Antioxidative Activity and Emulsifying Properties of Gelatin from Cuttlefish (*Sepia pharaonis*) Skin Modified with Phenolic Compounds and Fatty Acids

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ชื่อวิทยานิพนธ์	กิจกรรมการต้านปฏิกิริยาออกซิเดชั่นและสมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินจากหนังหมึกกระดอง (*Sepia pharaonis*) ที่ผ่านการดัดแปลงด้วยสารประกอบฟีนอลิกและกรดไขมัน
ผู้เขียน	นายทนง เอี่ยวศิริ
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา	2553

บทคัดย่อ

จากการศึกษาการสกัดและจำแนกลักษณะของเจลาตินหนังหมึกกระดองโดยนำหนังหมึกกระดองผ่านการฟอกสีด้วยไฮโดรเจนเปอร์ออกไซด์ที่ระดับความเข้มข้นร้อยละ 2 และ 5 นาน 24 และ 48 ชั่วโมง ก่อนการสกัดเจลาตินด้วยน้ำกลั่นที่อุณหภูมิ 60 องศาเซลเซียส นาน 12 ชั่วโมง พบว่า การฟอกสีหนังหมึกกระดองสามารถปรับปรุงสีของเจลาตินเจลที่ได้ โดยมีการเพิ่มขึ้นของค่า L* และการลดลงของค่า a* การฟอกสีสามารถเพิ่มค่าความแข็งแรงเจล สมบัติการเป็นอิมัลซิไฟเออร์และการเกิดโฟมของเจลาตินที่ได้ การฟอกสีโดยใช้ไฮโดรเจนเปอร์ออกไซด์ที่ระดับความเข้มข้นร้อยละ 5 นาน 48 ชั่วโมง มีผลให้ผลผลิต เจลาตินสูงสุด (ร้อยละ 49.65 และ 72.88 สำหรับหนังหมึกกระดองส่วนหลังและส่วนท้อง ตามลำดับ) เจลาตินที่สกัดได้หลังการฟอกสีประกอบด้วยโปรตีนที่มีน้ำหนักโมเลกุลเท่ากับ 97 กิโลดาลตัน และมีปริมาณคาร์บอนิลที่เพิ่มขึ้น จากการศึกษาด้วยเทคนิค Fourier transform infrared (FTIR) พบว่า เจลาตินที่ผ่านการฟอกสีเกิดการเชื่อมประสานกันระหว่างโมเลกุล และการสูญเสียสภาพสูงกว่าชุดควบคุม ไฮโดรเจนเปอร์ออกไซด์อาจเหนี่ยวนำให้เกิดการออกซิเดชั่นของเจลาตินเป็นผลให้เกิดการเชื่อมประสานกันของโมเลกุลซึ่งมีสมบัติเชิงหน้าที่ดีขึ้น

จากการดัดแปลงเจลาตินจากหนังหมึกกระดองโดยใช้สารประกอบฟีนอลิกชนิดต่างๆ ได้แก่ กรดคาเฟอิก กรดเฟอรูลิก และกรดแทนนิกที่ผ่านการออกซิเดชั่น พบว่าสารประกอบฟีนอลิกที่ผ่านการออกซิเดชั่นสามารถเข้ายึดจับกับเจลาตินได้ ซึ่งบ่งชี้จากหมู่อะมิโนอิสระที่ลดลงและการเพิ่มขึ้นของปริมาณสารประกอบฟีนอลิก ($P<0.05$) จากผลของ FTIR พบว่าเจลาตินที่ผ่านการดัดแปลงประกอบด้วยวงแหวนอะโรมาติกและหมู่ไฮดรอกซิล การดัดแปลงเจลาตินด้วยฟีนอลิกที่ผ่านการออกซิเดชั่นสามารถเพิ่มฤทธิ์การต้านออกซิเดชั่นได้ แต่ส่งผลให้ความไม่ชอบน้ำที่ผิวหน้าของเจลาตินลดลง อย่างไรก็ตามการใช้ฟีนอลิกที่ผ่านการออกซิเดชั่นที่ระดับร้อยละ 5 ไม่ส่งผลต่อสมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินดัดแปลงที่ได้เมื่อเทียบกับเจลาตินชุดควบคุม

เจลาตินที่ผ่านการดัดแปลงด้วยกรดแทนนิกที่ผ่านการออกซิเดชั่น (OTA) ที่ระดับร้อยละ 5 สามารถยับยั้งการเกิด TBARS ได้อย่างมีประสิทธิภาพกว่าเจลาตินชุดควบคุม

ชนิดพันธะระหว่างเจลาตินและสารประกอบฟีนอลิกมีบทบาทสำคัญต่อฤทธิ์การต้านปฏิกิริยาออกซิเดชั่นและสมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินดัดแปลงที่ได้ การเกิดพันธะชนิดนอนโควาเลนท์พบในการทำปฏิกริยาระหว่างเจลาตินกับกรดแทนนิก (TA) ที่พีเอช 7 ขณะที่พันธะแบบโควาเลนท์สามารถพบในการทำปฏิกิริยาระหว่างเจลาตินกับ OTA ที่พีเอช 9 โดยทั่วไปการเชื่อมประสานระหว่างกรดแทนนิกและเจลาตินโดยพันธะนอนโควาเลนท์พบในระดับที่สูงกว่าเมื่อเทียบกับพันธะโควาเลนท์ ซึ่งบ่งชี้จากปริมาณการสูญเสียหมู่อะมิโนอิสระ การเพิ่มขึ้นของปริมาณสารประกอบฟีนอลิก และการปรากฏของวงแหวนอะโรมาติกและหมู่ไฮดรอกซิลเมื่อตรวจสอบด้วย FTIR การดัดแปลงเจลาตินด้วยกรดแทนนิกด้วยพันธะนอนโควาเลนท์ส่งผลให้เจลาตินที่ได้ มีฤทธิ์การต้านปฏิกิริยาออกซเดชั่นที่สูงกว่าการจับด้วยพันธะโควาเลนท์ อย่างไรก็ตามเจลาตินที่จับกับกรดแทนนิกด้วยพันธะโควาเลนท์สามารถให้ความคงตัวและป้องกันการเกิดออกซิเดชั่นของระบบอิมัลชั่นได้ดีกว่าเจลาตินที่จับกับกรดแทนนิกด้วยพันธะชนิดนอนโควาเลนท์

เมื่อสารประกอบเอสเธอร์ เอ็น-ไฮดรอกซีซักซินิไมด์ (*N*-hydroxysuccinimide) ของกรดไขมันอิ่มตัวชนิดต่างๆ ได้แก่ กรดคาพริก (C10:0) กรดลอริก (C12:0) และกรดไมริสติก (C14:0) ทำปฏิกิริยากับเจลาตินหนังหมึกกระดอง พบว่า กรดไขมันอิ่มตัวสามารถจับกับเจลาตินซึ่งสังเกตได้จากปริมาณหมู่อะมิโนอิสระที่ลดลง จากผลของ FTIR พบการปรากฏของหมู่แอลคิลในเจลาตินที่ผ่านการดัดแปลง การเชื่อมต่อของกรดไขมันบนเจลาตินโดยเฉพาะอย่างยิ่งกรดไขมันสายโซ่ยาวสามารถเพิ่มความไม่ชอบน้ำที่พื้นผิวของเจลาติน ส่งผลให้กิจกรรมพื้นผิว ได้แก่ สมบัติการเกิดโฟมและสมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินเพิ่มขึ้นเมื่อเทียบกับเจลาตินชุดควบคุม ($P<0.05$) อิมัลชั่นที่เตรียมจากเจลาตินที่ผ่านการดัดแปลงด้วยเอสเธอร์ของกรดไมริสติกประกอบด้วยอนุภาคไขมันที่มีขนาดเล็กและมีความคงตัวตลอดระยะเวลาการเก็บ 10 วันที่อุณหภูมิ 30 องศาเซลเซียสเมื่อเทียบกับอิมัลชั่นที่ได้จากเจลาตินชุดควบคุม และอิมัลชั่นที่ได้มีความคงตัวในช่วงพีเอช 3-8 และในสภาวะที่มีเกลือ 0-500 มิลลิโมลาร์และการให้ความร้อนที่ 50-90 องศาเซลเซียส นาน 30 นาที

จากการศึกษาการดัดแปลงเจลาตินจากหนังหมึกกระดองด้วยกรดลิโนเลอิกที่ผ่านการออกซิเดชั่น (OLA) ที่สภาวะต่างๆ พบว่า OLA สามารถจับกับเจลาตินและเหนี่ยวนำให้เกิดหมู่คาร์บอนิล และการเพิ่มขึ้นของขนาดอนุภาคของเจลาติน จากผลของ FTIR พบการเปลี่ยนแปลงทางโครงสร้างและการเพิ่มขึ้นของหมู่แอลคิลในเจลาตินดัดแปลง นอกจากนี้ OLA ยังสามารถเพิ่มความไม่ชอบน้ำที่พื้นผิวของเจลาติน ซึ่งส่งผลให้เจลาตินมีกิจกรรมพื้นผิวได้แก่ สมบัติการ

เกิดโฟมและสมบัติการเป็นอิมัลซิไฟเออร์ที่เพิ่มขึ้น ระดับการออกซิเดชั่นของกรดลิโนเลอิกมีบทบาทสำคัญที่ส่งผลต่อคุณลักษณะและความสามารถเชิงพื้นผิวของเจลาตินดัดแปลง กรดลิโนเลอิกที่ผ่านการออกซิเดชั่นที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 9 ชั่วโมง สามารถเพิ่มความไม่ชอบน้ำที่พื้นผิวและกิจกรรมพื้นผิวของเจลาตินที่ดัดแปลงที่ได้สูงสุด และอิมัลชั่นที่เตรียมได้ประกอบด้วยอนุภาคไขมันที่มีขนาดเล็กและมีความคงตัวในสภาวะต่างๆ (พีเอช ปริมาณเกลือ และความร้อน)

โดยทั่วไปการเติม OLA ในการดัดแปลงเจลาตินมีผลให้เจลาตินมีผิวหน้าที่ไม่ชอบน้ำและสมบัติการเป็นอิมัลซิไฟเออร์ที่เพิ่มขึ้น ขณะที่การเติม OTA เพียงอย่างเดียวลงในเจลาตินสามารถเพิ่มฤทธิ์การยับยั้งปฏิกิริยาออกซิเดชั่น แต่มีผลต่อสมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินที่ได้ การใช้ OLA ในอัตราส่วน 10:1 (OLA:ปริมาณอะมิโนอิสระ) ร่วมกับ OTA ที่ระดับความเข้มข้นร้อยละ 5 (ต่อปริมาณโปรตีน) ในการดัดแปลงเจลาตินสามารถเพิ่มทั้งฤทธิ์ในการต้านปฏิกิริยาออกซิเดชั่น และ สมบัติการเป็นอิมัลซิไฟเออร์ของเจลาตินดัดแปลงที่ได้เมื่อเปรียบเทียบกับเจลาตินที่ไม่ผ่านการดัดแปลง โดยเจลาตินที่ผ่านการดัดแปลงด้วย OTA และ OLA สามารถรักษาความคงตัวของอิมัลชั่นจากปฏิกิริยาออกซิเดชั่นและการแยกชั้นได้อย่างมีประสิทธิภาพเมื่อเปรียบเทียบกับเจลาตินทางการค้า ดังนั้นเจลาตินจากหนังหมึกกระดองที่ผ่านการดัดแปลงมีสมบัติหลายประการซึ่งสามารถประยุกต์ใช้เป็นสารเติมแต่งอาหารที่มีประสิทธิภาพ

Thesis Title	Antioxidative activity and emulsifying properties of gelatin from cuttlefish (*Sepia pharaonis*) skin modified with phenolic compounds and fatty acids
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ABSTRACT

Gelatin from cuttlefish skin was extracted and characterized. Cuttlefish (*Sepia pharaonis*) skin was subjected to bleaching with 2 and 5% H_2O_2 for 24 and 48 h prior to gelatin extraction with distilled water at 60°C for 12 h. Bleaching not only improved the color of gelatin gel by increasing the L*-value and decreasing a*-value but also enhanced bloom strength, emulsifying and foaming properties of resulting gelatin. Bleaching of gelatin with 5% H_2O_2 for 48 h resulted in the highest yield (49.65 and 72.88 % for dorsal and ventral skin, respectively). Gelatin from bleached skin contained protein with molecular weight of 97 kDa and had increased carbonyl content. Fourier transform infrared (FTIR) spectroscopic study showed higher intermolecular interactions and denaturation of gelatin from bleached skin than that of the control. Hydrogen peroxide most likely induced the oxidation of gelatin, resulting in the formation of gelatin cross-links with the improved functional properties.

Modification of cuttlefish skin gelatin with different phenolic compounds including caffeic acid, ferulic acid and tannic acid at different concentrations was carried out to improve antioxidative activity. Oxidized phenolic compounds were attached to gelatin as indicated by the decrease in amino groups and increase in total phonolic content. Based on FTIR spectroscopic result, aromatic ring and hydroxyl group were present in gelatin after modification. Modification of gelatin with oxidized phenolic compounds increased antioxidative activity but decreased surface hydrophobicity of resulting gelatin ($P<0.05$). However, gelatin modified with 5% oxidized tannic acid (OTA) had no effect on emulsifying property, compared with the control gelatin ($P<0.05$). Gelatin modified by 5% OTA could inhibit the formation

of TBARS in the emulsion more effectively than the control gelatin.

Type of interaction between gelatin and phenolic compound played an important role in the antioxidative activity and emulsifying properties of modified gelatin. The non-covalent interaction was found between cuttlefish skin gelatin and tannic acid (TA) at pH 7, whereas covalent interaction was prevalent in gelatin modified with OTA at pH 9. Degree of tannic acid incorporation into gelatin via non-covalent interaction was more pronounced than that with covalent interaction as evidenced by lowered free amino group content and increased total phenolic content and hydroxyl group and aromatic ring determined by FTIR. Gelatin modified with TA via non-covalent interaction showed the higher antioxidative activity than that with covalent interaction ($P<0.05$). However, gelatin modified with OTA via covalent interaction rendered the emulsion with high stability and could inhibit lipid oxidation of menhaden oil-in-water emulsion more effectively than that with non-covalent interaction.

When the *N*-hydroxysuccinimide esters of different saturated fatty acids including capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) were incorporated into cuttlefish skin gelatin, covalent attachment of fatty acids into gelatin was observed as evidenced by the decrease in amino groups. FTIR results indicated the presence of alkyl group in modified gelatin. Attachment of fatty acid, especially with longer chain, into gelatin resulted in the increases in surface hydrophobicity and increase in surface activity including foaming and emulsifying properties, compared with the control gelatin ($P<0.05$). Emulsion stabilized by gelatin modified with *N*-hydroxysuccinimide esters of C14:0 had the smaller mean particle diameter with higher stability during storage for 10 days at 30°C, compared with that stabilized by the control gelatin ($P<0.05$). Emulsion stabilized by modified gelatin remained stable at various pH (3-8), and salt concentrations (NaCl 0-500 mM). It was also stable after heat treatment at 50-90°C for 30 min.

Modification of cuttlefish skin gelatin with oxidized linoleic acid (OLA) prepared at different conditions was also studied. Interaction between OLA and gelatin led to the generation of carbonyl groups and the increase in particle size of resulting gelatin. FTIR spectroscopic study revealed the structural changes and the

incorporation of alkyl group into modified gelatin. Gelatin with modified OLA had the increased surface hydrophobicity and improved surface activity including foaming and emulsifying properties. The degree of oxidation of linoleic acid played a key rule in the characteristic and surface activity of modified gelatin. Gelatin modified with OLA at 70°C for 9 h showed the highest surface hydrophobicity and surface activity. Emulsion stabilized by this modified gelatin had the smaller mean particle diameter with higher stability toward different harsh environments (pH, heat and salt), compared with the control gelatin.

In general, attachment of OLA into gelatin increased surface hydrophobicity and emulsifying property, whereas incorporation of OTA into gelatin increased antioxidative activity but decreased surface hydrophobicity. Attachment of OLA at a ratio of OLA-to-free amino group of 10:1 and OTA at a concentration of 5% increased both antioxidative activity and emulsifying property of modified gelatin. The emulsion stabilized by gelatin modified with both OLA and OTA was more stable to lipid oxidation and phase separation than the commercial bovine gelatin. Therefore, modified cuttlefish skin gelatin can possess multi-functions, which can be applied as additive in various foods.

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Tanong Aewsiri

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Gelatin is a denatured form of collagen and can be used widely in medical, pharmaceutical and food industries (Cho *et al.*, 2005). Because of its unique functional properties, gelatin has been utilized in food products as stabilizer, texturizer, water binding agent, foaming agent and emulsifier (Schrieber and Gareis, 2007). Gelatin with surface/interfacial activity can adsorb on oil-water or air-water interfaces, where various hydrophobic segment penetrating into the oil or air phase (Olijve *et al.*, 2001). Moreover, gelatin can form a continuous viscoelastic membrane-like film around oil droplets or air cells via noncovalent intermolecular interactions, resulting in the high stability in the food emulsion and foam products (Damodaran, 1997). Gelatin is commercially made from skins and skeletons of bovine and porcine by alkaline or acidic extraction (Gilsenan and Ross-Murphy, 2000). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot/mouth diseases have led to the major concern of human health and thus by-products of mammalians are limited for production of collagen and gelatin as the functional food, cosmetic and pharmaceutical products (Cho *et al.*, 2005). Additionally, porcine gelatin can be of objection from some religions. As a consequence, the increasing interest and attempt have been paid to other gelatin sources, especially skin and bone, by-product from seafood processing plants. So far, gelatin from skin from different fish species has been intensively extracted and characterized (Muyonga *et al.*, 2004b).

Gelatin is usually composed of a few hydrophobic amino acids (proline and leucine) and a large portion of hydrophilic amino acids (lysine, serine, arginine, hydroxyproline, aspartic and glutamic acids). Low hydrophobicity of gelatin therefore lowers its ability to function as a surface active agent (Toledano and Magdassi, 1997). To improve the surface activity of gelatin, the incorporation of hydrophobic domains

can be an effective means to bring about amphiphilic characteristic of gelatin (Kato and Nakai, 1980; Toledano and Magdassi, 1997). Covalent attachment of fatty acids into protein by reaction between *N*-hydroxysuccinimide esters of fatty acids with free amino groups of proteins could improve surface activity of protein (Magdassi *et al.*, 1996). Wierenga *et al.* (2003) reported that attachment of C10 alkyl chain to ovalbumin caused an increase in surface activity. Toledano and Magdassi (1998) showed that bovine gelatin modified with fatty acid ester had the higher foaming and emulsifying properties in comparison with native gelatin. Moreover, the lipid oxidation products could modify amino acids of proteins (Stadtman and Berlett, 1997). Alkyl and peroxyl radical decomposed from hydroperoxide can directly interact with side chains of proteins (Kato *et al.*, 1992). The secondary lipid oxidation products, such as aldehydes, react mainly with amino acids via condensation reaction to form Schiff's bases or by Michael addition reactions (Liu *et al.*, 2003; Stadtman and Berlett, 1997). Therefore, the introduction of lipid oxidation products to gelatin might provide the hydrophobic domain for gelatin, thereby facilitating the migration of gelatin to the interface.

Interactions of protein with phenolic compounds lead to changes in the proteins solubility and hydrophilic/hydrophobic nature. Such an interaction affects functional properties of proteins such as emulsion formation and gelling properties (Kroll *et al.*, 2003; Rawel *et al.*, 2002a). Due to cross-linking and complex formation, the conformation of protein is changed and the exposure of some additional hydrophobic regions previously buried takes place. Nevertheless, the increasing number of hydroxyl groups in the phenols bound can increase hydrophilicity (Rawel *et al.*, 2002a; Rawel *et al.*, 2002b). The interaction between proteins and phenolic compounds can also influence the antioxidant activity of phenolic compounds. Protein-phenolic complexes may act as radical scavengers and radical sinks (Rohn *et al.*, 2004; Rohn *et al.*, 2005).

Cuttlefish has become one of important fishery products of Thailand, mainly exported to different countries all over the world. During processing, de-skinning is a common practice to obtain desirable products, resulting in the generation of skin as a byproduct. Skin has the low market value and in commonly used as animal feed. The extraction of gelatin from cuttlefish skin could increase its

profitability. Additionally, the modification of cuttlefish skin gelatin by an appropriate means or combined methods could augment the use of gelatin from cuttlefish skin as surface active agent, which may function as antioxidant to retard lipid oxidation in emulsion food system. The information gained will be of benefit for both seafood processing industry as well as additive-related industries.

1.2 Review of literature

1.2.1 Cephalopod and cuttlefish

Cuttlefish belong to the family Sepiodia, class Cephalopoda, which forms part of phylum Mollusca. Sepioidea is characterized by a thick internal calcium-containing shell called the cuttlebone. Cuttlefish has the length ranging from 2.5 to 90 cm and have a somewhat flattened body bordered by a pair of narrow fins (Figure 1). They feed mainly on crustaceans as well as small fishes. Cuttlefish has been used as human food, as a source of ink, and the cuttlebone is used as a dietary supplement for cage birds. They grow quickly and live in the deeper water. The new adult cuttlefish are ready to spawn at 18- 24 months of age.

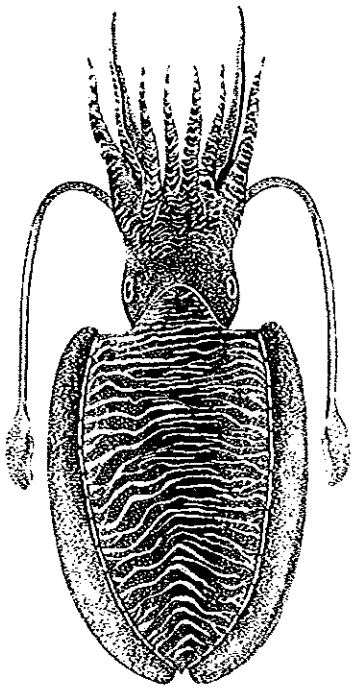


Figure 1. Cuttlefish (*Seapia pharaonis*)

Source: Department of Fisheries (2006)

1.2.1.1. Cephalopod tissue

1.2.1.1.1. Skin tissue

The skin (epidermis) on the surface of *Todarodes pacificus* is composed of four layers (Figure 2). The first and second layers have nuclei and black chromatopores. The third layer is composed of many nuclei. The fourth layer is typical muscle fiber connective tissue. The thickness of each layer varies. The first layer is 60-100 μm; the second layer is 150-270 μm; the third layer is 50-60 μm and the fourth layer is 30-40 μm (Sugiyama *et al.*, 1989).

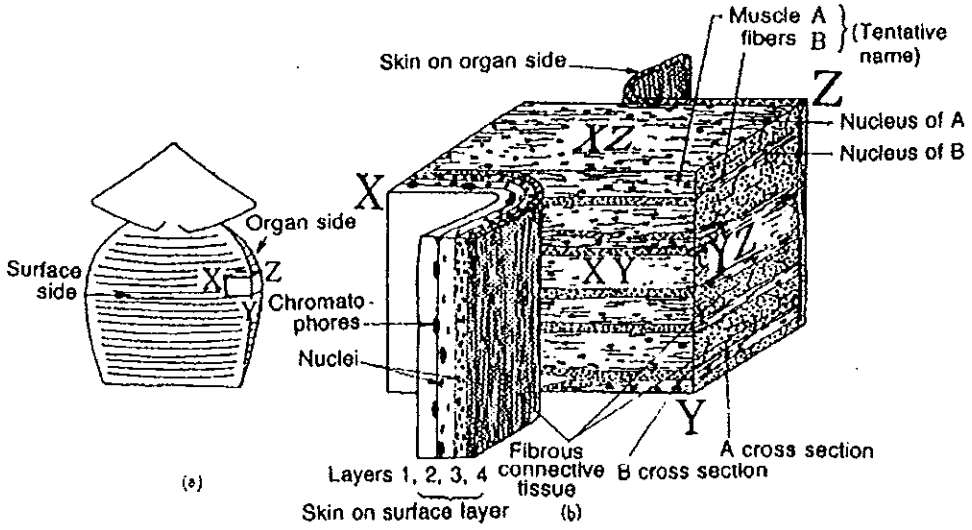


Figure 2. Tissue structure of mantle muscles of *T. Pacificus*
Source: Sugiyama *et al.* (1989)

A chromatophore is a container for the color particles. Each color is indicated by the three types of chromatophore cells including erythrophores, melanophores, and xanthophores. The first type is the erythrophores, which contains reddish pigments such as carotenoids and pteridines. The second type is the melanophores, which contains black and brown pigments such as the melanins. The third type is the xanthophores which contains yellow pigments in the forms of carotenoids. It consists of a cell with an expandable membrane as a wall (Kreuzer, 1984). Several muscle fibers are radially attached to the membrane and each fiber is

connected with a nerve. If the animal is on a light surface, the muscle attached to the cell wall are relaxed, the chromatophores appear small and round. The skin of the animal appears light. When the animal moves into a dark background, the shade of the required color are controlled by a nerve system. Instantly, the muscles contract and expand the walls of the chromatophores in all directions. Each chromatophore cell can now cover up to 60 times the surface than the small, contracted cell. The pigment flows to the periphyry of the expanded cell covering the whole area. The skin now becomes dark (Kreuzer, 1984).

1.2.1.1.2. Muscle tissue

The meat of cuttlefish is mainly from its mantle. The mantle is composed of five layers of tissue (Figure 3) (Otwell and Hamann, 1979). The middle layer contains the muscle tissue with the muscle fibers group (Kreuzer, 1984). The characteristic feature of the cephalopod muscles is their very small size. The elongated cells of myofibril are surrounding a central core which contains sarcoplasmic proteins. The muscle fibers are obliquely striated and covered with a thin sarcolemma.

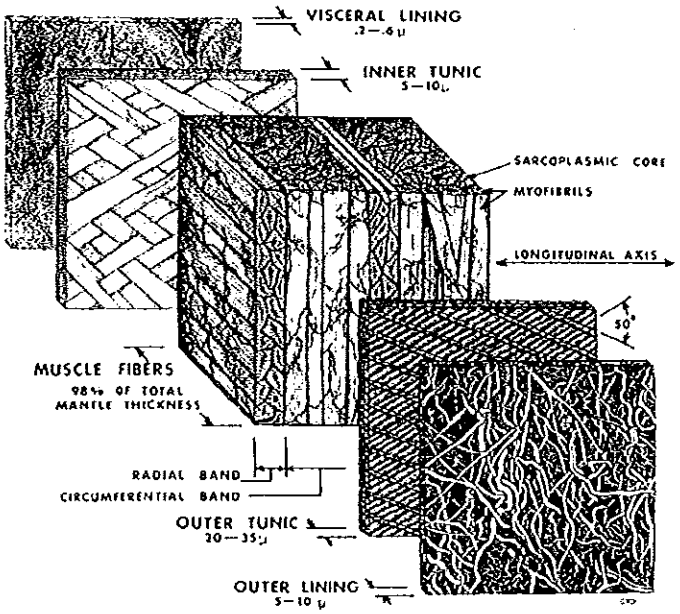


Figure 3. Tissue composition in the mantle of cephalopod

Source: Otwell and Hamann (1979)

The muscle layer comprises about 98% of the total thickness of the mantle. The muscle is covered on each side by a layer of connective tissue called the outer and the inner tunic. The outer side of most of each tunic is covered by a thin layer tissue, the outer lining, which connects the outer tunic with the skin. It consists of randomly oriented fibers. The inner lining is the inner surface of the mantle (Otwell and Hamann, 1979). The different layers possess different chemical and physical properties (Okuzumi and Fujii, 2000).

1.2.1.2 Chemical compositions of cephalopod

The chemical composition of cephalopod is dependent on species, growth stage and season. Mantle is the main edible portion of cephalopod constituting about 35-44.7 % of total body (Kreuzer, 1984). Proximate composition of mantle muscle of squid and cuttlefish is shown in Table 1.

Table 1. Proximate composition of mantle muscle of squid and cuttlefish (% on wet basis)

Species	Moisture	Protein	Lipid	Ash
Todarodes pacificus	76.6	20.6	1.88	1.59
Ommastrephesbartrami	77.1	20.9	1.33	1.71
Nototodaru sloani gouldi	78.4	18.7	1.66	1.78
Illex argentinus	78.8	18.2	2.03	1.71
Loligo opalescens	77.0	19.6	2.74	1.52
Sepia esculenta	81.5	15.6	1.28	1.56
Sepia pharaonis	76.4	20.2	1.36	1.86

Source: Kreuzer (1984)

Protein is an important component of cephalopod mantle. The main proteins of the muscle are myofibrillar (77-85%), sarcoplasmic (2.0%) and stroma proteins (12-13%) (Okuzumi and Fujii, 2000). Myofibrillar proteins are the main constituents in squid mantle. Squid protein consists of a thick filament called myosin and a fine filament called actin. The thick filament of squid is formed from a composite core of protein called paramyosin, unique to invertebrates, around which the myosin is coiled in a structure that is unique to squid. As a result, squid has a

longer and thicker filament than vertebrate (Okuzumi and Fujii, 2000). Sarcoplasmic proteins are soluble at low ionic strength even water. Sarcoplasmic protein is composed of proteinases that have the highest activity at weak alkaline, as well as TMAO demethylase, that causes the formation of dimethylamine and formaldehyde in frozen cuttlefish. Collagen content of fish and seafood depends on the species, feeding regime, and state of maturity. The structure of collagen fibrils evidently influences the physical properties of the tissue, mainly solubility and texture (Okuzumi and Fujii, 2000).

Lipid is a minor composition of cephalopod. The squid mantle contains only 1.0-2.0% (Okuzumi and Fujii, 2000). The lipid profile of cephalopod is clearly distinct from other species since it was rich in phospholipids. Triglyceride has been reported as a minor component of the flesh of cephalopods (Jangaard and Acman, 1965). Southgate and Lou (1995) reported that adult cephalopods are rich in long chain polyunsaturated fatty acid (PUFA). Their oil is a good source of PUFA, which is often used in aquaculture to supplement feed.

1.2.2 Collagen and gelatin

1.2.2.1 Collagen

Collagen is abundant in tendons, skin, bone, the vascular system of animals, and the connective tissue sheath surrounding muscle, contributing to toughness of muscle. About 10% of mammalian muscle protein is collagen but the amount in fish is generally much less (Foegeding *et al.*, 1996; Karim and Bhat, 2009). The collagen monomer is a long cylindrical protein about 2,800 Å long and 14-15 Å in diameter (Foegeding *et al.*, 1996). The triple helix of collagen assembles from specific polypeptide chain (α chains), which has Gly-X-Y repeat with the frequent occurrence of proline and hydroxyproline in the X and Y position, respectively. Hydroxyproline and hydroxylysine are found only in position Y, while proline can be found in either the X- or Y- position (Fratzl, 2008). Each α-chain coil is a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form tropocollagen. The triple helix is held together by hydrogen bonding (Figure 4). Each α-chain contains ~1,000 amino acid residues and varies in amino acid

compositions (Wong, 1989) and has a molecular mass of about 100,000 Da, yielding a total molecular mass of about 300,000 Da for collagen. (Foegeding *et al.*, 1996).

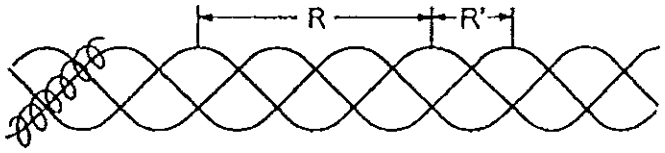


Figure 4. Schematic representation of the conformation of tropocollagen

Source: Burghagen (1999)

Various types of collagen are observed among different organs and connective tissue layers of muscular tissue (Table 2).

Table 2. Types of collagen

Type	Peptide chains[a]	Molecular composition	Occurrence
I	α^1, α^2	$[\alpha^1(I)]_2\alpha^2(I)$	Skin, tendons, bones, muscle (epimysium)
II	α^1	$[\alpha^1(II)]_3$	cartilage
III	α^1	$[\alpha^1(III)]_3$	Fetal skin, cardio vascular system, synovial membranes, inner organs, muscle
IV	α^1, α^2	$[\alpha^1(IV)]_3(?)$[b]	Basal membranes, capsule of lens, glomeruli, lung, muscle (endomysium)
V	αA, αB, αC	$[\alpha B]_2\alpha A$ or $[\alpha B]_3+(\alpha A)$ or $[\alpha C]_3(?)$	Placental membrane, cardiovascular system, lung, muscle (endomysium), secondary component of many tissues

[a] Since the α chains of various types of collagen differ, they are called $\alpha^1(I)$, $\alpha^1(II)$, αA etc.

[b] (?) Not completely elucidated.

Source: Burghagen (1999)

Polypeptides of collagen are mostly helical but differ from the typical α-helix due to the abundance of hydroxyproline and proline, which interfere with α-

helical structure (Foegeding *et al.*, 1996). Collagen molecules link end to end and adjacently form collagen fibers as shown in Figure 5.

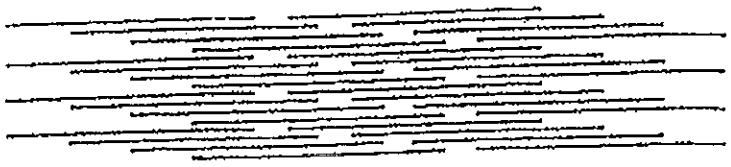


Figure 5. Overlap structure of the collagen
Source: Foegeding *et al.* (1996)

Collagen possesses high contents of glycine (33%), proline (12%) and the occurrence of 4-hydroxyproline (12%) and 5-hydroxylysine (1%) is its characteristics (Burghagen, 1999). Glycine generally represents mainly one-third of the total residues, and it is distributed uniformly at every third position throughout most of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 or so amino acid residues from the N-terminus and the first 10 or so from the C-terminus. These end portions are termed "telopeptides". Collagen is the only protein that is rich in hydroxyproline; however, fish collagens contain less of this amino acid than do mammalian collagens (Hulmes, 2008). Collagen is generally almost devoid of tryptophan.

The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule, and collagen that has small concentrations of both imino acids, denatures at lower temperatures than do those with high concentrations (Foegeding *et al.*, 1996). In general, fish collagens have lower imino acid contents than mammalian collagens, and this may be the reason for the denaturation at low temperature (Karim and Bhat, 2009). The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22–25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatin (cod) (Muyonga *et al.*, 2004b). The source and type of collagen will influence the properties of the resulting gelatins.

1.2.2.2 Gelatin

Gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen. The process involves the disruption of non-covalent bonds and it is partially reversible in agreement with the gelling properties of gelatin (Karim and Bhat, 2009). Collagen fibrils shrink to less than one-third of their original length at a critical temperature, known as the shrinkage temperature. This temperature varies, depending on species from which the collagen is derived (Burghagen, 1999). This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. Essentially the same type of molecular change occurs when collagen is heated in solution, but at a much lower temperature (Foegeding *et al.*, 1996). The midpoint of the collagen-to-gelatin transition is defined as the melting temperature (Figure 6). During the collagen-to-gelatin transition, many covalent bonds are broken along with some covalent inter- and intra-molecular bonds (Schiff's base and aldo condensation bonds). This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin. These changes lead to the denaturation of the collagen molecule but not to the point of a completely unstructured product (Foegeding *et al.*, 1996).

After gelatin is produced and the temperature is lowered to below the critical value, there is a partial renaturation of the collagen molecule, involving what is called the "Collagen fold". Apparently, those parts of collagen that are rich in proline and hydroxyproline residues regain some of their structure, following which they can apparently interact (Foegeding *et al.*, 1996). When many molecules are involved, a three-dimensional structure is produced and responsible for the gel observed at low temperatures. The strength of the gel formed is proportional to the square of the concentration of gelatin and directly proportional to molecular weight (Cho *et al.*, 2004). Circular dichroism analysis reveals that gelling involves a refolding of denatured collagen chains into the typical triple helix conformation and conversely unfolding upon reheating. The folding process seems to be directly related in the stabilization of the gels without disregarding its role in triggering the gelation process (Gómez-Guillén *et al.*, 2002).

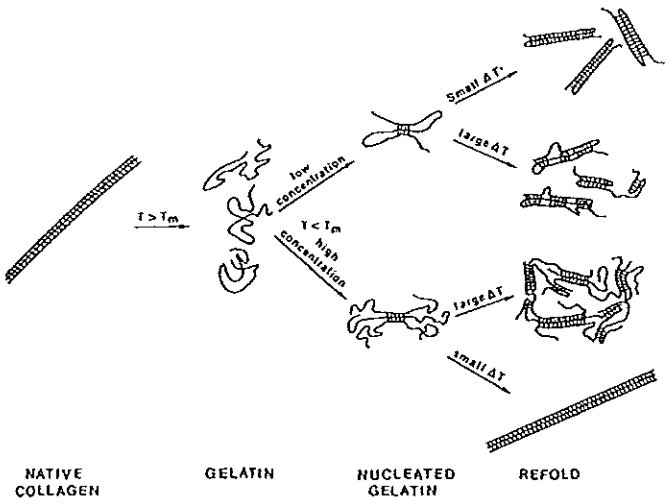


Figure 6. Collagen conversion into gelatin

Source: Wong (1989)

1.2.2.2.1 Fish gelatin

Gelatin from marine sources (warm- and cold-water fish skins, bones, and fins) is a possible alternative to bovine gelatin (Kim and Mendis, 2006). One major advantage of marine gelatins is that they are not associated with the risk of outbreaks of bovine spongiform encephalopathy (BSE). Fish gelatin is acceptable for Islam, and can be used with minimal restrictions in Judaism and Hinduism (Cho *et al.*, 2005). Furthermore, fish skin, which is a major byproduct of the fish-processing industry, causing waste and pollution, could provide a valuable source of gelatin (Badii and Howell, 2006). Fish skin contains a large amount of collagen. Nagai and Suzuki (2000) reported that the collagen contents in the skin of Japanese sea-bass, chub mackerel, and bullhead shark were 51.4, 49.8, and 50.1% (dry basis), respectively. Production of fish gelatin is actually not new as it has been produced since 1960 by acid extraction (Norland, 1990). Gelatin has been extracted from skins and bones of various cold-water (e.g., cod, hake, Alaska pollock, and salmon) and warm-water (e.g., tuna, catfish, tilapia, Nile perch, shark and megrim) fish as shown in Table 3.

Table 3. Sources of fish gelatin

Fish species	References
Unicorn leatherjacket (*Aluterus monoceros*)	Ahmad and Benjakul (2011)
Bamboo shark (*Chiloscyllium punctatum*), blacktip shark (*Carcharhinus limbatus*)	Kittiphattanabawon *et al.* (2010b)
Baltic cod (*Gadus morhua*), salmon (*Salmo salar*), herrings (*Clupea harengus*)	Kołodziejska *et al.* (2008)
Catfish (*Ictalurus punctatus*)	Liu *et al.* (2008)
Grass carp (*Ctenopharyngodon idella*)	Kasankala *et al.* (2007)
Atlantic salmon (*Salmo salar*)	Arnesen and Gildberg (2007)
Skate (*Raja kenojei*)	Cho *et al.* (2006)
Yellowfin tuna (*Thunnus albacares*)	Chiou *et al.* (2006)
Bigeye snapper (*Priacanthus macracanthus*), brownstripe red snapper (*Lutjanus vitta*)	Jongjareonrak *et al.* (2006c)
Sin croaker (*Johnius dussumieri*), shortfin scad (*Decapterus macrosoma*)	Cheow *et al.* (2006)
Horse mackerel (*Trachurus trachurus*)	Badii and Howell (2006)
Alaska pollock (*Theragra chalcogramma*)	Zhou and Regenstein (2005)
Nile perch (*Lates niloticus*)	Muyonga *et al.* (2004a)
Flounder (*Platichthys flesus*)	Fernández-Díaz *et al.* (2003)
Black tilapia (*Oreochromis mossambicus*), red tilapia (*Oreochromis nilotica*)	Jamilah and Harvinder (2002)
Megrim (*Lepidorhombrus boscii*) (Risso), Hake (*Merluccius merluccius*)	Gómez-Guillén *et al.* (2002)

1.2.2.2.2 Extraction of fish gelatin

Generally, gelatin manufacturing processes consist of three main stages: pretreatment of the raw material, extraction of the gelatin, and purification and drying (Karim and Bhat, 2009). Depending on the method in which the collagens are pretreated, two different types of gelatin (each with differing characteristics) can be produced. Type A gelatin (isoelectric point at pH 6–9) is produced from acid-treated collagen, and type B gelatin (isoelectric point at approximately pH 5) is produced from alkali-treated collagen (Stainsby, 1987). Acidic treatment is most suitable for the less covalently cross-linked collagens found in pig and fish, while alkaline treatment is suitable for the more complex collagens found in bovine hides. The extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin. This depends on the processing parameters (temperature, time, and pH), the pretreatment, and the properties and preservation method of the starting raw material (Karim and Bhat, 2009).

Fish gelatin has been extracted using a number of different methods as summarized in Table 4. The direct procedures used for preparing fish gelatin typically involve a mild chemical pretreatment of the raw material and mild conditions during the extraction process. In general, a mild acid pretreatment of the fish skin is used prior to gelatin extraction. Procedure for extracting gelatin with high gelling capacity from fish skins was essentially based on a mild alkaline or acid pretreatment for collagen swelling, followed by extraction in water at moderate temperatures (45°C) (Gómez-Guillén and Montero, 2001). The entire process takes about 24 h. Because of the acid or alkaline lability of the cross-links found in fish skin collagen, mild treatment is sufficient to produce adequate swelling and to disrupt the non-covalent intra- and intermolecular bonds (Montero *et al.*, 1990; Norland, 1990). Subsequent thermal treatment above 40°C (above helix-to-coil transition temperatures for fish gelatins) destroys hydrogen bonding and cleaves a number of covalent bonds, which destabilizes the triple-helix via a helix-to-coil transition and results in conversion to soluble gelatin (Djabourov *et al.*, 1993). High-molecular weight polymers may occur in the resulting gelatin through the possible persistence of cross-links, depending on the nature and degree of solubilization. However, pretreatment and extraction with strong condition might be required for gelatin extraction. Gómez-Guillén *et al.* (2002) reported that gelatin was not extracted by heating the squid skin (*Dosidicus gigas*) in water at 45°C. Therefore, heating the squid skin was performed at 80°C overnight. However, the yield of squid gelatin was only 2.6% which was lower than that found in sole (8.3%), megrim (7.4%), cod (7.2%) and hake (6.5%) using a milder procedure. Therefore, invertebrate collagens present a really high cross-linking degree, compared to vertebrate collagens, mainly due to the high increase in lysine hydroxylation that participate in different types of cross-links via Schiff-base formation. Moreover, hydroxylysine is normally glycosylated in invertebrate collagens, and the sugar moieties contribute to the establishment of additional covalent cross-links (Gómez-Guillén *et al.*, 2002).

Table 4. Procedures employed for fish gelatin extraction

Fish species	Extraction procedure	References
Bamboo shark (*Chiloscyllium punctatum*), blacktip shark (*Carcharhinus limbatus*)	Skin was soaked in 0.1M NaOH (1:10 w/v) with gentle stirring for 6 h, followed by washing with tap water until neutral basic pH was obtained. Pretreated skins were demineralized using 1 M HCl (1:10 w/v) with gentle stirring for 1 h, followed by washing with tap water until neutral basic pH was obtained. The skin samples were swollen using 0.2 M acetic acid (1:10 w/v). Gelatin in prepared skin was extracted using warm water at 45°C for 6 h.	Kittiphattanabawon *et al.* (2010b)
Atlantic salmon (*Salmo salar*) and Atlantic cod	Skins were washed in cold-water (8°C) and incubated in cold NaOH solutions (0.04 M) for 30 min. Pretreated skins were soaked in 0.12 M H_2SO_4 and 0.005 M citric acid for 30 min. After washing in cold-water, a two-step gelatin extraction was performed by gentle stirring, first in 1 L water at 56 °C for 2 h and then in 1 L at 65°C for 2 h.	Arnesen and Gildberg (2007)
Channel catfish (*Ictalurus punctatus*)	The skins were treated with NaOH (1:6 w/v) for various times and rinsed with tap water. The samples were treated with acetic acid (1:6 w/v) for various times, followed by rinsing with tap water (1:6 w/v) (three times). Gelatin was extracted in a water bath for different times.	Yang *et al.* (2007)

Table 4. Procedures employed for fish gelatin extraction (Continued)

Fish species	Extraction procedure	References
Grass carp (*Ctenopharyngodon idella*)	Skins were treated with 0.1–3.0% HCl and incubated at 7°C. Then, skin samples were washed for removal of excessive chemicals before extraction. Gelatin was extracted using water at 40–80°C.	Kasankala *et al.* (2007)
Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*)	Skins were soaked in 0.2 M NaOH (1:10, w/v) at 4°C with gentle stirring, followed by washing with tap water until neutral basic pH was obtained. Pretreated skins were soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature (25°C) with gentle stirring to swell the collagenous material in fish skin matrix. Gelatin extraction was performed using distilled water with a skin/water ratio of 1:10 (w/v) at 45°C (12 h) with a continuous stirring.	Jongjareonrak *et al.* (2006c)
Yellowfin tuna (*Thunnus albacares*)	Skins was treated with 8 volumes (v/w) of 1–3% NaOH at 10°C for 1–5 days to remove the non-collagen protein and subcutaneous tissue. Then, pretreated skins were neutralized with 6 N HCl. Gelatin was extracted with distilled water at 40–80°C for 1–9 h.	Cho *et al.* (2005)
Dover sole (*Solea vulgaris*)	Fish skins were treated with 50 mM acetic acid for 3 h. Gelatin was extracted with distilled water at 45°C.	Gómez-Guillèn *et al.* (2005)

Table 4. Procedures employed for fish gelatin extraction (Continoued)

Fish species	Extraction procedure	References
Flounder (*Platichthys flesus*)	Fish skins were pretreated by mild acid and gelatin was extracted using water at temperatures below 50 °C.	Fernández-Díaz *et al.* (2003)
Nile perch (*Lates niloticus*)	Skins were pretreated using 0.01 M H_2SO_4 solution (pH of 2.5–3.0). For bones, de-mineralization was performed using 3% HCl prior to pretreatment. Gelatin was extracted using distilled water at 60°C	Muyonga *et al.* (2004a)
Black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*)	Fish skins were soaked in 0.2% (w/v) NaOH solution for 40 min, followed by 0.2% H_2SO_4 acid and 1.0% citric acid. Rinsing with distilled water was carried out between soakings. Gelatin was extracted in distilled water at 45 °C for 12 h.	Jamilah and Harvinder (2002)
Squid (*Dosidicus gigas*)	The squid skins were treated with 0.05 M acetic acid. Gelatin was extracted in distilled water at 80°C	Gómez-Guillèn *et al.* (2002)
Megrim (*Lepidorhombus boscii*)	The skins were treated with 0.8 M NaCl and 0.2 M NaOH, followed by swelling with 0.05 M acetic acid. Gelatin was extracted in distilled water at 45 °C	Montero and Gómez-Guillén (2000)

The lower extraction yield of fish gelatin could be due to the loss of extracted collagen through leaching during the series of washing steps or due to incomplete hydrolysis of the collagen (Jamilah and Harvinder, 2002). In addition, it has been reported that endogenous heat-stable proteases are involved in the degradation of gelatin molecules (specifically the β- and α-chains) during the extraction process at elevated temperatures, which contribute to the low Bloom strength (Intarasirisawat *et al.*, 2007). Nalinanon *et al.* (2008) found that the addition of an appropriate protease inhibitor together with the pepsin-aided process might be an effective means to obtain a higher yield with negligible hydrolysis of the peptides. Extraction of gelatin from the bigeye snapper using the pepsin-aided process in combination with a protease inhibitor (pepstatin A) markedly increased the yield from 22.2 to 40.3%.

Generally, the extraction yield of gelatin from skins ranged from about 5.5 to 21% of the starting material (Giménez *et al.*, 2005a; Giménez *et al.*, 2005b; Muyonga *et al.*, 2004b). The variation in such values depends on the differences in both proximate composition of the skins and the amount of soluble components in the skins (Muyonga *et al.*, 2004b), as these properties vary with the species and the age of the fish. In addition, the variation in the extraction method can also have an effect on yields (Arnesen and Gildberg, 2007). Higher extraction temperature resulted in the higher yield of gelatin, however the higher degradation took place (Kittiphattanabawon *et al.*, 2010a).

1.2.2.3 Composition of gelatin

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight (Kantaria *et al.*, 1999). On a dry weight basis, gelatin consists of 98 to 99% protein. The molecular weight of these large proteins typically ranges between 20,000 and 250,000 Da. However, some aggregates weigh in the millions (Poppe, 1997). Coils of amino acids are joined together by peptide bonds. The predominant amino acid sequence is Gly-Pro-Hyp (Fratzl, 2008). Gelatin contains relatively high levels of these following amino acids: glycine (Gly) 26-34%; proline (Pro) 10-18%; and hydroxyproline (Hyp) 7-15% (Poppe, 1997). Other significant amino acids include alanine (Ala) 8-11%; arginine (Arg) 8-9%; aspartic acid (Asp) 6-7%; and

glutamic acid (Glu) 10-12% (Poppe, 1997). Table 5 shows the typical amino acid composition of gelatin from different sources.

Table 5. Amino acid composition of fish gelatins in comparison with pork gelatin (residues/1000 total amino acid residues)

Amino acid	Cod[a]	Hake[b]	Megrim[a]	Tilapia[b]	Giant squid[c]	Pork[d]
Ala	96	119	123	123	82	112
Arg	56	54	54	47	61	49
Asx	52	49	48	48	61	46
Cys	0	0	0	0	10	0
Glx	78	74	72	69	83	72
Gly	344	331	350	347	332	330
His	8	10	8	6	7	4
Hyl	6	5	5	8	17	6
Hyp	50	59	60	79	74	91
Ile	11	9	8	8	11	10
Leu	22	23	21	23	27	24
Lys	29	28	27	25	12	27
Met	17	15	13	9	10	4
Phe	16	15	14	13	10	14
Pro	106	114	115	119	89	132
Ser	64	49	41	35	43	35
Thr	25	22	20	24	26	18
Trp	0	0	0	0	0	0
Tyr	3	4	3	2	8	3
Val	18	19	18	15	37	26
Imino acid	156	173	175	198	163	223

Cod and hake are examples of cold-water fish, whereas megrim and tilapia are examples of warm-water fish. Giant squid is example of invertebrate

[a] Gómez-Guillén *et al.* (2002)

[b] Sarabia *et al.* (2000)

[c] Giménez *et al.* (2009)

[d] Eastoe and Leach (1977)

Generally, gelatin from fish skin shows a wider variety in amino acid compositions than those of mammalian gelatin. Fish gelatin has lower imino acid contents (proline and hydroxyproline) than mammalian gelatin (Grossman and Bergman, 1992). The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22–25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatin (cod) (Muyonga *et al.*, 2004b). Avena-Bustillos *et al.* (2006) reported that cold-water fish gelatins have significantly lower hydroxyproline, proline, valine, and leucine residues than mammalian gelatins, but possess more glycine, serine, threonine, aspartic acid, methionine, and histidine residues. However, both cold-water fish and mammalian gelatins have the same proportion of alanine, glutamic acid, cysteine, isoleucine, tyrosine, phenylalanine, homocysteine, hydroxylysine, lysine, and arginine residues (Avena-Bustillos *et al.*, 2006). Gelatin is not a nutritionally complete protein. It contains no tryptophan and is deficient in isoleucine, threonine, and methionine (Potter and Hotchkiss, 1998). Sulfur-containing amino acids, cysteine and cystine, are also deficient in gelatin. Water varies between 6 and 9% (Potter and Hotchkiss, 1998).

1.2.2.4 Gelatin structure

1.2.2.4.1 Primary structure

The primary structure of gelatin closely resembles the parent collagen. Small differences are due to raw material sources together with pretreatment and extraction procedures. These can be summarized as follows (Johnston-Banks, 1990):

1. Partial removal of amide groups of asparagines and glutamine, resulting in an increase in the contents of aspartic acid and glutamic acid. This increases the number of carboxyl groups in the gelatin molecule and thus lowers the isoelectric point. The degree of conversion is related to the severity of the pretreatment process.

2. Conversion of arginine to ornithine in more prolonged treatments experienced during long liming processes. This takes place by removal of a urea group from the arginine side-chain.

3. There is a tendency for trace amino acids, such as cysteine, tyrosine, isoleucine, serine, etc., to be found in lower proportions than in their parent collagens.

This is due to the inevitable removal of some telopeptide during cross-link cleavage, which is then lost in the pretreatment solutions.

1.2.2.4.2 Secondary structure

Gelatin is not completely polydispersed, but has a definite molecular weight distribution pattern corresponding to the α-chain and its oligomers (Table 6). One to eight oligomers may be detected in solution, but it is possible that higher numbers exist. Doublets, known as β-chains, are formed from both α1- and α2-chains, giving rise to β11- and β12-molecules (Johnston-Banks, 1990). Oligomers of three α-chains will mainly exist as intact triple helix, but a certain proportion will exist as extended α-polymers bonded randomly by end-to-end or side-to-side bonds (Johnston-Banks, 1990). The structure of oligomers of greater than four α-chain units obviously becomes increasingly more complex. Molecular-weight spectra normally relate with physical properties of gelatin (de Wolf, 2003; Karim and Bhat, 2009).

Table 6. Molecular weight distribution showing the major structural components of gelatin

Molecular fraction	Description
Q	Very high molecular weights, of 15-20 x 106 daltons and thought to be branched in character owing to their inability to penetrate the gel successfully.
1-4	Oligomers of α-chains, levels of five to eight.
X	Oligomers of four α-chains.
γ	285,000 daltons, i.e. 3 x α-chain.
β	190,000 daltons, i.e. 2 x α-chain.
α	95,000 daltons.
A-peptide	86,000 daltons.
α-, β- and γ- peptides	Seen as tailing their parent peaks.

Source: Johnston-Banks (1990)

Differences can be detected between commercial gelatin from the different raw materials. In general, the sum of the α- and β-fractions, together with their larger peptides, is proportional to the bloom strength, and the percentage of higher molecular weight material is related with the viscosity (Karim and Bhat, 2009). The setting time is increased for the peptide fractions below α-chain, but a certain proportion of the very high molecular weight "Q" fraction can reduce the setting time

markedly (Johnston-Banks, 1990). The melting point also increases with higher molecular weight content (Cho *et al.*, 2004; Karim and Bhat, 2009).

1.2.2.5 Functional properties of gelatin

The functional properties of gelatin are related to their chemical characteristics. The gel strength, viscosity, setting behavior and melting point of gelatin depend on their molecular weight distribution and the amino acid composition (Karim and Bhat, 2009).

1.2.2.5.1 Gelation

Gelatin is categorized as a physical gel and the interactions or bonds between the chains that make up the material are physical in nature (van der Waal's interactions and hydrogen bonds) (Karim and Bhat, 2009). Gel strength and gel melting point are the major physical properties of gelatin gels. These are governed by amino acid composition, as well as by molecular weight and the ratio of α/β-chains present in the gelatin (Cho *et al.*, 2004). It is generally recognized that the imino acids, proline and hydroxyproline, are important in gelation (Kittiphattanabawon *et al.*, 2005). Since super-helix structure of the gelatin is stabilized by hydrogen bonds, gelatin with high levels of imino acids tends to have higher gel strength and melting point. This results from the presence of pyrrolidine rings of the imino acids in addition to the hydrogen bonds formed between amino acid residues (Te Nijenhuis, 1997). In addition, the molecular weight distribution is also important in determining the gelling behavior of gelatin. According to Johnston-Banks (1990), the sum of intact α and β fractions together with their peptides is proportional to the gel strength, while the viscosity, setting rate and melting point increase with increasing in the amount of the high molecular weight (greater than γ) fraction. While the amino acid composition is mainly dependent on the source or species (Muyonga *et al.*, 2004a), the molecular weight distribution of gelatin depends to a large extent on the extraction process (Zhou *et al.*, 2006). During conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds and therefore the higher the proportion of peptides with molecular weight less than α are generated. Kittiphattanabawon *et al.* (2010b) reported that the decreased bloom strength and increased setting time of gels of gelatin extracted from bamboo

shark and blacktip shark associated with the degradation of α-, β- and γ-chain during heating in gelatin extraction. Older animal collagen is more cross-linked and a m ore severe process is required to denature it to form gelatin (Muyonga *et al.*, 2004a). There are differences in the extent and type of cross-linking found in bones and skins (Kittiphattanabawon *et al.*, 2005). This may also affect solubilization and transformation of collagen to gelatin may result in differences in properties between gelatins extracted from both sources.

1.2.2.5.2 Emulsifying and foaming properties

Gelatin, and to some extent also collagen, is used as a foaming, emulsifying, and wetting agent in food, pharmaceutical, medical, and technical applications due to its surface active properties. Gelatin is surface active and it is capable of acting as an emulsifier in oil-in-water emulsions (Lobo, 2002). The hydrophobic areas on the peptide chain are responsible for giving gelatin its emulsifying and foaming properties (Cole, 2000; Galazka *et al.*, 1999). The versatility of the emulsifying and foaming properties of gelatin is particularly valued in products like emulsified powders (Kläui *et al.*, 1970). In such products, its surface active and film-forming characteristics can be successfully exploited during the emulsification process. Its stabilization and gelation characteristics are useful during the subsequent drying and encapsulation stages. In marshmallows, the gel-forming properties of gelatins are used to stabilize the foam upon cooling. In most applications, gelatin is chosen not only for its surface active properties, but rather because of its unique combination of surface active, chemical, rheological, and gelling properties. In gelatin-foamed foods and ice creams, the unique gel melting behavior in the range of 10–30°C results in the melting of gelatin gels in the mouth (de Wolf, 2003). Surh *et al.* (2006) reported that fish gelatin at protein concentrations ≥4.0 wt%, both low molecular weight fish gelatin [LMW-FG] and high-molecular weight fish gelatin [HMW-FG], could yield emulsions with monomodal particle size distributions and small mean droplet diameters ($d_{43} \sim 0.35$ μm for LMW-FG and 0.71 μm for HMW-FG). The number of large droplets and the amount of destabilized oil was less in the HMW-FG emulsions than in the LMW-FG emulsions. Thickness of an adsorbed gelatin membrane, which increases with increasing molecular weight, is associated with emulsifying properties. Emulsion stabilized by low- and high-molecular weight

fish gelatins were fairly stable when subjected to high salt concentrations (250 mM sodium chloride), thermal treatments (30 and 90°C for 30 min), and different pH values (pH 3–8). However, gelatin is generally a weaker emulsifier than other surface active substances such as globular proteins and gum arabic. Therefore, it was either hydrophobically modified by the attachment of nonpolar side-groups (Toledano and Magdassi, 1998), or used in conjunction with anionic surfactants to improve its effectiveness as an emulsifier (Surh *et al.*, 2005).

1.2.2.5.3 Film-forming properties

Films from fish gelatin and their characterization have been carried out. It has been observed that all fish gelatins exhibited excellent film-forming properties (Avena-Bustillos *et al.*, 2006; Gómez-Guillén *et al.*, 2007; Jongjareonrak *et al.*, 2006a; Jongjareonrak *et al.*, 2006b). The properties of film, especially mechanical property, were governed by peptide chain length. Hoque *et al.* (2011) reported that gelatin from cuttlefish skin had the lower tensile strength and elongation at breake when it was hydrolysed to obtain the degree of hydrolysis of 0.4%. Film could not be formed when gelatin with excessive hydrolysis was used as film forming material. In general, gelatin films from the skins of a warm-water fish species, such as the Nile perch, showed stress and elongation at break similar to that of bovine bone gelatin (Avena-Bustillos *et al.*, 2006). Fish gelatin film, however, exhibits lower water vapor permeability than bovine gelatin. Films from tuna skin gelatin plasticized with glycerol showed lower water vapor permeability (WVP) compared to values reported for pigskin gelatin (Muyonga *et al.*, 2004b). The lower WVP values (compared to those from bovine or porcine) reported for films based on fish gelatins from several species can be explained in terms of the amino acid composition. Fish gelatins are known to have much higher hydrophobicity due to lower proline and hydroxyproline contents, as the hydroxyl group of hydroxyproline is normally available to form hydrogen bonds with water (Gómez-Guillén *et al.*, 2007). Gelatin films prepared from cold-water fish and warm-water fish also show different WVPs. WVP of cold-water fish gelatin films was significantly lower than warm-water fish, and this was attributed to increased hydrophobicity associated with the lower amounts of proline and hydroxyproline in cold-water fish gelatins. This result suggested that the lower

WVP of fish gelatin films can be particularly useful for applications related to lower water loss from encapsulated drugs and from refrigerated or frozen food systems.

1.2.2.5.4 Sensory properties

Choi and Regenstein (2000) studied the physicochemical differences between pork and fish gelatin and the effect of melting point on the sensory characteristics of a gelatin–water gel. Quantitative descriptive analysis (QDA) was performed to determine the effect of the melting point on the sensory characteristics of gelatin gels. Flavored fish gelatin dessert gel product had less undesirable off-flavors and off-odors, with more desirable release of flavor and aroma than the same product produced with pork gelatin. The lower melting temperature of fish gelatin seems to assist in the release of fruit aroma, fruit flavor, and sweetness. In contrast, since pork gelatin melts more slowly than fish gelatin in the mouth, the perceived viscosity of pork gelatin might be expected to be higher than that of the fish gelatin under the same conditions (Karim and Bhat, 2009).

1.2.3 Food emulsion and the role of proteins

An emulsion can be defined as a thermodynamically unstable system consisting of one liquid, in the form of small spherical droplets, dispersed in another immiscible liquid (Dickinson, 2010). The individual droplets are considered as the dispersed phase, and the surrounding liquid as the continuous phase (Pomeranz, 1991). For most food emulsions, the diameters of the droplets range from 0.1 to 100 microns (Walstra, 1996). Oil-in-water or O/W emulsions consist of oil droplets dispersed in an aqueous phase, while water-in-oil or W/O emulsions are opposite. The thermodynamic instability of these emulsions is due to the fact that the contact between oil and water molecules, which are different in polarity, is thermodynamically unfavorable (Israelachvili, 1992). However, a kinetically stable emulsion can be formed by adding an emulsifier and/or a thickening agent before homogenization (McClements, 1999). Emulsifiers that have both polar and non-polar groups on their molecules can absorb to the interface forming a protective membrane to prevent droplet aggregation, whereas thickening agents can increase the viscosity of the continuous phase to retard droplet movement (McClements, 1999). A wide variety of synthetic and natural emulsifiers

are legally acceptable for utilization in food emulsions, including small molecule surfactants, phospholipids and biopolymers (Stauffe, 1999).

Proteins extracted from a variety of natural sources are surface active molecules that can be used as emulsifiers in foods because of their ability to facilitate the formation, improve the stability and produce desirable physicochemical properties in oil-in-water emulsions, e.g. soy, whey, casein, fish, meat and plant proteins (Dickinson, 2003; McClements, 2004). Proteins adsorb to the surfaces of freshly formed oil droplets created by homogenization of oil-water-protein mixtures, where they facilitate further droplet disruption by lowering the interfacial tension and retard droplet coalescence by forming protective membranes around the droplets via noncovalent interactions (Walstra, 2003) and the sulfhydryl-disulfide interchange reaction (Damodaran, 1997). These interactions, apart from making the protein irreversibly adsorbed to the interface, provide a highly viscoelastic film that resists coalescence (Dickinson and Lopez, 2001). The ability of proteins to generate repulsive interactions (e.g., steric and electrostatic) between oil droplets and to form an interfacial membrane that is resistant to rupture also plays an important role in stabilizing the droplets against flocculation and coalescence during long-term storage (Tcholakova *et al.*, 2003).

1.2.3.1 Molecular factors affecting emulsifying property

1.2.3.1.1 Solubility

Solubility of proteins plays a role in their emulsifying properties; however, 100% solubility is not an absolute requirement. While highly insoluble proteins do not perform well as emulsifiers, no reliable relationship exists between solubility and emulsifying properties in the 25 – 80% solubility range (Liao and Mangino, 1987). However, since highly insoluble proteins may precipitate at the oil-in-water interface, which may promote coalescence, some degree of solubility may be necessary in order to form a stable elastic film at the interface (Damodaran, 1997).

1.2.3.1.2 Surface hydrophobicity

The emulsifying properties of proteins show a positive correlation with surface hydrophobicity (Kato and Nakai, 1980), but not with mean hydrophobicity of amino acid residues. Proteins having many hydrophobic patches or cavities on their surface are potentially unstable at the oil-in-water interface.

Although proteins are unstable and undergo conformational change at the oil-in-water interface, they are not extensively denatured as it occurs at the air/water interface. The interfacial free energy at the oil-in-water interface is considerably lower than that at the air/water interface; this lower interfacial energy is probably insufficient to overcome the activation energy barrier for complete unfolding of proteins at the oil-in-water interface (Damodaran, 1997). Apart from hydrophobicity, molecular flexibility is another important factor affecting the emulsifying properties of proteins (Damodaran, 1997).

1.2.3.1.3 Heat denaturation

Controlled heat denaturation of proteins prior to emulsification, which does not result in insolubilization, can improve the emulsifying properties of proteins (Dickinson and Hong, 1994; Kato *et al.*, 1983). This is probably due to an increase of surface hydrophobicity and flexibility of heat-treated proteins. Matsumura *et al.* (1994) showed that α-lactalbumin in the molten globule state unfolded more readily than the native protein at interfaces. Moreover, in competitive adsorption experiments involving native β-lactoglobulin and the molten globule state α-lactalbumin, the latter preferentially adsorbed to the oil-in-water interface than the former (Damodaran, 1997). Excessive heat treatment that leads to aggregation and/or insolubilization of proteins impairs their emulsifying properties (Damodaran, 1997).

1.2.3.1.4 Competitive adsorption

Typical food proteins are mixtures of several proteins. Thus, competitive adsorption among the proteins is expected, and hence the composition of the interfacial film and its stability will be dependent on the initial ratio of protein components in the bulk phase (Damodaran, 1997). Whey protein exhibited selective adsorption at the oil-in-water interface (Shimizu *et al.*, 1981). This selectivity was affected by the pH. At pH 3, 48% of the proteins in the film at the oil-in-water interface was α-lactalbumin, which decreased to about 10% at pH 9; in contrast, the surface composition of β-lactoglobulin increased from 13% at pH 3 to about 62% at pH 9. In the case of caseinate-stabilized emulsions, the composition of α_{s1}- and β-caseins at the oil-in-water interface immediate after formation of the emulsion

was almost the same as that of the initial bulk solution; however, during aging, the ratio of the β-casein to α_{s1}-casein in the adsorbed film increased, presumably because of displacement of α_{s1}-casein by β-casein (Dickinson *et al.*, 1988). Because α_{s1}-casein and β-casein are similar proteins and thermodynamically compatible, they can displace each other from the oil-in-water interface (Dickinson *et al.*, 1988). However, gelatin-caseinate mixtures showed different phenomenon. For gelatin to caseinate weight ratio of less than 2:1, the interfacial film of freshly formed emulsion contained only caseinate (Castle *et al.*, 1986). On the other hand, in the case of gelatin-β-lactoglobulin mixtures, a significant amount of gelatin was able to adsorb to the oil-in-water interface even at high ratios of β-lactalbumin to gelatin in the bulk phase. This must be related to differences in thermodynamic compatibility of the mixture of gelatin with β-casein and β-lactoglobulin at the interface (Damodaran, 1997).

1.2.3.1.5 Low molecular weight surfactants

Typical food emulsifiers include low molecular weight surfactants, such as phospholipids and mono- and diglycerides. Competitive adsorption between proteins and these low molecular weight surfactants can influence the stability of food emulsions (Damodaran, 1997). Because low molecular weight surfactants are more surface active than the macromolecular protein surfactants, they effectively compete for adsorption at interfaces. When added to protein-stabilized emulsions, they displace proteins from the interface (Pugnaloni *et al.*, 2004). The displacement of the protein from the interface decreases interparticle electrostatic and steric repulsions and reduces the thickness of the protein layer and the viscoelastic properties of the protein film. These adverse changes induce instability and rapid coalescence of emulsion particles. Notably, the low molecular weight surfactants affect the stability but not the droplet size (Van Der Meeren *et al.*, 2005). The time of addition of the low molecular weight surfactant also has an effect on the stability of the emulsion. Generally, when a low molecular weight surfactant is added prior to emulsification, only partial displacement of the protein occurs. When it is added after emulsification, the complete displacement occurs, depending on the amount added (Damodaran, 1997).

1.2.3.1.6 Conformation of adsorbed protein

Conformational changes in proteins at interfaces occur at different degrees, since the conformation of a protein is the manifestation of its interaction within itself and with the environment. Any change in a protein's environment must cause conformational change. This must involve a net decrease in its free energy compared to that in the bulk phase. Protein configuration at the interface is usually depicted in terms of "trains," "loops," and "tails" (Figure 7) (Damodaran, 1997). The trains are the hydrophobic segments that lie flat on the interface, making contact with both the aqueous and oil phases (only the nonpolar side chains of amino acids are oriented to-wards the oil phase); the loops are the polypeptide segments between the trains that are suspended into the aqueous continuous phase; and the tails are the N- and C-terminal segments invariably suspended into the aqueous phase. The relative distribution of trains, loops, and tails in the adsorbed molecule determines the properties of emulsions. If trains are the predominant configuration, the protein should be highly flexible and highly hydrophobic (e.g., β-casein). The stability of the emulsions of such proteins, however, may be no better than those that form more loops, e.g., globular proteins. The presence of loops extended into the continuous phase will exert steric repulsion against flocculation and coalescence (Damodaran, 1997).

The relative amounts of trains and loops in an adsorbed protein depends at least on two factors: the rate of adsorption and the rate of unfolding at the interface. If the rate of adsorption is faster than the rate of unfolding of the protein at the interface, as it is at high protein concentration, then because of the limited availability of unoccupied area at the interface, the protein may only partially unfold or only a limited number of trains may form; a majority of the polypeptide may assume the loop configuration. Thus, in a typical emulsion formed with 3-5% protein solution, a major fraction of the protein at the interface may be in the loop configuration (Damodaran, 1997).

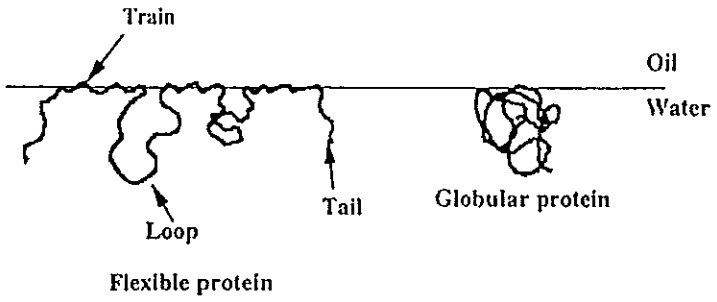


Figure 7. Various protein configurations at liquid interfaces
Source: Douillard (1994)

1.2.4 Lipid oxidation

Oxidation of lipids is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Lipid oxidation is mainly associated with the rejection by consumer due to the off-odor and off-flavor. The direct reaction of a lipid molecule with a molecule of oxygen, termed autoxidation, is a free-radical chain reaction and mainly involves in food deterioration. The mechanism of autoxidation can be distinguished in three distinct steps: initiation, propagation and termination (Jadhav *et al.*, 1996).

1.2.4.1 Initiation

The autoxidation of fat is thought to be initiated with the formation of free radicals. Initiation reactions take place either by the abstraction of hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond. The rearrangement of the double bonds results in the formation of conjugated diene (-CH=CH-CH=CH-), showing a characteristic UV absorption at 232-234 nm (Nakayama *et al.*, 1994).

The formation of lipid radical (R•) is usually mediated by trace metals, irradiation, light or heat [1]. Also, lipid hydroperoxide, which exists in trace quantities prior to the oxidation, breaks down to yield radicals as shown by Eqs. [2] and [3]. Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids (Jadhav *et al.*, 1996).

$$RH + \text{initiator} \rightarrow R^{\bullet} + H^{\bullet} \quad [1]$$

$$ROOH \rightarrow RO^{\bullet} + HO^{\bullet} \quad [2]$$

$$2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \quad [3]$$

1.2.4.2 Propagation

In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation process occurs by chain reactions that consume oxygen and yields new free-radical species (peroxy radicals, $ROO^{\bullet}$) or by the formation of peroxides (ROOH) as in Eqs. [4] and [5] (Jadhav *et al.*, 1996). The product $R^{\bullet}$ and $ROO^{\bullet}$ can further propagate free-radical reactions.

$$R^{\bullet} + {}^3O_2 \rightarrow ROO^{\bullet} \quad [4]$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad [5]$$

Lipid peroxy radicals ($ROO^{\bullet}$) initiate a chain reaction with other molecules, resulting in the formation of lipid hydropeoxides and lipid free radicals. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available. Lipid hydroperoxides may also be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase. Lipid hydroperoxide, the primary products of autoxidation, are odorless and tasteless (Jadhav *et al.*, 1996).

1.2.4.3 Termination

A free radical is any atom with unpaired electron in the outermost shell. Free radicals are electrically neutral, and salvation effects are generally very small. Owing to the bonding-deficiency and structural unstability, radicals therefore tend to react whenever possible to restore normal bonding. When there is a reduction in the amount of unsaturated fatty acids present, radicals bond to one another, forming a stable nonradical compounds [6-8]. Thus the termination reactions lead to

interruption of the repeating sequence of propagating steps of the chain reaction (Jadhav *et al.*, 1996).

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R \quad [6]$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \quad [7]$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \quad [8]$$

1.2.5 Antioxidants

Antioxidant in food is defined as any substance which is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due to oxidation (Gordon, 2001). In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are functional at very low concentrations, 0.01% or less (Rajalakshmi and Narasimhan, 1996). The use of antioxidants in food products is controlled by laws and regulations of the country or by international standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as generally recognized as safe (GRAS) substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee for Food Additives (JECFA) and the European Community's Scientific Committee for Food (SCF).

1.2.5.1 Classification of food antioxidants

1.2.5.1.1 Primary antioxidants

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also interact with the lipid radicals, forming lipid-antioxidant complexes. Many of the naturally occurring phenolic compounds like flavonoids, eugenol, vanilin and rosemary antioxidant also have chain-breaking properties (Rajalakshmi and Narasimhan, 1996). Protein hydrolysate from many plant and animal sources also possess the primary antioxidative activity (McCarthy *et al.*, 2001; Sakanaka *et al.*, 2004). Primary antioxidants are effective at very low concentrations but at higher levels they may become prooxidants (Rajalakshmi and Narasimhan, 1996).

1.2.5.1.2 Secondary antioxidants

Secondary or preventive antioxidants such as thiopropionic acid and dilauryl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996). Other polyvalent acids such as tartaric, malic, gluconic, oxalic, succinic and dehydroglutaric acids, as well as sodium triphosphate and pyrophosphate also show the synergistic properties similar to those of citric acid (Yanishlieva and Marinova, 2001). Flavonoids and related compounds and amino acids function as both primary antioxidants and synergists. Nitrites and nitrates, which are used mainly in meat curing, probably function as antioxidants by converting heme proteins to inactive nitric oxide forms and by chelating the metal ions, especially non-heme iron, copper and cobalt that are present in meat (Rajalakshmi and Narasimhan, 1996). β-carotene and related carotenoids are effective quenchers of singlet oxygen and also prevent the formation of hydroperoxides.

1.2.5.2 Mode of action of antioxidants in food

1.2.5.2.1 Radical scavenger

Antioxidants can retard or inhibit lipid oxidation by inactivating or scavenging free radicals, thus inhibiting initiation and propagation reactions. Free radical scavengers or chain-breaking antioxidants are capable of accepting a radical from oxidizing lipids species such as peroxyl (ROO•) and alkoxyl (RO•) radicals to form stable end products (Decker, 1998). Antioxidants can scavenge free radical either as hydrogen donors [9-11] or as electron donors [12-13] (Namiki, 1990; Osawa, 1994).

Hydrogen donor

$ROO^{\bullet} + AH$	$\rightarrow$	$ROOH + A^{\bullet}$	[9]
$RO^{\bullet} + AH$	$\rightarrow$	$ROH + A^{\bullet}$	[10]
$R^{\bullet} + AH$	$\rightarrow$	$RH + A^{\bullet}$	[11]

Electron donor

$ROO^{\bullet} + A^{\bullet}$	$\rightarrow$	$(AH\text{-}ROO)^{\bullet}$	[12]
$(AH\text{-}ROO)^{\bullet} + ROO^{\bullet}$	$\rightarrow$	Stable product	[13]

The free antioxidant radicals (A$^{\bullet}$) may undergo additional reactions that remove radical from the system. Termination reactions of antioxidant with other free antioxidant radicals or lipid radicals can form nonradical species [15-16].

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \quad [14]$$

$$RO^{\bullet} + A^{\bullet} \rightarrow ROA \quad [15]$$

$$A^{\bullet} + A^{\bullet} \rightarrow AA \quad [16]$$

Antioxidants may act as hydrogen donors to the phenoxy radicals, which are stabilized by resonance delocalization of the unpaired electron in the aromatic ring and are further stabilized by bulky group at the ortho position as shown in Figure 8 (Shahidi and Wanasundara, 1992).

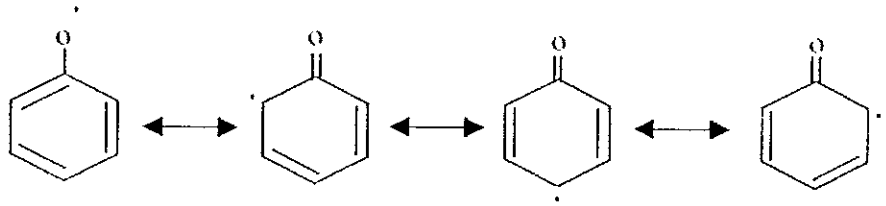


Figure 8. Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals

Source: Shahidi and Wanasundara (1992)

1.2.5.2.2 Peroxide decomposer

Peroxide decomposers are secondary or preventative antioxidants which react with hydroperoxides and prevent their decomposition into free radicals for initiation of new chain reaction (Yanishlieva and Marinova, 2001). Some phenols, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Namiki, 1990).

1.2.5.2.3 Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of converting triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in

the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Thus, the singlet oxygen can react with a lipid molecule to yield a hydroperoxide. Singlet oxygen reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleates (Jadhav *et al.*, 1996). Lipid oxidation initiated by xanthine oxidase can be inhibited by β-carotene because of its ability to quence singlet oxygen (Namiki, 1990; Rajalakshmi and Narasimhan, 1996). The maillard reaction derived from xylose-lysine, tryptophan-glucose and glucose-glycine model systems had a high scavenging effect on active oxygen (Yen *et al.*, 2002; Yoshimura *et al.*, 1997).

1.2.5.2.4 Lipoxygenase inhibitor

Lipoxygenase is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas *et al.*, 1999). Free-radical intermediates occur during lipoxygenase catalysis, and these can lead to cooxidation of easily oxidized compounds, e.g. carotenoids and polyphenols (Rajalakshmi and Narasimhan, 1996).

1.2.5.2.5 Synergists

1.2.5.2.5.1 Chelating agents

Chelating agents are not antioxidants, however, they play a valuable role in stabilizing foods. Chelating agents that improve the shelf-life of lipid containing food are EDTA, citric acid and phosphoric acid derivatives (Jadhav *et al.*, 1996). Chelating agents form stable complexes with prooxidant metals such as iron and copper. Chelating agents bind metal ions and forms sigma bonds with a metal. It is considered as an effective secondary antioxidant because of the stabilized oxidation form of the metal ion. An unshared pair electron in their molecule structure promotes the chelating action (Jadhav *et al.*, 1996; Nakayama *et al.*, 1994; Rajalakshmi and Narasimhan, 1996). The Maillard reaction prepared from glucose-glysine, glucoselysine and fructose-lysine exhibited metal chelating property (Yoshimura *et al.*, 1997).

1.2.5.2.5.2 Reducing agents or oxygen scavengers

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system (Rajalakshmi and Narasimhan, 1996). Ascorbic acid is a strong reducing agent, readily losing H+ to become dehydroascorbic acid, which also has vitamin C activity. However, vitamin C activity is lost, when the lactone ring of dehydroascorbic acid is hydrolyzed to yield diketogluconic acid (Gordon, 2001). Moreover, ascorbic acid can act as a synergist with tocopherols by regenerating or restoring their antioxidant properties (Niki, 1987). Ascorbic acid and its derivatives also function as oxygen scavengers (Yanishlieva and Marinova, 2001).

1.2.6 Antioxidative activity and the application of plant phenolics

Natural antioxidants are primarily plant polyphenolic compounds that may occur in all parts of the plant (Shahidi and Wanasundara, 1992). Common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acid (Pratt and Hudson, 1990). Phenol compounds are included in the category of radical scavenger (Shahidi and Wanasundara, 1992). The antioxidant properties of phenolics are mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al.*, 1997). These phenolic compounds, during lipid oxidation, act in various ways, such as binding metal ions, scavenging radicals or decomposition of peroxides (Moure *et al.*, 2006).

Their antioxidant effectiveness depends on the stability in different systems, as well as number and location of hydroxyl groups. Shahidi and Wanasundara (1992) and Abdille *et al.* (2005) reported that phenolic compounds with ortho- and para-dihydroxylation or with a hydroxyl and a methoxy group are more effective than simple phenolics. Furthermore, the antioxidative activity generally increased with increasing phenolic compound content (Abdille *et al.*, 2005; Shahidi and Wanasundara, 1992). In many *in vitro* studies, phenolic compounds demonstrated higher antioxidant activity than antioxidant vitamins and carotenoids (Re *et al.*, 1999; Vinson *et al.*, 1995).

Plant phenolic can be used as the safe natural antioxidants in different food systems. Jayaprakasha *et al.* (2001) reported the antioxidative activity of grape seed extracts, which prevented the peroxidation of linoleic acid in model system. Many classes of phenolic compounds in barley showed strong antioxidant activities (Liu and Yao, 2007). Yu *et al.* (2002) demonstrated that extracts from the hard winter wheat (*Triticum aestivum)* variety Trego was able to suppress the oxidation of fish oils, and its capacity was comparable to tocopherol. Phenolic compounds from extra virgin olive oil (EVOO) at 400 ppm showed comparable antioxidant effect as 100 ppm 1:1 mixture of BHT:BHA during thermal oxidation of tuna lipids at 40 and 100°C (Medina *et al.*, 1999). The use of phenolic compounds from plant sources as natural antioxidants in a number of edible oils, such as corn, cottonseed, fish, olive, peanut, rapeseed, soybean, and sunflower oils was reported (Yanishlieva and Marinova, 2001). Goli *et al.* (2005) reported that pistachio hull extracts at a concentration of 0.06% (w/w) was as effective as BHA and BHT at 0.02% in inhibiting oxidation of soybean oil at 60°C. Methanolic extracts of wild rice hulls were shown to inhibit lipid oxidation in ground beef as measured by the thiobarbituric acid reactive substances (TBARS) (Asamarai *et al.*, 1996). Tea catechins were found to be more efficient than α-tocopherol (both applied at 300 mg/kg level) in inhibiting minced muscle lipid oxidation in fresh meats, poultry and fish (Tang *et al.*, 2001). Huang and Frankel (1997) reported the antioxidative efficiency of isolated catechins from green tea leaves. The extracts included (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin gallate (EGCg). The activity of catechins in model systems was on the order of EC<ECg<EGC<EGCg. At similar molar concentrations, the activity of these compounds was superior to those of BHA and α-tocopherol in lard (Namiki, 1990).

Additionally, Lau and King (2003) reported that the addition of grape seed extract to dark poultry meat patties at 1.0% and 2.0% effectively inhibited the development of TBARS. Treated samples had 10-fold lower TBARS values compared to untreated control. The addition of 2% orange fiber to bologna sausages significantly retarded the development of TBARS over 28-days of storage at 4°C when compared to untreated sausages (Fernández-López *et al.*, 2004). Pazos *et al.*

(2005) reported that phenolics from grape pomace were as effective as propyl gallate in fish oil-in-water emulsions.

1.2.7 Protein interactions with phenolic compound

Polyphenols are widespread in the plant kingdom and they can interact with proteins, leading to the inhibition of the enzymes, decrease of the protein digestibility, and the induction of protein precipitation. Such an interaction can also affect protein functionality (Rawel *et al.*, 2001; Sarker *et al.*, 1995). Polyphenols can interact non-covalently or covalently with proteins. Non-covalent interactions including hydrogen bonds and hydrophobic forces stabilize tannin-protein complexes (Chen and Hagerman, 2004). The non-covalent interaction may occur between polyphenols and many different functional groups of proteins by hydrogen bonding and by hydrophobic bonding; in some cases, ionic bonding may be possible. Covalent attachment can occur depending on the polarity of the polyphenol (Kroll *et al.*, 2003; Prigent *et al.*, 2008; Rawel *et al.*, 2002a; Siebert, 2006). The interaction may occur via multisite interaction (several phenolic compounds bound to one protein molecule) or multidentate interaction (one phenolic compound bound to several protein sites or molecules). The type of interaction depends on the type and the molar ratio of both phenolic compound and protein (Kroll *et al.*, 2003; Prigent *et al.*, 2008). Tannins can interact with proteins non-specifically such as bovine serum albumin (BSA) and tannins, or specifically such as gelatin interaction with tannins (Frazier *et al.*, 2003). The interaction may occur co-operatively which means that tannins already bound to protein have an effect on the new binding interaction (Frazier *et al.*, 2003).

In the situation in which the number of polyphenol ends equals the number of protein binding sites, the largest network can be produced (Siebert *et al.*, 1996). The complexation between polyphenols and proteins can be reversible or irreversible, leading to soluble and insoluble complexes (Luck *et al.*, 1994). In general, the increase in polyphenol concentration favors the formation of the polyphenol-protein insoluble aggregates (Mateus *et al.*, 2004). Interaction will occur if the protein has a number of binding sites for tannins and protein-tannin complex formation can be enhanced (Sarker *et al.*, 1995).

1.2.7.1 Non-covalent interaction between proteins and phenolic compounds

1.2.7.1.1 Hydrogen bonding

During protein-polyphenol interaction, a strong hydrogen bond is formed between the carbonyl groups of the amino acids or peptide backbone and the isolated phenolic hydroxyl group in the polyphenol (De Freitas and Mateus, 2001; Hagerman *et al.*, 1998). Hydrogen bond is important in the complex formation (Luck *et al.*, 1994). The hydrogen bond between phenolic hydroxyl group and the peptide carbonyl groups of the protein are in aqueous media solvated with hydrogen-bonded water molecules (Kroll *et al.*, 2003). Therefore, these solvent bonds have to be broken before interaction can take place.

Interaction between polyphenol and protein is affected by the shape of polyphenol, projection of phenolic hydroxyl groups, addition of galloyl groups, and position of peripheral groups imposed by the stereochemistry of the pyranic ring of the polyphenol molecule (Kroll *et al.*, 2003; Rawel *et al.*, 2001). In addition, the degree of the polymerization and esterification may affect the binding affinities (Bacon and Rhodes, 1998). Phenolic hydroxyl groups on both A- and B-rings in the polyphenol molecule play an important role in the protein-phenol complex formation (Kawamoto *et al.*, 1996; Simon *et al.*, 2003). Phenolic hydroxyl groups that are located close to other hydrogen-bonding groups (1,2-dihydroxy and 1,2,3- trihydroxy groups) generally form hydrogen bonds with themselves, and this reduces their capacity to form external hydrogen bonds with proteins. The binding affinity parallels with the number of 1,2-dihydroxy rings in the polyphenol molecule (Charlton *et al.*, 2002).

The stability of the polyphenol-protein complexes are governed by many groups present in the amino acids (Prigent *et al.*, 2003; Vergé *et al.*, 2002). Frazier *et al.* (2003) concluded that the structure and the flexibility of the polyphenols do not appear to have any effect on the binding mechanism. On the contrary, the structure of protein has an effect on binding mechanism. Proline and arginine residues in proteins can form hydrogen bonds. Arginine has a long flexible hydrophobic side chain with the hydrogen-bond donating guanido group. The enhanced ability of the proline-rich proteins to interact with the phenolic compounds is related to their

flexible secondary structure, the greater extent of hydrogen bonding due to the increased accessibility of the peptide bond, and also to the fact that the carbonyl group of tertiary amides is a better hydrogen bond acceptor than the carbonyl group of primary or secondary amides (Luck *et al.*, 1994; Murray *et al.*, 1994; O'Connell and Fox, 2001).

In general, the polyphenol-protein associations are thought to involve in cross-linking of protein molecules by the polyphenol through hydrogen bonding. Phenolic compounds act as a polydentate ligand on the protein surface involving hydrogen bonding as well as hydrophobic effects. The aromatic groups of polyphenols are supposed to be involved in a face-to-face stacking with amino acid residues of linear proteins, whereas the interaction with globular proteins, such as α-lactalbumin and BSA, probably involves only surface exposed residues (Carvalho *et al.*, 2004). The interaction between proteins and phenols are usually more effective in pure aqueous solutions than in organic solutions (Hagerman *et al.*, 1998). In solution, the complex is formed through electrostatic forces (for example phenylalanine residues), hydrophobic stacking reinforced by hydrogen bonding (proline rings, arginine) (Murray *et al.*, 1994).

Largest particles are formed when the number of polyphenol ends equals the number of protein binding sites (Figure 9) (Siebert *et al.*, 1996). In this figure, polyphenols are depicted to have two ends that can bind protein, while proteins have a fixed number of polyphenol binding sites. In the presence of excess of protein relative to polyphenol, each polyphenol molecule should be able to interact through hydrogen bonding with two protein molecules, but these proteins would not interact with each other, resulting in protein dimmers and smaller aggregates. With excess polyphenol relative to protein, all of the protein interaction sites would be occupied and free polyphenol end would have a small possibility to find a free interaction site on a protein molecule, resulting in small aggregates. The tendency for polyphenols to cross-link protein molecules is supposed to be higher at low polyphenol concentration (De Freitas and Mateus, 2001).

Larger polyphenol molecules bind more tightly to the proteins due to their increased amount of phenolic hydroxyl groups, compared to the smaller polyphenol molecules (De Freitas and Mateus, 2001; Sarni-Manchado *et al.*, 1999).

However, the smaller polyphenols can bind to proteins but they cannot cross-link them to generate larger complexes. In larger polyphenols, there are more phenolic groups (especially ortho groups) and aryl rings able to bind and precipitate proteins without any major conformational restriction (Baxter *et al.*, 1997; De Freitas and Mateus, 2001).

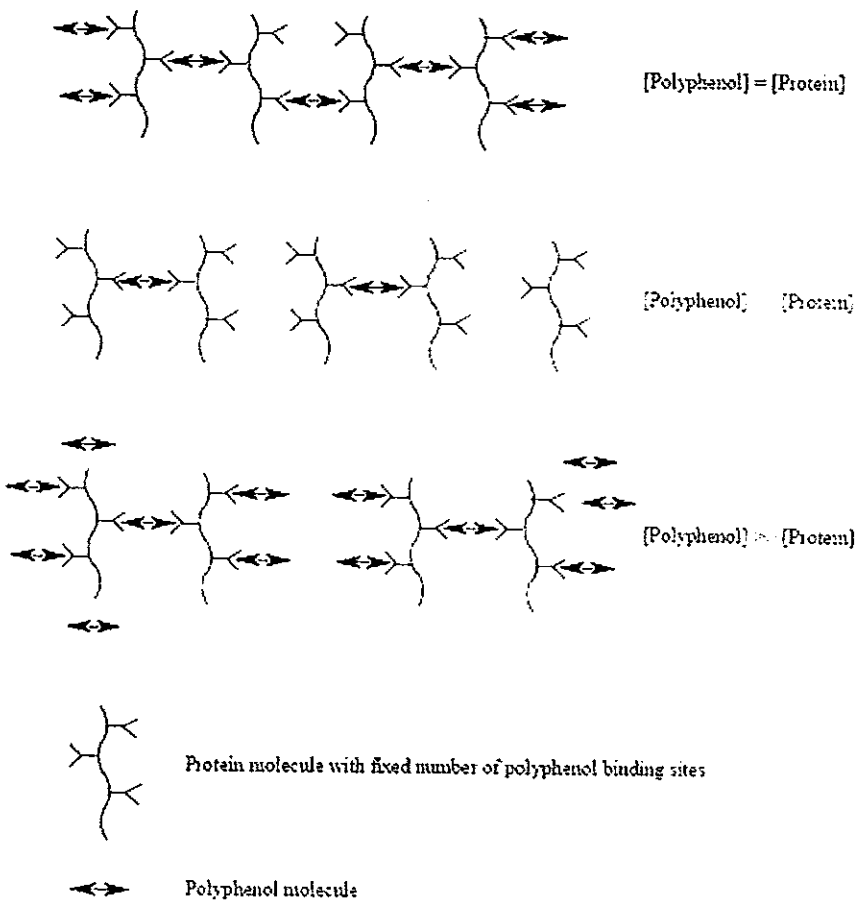


Figure 9. Mechanism of the polyphenol-protein interaction
Source: Siebert *et al.* (1996)

Interaction between proteins and polyphenols can be influenced by various factors, such as the solution conditions (solvent composition, ionic strength, and pH), temperature, and time (De Freitas and Mateus, 2001; Kawamoto and Nakatsubo, 1997; Prigent *et al.*, 2003; Ricardo Da Silva *et al.*, 1991). Protein structure has an influence on the formation of complexes with polyphenols through hydrogen

bonding (De Freitas and Mateus, 2001; Hagerman *et al.*, 1998; Naczk *et al.*, 2001; Siebert *et al.*, 1996). The protein-polyphenol complex formation is usually strongest just below the isoelectric point of proteins where the protein-protein electrostatic repulsion is minimized (Hagerman and Butler, 1981).

1.2.7.1.2 Hydrophobic interactions

The protein-polyphenol complexes are also stabilized by hydrophobic interactions. In hydrophobic bonding, the aliphatic (basic) and aromatic side chains (hydrophobic regions) of amino acids can interact with the aromatic nuclei of the polyphenol and the complexes can be formed in which proline residues play a key role (Baxter *et al.*, 1997; Hagerman *et al.*, 1998; Lu and Bennick, 1998; Wróblewski *et al.*, 2001). The proline residues (pyrrolidine rings) are good hydrophobic binding sites since they provide an open, flat, rigid, hydrophobic surface favorable for association with other flat, hydrophobic functions, such as aromatic rings in the polyphenols (Charlton *et al.*, 2002; Luck *et al.*, 1994; Murray *et al.*, 1994). The hydrophobic interaction usually occurs between the least sterically hindered amino acids and phenolic rings available for intermolecular interaction (Murray *et al.*, 1994; Oh *et al.*, 1980). Besides proline residues, histidine, arginine, phenylalanine, tryptophan, lysine, cysteine, and methionine can participate in the interaction reaction (Baxter *et al.*, 1997; Charlton *et al.*, 2002; Suryaprakash *et al.*, 2000). In histidine residues, imidazole ring interacts with aromatic components of polyphenols. Polyphenols interaction with tryptophan is most likely due to the partial positive charge present at the –NH group of the indole moiety (Suryaprakash *et al.*, 2000). Lysine, as well as arginine, has hydrophobic section in the side chain which may take part in the interaction. In addition, lysine has positive charge near neutral pH leading to interaction with the carboxylate group or the aromatic ring of the polyphenol (Suryaprakash *et al.*, 2000).

The binding capacity depends not only on the molecular size of the polyphenol but also on the number and the stereospecificity of the separate sites on the polyphenol molecule, which is able to associate with proteins. Polyphenols can bind more than one location at the same time, which may strengthen the interaction between the polyphenol and the protein (Charlton *et al.*, 2002). The polyphenol should present sufficient size and adequate composition to be able to bind

simultaneously more than one site of the protein surface, acting as a polydentate ligand. The regular increase in the number of aromatic and pyranic rings with the molecular weight of polyphenol (such as procyanidin) provides a multiplicity of sites of potentially hydrophobic nature to participate in interactions, presumably stabilized by hydrogen bonds from the *o*-dihydroxyphenol group (De Freitas and Mateus, 2001). Phenolic rings A and D are important for face-to-face stacking with proline residues (Charlton *et al.*, 2002).

Precipitation of the protein-polyphenol complexes is likely to occur via interactions between exposed phenolic rings on one complex, and proline or phenolic rings on the other (Charlton *et al.*, 2002). The interaction phenomenon takes place in the beginning of reactions, leading to the formation of stable complexes (Ricardo Da Silva *et al.*, 1991). Figure 10 shows the mechanism of the protein-polyphenol interaction with the different protein and polyphenol concentration through hydrophobic interactions (Charlton *et al.*, 2002; De Freitas and Mateus, 2001; Siebert *et al.*, 1996).

Initially added polyphenols bind to the protein, and soluble complexes are formed. In general, several polyphenol molecules can bind to the same protein. When more polyphenol is added, until the second stage, where there is enough polyphenol bound to the protein to act as a linker between two protein molecules, reaches. The protein then forms a polyphenol-coated dimer, which start to precipitate. In the final stage, the complex aggregates into either small particles or large particles. Larger protein molecules tend to bind polyphenol more tightly because increasing length of protein allows it to fold and "wrap around" the polyphenol molecule, form intramolecular interactions, and interact at several places at the same time (Charlton *et al.*, 2002). Usually the number of amino acids blocked in the protein by phenolic compounds is higher than the amount of phenolic compound bound (Rawel *et al.*, 2002a). Therefore, some protein groups must be involved in intra- and intermolecular cross-linking through hydrophobic interactions (De Freitas and Mateus, 2001; Kroll *et al.*, 2003; Rawel *et al.*, 2002a). Charlton *et al.* (2002) concluded that polyphenols bind through hydrophobic interactions reversibly and relatively weakly at each individual binding site.

Procyanidins tend to have poor affinity for small, tightly folded globular proteins (myoglobin, lysozyme, BSA), whereas they bind preferentially to proteins that have either random-coil or collagen-like helical conformations (De Freitas and Mateus, 2001; Hagerman and Butler, 1981). In addition, it may also be due to the fact that globular proteins lack proline (at least on their surface).

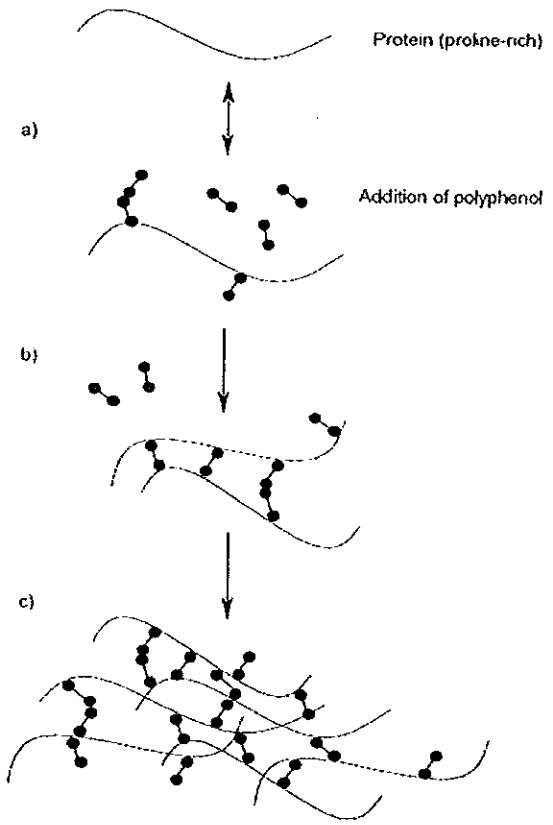


Figure 10. Protein-polyphenol interaction leading to the complex formation and precipitation: a) reversible hydrophobic interaction between protein and polyphenol, giving a soluble complex; b) on addition of more polyphenol, two peptides are cross-linked (at least two polyphenol/protein interactions) and the complex becomes insoluble; c) further aggregation (phase separation) of the insoluble complexes occurs

Source: Charlton *et al.* (2002)

1.2.7.2 Covalent interaction between proteins and phenolic compounds

The covalent binding of the phenolic compounds to the proteins occurs through oxidized phenolic substances (quinones). These quinones can be formed enzymatically or non-enzymatically.

1.2.7.2.1 Enzymatic oxidation

Quinones can be formed via the action of two types of enzymes: polyphenol oxidases and peroxidases. Quinones are produced by peroxidases via the formation of radicals. Peroxidases require the presence of hydrogen peroxide (Sroka and Cisowski, 2003), which thus makes their role in foods limited, compared to the action of polyphenol oxidases (Kroll *et al.*, 2003).

Polyphenol oxidases (EC 1.14.18.1) are divided into catechol oxidases and laccases (Mayer and Staples, 2002). Both enzymes can oxidize phenolic substrates using molecular oxygen (Mayer, 1986). Catechol oxidases can catalyze the oxidation of *o*-diphenols to *o*-quinones using their catecholase activity (Mayer, 1986). Furthermore, when they also possess the so-called cresolase activity, catechol oxidases may convert monophenols to *o*-diphenols (Mayer, 1986; Rodríguez-López *et al.*, 2001) (Figure 11). Laccases are able to oxidize a broader range of substrates than catechol oxidases, including *p*-diphenols (Mayer and Staples, 2002) and non phenolic compounds e.g. phosphorothiolates (Amitai *et al.*, 1998). They are also able to catalyze other reactions than oxidation, such as demethylation and (de-)polymerization of phenolic compounds (Mayer, 1986).

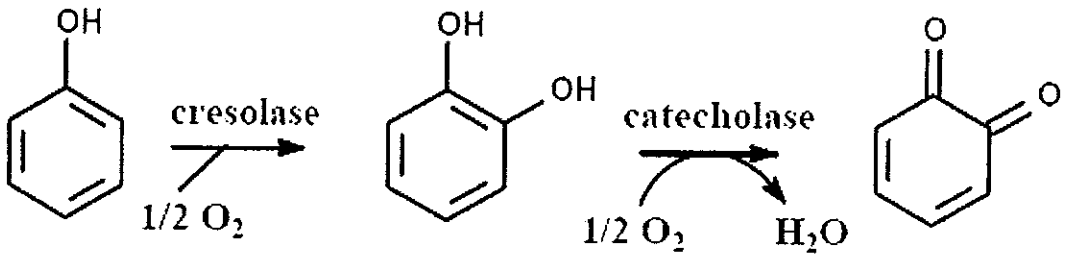


Figure 11. Cresolase and catecholase mechanisms of polyphenol oxidase

Source: Matheis and Whitaker (1984)

Catechol oxidase is usually called tyrosinase in mammals and mushrooms. Tyrosinases from mammals are relatively specific for tyrosine and DOPA (dihydroxyphenylalanine), whereas catechol oxidases from fungi and higher plants show activity on a wider range of mono- and *o*-diphenols (Mayer and Staples, 2002).

1.2.7.2.2 Non-enzymatic oxidation

The formation of quinones may also occur in the absence of an enzyme. Increasing the pH induces the deprotonation of the phenolic hydroxyl group, eventually leading to the formation of quinones (Yabuta *et al.*, 2001). This method is used in food industry e.g. to produce black olives (García *et al.*, 1996).

Quinones can bind covalently to the proteins at nucleophilic functional groups, such as lysine, methionine, histidine, cysteine, tyrosine, and tryptophan residues, limiting the digestibility of the protein molecule (Carbonaro *et al.*, 1996; Hurrell *et al.*, 1982; Rawel *et al.*, 2001). The formation of the quinone-protein complexes, as well as formation of the other polyphenol-protein complexes, can lead to polymerization (Kroll *et al.*, 2003). Quinones are electrophilic and can form cross-links with either sulfhydryl or amino groups of proteins (O'Connell and Fox, 2001). The protein-quinone interaction can change the isoelectric point of protein to the lower pH values due to the introduction of carboxylic groups following the covalent attachment of the phenolic acids, and by the parallel blocking of the lysine residues in protein (Rawel *et al.*, 2001; Rawel *et al.*, 2002b).

The reactivity of the phenolic compounds with the tryptophan residues has been shown to increase with the higher number of hydroxyl groups, and the reactivity depends also on the position of the hydroxyl groups (Rawel *et al.*, 2001; Rawel *et al.*, 2002b). The reaction between tryptophan residues in the protein and the hydroxyl substituents on the B- and C rings (positions 3, 3', 5') of the quinones occur through the enol-keto tautomerization across the 2-3 bond as shown in Figure 12 (Rawel *et al.*, 2001; Rohn *et al.*, 2005).

In addition, the quinones can react very efficiently with lysine residues in proteins (Figure 13) (Hurrell *et al.*, 1982; Rawel *et al.*, 2001). Phenol-protein complexes formed under oxidizing conditions are more stable than those formed in the absence of oxidants (Chen and Hagerman, 2004). In addition, quinones can

oxidize the functional groups of the proteins (Kroll *et al.*, 2003) and semiquinone radical is also able to react with proteins (Hagerman *et al.*, 2003).

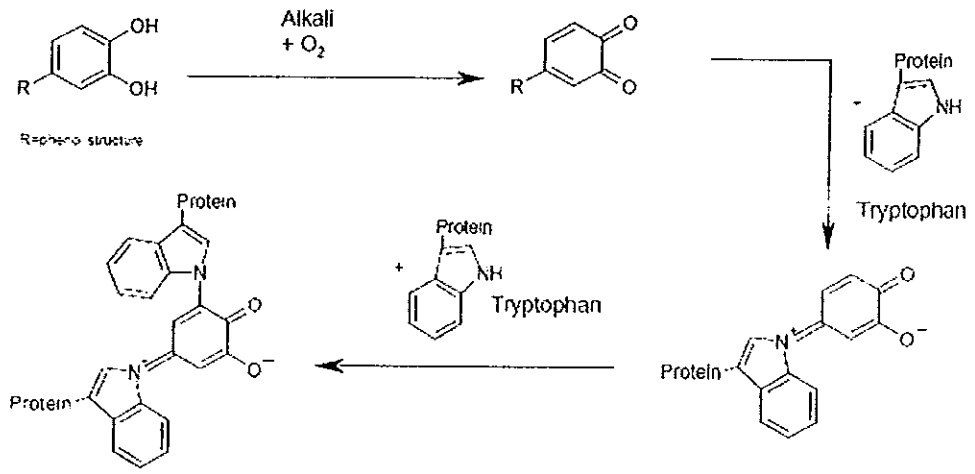


Figure 12. Interaction reaction between tryptophan residues of the protein and quinone form of polyphenol, leading to polymerization, complex formation

Source: Rawel *et al.* (2001)

The covalent interaction between oxidized phenolic compounds and amino acid residues of the proteins allows the formed complex to act as antioxidants as long as there are free phenolic hydroxyl groups in one of the phenolic rings (Almajano and Gordon, 2004; Arts *et al.*, 2002; Rohn *et al.*, 2004). However, the antioxidant activity of the quinone-protein complexes is less than the antioxidant activity of free phenolic compounds (Rohn *et al.*, 2004). The degree of masking of antioxidant capacity depends on both the type of polyphenol and the type of protein (Arts *et al.*, 2002). The cross-linking and polymerization is partly responsible for the loss of the antioxidative ability of covalently bound phenolic compounds. With an increasing amount of proteins present, the reactive sites of phenolic compound are more involved in the protein-phenolic-protein reactions (Rohn *et al.*, 2004). Additionally, steric hindrance could also be responsible for the decreased activity.

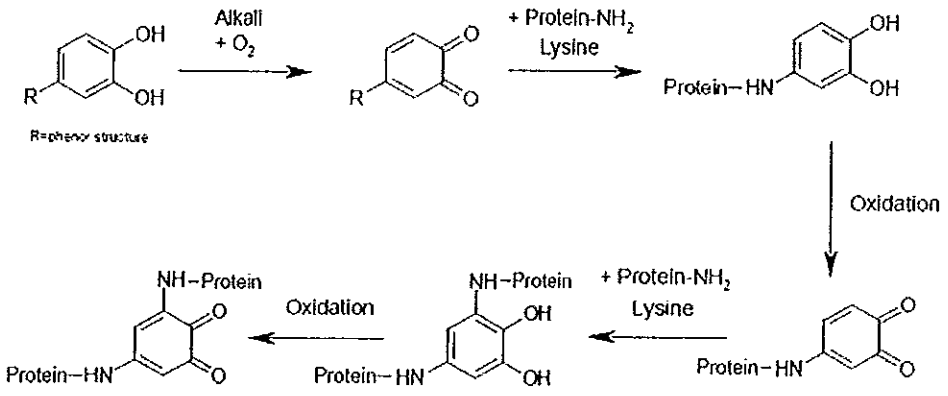


Figure 13. Interaction reaction between lysine side chain of protein and quinone form of polyphenol, leading to polymerization, complex formation

Source: Rawel *et al.* (2001)

1.2.7.3 Effect of the protein-polyphenol interaction on protein functional properties

Interactions of proteins with phenolic compounds lead to the changes in the proteins solubility (increasing the content of denatured proteins) and hydrophilic/hydrophobic nature. That can affect protein functional properties such as emulsion formation and gelling properties (Kroll *et al.*, 2003; Rawel *et al.*, 2001; Rubino *et al.*, 1996). The phenolic compounds can either increase or decrease proteins hydrophobicity, depending on the participating amino acids, which can furthermore promote a greater or lower affinity of the protein for an oil phase (Neucere *et al.*, 1978; Prigent *et al.*, 2008; Rawel *et al.*, 2001; Rawel *et al.*, 2002b). The influence of phenolic compounds on the hydrophobicity of the proteins depends on phenolic compound used as well as on the protein (Kroll *et al.*, 2003; Rawel *et al.*, 2002a; Rawel *et al.*, 2002b). Due to cross-linking and complex formation, the conformation of protein is changed and can furthermore increase the exposure of some additional hydrophilic regions previously buried (Kroll *et al.*, 2003; Rawel *et al.*, 2002a; Rawel *et al.*, 2002b). Moreover, the covalent blocking of the hydrophobic residues, such as tryptophan, the introduction of hydrophilic carboxyl residues (phenolic acids), and the increasing number of hydroxyl groups in the phenols bound can increase hydrophilicity (Rawel *et al.*, 2002a; Rawel *et al.*, 2002b). On the contrary, the

decrease in solubility and the increase in hydrophobicity is due to blocking of hydrophilic amino groups of protein (such as lysine) (Kroll *et al.*, 2003).

The interaction between proteins and phenolic compounds can also influence the antioxidant activity of phenolic compounds. Protein-phenolic complex may inhibit protein oxidation by binding to the proteins or the formed complexes may act as radical scavengers and radical sinks (Hagerman and Butler, 1981; Rohn *et al.*, 2004; Siebert *et al.*, 1996).

1.2.8 Protein interactions with lipid

Proteins and lipids constitute as the major food components. Their individual contributions to the functional and nutritional properties of foods are well established (Damodaran, 1997). Protein–lipid interactions are also known to be responsible for the desired biological structures in living cells and tissues. Naturally occurring protein–lipid complexes serve a wide variety of functions in cellular membranes and in the transport and metabolism of lipids (Shimizu *et al.*, 1986). Some examples of protein-lipid complexes of animal origin are found in the cell membranes and lipoproteins of plasma, milk fat globule membrane and egg yolk (Meng *et al.*, 2006). Examples of plant origin include some fruits, vegetables, wheat grains, peanuts and oil bodies of oil seeds such as soybean, sunflower seed, and rapeseed (Meng *et al.*, 2006). In food technology, the interactions between proteins and lipids occur during the processing and storage of food products such as cheeses, milk creams, mayonnaise, doughs, bakery products and several meat products (Pokorny *et al.*, 1993). These interactions lead to formation of 'induced' protein-lipid complexes. Interaction between protein and lipid affect the quality of food, but they also have an impact on the charge and conformation of the protein three-dimensional structure (exposure of hydrophobic groups, changes in secondary structure and disulfide groups), loss of enzyme activity, and changes in the nutritive value (loss of essential amino acids) (Howell, 1991). In addition, the modified proteins will have different functional properties from those of their unmodified molecules; their emulsifying, foaming, gelling, and water binding properties may be affected as well as the texture of food will be changed (Levine *et al.*, 1996).

Improvements of functional and physicochemical properties of proteins via their interaction with lipids have been reported. In the processing of ice cream, lipids interact with caseins to form protein-lipid complexes, whereas in butter making, fat globules concentrate at an air-water interface and interact with proteins to form complexes at the interface (Evans, 1986). The presence of protein-lipid complexes in dough and the bread gluten network, in which the polar ends of lipids are bound to gliadin by hydrophilic interactions and the nonpolar ends are bound to glutenin by hydrophobic interactions; cements the gluten network and contributes to the structure of the gas-retaining complexes that are essential for good gas retention, adequate loaf volume, and satisfactory bread structure (Chobert and Haertle, 1997). Soy films and soymilk are examples of complexes of lipids with the major soybean globular proteins, the 7S and 11S globulins. Microscopic examination of soy films revealed a structure consisting of a continuous protein matrix in which lipid droplets are dispersed (Beckwith, 1984). In addition, the interaction between lipid and protein could improve the surface activity of ovalbumin as evidenced by the smaller oil droplets size in oil-in-water emulsion (Mine, 2002).

1.2.8.1 Type of protein-lipid interaction

The interaction between protein and lipid was involved via non covalent interaction (hydrogen bonding, electrostatic, hydrophobic and van der Waals force) and covalent interaction. The interactions may vary depending on pH, ionic strength and temperature. Moreover, more than one type of bonding may be involved in the interactions in the same protein–lipid complex. The structural linkages between lipids and proteins in naturally occurring protein–lipid complexes cannot be readily dissociated by simple manipulations of pH, ionic strength, and ultracentrifugal force fields (Alzagtat and Alli, 2002; Kamat *et al.*, 1978).

1.2.8.1.1 Non-covalent interaction between proteins and lipid

1.2.8.1.1.1 Dispersion interaction (van der Waals force)

Dispersion interactions are non-specific short-range forces resulting from interactions between induced dipoles and adjacent atoms or molecules, and therefore are related to the polarizability of the atoms or molecules. The interactions are very weak individually but collectively may be very important in stabilizing domains of protein, and are considered to be quite different from hydrophobic

interactions (Kinsella, 1984). These forces are particularly sensitive to intermolecular distances, and are important in the interaction of non-polar groups; the CH_2 groups of protein side-chains can attract CH_2 groups present in phospholipids or fatty acids to form protein–lipid complexes (Howell, 1991). Van der Waals dispersion force also exists between the non-polar ends of both lipids and proteins in protein–lipid complexes such as in Danielli's model for cell membrane structure (Pomeranz, 1973); the polar ends of the lipids are bound to the proteins by either electrostatic or hydrogen bonds.

1.2.8.1.1.2 Electrostatic bonding

Electrostatic bonding takes place between (a) positively charged groups (e.g. choline) of a phospholipid and the negatively charged group (e.g. aspartyl, glutamyl) of a protein, and/or (b) a negatively charged phosphate group of a lipid (e.g. phospholipid) and a positively charged group (e.g. lysyl or guanidyl, amyl) of a protein (Ericsson, 1990). In addition, electrostatic forces or dipole interactions (Howell, 1991) occur between uncharged but polar molecules; in these molecules electrons are distributed in such a way that there is an excess of negative charge in one area and an excess of positive charge in another, such as with the interaction of phospholipid with proteins in the cell membranes (Karel, 1973). Electrostatic bonds are also involved in dough and bread making, between polar amino acids residue of protein and polar lipids (Lasztity, 1996). In protein–lipid complexes such as between whey protein with phospholipids, the electrostatic bonding is affected by pH (Cornell, 1991); at acidic pH, whey protein carry significant positive charges which can bind to the negatively charged of phospholipids, while at neutral pH, where the phospholipids and the protein carry a net negative charge, there is electrostatic repulsion and a reduced tendency to interact.

1.2.8.1.1.3 Hydrogen bonding

Hydrogen bonding occurs between the hydrogen of the hydroxyl groups of fatty acids, di and monoglyceride, or the head groups of phospholipid such as phosphatidylamine and phosphatidylserine and the carbonyl groups of proteins (Howell, 1991). The hydrogen bonding between these groups is much stronger than the interaction between water and the non-polar groups. Hydrogen bonding is particularly important in the structural organization of protein–lipid complexes, such

as the interactions of proteins with phospholipids in milk fat globules membrane (Aynié *et al.*, 1992; McWilliams, 1989).

1.2.8.1.1.4 Hydrophobic interaction

Hydrophobic interactions are endothermic and entropy-driven and their strength increases with temperature. This effect of temperature on hydrophobic interactions, suggests its importance in the thermostability of proteins (Damodaran, 1997). Hydrophobic interactions between protein and oil in aqueous system alter protein structure by decreasing the protein intramolecular hydrophobic bonds and explain partial protein unfolding at the oil–water interface of the system (Alzagtat and Alli, 2002). When proteins unfold, they expose reactive amino acids that are capable of forming hydrophobic and disulfide bonds with their neighbours, thus generating a highly viscoelastic membrane (McClements, 1999). Hydrophobic interactions between protein and lipids are believed to be important in protein aggregation and are considered to be critical in stabilizing induced protein–lipid complexes; for example, in bread making, lipids interact through hydrophobic bonds with glutenin, and through hydrophilic bonds with gliadin (Finney, 1971).

1.2.8.1.2 Covalent interaction between proteins and lipid

The covalent bond is important in the interaction between protein and lipid, in which the protein-lipid complex with more stability in both biological system and food products was formed (Pokorny *et al.*, 1993). The covalent interaction between lipid and protein can be induced by using the oxidative lipid substrate or preparing by chemical reagent.

1.2.8.1.2.1 Covalent interaction of protein with oxidized lipid

Oxidation of polyunsaturated fatty acid leads to the formation of radicals, lipid hydroperoxides, and secondary lipid oxidation product such as aldehydes and ketones, which could covalently modify side chains and polypeptide backbone of protein molecule, resulting in cross-linking, fragmentation, aggregation, and conformational changes of the resulting protein (Refsgaard *et al.*, 2000). Alkyl and peroxyl radical decomposed from hydroperoxide can directly interact with side chains of proteins (Kato *et al.*, 1992). The secondary lipid oxidation products, such as aldehydes, react mainly with amino acids via condensation reaction to form Schiff's bases or by Michael addition reactions (Liu *et al.*, 2003; Stadtman and Berlett, 1997).

1.2.8.1.2.1.1 The primary lipid oxidation product

Free radicals produced by cleavage of the primary oxidation products, mainly hydroperoxides, can modify proteins by inducing protein free radicals and the protein oxidation. Oxidation reactions have an impact on the charge and conformation of the protein three-dimensional structure, loss of enzyme activity, and changes in the nutritive value (loss of essential amino acids) (Gerrard *et al.*, 2002; Howell *et al.*, 2001). Oxidation of the proteins and amino acids is affected by many environmental factors such as pH, temperature, water activity, and the presence of catalysts or inhibitors. In addition, the three-dimensional structures of each protein affect the interaction with lipids (Aynié *et al.*, 1992). Casein with disordered random-coil and flexible structure tends to cross-link in the presence of oxidized lipids more readily than the globular and more compact whey proteins (Sharma *et al.*, 2002).

The most sensitive amino acids toward oxidation are heterocyclic amino acids. In addition, amino and phenolic groups of amino acids are susceptible to oxidation (Viljanen *et al.*, 2004). Tryptophan, histidine, and proline, lysine, cysteine, methionine, and tyrosine are prone to oxidation where the hydrogen atom is abstracted either from OH-, S-or N-containing groups (Alaiz *et al.*, 1995; Doorn and Petersen, 2002) (Figure 14). Interaction of proteins with oxidized lipid may result in a loss of these amino acids. Oxidative cleavage of the peptide main chain and the oxidation of the side chains of lysine, proline, arginine, and threonine yields carbonyl derivatives (Stadtman and Berlett, 1997).

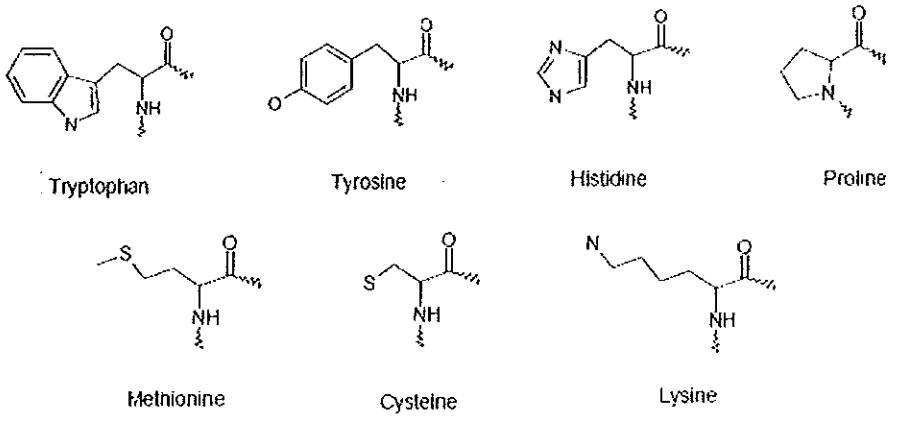


Figure 14. Structure of amino acid residues prone to oxidation

Resource: Doorn and Petersen (2002)

Protein oxidation occurs via free-radical reactions in which peroxyl radicals (ROO·) formed during lipid oxidation can abstract hydrogen atoms from protein molecules (PH) [17]. Consequently, protein radicals are formed (P·) and they can in turn create a protein net (P-P) due to the cross-linking [18].

$$ROO\cdot + PH \rightarrow P\cdot + ROOH \quad [17]$$

$$P\cdot + P\cdot \rightarrow P\text{-}P \text{ (protein net)} \quad [18]$$

It is also postulated that the protein oxidation process can occur via non-covalent complex formation by both electrostatic and hydrophobic attractions between lipid hydroperoxide (ROOH) or secondary lipid oxidation products (mainly aldehydes and ketones, breakdown products of lipid hydroperoxides), and the nitrogen or sulfur centers of reactive amino acid residues of the protein (PH) [19] (Aubourg, 1999; Kikugawa *et al.*, 1991).

$$ROOH + PH \rightarrow [ROOH\text{---}HP] \rightarrow RO\cdot + P\cdot + H_2O \quad [19]$$

Moreover, covalent bonds between lipid and protein moieties are also produced. These may recombine via the formation of protein copolymers, with various lipid free radicals to form protein–lipid complexes, for example, in the oxidation of meat and fish during storage (Pokorny *et al.*, 1993). Lipid free radical with unpaired electron could complex with amino acid of protein via covalent interaction to form the stable produce. The imine bonds formed between a lipid carbonyl group and an amine group of the protein moiety (primarily lysine) are typical examples of covalent bond formation (Figure 15).

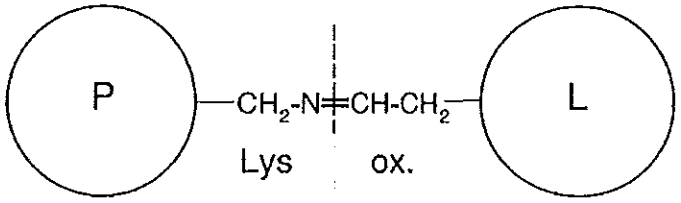


Figure 15. Example of protein-lipid covalent bonds. L = lipid phase; Lys = residue of bound lysine; ox. = residue of a carbonyl chain of oxidized lipid; P = protein

Resource: Pokorny and Kolakowska (2003)

1.2.8.1.2.1.2 The secondary lipid oxidation product

Secondary lipid oxidation product, especially reactive aldehydes including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and 4-hydroxyhexenal (HHE), react mainly with amino acids via condensation reaction to form Schiff's bases and by Michael addition reactions (Stadtman and Berlett, 1997) (Figure 16). Reactive aldehydes show high reactivity with the sulfhydryl group of cysteine, the imidazol group of histidine and the ε-amino group of lysine and subsequently alter structure and functional properties of protein (Esterbauer *et al.*, 1991).

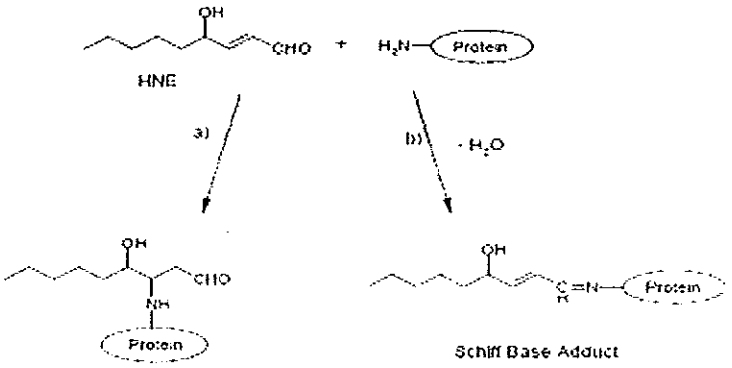


Figure 16. Interaction reaction between the secondary lipid oxidation product and amino acid: a) Michael addition to cysteine, lysine, and histidine side chains; b) Schiff' base formation followed by displacement of HNE (4-hydroxy-2-noneal) from protein

Source: Bruenner *et al.* (1995)

Especially lysine residues (contains free amino group) in the proteins can interact with aldehydes and lead to Schiff base formation which is typical product for non-enzymatic browning (Gerrard *et al.*, 2002). MDA reacts with lysine residues of proteins to form stable carbonyl derivatives (Burcham and Kuhan, 1996). The α, β-unsaturated aldehydes, such as HNE can undergo Michael addition type reactions with side chain of lysine, cysteine and histidine (Esterbauer *et al.*, 1991).

The Schiff bases or Michael adducts formed might subsequently be involved in inter- and inter-molecular cross-linking with amino acid residues such as lysine, cysteine, or histidine residues (Liu *et al.*, 2003; Stadtman and Berlett, 1997) (Figure 17). The formed cross-links can further stabilize the absorbed proteins. First the Schiff base is formed between lysine and aldehyde, and then Michael addition of side-chain nucleophiles (cysteine and histidine) will occur.

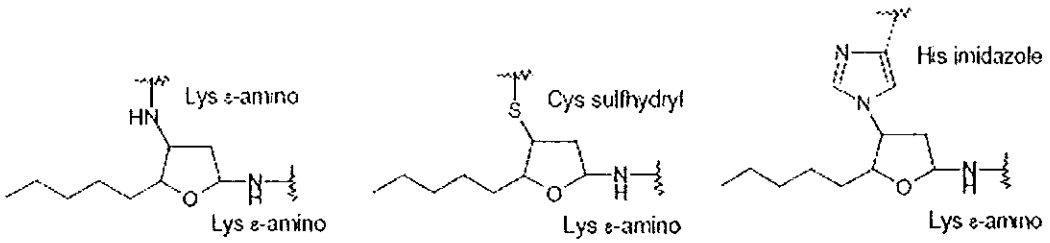


Figure 17. Formation of cross-links between different amino acid residues
Soruce: Nadkarni and Sayre (1995)

1.2.8.1.2.2 Covalent attachment of free fatty acid to protein by *N*-hydroxysuccinimide

Covalent interaction between protein and free fatty acid can be performed by using *N*-hydroxysuccinimide (Magdassi *et al.*, 1996). Generally, *N*-hydroxysuccinimide (NHS) is used as an activating reagent for carboxylic acid. Fatty acid binding with *N*-hydroxysuccinimide ester (activated acid) can react with free amino group of protein via amide bond, while *N*-hydroxysuccinimide was removed (Figure 18). Incoporation of fatty acid, which is natural hydrophobic domain, can increase surface hydrophobicity of modified gelatin. Lin and Chen (2006) reported that attachment of hydrophobic group to bovine gelatin caused an increase in surface

activity of resulting gelatin. Toledano and Magdassi (1998) showed that bovine gelatin modified with *N*-hydroxysuccinimide ester fatty acid had the higher foaming and emulsifying properties in comparison with native gelatin. The increase in surface hydrophobicity of modified gelatin was associated with the degree of modification and the chain length of fatty acid used (Toledano and Magdassi, 1998).

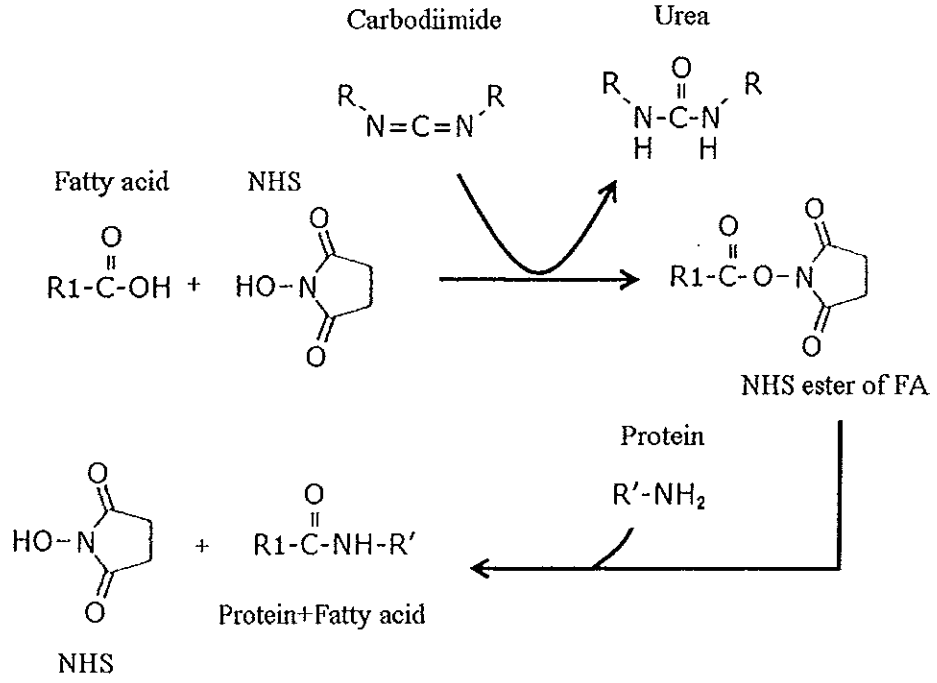


Figure 18. The synthesis of *N*-hydroxysuccinimide ester of fatty acid and its reaction with protein

Source: Lapidot *et al.* (1967)

1.2.8.2 Effect of the protein–lipid interaction on protein functional properties and quality of food

The functional characteristics and quality of protein are affected by the interactions between proteins and lipids. The common functional properties of proteins that are considered to contribute most to the characteristics of processed food include gelation, foaming, emulsification and solubility. Moreover, quality of protein including nutritional value and texture are also affected by protein-lipid interaction

1.2.8.2.1 Gelation

Protein gel networks are affected by the presence of lipids (Dickinson and Yamamoto, 1996; King *et al.*, 1984). Under some conditions, the interactions of lipids with proteins can improve gelling properties. However, lipids might affect the water holding capacity of gel proteins (Seal, 1980). Protein gels, in the presence of lipid-containing small fat globules with a narrow particle size distribution, have smooth texture and higher gel strength (Sikorski, 2001). Heat set protein gel in the presence of lipids has greater gel strength than protein gel in the absence of lipids; this behaviour is consistent with a reinforcement of the protein gel network by 'filler' particles of lipids in the matrix of the gel (van Vliet, 1988). Whey protein–lecithin gels showed increase gel strength which was attributed to the complexation between lecithin and whey protein; the layer of aqueous protein surrounding the droplets of lecithin act as an impermeable layer and modified the gel properties (Dickinson and Yamamoto, 1996). Farnum *et al.* (1976) reported that the interaction of soybean protein isolate and soybean oil could affect the properties of gels through the formation of protein matrix or structure network in which fat droplets are trapped.

1.2.8.2.2 Solubility

Interaction of protein with lipid more likely induced the decrease in solubility of protein (Liang, 1999). Polar group of lipid, such as phosphate group of phospholipid or hydroxyl group of mono-, diglyceride, could bind with polar amino acid residues of protein though hydrogen bonding, leading to the loss in water binding capacity of protein (Howell, 1991). In addition, the lipid oxidation products could contribute to the cross-linking or aggregation of protein, resulting in lowered protein solubility (Wu *et al.*, 2010).

1.2.8.2.3 Surface activity

Many proteins can act as surfactant, such as foaming agent and emulsifiers, by lowering the interfacial tension between the phases and forming cohesive interfacial films that surround air bubble or oil droplets (Phillips *et al.*, 1994). The surface activity of proteins depend on their solubility, hydrophilic/hydrophobic balance, molecular flexibility and the nature and proportion of lipid and other non-protein components in the system (Tornberg *et al.*, 1990). It has been shown that the surface activity of proteins closely depends on their surface

hydrophobicity (Kato *et al.*, 1983). Modification of protein with lipid affects the surface hydrophobicity of resulting gelatin. Wu *et al.* (2010) reported that soy protein modified by lipid peroxidation product acrolein had the decrease in surface hydrophobicity. Oxidized lipids might bind to hydrophobic amino acids of protein, resulting in the decrease in surface hydrophobicity of modified protein or protein aggregation (Liang, 1999), leading to the loss of surface activity. However, oxidative modification of protein could change the protein conformation via the exposure of hydrophobic groups. Due to protein partial unfolding, the surface hydrophobicity increases. Moreover, the attachment of oxidized lipid to protein may lead to the increase in its hydrophobicity, causing the changes in the surface activity, foaming ability and emulsifying properties of protein (Kato and Nakai, 1980; Toledano and Magdassi, 1997). Amarnath *et al.* (1998) reported that reactive aldehydes can form imines with lysine residues and cyclize to pyridinium rings, resulting in increases the hydrophobicity and opens the pathway for cross-linking. In animal origin, lipoproteins are capable of forming strong films around oil droplets, and some lipoproteins, including those of eggs, are excellent emulsifiers (Karel, 1973). Low density lipoprotein (LDL) has been shown to have high emulsifying activity and emulsifying stability (Mizutani and Nakamura, 1987); the lipid component around the apoprotein of LDL could provide hydrophobic environments that enhance the binding of the apoprotein to the oil droplet surface during emulsion formation.

1.2.8.2.4 Textural property

Fats and oils are added to food raw materials during culinary operations, among other reasons, to improve the flavor and texture of the food. The surface properties of lipoproteins formed, and even of the lipid films and droplets, assist in retaining gases during dough fermentation, thus improving the volume, softness, appearance, and acceptability of many bakery products (Chobert and Haertle, 1997). During chewing of high fat foods, fats are emulsified into a relatively thick fluid. Consumers perceive the viscosity as highly acceptable and appreciate the texture of such a product. On the contrary, the interaction products of oxidized lipids with food proteins are not easily moistened and emulsified, and result in deterioration of the texture after food ingestion. The functional properties of beef proteins are deteriorated by oxidized lipids (Farouk and Swan, 1998). The feeling of the chewed

morsel is rated lower because of the lower viscosity, which is due to a higher content of insoluble fractions. The content of salt-soluble proteins decreased proportionally to increasing lipid peroxidation in frozen fish (Sarma *et al.*, 1998).

1.2.8.2.5 Antioxidative acitivty

Modification of proteins by lipid oxidation products is not necessarily a negative consequence of oxidation since some oxidized lipid-amino acid reaction products have been shown to inhibit both lipid and protein oxidation (Alaiz *et al.*, 1996; Alaiz *et al.*, 1997; Zamora *et al.*, 1997). Complexes of oxidized lipids with proteins show moderate antioxidant activity, such as those with carnosine or histidine (Decker *et al.*, 2001). Products, such as pyrroles, derived from interaction between lipid oxidation products and proteins amino acids may also have antioxidant potential (Alaiz *et al.*, 1996; Hidalgo *et al.*, 2001; Hidalgo *et al.*, 2003; Zamora *et al.*, 1997). These modified proteins have been shown to act better inhibitors than their unmodified counterparts. Ahmad *et al.* (1996) reported that the antioxidant activity of the modified amino acids (by lipid oxidation products) is related to the polarity of the compounds. The antioxidant activity also depends on different substituents in the oxidation products. The size and the type of heterocyclic rings seem to be important factor. The antioxidant activity of heterocyclic compounds has been shown to increase with increasing size of rings, and with increasing number of hetero atoms present (Viljanen *et al.*, 2004). In addition, the polymerization increased the antioxidant activity, compared to monomers. The modified proteins, which are produced as a final step in the lipid oxidation process, are delaying the whole oxidative stress by competing effectively with other nucleophiles in reacting with reactive oxygen species produced. All proteins may exert antioxidant effect after reaction with lipid oxidation products, which might either increase their ability to sequester metals or generate new molecules that are antioxidants by themselves (Hidalgo *et al.*, 2001).

1.2.8.2.6 Nutritive value

Generally, natural protein-lipid complex are easily digestible because they are enzymatically hydrolyzed nearly completely before absorption in the intestines. Their nutritional value is equal to those of the respective lipids and proteins bound in protein-lipid complex, when consumed separately. However, the nutritional value of protein interacted with oxidized lipid is substantially lower than that of the

original proteins-lipid complex (Alzagtat and Alli, 2002). The main reason is the lower digestibility; most covalent bonds formed in the interactions are not attacked by proteases under the conditions of digestion. The ε-amino group of bound lysine is particularly sensitive to interactions with carbonylic oxidation products (Refsgaard *et al.*, 2000), and the resulting imine bonds substantially reduce the lysine availability. Moreover, the other amino acids, such as tyrosine, tryptophan, and methionine, are also partially converted into unavailable products (Refsgaard *et al.*, 2000).

1.3 Objectives

1. To study the extracting conditions, functional properties and characteristics of gelatin from cuttlefish skin.
2. To investigate the impact of different phenolic compounds on modification of cuttlefish skin gelatin and its emulsifying properties and antioxidative activity.
3. To determine the emulsifying properties and antioxidative activity of cuttlefish skin gelatin modified with tannic acid via non-covalent and covalent interaction and to elucidate its prevention of lipid oxidation in oil-in-water emulsion.
4. To investigate the surface activities and characteristics of cuttlefish skin gelatin modified with *N*-hydroxylsuccinimide ester of fatty acids.
5. To study the surface activities and characteristics of cuttlefish skin gelatin modified with oxidized linoleic acid.
6. To determine the antioxidative activity and emulsifying property of gelatin modified with oxidized linoleic acid and oxidized tannic acid, and to elucidate its prevention of lipid oxidation in oil-in-water emulsion.

CHAPTER 2

Functional properties of gelatin from cuttlefish skin as affected by bleaching using hydrogen peroxide

2.1 Abstract

Functional properties of gelatin from dorsal and ventral skin of cuttlefish with and without bleaching by H_2O_2 at different concentrations (2 and 5% (w/v)) for 24 and 48 h were studied. Gelatin from skin bleached with 5% H_2O_2 for 48 h showed the highest yield (49.65 and 72.88 % for dorsal and ventral skin, respectively). Bleaching not only improved the color of gelatin gel by increasing the L*-value and decreasing a*-value but also enhanced gel strength, emulsifying and foaming properties of resulting gelatin. Gelatin from bleached skin contained protein with molecular weight of 97 kDa and had an increased carbonyl content. Fourier transform infrared spectroscopic study showed higher intermolecular interactions and denaturation of gelatin from bleached skin than that of the control. These results indicated that hydrogen peroxide most likely induced the oxidation of gelatin, resulting in the formation of gelatin cross-links with the improved functional properties.

2.2 Introduction

Gelatin is a denatured form of collagen and can be used widely in food and pharmaceutical industries (Cho *et al.*, 2005). Gelatin is commercially made from skins and skeletons of bovine and porcine by alkaline or acidic extraction (Gilsenan and Ross-Murphy, 2000). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot/mouth diseases have led to the major concern of human health and thus by-products of mammalians are limited for production of collagen and gelatin as the functional food, cosmetic and pharmaceutical products

(Cho *et al.*, 2005). Additionally, porcine gelatin can be of objection from some religions. As a consequence, the increasing interest and attempt have been paid to other gelatin sources, especially fish skin and bone from seafood processing waste. So far, gelatin from skin from different fish species has been intensively studied (Gómez-Guillén *et al.*, 2002; Muyonga *et al.*, 2004a). However, gelatin from marine resources had the poorer gel strength, compared with mammalian gelatin, due to the lower imino acid content. Therefore, gel strength of fish gelatin has been improved by chemical modification (e.g. $MgSO_4$, glycerol) or enzyme modification (e.g. transglutaminase) (Fernández-Díaz *et al.*, 2001).

Cuttlefish has become one of important fishery products of Thailand, mainly exported to different countries all over the world. During processing, de-skinning is a common practice to obtain desirable products, resulting in the generation of skin as a byproduct. Skin has the low market value and used as animal feed. The extraction of gelatin from cuttlefish skin could increase its profitability. Nevertheless, the pigments in skin may pose the color problem and bleaching could be performed prior to gelatin extraction. Hydrogen peroxide is a potent oxidant that is widely used as bleaching agent in seafood processing (Kołodziejska *et al.*, 1999; Thanonkaew *et al.*, 2008). Kołodziejska *et al.* (1999) reported that soaking squid skin in 1% H_2O_2 in 0.01 M NaOH for 48 h could improve the color of resulting collagen. The decomposition of H_2O_2 in an aqueous solution occurs by dissociation and homolytic cleavage of O–H or O–O bonds with the formation of highly reactive products: hydroperoxyl anion (HOO^-), hydroperoxyl ($HOO^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals, which can react with many substances including chromatophores (Perkins, 1996). Wash water containing H_2O_2 also showed the gel enhancing effect in surimi via the induced protein oxidation (Phatcharat *et al.*, 2006). Currently, no information regarding the use of H_2O_2 as the bleaching agent in cuttlefish skin prior to gelatin extraction and its effect on the functional properties and yield of gelatin has been reported. The objectives of this work were to study the effect of H_2O_2 pretreatment on bleaching of cuttlefish skin and to investigate its impact on functional properties of resulting gelatin.

2.3 Materials and Methods

2.3.1 Chemicals

β-Mercaptoethanol (β-ME), bovine serum albumin and protein markers were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Hydrogen peroxide (H_2O_2), *p*-dimethylamino-benzaldehyde and Tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from El-astin products Co., INC. (Owensville, MO, USA). Food grade bovine bone gelatin (98% protein content, 80-250 Bloom) was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

2.3.2 Collection and preparation of cuttlefish skin

Dorsal and ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20°C until use. Storage time was not greater than 2 months.

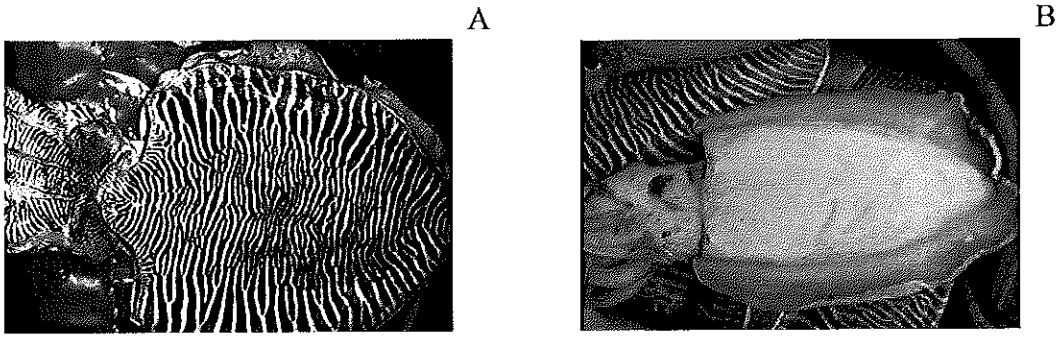


Figure 19. Dorsal (A) and ventral (B) skin of cuttlefish (*Sepia pharaonis*)

2.3.3 Extraction of gelatin from cuttlefish skin without and with bleaching

Gelatin was prepared according to the method of Gómez-Guillén *et al.* (2002) with some modifications. Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) for 6 h with a gentle stirring at room temperature (26-28°C). The solution was changed every 1 h to remove non-collagenous proteins for up to 6 h. Alkali treated skins were then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 2 and 5% H_2O_2 using a sample/solution ratio of 1:10 (w/v) for 24 and 48 h at 4°C. Bleached samples were washed with 10 volume of water for 3 times. The alkali treated skin without bleaching was used as the control. Gelatin was extracted from skin with and without bleaching using distilled water at 60°C for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extracts were centrifuged at 8,000 x g for 30 min using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ µP/Dura Dry™ µP, FTS® System, Inc., Stone Ridge, New York, USA). The yield of gelatin obtained was calculated and expressed as the percentage of dry matter of gelatin relative to dry matter of cuttlefish skin. Gelatins were subjected to analyses.

2.3.4 Proximate analyses

Cuttlefish skin and selected cuttlefish skin gelatin were subjected to proximate analysis. Moisture, ash, fat and protein contents were determined according to the AOAC (2000). Hydroxyproline content was analysed according to the method of Bergman and Loxley (1963) with a slight modification. The samples were hydrolysed with 6 M HCl at 110°C for 24 h in an oil bath (model B-490, BUCHI, Flawil, Switzerland). The hydrolysate was clarified with activated carbon and filtered through Whatman No. 4 filter paper. The filtrate was neutralised with 10 M and 1 M NaOH to obtain the pH of 6.0–6.5. The neutralised sample (0.1 ml) was transferred into a test tube and isopropanol (0.2 ml) was added and mixed well; 0.1 ml of oxidant solution (mixture of 7% (w/v) chloramine T and acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)) was added and mixed thoroughly; 1.3 ml of Ehrlich's reagent solution (mixture of solution A (2 g of *p*-dimethylamino-benzaldehyde in 3 ml of 60% (v/v)

perchloric acid (w/v)) and isopropanol at a ratio of 3:13 (v/v)) were added. The mixture was mixed and heated at 60°C for 25 min in water bath (Memmert, Schwabach, Germany) and then cooled for 2–3 min in a running water. The solution was diluted to 5 ml with isopropanol. Absorbance was measured against water at 558 nm. A hydroxyproline standard solution, with concentration ranging from 10 to 60 ppm, was prepared. Hydroxyproline content was calculated and expressed as mg/g sample.

2.3.5 Determination of carbonyl content

Carbonyl content of gelatin was determined according to the method of Liu *et al.* (2000). Gelatin solution (0.5 mL, 4 mg protein/ml) was added with 2.0 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl. The mixture was allowed to stand for 1 h at room temperature. Thereafter, 2 mL of 20% (w/v) TCA were added to precipitate protein. The pellet was washed twice with 4 mL of ethanol:ethylacetate (1:1, v/v) mixture to remove unreacted DNPH, blow-dried, and dissolved in 1.5 mL of 0.6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). The absorbance of solution was measured at 370 nm using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). A molar absorptivity of 22,400 M^{-1} cm^{-1} was used to calculate carbonyl content (Levine *et al.*, 1990).

2.3.6 Determination of functional properties

2.3.6.1 Determination of gel strength

Gel strength of gelatin was determined according to the method of Gómez-Guillén *et al.* (2002) with a slight modification. Gelatins was dissolved with 30 mL of distilled water (60°C) in 50 mL-beaker (PYREX®, Corning, NY, USA) with the inner diameter of 3.8 cm for 30 min to obtain a final concentration of 6.67% (w/v). Gelatin solution was kept at 5°C for 18 h prior to the measurement. Gel strength of sample (2.7 cm height) was measured at 8–10°C using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN, cross-head speed 1 mm/sec, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon plunger. Maximum force (in grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded.

2.3.6.2 Determination of color

Gelatin gel (6.67%, w/v) was prepared as described previously. Color of gel samples was determined using a colorimeter (ColorFlex, HunterLab Reston, VA, USA.). CIE L* (lightness), a*(redness/greenness) and b* (yellowness/blueness) values were measured.

2.3.6.3 Determination of emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and gelatin solution (1 % protein, 6 mL) were homogenized using a homogenizer model T25 basic (IKA LABORTECNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 50-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formula:

$$EAI\ (m^2/g) = (2\times 2.303\ A)DF/l\phi C$$

$$ESI\ (min) = A_0 \times \Delta t / \Delta A$$

where $A = A_{500}$, DF= the dilution factor (100), l = path length of cuvette (cm), ϕ = oil volume fraction (0.25) and C = protein concentration in aqueous phase (mg/mL), $\Delta A = A_0 - A_{10}$ and Δt = 10 min

2.3.6.4 Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi *et al.* (1995)with a slight modification. Gelatin solution with 1% protein concentration was transferred into 100 mL-cylinders (PYREX®, Corning, NY, USA). The mixtures were homogenized for 1 min using a homogenizer model T25 basic (IKA LABORTECNIK, Selangor, Malaysia) at 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T/V_o) \times 100$$

$$FS\ (\%) = (V_t/V_o) \times 100$$

where V_T is total volume after whipping; V_o is the original volume before whipping and V_t is total volume after leaving at room temperature for different times (30 and 60 min).

2.3.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of gelatin samples extracted from both dorsal and ventral skins with and without bleaching were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970) using 10% separating gel and 4% stacking gel. Gelatin solution was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) β-ME) at the ratio of 1:1 (v/v). Fifteen μg of protein determined by the Biuret method (Robinson and Hodgen, 1940) were loaded onto the gel. Electrophoresis was conducted using the Protean II xi vertical cell and the 1000 powerpac (Bio-Rad laboratories, Hercules, CA) at a constant current of 15 mA. Gels were stained using 0.05% Coomassie Brilliant Blue R250 dissolved in 15% (v/v) methanol and 5% (v/v) acetic acid and de-stained with 30% (v/v) methanol and 10% (v/v) acetic acid. Protein markers including myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa) were used for estimation of molecular weight.

2.3.8 Fourier transform infrared (FTIR) spectroscopy

Sample was dissolved in water and cast into film prior to analysis. Spectra of gelatins from bleached and unbleached cuttlefish skin were obtained by using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulfate (DLATGS) detector. The Horizontal Attenuated Total Reflectance Accessory (HATR) was

mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–600 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.3.9 Amino acid analysis

Cuttlefish skin gelatin were hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.3.10 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

2.3.11 Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

2.4 Results and Discussion

2.4.1 Proximate analyses of dorsal and ventral skin of cuttlefish and their gelatin

Proximate composition of cuttlefish skin and its gelatin is shown in Table 7. Cuttlefish skin had a high content of moisture (86.63% for dorsal skin and

83.93% for ventral skin, respectively). The ash content of dorsal skin was greater than that of ventral skin. Conversely, a lower protein content was observed in dorsal skin than that found in ventral skin. The hydroxylproline contents in dorsal and ventral gelatin were 8.82 and 12.57 mg/g sample, respectively. Hydroxyproline contented in fish skin was 19.5 mg/g sample for bigeye snapper skin (Kittiphattanabawon *et al.*, 2005), 14.6 mg/g sample for cod (Sadowska *et al.*, 2003) and 24.2 mg/g sample for carp skin (Zhang *et al.*, 2009). Different hydroxyproline contents depend upon the species (Rigby, 1968). When gelatin was extracted from the dorsal and ventral skin bleached with 5% H_2O_2 for 48 h, it was noted that the higher protein and hydroxyproline contents were observed in ventral skin, compared with those of dorsal skin. Increase in hydroxyproline content in gelatin samples was generally accompanied with an increased protein content.

Table 7. Proximate composition and hydroxyproline content of dorsal and ventral skin of cuttlefish and their gelatin

Samples	Proximate composition (% wet wt.)				Hydroxyproline** (mg/g sample)
	Moisture	Protein	Fat	Ash	
Skin					
Dorsal skin	86.63±2.05	10.05±0.82	0.43±0.09	3.05±0.58	8.82±0.52
Ventral skin	83.93±1.54	12.07±0.68	0.57±0.04	1.14±0.80	12.57±0.97
Gelatin*					
Dorsal skin	12.89±0.81	83.25±1.47	0.48±0.07	2.93±0.23	28.73±1.51
Ventral skin	11.17±1.49	87.97±1.79	0.94±1.25	0.53±0.11	45.24±3.57

Mean±SD (n=3).

*Gelatin was extracted from dorsal and ventral cuttlefish skin with prior bleaching using 5 % H_2O_2 for 48 h

** Expressed as dry weight basis

2.4.2 Yield

Yields of gelatins extracted from dorsal and ventral cuttlefish skin without bleaching were 36.82 and 59.69 %, respectively (Table 8). Generally, dorsal skin showed the lower yield than did ventral counterpart ($p<0.05$). Both dorsal and ventral skins might have the different structural complexation, in which the gelatin extraction from both skins was different. Nevertheless, the increases in yield of gelatin were observed as the skin was bleached with 5% H_2O_2 ($p<0.05$). Longer

bleaching time (48 h) resulted in the higher yield in comparison with the shorter time (24 h). The highest yield was observed in gelatin extracted from skin bleached with 5% H_2O_2 for 48 h (49.65 and 72.88 % for dorsal and ventral skin, respectively). H_2O_2 was found to break the hydrogen bond of collagen (Courts, 1961). Donnelly and McGinnis (1977) reported that tissue containing collagen was liquefied through agitation with H_2O_2 for 4-24 h. In the presence of sufficient amount of H_2O_2 (5%), hydrogen bonds of collagen molecules in cuttlefish skin might be broken, resulting in an increased efficiency in gelatin extraction as evidenced by the increased yield. However, no increases in yield were observed in gelatin from skin bleached with 2 % H_2O_2 for both 24 and 48 h, compared with that of the control (without bleaching) ($p>0.05$).

Table 8. Yield and carbonyl content of gelatins extracted from dorsal and ventral cuttlefish skin with and without bleaching by H_2O_2 at different concentrations for various times

Samples	Treatment	Yield (%)	Carbonyl content (nmole/mg protein)
Dorsal skin gelatin	control	36.82 ± 1.53a*	3.91 ± 0.59a
	2% H_2O_2 24 h	37.14 ± 2.01a	5.74 ± 0.48b
	2% H_2O_2 48 h	36.83 ± 1.44a	5.91 ± 0.47b
	5% H_2O_2 24 h	45.00 ± 2.67b	8.33 ± 0.50c
	5% H_2O_2 48 h	49.65 ± 0.84c	8.53 ± 0.36c
Ventral skin gelatin	control	59.69 ± 3.74a	2.49 ± 0.28a
	2% H_2O_2 24 h	58.91 ± 1.47a	6.11 ± 0.62b
	2% H_2O_2 48 h	59.78 ± 1.95a	6.23 ± 0.14b
	5% H_2O_2 24 h	68.44 ± 2.59b	6.45 ± 0.15b
	5% H_2O_2 48 h	72.88 ± 1.20c	6.57 ± 0.08b

Mean±SD (n=3).

*Different letters in the same column within the same gelatin indicate significant differences ($p<0.05$).

2.4.3 Carbonyl content

Carbonyl content of gelatin from cuttlefish skin is shown in Table 8. Carbonyl content is one of the most reliable measures of protein oxidation (Levine *et al.*, 1990). Gelatins from both dorsal and ventral skin bleached with 5% H_2O_2 showed the marked increases in the carbonyl content, compared with those from unbleached

counterpart ($p<0.05$). However, bleaching with 2% H_2O_2 showed no impact on carbonyl content of resulting gelatin from both dorsal and ventral skins ($p>0.05$). For the control gelatin, that from dorsal skin contained a higher carbonyl content than that from ventral counterpart ($p<0.05$). Hawkins and Davies (1997) found that hydroxyl radical, generated from a Fe(II)-H_2O_2 redox couple, attacked collagen, resulting in the generation of carbonyl compounds. The peroxide decomposition products such as the hydroxyl radicals and superoxide anion radicals (O_2^-) are thought to cause the oxidation of protein and responsible for the conversion of some amino acid residues to carbonyl derivatives (Butterfield and Stadtman, 1997). Carbonyl content in gelatin increased with increasing concentration of H_2O_2 used for bleaching. However, bleaching time showed no effect on the carbonyl content in resulting gelatin, regardless of skin portion and H_2O_2 concentration. The most sensitive amino acids toward oxidation are heterocyclic amino acids. In addition, amino and phenolic groups of amino acids are susceptible to oxidation. Not only tryptophan, histidine and proline, but also lysine, cysteine, methionine and tyrosine are prone to oxidation, where hydrogen atom is abstracted either from OH-, S or N-containing groups (Doorn and Petersen, 2002). Oxidation of protein is associated with the alteration of protein structure, peptide chain scission, formation of amino acid derivatives and polymers, the decreases in solubility, and changes in the functional properties (Decker *et al.*, 1993). Susceptibility of proteins to oxidation induced by H_2O_2 in cuttlefish skin from dorsal portion was greater than that found in ventral portion. This suggested the different composition of proteins in both portions.

2.4.4 Gel strength of gelatin gel

The effect of bleaching of cuttlefish skin with H_2O_2 on gel strength of gelatin gels is shown in Figure 20. The lowest gel strength was observed in gels of the control gelatin from dorsal and ventral skin (35.15 and 30.04 g, respectively). This result was in agreement with Gómez-Guillén *et al.* (2002) who found that gelatin gel extracted from squid skin was extremely soft and showed little cohesive forces (~10 g). According to Holzer (1996), the gel strength of commercial gelatin expressed as bloom value, ranges from 100 to 300 g but gelatin with bloom values of 250–260 g are most desired. Gelatin from skin of both portions bleached with 2 and 5% H_2O_2 for

24 and 48 h resulted in the marked increases in gel strength ($p<0.05$). Gel strength of gelatin gel from ventral skin increased with increasing H_2O_2 concentration and bleaching time ($p<0.05$). However, no pronounced effect of both H_2O_2 concentration and bleaching time on gel strength of gelatin from dorsal skin ($p>0.05$) was noticeable. The highest gel strength of gelatin from dorsal and ventral skins bleached in 5% H_2O_2 for 48 h (125.65 and 136.90 g for dorsal and ventral skin gelatin, respectively) was obtained. Gel strength of resulting gelatins was about 4 times higher than that of control. This result suggested that H_2O_2 might induce the oxidation of protein with the concomitant formation of carbonyl groups. Those carbonyl groups might undergo the Schiff's base formation with the amino groups, in which the protein cross-links were most likely formed (Stadtman and Berlett, 1997).

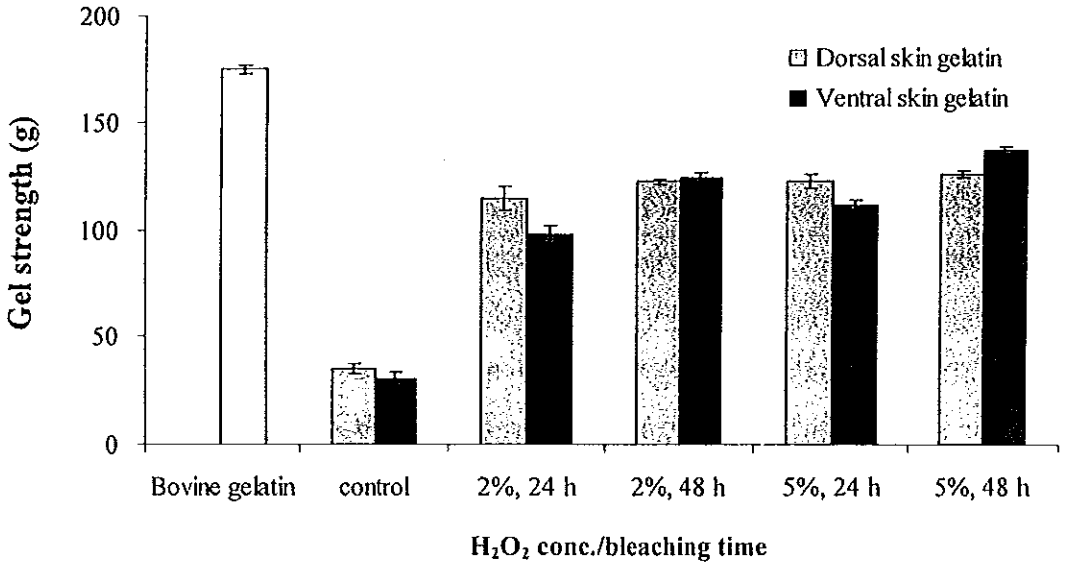


Figure 20. Gel strength of gelatin extracted from dorsal and ventral cuttlefish skin with and without bleaching by H_2O_2 at the different concentrations for various times. Bars represent the standard deviation (n=3).

Moreover, $OH^{\bullet}$ can abstract H atoms from amino acid residues to form a carbon-centered radical derivative, which can react with one another to form –C-C- protein cross-linked products (Stadtman, 1998). The larger protein aggregates were mostly associated with the improved gel strength. However, the gel strength from

cuttlefish skin gelatin gel of all treatments was lower than that of bovine bone gelatin gel, most likely due to lower hydroxyproline content of the former. Therefore, gelatin from cuttlefish skin could be more advantageous to be used as an emulsifier or foaming agent, rather than gelling agent, especially after appropriate modification.

2.4.5 Color of gelatin gel

L*, a* and b*-values of gelatin gels from cuttlefish skin with and without bleaching under different conditions are presented in Table 9. Gelatin gel from skin without bleaching was higher pink-purple in color as indicated by lower L*-value but higher a*-value, when compared with gelatin gel from bleached skin. Thus, soaking cuttlefish skin in 2 or 5% H_2O_2 solution could improve the color of gelatin gel by increasing L*-value and decreasing a*-value. For dorsal skin, increases in H_2O_2 concentration and bleaching time resulted in the increases in L* and lowered a*-value of resulting gelatin ($p<0.05$). For ventral skin, a*-value of gelatin gel was decreased with increasing H_2O_2 concentration and bleaching time ($p<0.05$), whereas slight changes in L*-value were found ($p<0.05$). In general, the control gel from dorsal skins had the higher color intensity than did the gel from ventral skins, most likely due to the higher content of chromatophore in the former. Thus, higher H_2O_2 concentration was necessary for improvement of colors of gelatin from dorsal skin in comparison with ventral skin. H_2O_2 is widely used in cephalopod industry as the bleaching agent. The cephalopod needs to be bleached because the flesh could be stained by ink, viscera and color pigments during handling and processing (Thanonkaew *et al.*, 2008). Oxidizing agents, derived from the decomposition of hydrogen peroxide, were able to destroy the chromophore. Hydroperoxyl anion is a strong nucleophile which, during bleaching, is able to break the chemical bonds that make up the chromophore. This changes the molecule into a different substance that either does not contain a chromophore, or contains a chromophore that does not absorb visible light (Perkins, 1996). On the other hand, hydroperoxyl and hydroxyl radical ($OH^{\bullet}$) generated by the decomposition of hydrogen peroxide may induce free radical, causing the oxidation of protein, changes in protein structure and functional properties of gelatin. As a result, bleached skin contained a low content of chromophore, or still had the chromophore, which was colorless.

Table 9. Color (L*, a* and b*-values) of gel from gelatin extracted from dorsal and ventral of cuttlefish skin with and without bleaching by H_2O_2 at different concentrations for various times

Samples	Treatment	Color[#]		
		L*	a*	b*
Dorsal skin gelatin	control	14.12 ± 0.30a[##]	18.08 ± 0.71d	8.86 ± 0.38a
	2% H_2O_2 24 h	41.66 ± 0.15b	12.61 ± 0.46c	19.81 ± 0.12e
	2% H_2O_2 48 h	41.43 ± 0.09b	6.80 ± 0.09b	19.17 ± 0.02d
	5% H_2O_2 24 h	48.68 ± 0.07c	6.34 ± 0.16b	13.68 ± 0.04c
	5% H_2O_2 48 h	48.89 ± 0.04c	5.87 ± 0.13a	12.77 ± 0.01b
Ventral skin gelatin	control	47.78 ± 0.12a	11.20 ± 0.11d	15.56 ± 0.09e
	2% H_2O_2 24 h	63.65 ± 0.13c	-0.11 ± 0.36c	10.08 ± 0.06d
	2% H_2O_2 48 h	63.66 ± 0.05c	-1.02 ± 0.06a	5.77 ± 0.02a
	5% H_2O_2 24 h	63.16 ± 0.05b	-0.54 ± 0.03b	8.69 ± 0.03c
	5% H_2O_2 48 h	63.86 ± 0.04d	-1.06 ± 0.09a	6.35 ± 0.04b
Bovine bone gelatin		80.21 ± 0.19	-2.56 ± 0.18	14.39 ± 0.08

Mean±SD (n=3).

[#]6.67 % (w/v) gelatin

[##]Different letters in the same column within the same gelatin indicate significant differences ($p<0.05$).

2.4.6 Emulsifying properties of gelatin

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin from cuttlefish skin with and without bleaching are presented in Table 10. No differences in EAI of gelatin from dorsal skin were observed ($p>0.05$), irrespective of bleaching. For gelatin from ventral skin, bleaching using 2 and 5% H_2O_2 for 48 h resulted in the lower EAI ($p<0.05$), compared with 24 h bleaching and no bleaching. It was presumed that bleaching of ventral skin for a long time caused the aggregation of protein to a large extent. Aggregated proteins might be rigid and could not unfold rapidly at the interface and form the film around the oil droplet effectively.

For stability of emulsion, emulsion containing gelatin from bleached dorsal and ventral skin was more stable than that of the control ($p<0.05$). Larger and longer peptides could stabilize the protein film at the interface more effectively. However, proteins oxidized to a higher degree might possess the lower ability of stabilizing emulsion. It was noted that the longer bleaching time and higher H_2O_2 concentration led to the lower ESI of gelatin from all samples, except for gelatin from

dorsal skin, in which the highest ESI was obtained when the skin was bleached with 5% H_2O_2 for 48 h ($p<0.05$). Surh *et al.* (2006) found that the oil-in-water emulsion prepared with high molecular weight fish gelatin (~120 kDa) was more stable than that prepared with low molecular weight fish gelatin (~50 kDa). Thickness of an adsorbed gelatin membrane increased with increasing molecular weight. This was associated with the increased stability of emulsions to coalescence during homogenization (Lobo and Svereika, 2003). EAI and ESI of bovine bone gelatin were higher than those of all cuttlefish skin gelatin samples. This possibly resulted from the differences in the intrinsic properties of proteins, composition and conformation of protein between gelatins from both sources (Damodaran, 1997).

2.4.7 Foaming properties of gelatin

Foam expansion (FE) and foam stability (FS) of gelatin extracted from cuttlefish skin with and without bleaching are shown in Table 10. Gelatin from unbleached skin, both dorsal and ventral, had the slightly lower FE than gelatin extracted from bleached skin ($p<0.05$). However, bleaching condition had no marked impact on FE of resulting gelatin ($p>0.05$). The foaming ability of proteins is related to their film-forming ability at the air-water interface. In general, proteins that rapidly adsorb at the newly created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foam ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). At both 30 and 60 min, bleaching had no effect on FS of gelatin from ventral skin ($p>0.05$). Gelatin from dorsal skin bleached with 5% H_2O_2 for 48 h exhibited the highest FS at both 30 and 60 min ($p<0.05$). No differences in FS were noticeable when 2% H_2O_2 was used, regardless of bleaching time ($p>0.05$). Gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via interbubble gas diffusion contribute to instability of foams (Yu and Damodaran, 1991). Coalescence of bubbles occur because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). Thus, the foam stability could be improved by bleaching the skin of cuttlefish with H_2O_2 under the appropriate condition.

Table 10. Emulsifying and foaming properties of gelatin extracted from dorsal and ventral of cuttlefish skin without and with bleaching in H_2O_2 at different concentrations for various times

Samples	Treatment	Emulsion[#] activity index (m^2/g)	Emulsion[#] stability index (min)	Foam expansion[#] (%)	Foam stability[#] (%)	
					30 min	60 min
Dorsal skin gelatin	control	24.30 ± 0.51a*	15.14 ± 1.31a	170.00 ± 12.25a	89.00 ± 4.18aB**	76.00 ± 2.24aA
	2% H_2O_2 24 h	23.58 ± 1.20a	20.96 ± 2.97b	184.00 ± 8.94b	90.00 ± 0.00abB	81.00 ± 2.24abA
	2% H_2O_2 48 h	23.07 ± 2.56a	19.24 ± 2.81b	192.00 ± 4.47b	94.00 ± 2.24abB	82.00 ± 8.37abA
	5% H_2O_2 24 h	23.47 ± 1.23a	26.40 ± 1.71c	196.00 ± 5.48b	96.00 ± 2.24bcB	81.00 ± 2.24abA
	5% H_2O_2 48 h	22.05 ± 1.31a	17.84 ± 0.64ab	198.00 ± 8.94b	101.00 ± 8.22cB	86.00 ± 4.18bA
Ventral skin gelatin	control	23.04 ± 0.21 a	17.15 ± 0.29 a	174.00 ± 4.18a	89.00 ± 5.48aA	82.00 ± 2.74aA
	2% H_2O_2 24 h	23.50 ± 0.99 a	23.95 ± 0.86 d	182.00 ± 10.95ab	91.00 ± 5.48aB	82.00 ± 2.74aA
	2% H_2O_2 48 h	18.91 ± 1.06 b	21.10 ± 0.04 c	190.00 ± 10.00b	93.00 ± 2.74aB	85.00 ± 4.18aA
	5% H_2O_2 24 h	22.72 ± 1.10 a	21.20 ± 1.28 c	192.00 ± 4.47bc	92.00 ± 2.74aB	78.00 ± 6.71aA
	5% H_2O_2 48 h	18.17 ± 1.00 b	19.03 ± 1.19 b	202.00 ± 8.37c	94.00 ± 5.48aB	80.00 ± 7.07aA
Bovine bone gelatin		28.27 ± 0.71	31.23 ± 0.90	190.00 ± 12.25	98.00 ± 7.58B	90.00 ± 0.00A

Mean±SD (n=3).

[#]Protein at a level of 1% (w/v) was used for determination.

*Different letters in the same column within the same gelatin indicate significant differences ($p<0.05$).

**Different capital letters in the same row of foam stability indicated significant differences ($p<0.05$).

2.4.8 Protein patterns of gelatin with and without bleaching

Protein patterns of gelatins extracted from dorsal and ventral cuttlefish skin with and without bleaching using 5% H_2O_2 for 48 h are shown in Figure 21. Gelatins extracted from both bleached and unbleached skin had smear protein bands with molecular weight equivalent to γ-chain, α-chain and less than α-chain. Proteins with smear bands were generated during extraction. Muyonga *et al.* (2004a) reported that during conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds was obtained. Without bleaching, skin matrix was denser and the conversion of collagen to gelatin was less effective.

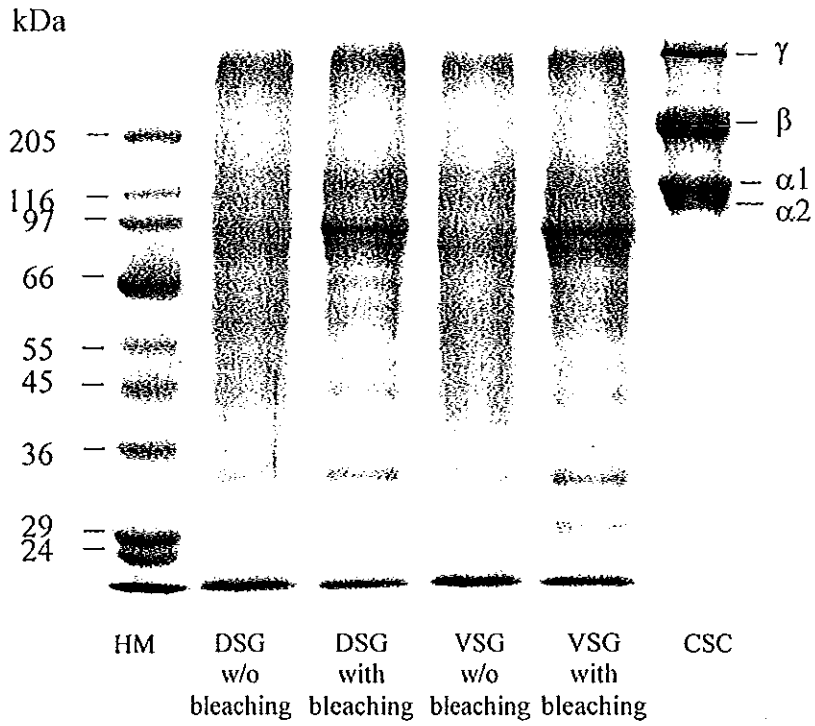


Figure 21. SDS-PAGE pattern of gelatin extracted from dorsal (DSG) and ventral (VSG) cuttlefish skin with and without bleaching using 5% H_2O_2 for 48 h. HM and CSC denote high MW protein markers and bovine collagen type I, respectively.

Gelatin from dorsal and ventral skin with prior bleaching contained proteins with molecular weight of about 97 kDa as the dominant component. This suggested that the peroxide decomposition products such as the hydroxyl radicals and superoxide anion radicals (O_2^-) were presumed to destroy H-bond stabilizing α-chains, resulting in the increased extractability. However, the MW of 97 kDa protein was slightly lower than that of α1 and α2. H_2O_2 might induce some fragmentation of α-chain, leading to slightly lower MW (97 kDa). Collagen extracted from both cuttlefish and squid skin was composed mainly of α-chains and low content of dimmer (β-components) and higher molecular weight aggregates (γ-components and other) (Gómez-Guillén *et al.*, 2002; Kołodziejska *et al.*, 1999; Nagai *et al.*, 2001).

2.4.9 FTIR spectra of gelatin

FTIR spectra of gelatin extracted from dorsal and ventral skin with and without bleaching in 5% H_2O_2 for 48 h are shown in Figure 22. FTIR spectroscopy has been used to study changes in the secondary structure of gelatin. A spectra of both dorsal and ventral skin gelatin displayed major bands at 3264 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), 1628 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-), 1550.49 cm^{-1} (amide II, representative of NH bending coupled with CN stretching) and 1240.01 cm^{-1} (amide III, representative of NH bending). FTIR spectra of cuttlefish skin gelatin were similar to those found in other gelatins (Muyonga *et al.*, 2004b). Similar spectra were observed between gelatin from skin with and without bleaching for both dorsal and ventral skin (Figure 22A, 22B). Bleaching skin with H_2O_2 resulted in the decreases in the intensity of amide A, I, II and III bands of gelatin from both dorsal and ventral skin. These changes are indicative of greater disorder (Friess and Lee, 1996) in gelatin and are associated with loss of triple helix state (Muyonga *et al.*, 2004b). The shift to lower wavenumber was observed in gelatin from ventral skin bleached with 5% H_2O_2. However, no shift was noticeable in gelatin from dorsal skin. The amine A, I and II peaks of gelatin extracted from bleached ventral skin (3264, 1628 and 1535 cm^{-1}, respectively) had lower wavenumbers than those of gelatin from unbleached counterpart (3289, 1629 and 1538 cm^{-1}, respectively).

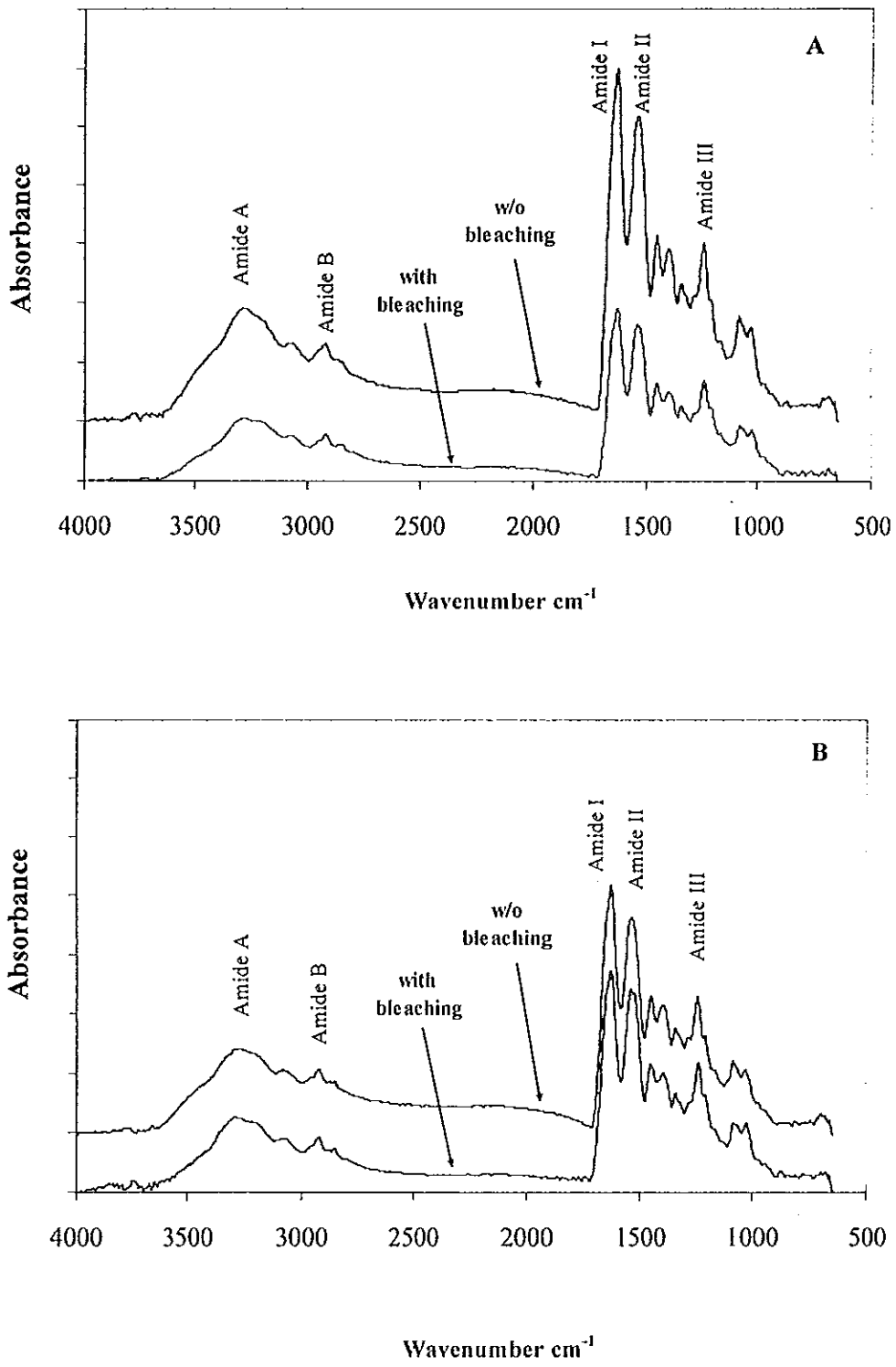


Figure 22. Fourier transform infrared (FTIR) spectroscopic spectra of gelatin extracted from dorsal (A) and ventral (B) cuttlefish skin with and without bleaching using 5% H_2O_2 for 48 h.

Muyonga *et al.* (2004b) reported that the amide I and II peak of collagen extracted from adult Nile perch was at a higher frequency than the young fish skin collagen, due to more intermolecular cross-links in the adult fish collagen. A shift of peaks to lower wavenumbers is associated with a lower molecular order (Payne and Veis, 1988). The amide I is the most useful peak for infrared analysis of the secondary structure of protein including gelatin (Surewicz and Mantsch, 1988). Yakimets *et al.* (2005) reported that the absorption peak at 1633 cm^{-1} was the characteristic for the coil structure of gelatin. The change in amide I band of gelatin suggested that the use of H_2O_2 might affect the helix coil structure of gelatin. This result suggested that hydrogen peroxide might induce the changes in secondary structure and functional groups of resulting gelatin, associated with the increased intermolecular interactions and denaturation of gelatin.

2.4.10 Amino acid composition of gelatin

The amino acid composition of gelatin extracted from ventral cuttlefish skin with prior bleaching using 5% H_2O_2, expressed as residues per 1000 total residues is shown in Table 11. Generally, glycine was a major amino acid in ventral cuttlefish skin gelatin (approximately 1/3 of the total amino acids). Relatively high contents of proline, glutamic acid, hydroxyproline, alanine and aspartic acid were found in the gelatin. However, ventral cuttlefish skin gelatin showed low content of threonine, methionine and histidine. There were negligible cysteine and no tyrosine in the gelatin. The imino acid content (proline and hydroxyproline) of gelatin from ventral cuttlefish skin was 194 residues per 1000 residues. The imino acid content was higher that that of gelatin from squid and giant squid (175 and 163 residues per 1000 residues, respectively) (Giménez *et al.*, 2009; Gómez-Guillén *et al.*, 2002) but lower than that of bigeye snapper, brownbanded bamboo shark and porcine skin gelatin (195, 208 and 223 residues per 1000 residues, respectively) (Eastoe and Leach, 1977; Jongjareonrak *et al.*, 2005; Kittiphattanabawon *et al.*, 2010). It is generally recognized that imino acids are key factors affecting functional properties of gelatin. Since super-helix structure of the gelatin is stabilized by hydrogen bonds, gelatin with high levels of imino acids tends to have higher gel strength and melting point (Ledward, 1986). Pyrrolidine rings of imino acids are involved in the hydrogen bond formation between

amino acid residues (Te Nijenhuis, 1997). Apart from Pro and Hyp, gelatin also contained a high content of Ala, Asp, Asn, Glu and Gln. Ala is found in non-polar regions, where the sequences of Gly–Pro–Y predominate and the third positions are normally occupied by Hyp or Ala (Ledward, 1986). Gelatin with a higher content of Pro, Hyp and Ala are considered to have higher viscoelastic properties and also had ability to develop triple-helix structures, which are important for stabilizing the structure of gelatin gel (Gómez-Guillén *et al.*, 2002).

Table 11. Amino acid composition of gelatin extracted from ventral cuttlefish skin.

Amino acid	Content (residues/ 1000 residues)
Alanine	83
Arginine	60
Aspartic acid/asparagine	72
Cysteine	1
Glutamine/glutamic acid	92
Glycine	314
Histidine	5
Isoleucine	20
Leucine	26
Lysine	12
Hydroxylysine	12
Methionine	7
Phenylalanine	12
Hydroxyproline	91
Proline	103
Serine	40
Threonine	7
Tyrosine	0
Valine	19
Total	**1000**
Imino acid*	194

* Imino acids include proline and hydroxyproline

2.5 Conclusion

Bleaching of cuttlefish skin with 5% H_2O_2 not only improved the color of resulting gelatin but also enhanced the gel strength effectively. Furthermore, bleaching could increase the yield of gelatin. Bleaching also improved emulsifying and foaming properties of resulting gelatin, mostly via the oxidation of gelatin molecule. Due to the higher proportion and functional properties of ventral skin as well as the much lower pigment content, this portion was used for further study.

CHAPTER 3

Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by oxidized phenolic compounds

3.1 Abstract

Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by different oxidized phenolic compounds including caffeic acid (OCA), ferulic acid (OFA) and tannic acid (OTA) at different concentrations were investigated. Oxidized phenolic compounds were covalently attached to gelatin as indicated by the decrease in amino groups. Fourier transform infrared spectroscopic study indicated the presence of aromatic ring and hydroxyl group of gelatin after modification. Modified gelatin had the increased antioxidative activity but the decreased surface hydrophobicity. Gelatin modified with 5% OTA had no change in emulsifying property. Emulsion stability and oxidative stability of menhaden oil-in-water emulsion stabilized by 0.5 and 1.0% gelatin without and with modification by 5% OTA were studied. Both gelatins at a higher concentration (1.0%) yielded emulsion with the smaller particle size. Modified gelatin inhibited the formation of TBARS in the emulsion more effectively than the control gelatin throughout the storage of 12 days.

3.2 Introduction

Proteins are widely used as emulsifiers in food products because of their ability to improve the stability of oil-in-water emulsions (McClements, 2004). Although low molecular mass surfactants, such as lecitin, mono- and diglycerides etc., are more effective than protein in reducing the interfacial tension, foams and emulsions formed by such surfactants are mostly unstable (Damodaran, 2005). In addition to lowering interfacial tension, protein can form a continuous viscoelastic

membrane-like film around oil droplets, whereas the low molecular mass surfactants cannot form such a viscoelastic film (Damodaran, 1997). A major potential advantage of proteins as emulsifiers in foods is their ability to protect lipids from iron catalysed oxidation (Hu *et al.*, 2003). At pH values below their isoelectric point (pI), proteins form positively charged interfacial membranes around oil droplets, which electronstatically repel any Fe^{2+} and Fe^{3+} ions present in the aqueous phase, thereby preventing oxidation of polyunsaturated lipids within the droplets (Surh *et al.*, 2005). Moreover, amino acids in protein can scavenge free radical and chelate prooxidative metals (Djordjevic *et al.*, 2008).

Gelatin is commercially made from skins and skeletons of bovine and porcine. It has been used extensively in the medical, food, photographic and other industries (Derkatch *et al.*, 1999). Gelatin is surface active and is capable of acting as an emulsifier in oil-in-water emulsions (Lobo, 2002; Müller and Hermel, 1994; Olijve *et al.*, 2001). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot/mouth diseases has led to the increasing interest of other gelatin sources, especially fish skin and bone from seafood processing. Nevertheless, gelatin from marine resources had the poorer emulsifying properties, compared with mammalian gelatin (Aewsiri *et al.*, 2008).

Plant phenolics are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), and can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Parr and Bolwell, 2000). Phenolic compounds can interact with proteins through non-covalent and covalent interaction. However, covalent bonding seems to play an important role in protein-phenol interaction, which is used to improve functional properties of proteins. Foam formation and stability of Tween 20/β-lactoglobulin mixed system was improved by (+)-catechin due to (+)-catechin-induced cross-linking of proteins in the adsorbed layer (Sarker *et al.*, 1995). Additionally, antioxidative activity of protein can be modified by phenolic compounds (Rohn *et al.*, 2004). Nevertheless, a little information regarding the modification of gelatin with phenolic compound and its impact on stability and lipid oxidation of emulsion system has been reported. Therefore, the objectives of this study were to investigate the effect of cuttlefish skin gelatin modified by oxidized phenolic compounds, including caffeic acid (OCA),

ferulic acid (OFA) and tannic acid (OTA), possessing different size and number of hydroxyl groups, on stability and lipid oxidation of fish oil emulsion.

3.3 Materials and Methods

3.3.1 Chemicals

Caffeic, ferulic acid, tannic acid, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-triazine (TPTZ), 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), Trolox and menhaden oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Folin-Ciocalteu's phenol reagent, sodium sulfite and ferric chloride were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

3.3.2 Preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20°C until use. Storage time was not longer than 2 months.

3.3.3 Extraction of gelatin from cuttlefish skin

Cuttlefish skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28 °C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5% H_2O_2 at 4°C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown,

CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ µP/Dura Dry™ µP, FTS® System, Inc., Stone Ridge, NY, USA). Gelatin extracted from cuttlefish skin consisted of 87.97% protein, 11.17% moisture, 0.94% fat and 0.53% ash contents as determined by AOAC (2000). The molecular weight of major proteins in cuttlefish skin gelatin was estimated to be 97 kDa as analyzed by SDS-PAGE using 4% stacking gel and 10% separating gel (Laemmli, 1970). pI of gelatin was estimated to be 4.5 using a zeta potential analysis (Kittiphattanabawon *et al.*, 2010).

3.3.4 Modification of cuttlefish skin gelatin by phenolic compounds

Cuttlefish skin gelatin was dissolved in distilled water containing 0.02 % sodium azide (NaN_3) to obtain a final concentration of 1.2 % protein. The pH of the gelatin solution was adjusted to 9 using 1 M NaOH. To prepare the solutions of phenolic compounds, caffeic acid, ferulic acid and tannic acid at the concentration of 3% (w/v) were dissolved in distilled water, followed by pH adjustment to 9 with 1 M NaOH. Solutions were then bubbled with oxygen at 40°C for 1 h to convert phenolic compounds into oxidized form. To 75 mL of gelatin solution, oxidized phenolic solutions at different concentrations (5, 10, 25 and 50 %, based on protein content) were added. Final volume was raised to 90 mL using distilled water (pH 9) to obtain a final concentration of 1% protein. The mixture was stirred continuously using a magnetic stirrer (RO 10 power IKAMAG®, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 200 rpm at room temperature for 12 h. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 24 h at room temperature (26-28°C) against 20 volumes of water to remove free phenolic compounds (unbound to proteins). The control was prepared in the same manner except that phenolic compound was excluded. Gelatin-phenolic complexes were subjected to analyses.

3.3.5 Determination of free amino group content

Free amino group content of samples was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µL), 2.0 mL of 0.2125 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath

(Model W350, Memmert, Schwabach, Germany) at 50°C for 30 min in dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and free amino group content was expressed in terms of L-leucine.

3.3.6 Determination of total phenolic content

Total phenolic content of samples was measured according to the method of Slinkard and Singleton (1977) with some modifications. Sample solution (1 mL) was 50-fold diluted with deionized water prior to mixing with 200 μL of the freshly prepared Folin–Ciocalteu reagent using a vortex mixer. After 3 min, 3 mL of sodium carbonate (15%, w/v) were added and the mixture was allowed to stand for 30 min at room temperature. The absorbance at 760 nm was measured using a UV–vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The blank was prepared by using deionized water instead of Folin-Ciocalteu reagent. Total phenolic content was expressed as A_{760} after blank substraction.

3.3.7 Determination of surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (4 mg/mL) was diluted in 10 mM phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5%. The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm using a RF-1501 *spectrofluorometer* (Shimadzu, Kyoto, Japan). The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

3.3.8 Fourier transform infrared (FTIR) spectroscopy

Spectra of gelatin modified without and with oxidized phenolic compounds were obtained using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulfate (DLATGS) detector (Bruker, Ettlingen, Germany). The Horizontal Attenuated Total

Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–600 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25°C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

3.3.9 Determination of emulsifying properties

Emulsion activity index (EAI) was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and gelatin solution (1 % protein, 6 mL) were homogenized using a homogenizer (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 50-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI at 0 and 10 min were calculated by the following formula:

$$EAI\ (m^2/g) = (2\times 2.303\ A)DF/l\phi C$$

where $A = A_{500}$, l = path length of cuvette (m), DF= the dilution faction (100), ϕ = oil volume fraction (0.25) and C = protein concentration in aqueous phase (g/m^3).

3.3.10 Determination of antioxidative activities

3.3.10.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan *et al.* (2008) with a slight modification. To the diluted sample (1.5 mL), 1.5 mL of 0.10 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol was added. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm

using a UV-vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The standard curve of Trolox (60-600 μM) was prepared in the same manner. The activity was expressed as μmol Trolox equivalent (TE)/ mg protein.

3.3.10.2 ABTS radical scavenging activity

ABTS free radical scavenging assay was determined according to the method of Re *et al.* (1999) with a slight modification. 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical was generated by reacting 7.4 mM ABTS and potassium persulfate (2.6 mM) at a ratio of 1:1 (v/v) and leaving at room temperature in the dark for 12 h. ABTS free radical solution was diluted by mixing 1 mL of ABTS solution with 50 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using an UV-vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). To 150 μL of sample, 2850 μL of ABTS•+ solution was added and mixed thoroughly using a vortex mixer (Vortex genie® 2, Scientific Industries, Bohema, NY, USA). The extent of quenching of the ABTS•+ was measured at 734 nm after 2 h incubation at room temperature in dark. The standard curve of Trolox (60 – 600 μM) was prepared in the same manner. The activity was expressed as μmol TE/ mg protein.

3.3.10.3 Ferric reducing antioxidant power (FRAP)

FRAP was determined as described by Benzie and Strain (1996) with a slight modification. A 2.85 mL of freshly prepared FRAP solution (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution and 25 mL of 300 mM acetate buffer, pH 3.6) was incubated at 37 °C for 30 min before mixing with 150 μL of sample. The mixture was allowed to react in dark at room temperature. Absorbance at 593 nm was measured after 30 min of reaction. The standard curve of Trolox (60 – 600 μM) was prepared in the same manner. FRAP was expressed as μmol TE/ mg protein.

3.3.10.4 Determination of Fe^{2+} chelating activity

Chelating activity toward Fe^{2+} was measured by the method of Boyer and McCleary (1987) with a slight modification. Diluted sample (4.7 mL) was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at

562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The chelating activity was calculated as follows:

Chelating activity = [(B-A) / B] x 100

where A is A_{562} of sample and B is A_{562} of the blank.

3.3.11 Effect of gelatin modified without and with OTA on emulsion stability and lipid oxidation of menhaden oil emulsion

Menhaden oil-in-water emulsions were prepared by homogenizing the mixture of menhaden oil and gelatin solution (1:9, v/v) at a speed of 10,000 rpm for 2 min (IKA LABORTECNIK, model T25 basic, Selangor, Malaysia). Gelatin modified without and with OTA (5% of protein) at concentrations of 0.5 and 1 % protein were used. These coarse emulsions were then passed through a two-stage high-pressure valve homogeniser (M-110P, Microfluidizer, Newton, MA, USA) at 10,000 psi for 2 times. NaN_3 (0.02 %) was added to the emulsions as an antimicrobial agent. The menhaden oil emulsions were then stored at room temperature (28-30°C) for 12 days. The samples were taken every two days for analyses.

3.3.11.1 Measurement of zeta potential and particle size

Zeta potential and particle size of emulsions were determined with ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. Prior to analysis, the samples were properly diluted with deionized water.

3.3.11.2 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Two mL of fish oil emulsion sample were dispersed in 10 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 x g for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The standard curve was prepared using malonaldehyde (2-10 ppm) and TBARS were expressed as mg malonaldehyde/l emulsion.

3.3.12 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

3.3.13 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

3.4 Results and Discussion

3.4.1 Changes in free amino group content of gelatin

Free amino group contents of cuttlefish skin gelatin modified by different oxidized phenolic compounds at various levels are shown in Table 12. Gelatin modified by oxidized phenolic compounds had the lower free amino group content than the control gelatin (without oxidized phenolic compound) ($p<0.05$). It indicated that nucleophilic amino groups might interact with electrophilic quinone, an oxidized form of phenolic compounds. A reaction of protein with oxidized phenolic compounds led to a decrease in free amino group content (Rawel *et al.*, 2001). Furthermore, the oxidized phenolic compound can react with other nucleophilic groups, such as tryptophan, cysteine, methionine, histidine, tyrosine and N-terminal proline of the protein molecule (Kroll *et al.*, 2003). The rate of loss in free amino group of gelatin depended on type and concentration of oxidized phenolic compounds used. At the same level of oxidized phenolic compounds, the higher loss in free amino groups was observed in gelatin modified by OCA ($p<0.05$), followed by that found in gelatin modified with OTA and OFA, respectively. In general, the loss in free amino groups was increased when the concentration of oxidized phenolic compounds used increased ($p<0.05$). OCA (MW ~ 180) exhibited the higher interaction with amino groups of gelatin, compared with OTA (MW ~ 1,701). The smaller molecules might disperse uniformly and could interact with amino groups of gelatin more effectively.

Although OFA (MW ~ 194) had the similar molecular mass to OCA, the loss in free amino groups of gelatin modified with OFA was lower. Ferulic acid, with one hydroxyl of caffeic acid replaced by $-OCH_3$, is considerably less reactive. Rawel *et al.* (2001) reported that ferulic acid cannot be oxidized to their corresponding quinone derivative. The formation of a semiquinone radical with lower reactivity was presumed, compared with OCA and OTA. Quinone is known as the electrophilic compound, which can interact with nucleophilic amino group of proteins (Kroll *et al.*, 2003).

Table 12. Free amino group content and emulsifying properties of cuttlefish skin gelatin modified with different oxidized phenolic compound at various levels

Oxidized phenolic compound	Concentration (% w/w of protein)	Free amino group content (mM)	Emulsion activity index (m^2/g)	
			0 min	10 min
Control (unmodified)		3.84 ± 0.12i*	20.86 ± 1.24f	10.58 ± 0.08h
Ferulic acid	5	3.61 ± 0.19gh	19.93 ± 0.48def	10.67 ± 0.50h
	10	3.61 ± 0.02gh	18.63 ± 0.63bcd	10.15 ± 0.47h
	25	3.55 ± 0.14g	18.63 ± 0.24bcd	8.95 ± 0.81fg
	50	3.34 ± 0.03f	18.75 ± 0.95bcd	7.92 ± 0.99def
Caffeic acid	5	3.03 ± 0.05e	18.72 ± 0.57bcd	8.82 ± 0.61efg
	10	2.73 ± 0.02d	18.48 ± 0.87bcd	7.77 ± 0.59de
	25	1.98 ± 0.05b	18.04 ± 0.68bc	7.23 ± 0.80cd
	50	1.46 ± 0.13a	17.99 ± 0.60bc	6.57 ± 0.38bc
Tannic acid	5	3.79 ± 0.17hi	20.25 ± 0.87ef	9.89 ± 0.28gh
	10	3.08 ± 0.05e	19.38 ± 0.97cde	7.19 ± 0.62cd
	25	2.68 ± 0.02d	17.44 ± 0.60b	5.63 ± 0.97b
	50	2.43 ± 0.02c	13.04 ± 0.68a	4.37 ± 0.68a

Mean±SD (n=4).

* Different letters in the same column indicate significant differences ($p<0.05$).

3.4.2 Changes in total phenol content

Total phenol contents of cuttlefish skin gelatin modified by different oxidized phenolic compounds at various levels determined by Folin–Ciocalteu method are expressed as A_{760} (Figure 23A). The increases in A_{760} were observed in gelatin modified with all oxidized phenolic compounds when the levels of introduced compounds increased ($p<0.05$). This result indicated that oxidized phenolic compounds were incorporated with gelatin molecules to a higher extent when the

higher level of oxidized phenols were introduced. At the same level, no differences in A_{760} were noticeable between gelatin modified with OCA and OTA ($p>0.05$). A_{760} of gelatin modified by OFA was lowest ($p<0.05$). This result was associated with the lowest loss in free amino group of gelatin modified by OFA. The result suggested that OFA could incorporate with amino group of protein to some extent.

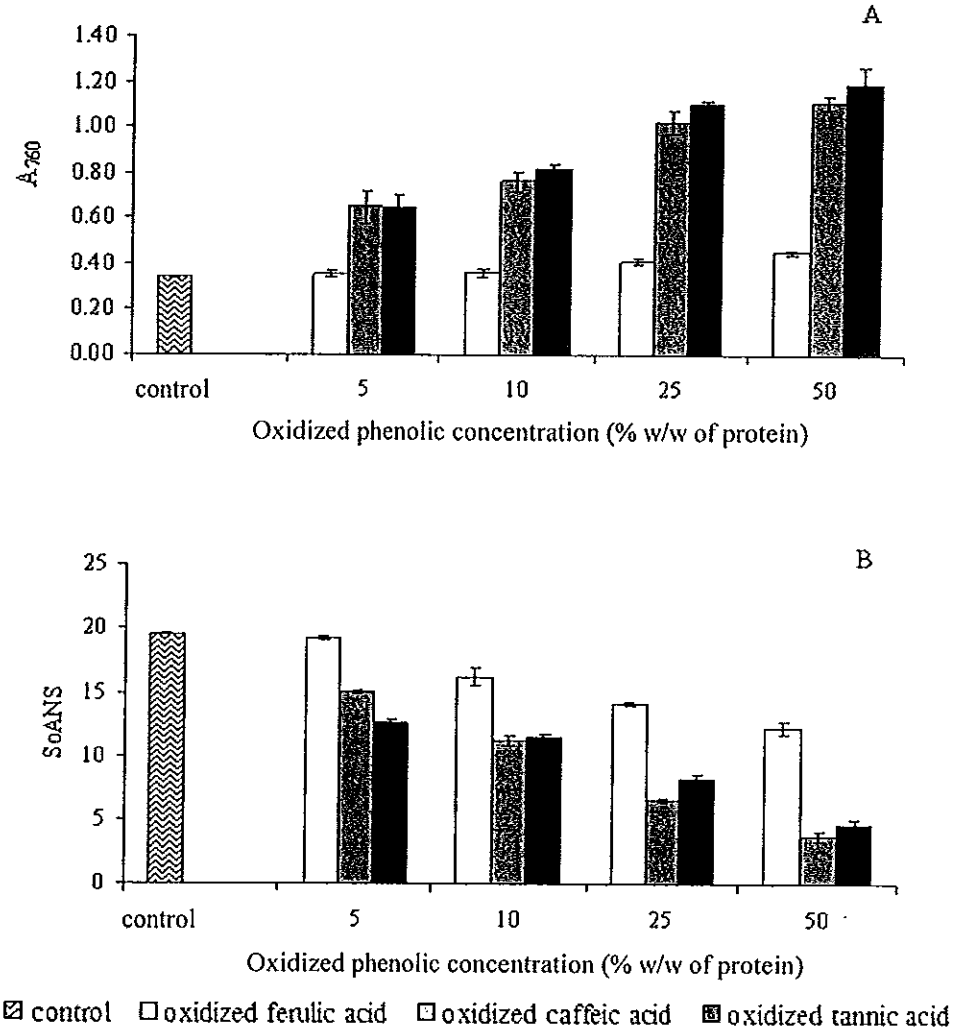


Figure 23. A_{760} (A) and surface hydrophobicity (B) of cuttlefish skin gelatin modified with different oxidized phenolic compounds at various levels. Bars represent the standard deviation (n=3).

Although the loss in free amino groups of gelatin modified with OTA was less than that of gelatin modified by OCA (Table 12), no differences in A_{760} were

observed between gelatin modified with both oxidized compounds. Tannic acid possessed a greater number of hydroxyl groups attached to the aromatic benzene ring as compared to caffeic acid. Those hydroxyl groups were converted to quinone, which were able to interact with amino group of gelatin molecules. During dialysis to remove unbound oxidized phenols, the reduction of free quinone groups to hydroxyl groups of phenolic compound attached to gelatin could probably take place to some degree. The similar number of reduced free hydroxyl groups between gelatin modified by OCA and OTA more likely contributed to similar reducing ability of Folin-Ciocalteu reagent, as indicated by the similar A_{760} between both modified gelatin. A_{760} observed in the control gelatin solution was more likely due to the presence of amino acid residues including tyrosine, tryptophan, cysteine, histidine and asparagines (Lowry *et al.*, 1951). Those amino acids were able to reduce Folin-Ciocalteu reagent as evidenced by the increase in A_{760}.

3.4.3 Changes in surface hydrophobicity

Changes in surface hydrophobicity of cuttlefish skin gelatin modified with different oxidized phenolic compounds at various levels are shown in Figure 23B. Generally, gelatin modified with oxidized phenolic compounds had a decrease in surface hydrophobicity, compared with the control gelatin ($p<0.05$). The surface hydrophobicity of gelatin modified with all oxidized phenolic compounds decreased with increasing concentrations of compounds used ($p<0.05$). ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as phenylalanine and tryptophan, and can be used to indicate the surface hydrophobicity of protein (Benjakul *et al.*, 1997). The decrease in surface hydrophobicity of resulting gelatin indicated that phenolic compounds bound with gelatin most likely contributed to the increased hydrophilicity at the surface of gelatin molecules. This result was in agreement with Rawel *et al.* (2002a) who reported that the surface hydrophobicity of soy protein decreased when reacted with phenolic compounds. The decrease in surface hydrophobicity of gelatin derivatives was possibly caused by hydroxyl and carboxyl groups of phenolic compounds attached to gelatin. Kroll *et al.* (2003) reported that the covalent attachment of the phenolic compound to proteins causes the blocking of the hydrophilic groups like amino and

thiol groups. On the other hand, there is also an increase in the amount of apolar groups (benzene ring) and polar groups (hydroxyl and carboxyl groups as in the case of the phenolic acids) being introduced. Among all phenolic compounds introduced, gelatin modified with OCA or OTA had the lower surface hydrophobicity than that modified by OFA ($p<0.05$). At the same amount of oxidized phenolic compounds introduced, gelatin modified with OTA and OCA exhibited the similar surface hydrophobicity ($p>0.05$). Thus, the introduction of oxidized phenolic compounds resulted in the altered hydrophobicity of resulting gelatin.

3.4.4 Changes in emulsifying properties

Emulsion activity index (EAI) of cuttlefish skin gelatin modified with oxidized phenolic compounds at various levels expressed as the turbidity of emulsion at wavelength of 500 nm is shown in Table 12. At the low level of oxidized phenolic compounds introduced (5 wt% of protein), gelatins modified with OCA showed the decrease in EAI both at 0 and 10 min when compared with the control ($p<0.05$). Nevertheless, no change in EAI of gelatin modified with 5% of OTA and OFA was observed ($p>0.05$). However, at the concentration of oxidized phenolic compounds introduced above 5% (w/w of protein), EAI of gelatin modified with all oxidized phenolic compounds decreased for both at 0 and 10 min. The decrease was more pronounced as the concentration of oxidized phenolic compounds increased. This was possibly associated with the decreases in surface hydrophobicity of gelatin modified with oxidized phenolic. Kato and Nakai (1980) reported that surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved. The decrease in surface hydrophobicity of gelatin modified with oxidized phenolic compound might lower the ability of gelatin to localize at the oil-water interface. Moreover, interactions of oxidized phenolic compounds with gelatin probably led to the aggregation of gelatin molecules. As result, gelatin might not unfold at the oil-water interface and form the film around the oil droplet effectively (McClements, 2004; Rawel *et al.*, 2002b). However, the improvement of foam and emulsion stability of proteins by phenolic compound has been reported (Viljanen *et al.*, 2005). The addition of blackberry and raspberry juices in oil-in-water emulsion using whey

proteins as emulsifier resulted in the enhanced stabilization of the interface formed during emulsification. Sarker *et al.* (1995) reported that (+)-catechin at 0.1 molar ratio could improve foam formation and stability of Tween 20/β-lactoglobulin mixed system, because of (+)-catechin-induced cross-linking of proteins in the adsorbed layer. Therefore, the incorporation of oxidized phenolic compounds into gelatin to a high level showed the detrimental effect on emulsifying property of modified gelatin.

3.4.5 Changes in antioxidative activities

3.4.5.1 DPPH radical scavenging activity

DPPH radical scavenging activities of gelatin modified with different oxidized phenolic compounds at various levels are depicted in Figure 24A. DPPH radical scavenging activity of the control gelatin was 12.49 μmol TE/ mg protein. When gelatin was incorporated with 5% OFA, OCA and OTA, the activity was increased to 48.76, 70.11 and 81.94 μmol TE/mg protein, respectively. These results indicated that phenolic compounds introduced to gelatin contributed to the increased antioxidative activity of gelatin. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability (Binsan *et al.*, 2008). The increase in DPPH radical scavenging activity was observed in all gelatins modified with all oxidized phenolic compounds when the higher concentrations of oxidized phenolic compound were used ($p<0.05$). The maximum activity was observed when gelatin was modified with 50% oxidized phenolic compound ($p<0.05$). This was in agreement with Rohn *et al.* (2004) who found that the antioxidative activity of bovine serum albumin (BSA) derivatized by covalent attachment of quercetin increased with increasing concentration of quercetin.

At the same concentration of oxidized phenolic compounds used, gelatin modified with OTA had the highest DPPH radical scavenging activity, followed by those modified with OCA and OFA, respectively. The result revealed that DPPH radical scavenging activity of gelatin modified by oxidized phenolic compounds was governed by the type of phenolic compound used and the degree of incorporation of phenolic compounds into gelatin. The result indicated that free quinones of oxidized phenolic compounds were mostly reduced to hydroxyl groups. Those hydroxyl groups could donate hydrogen to DPPH radicals.

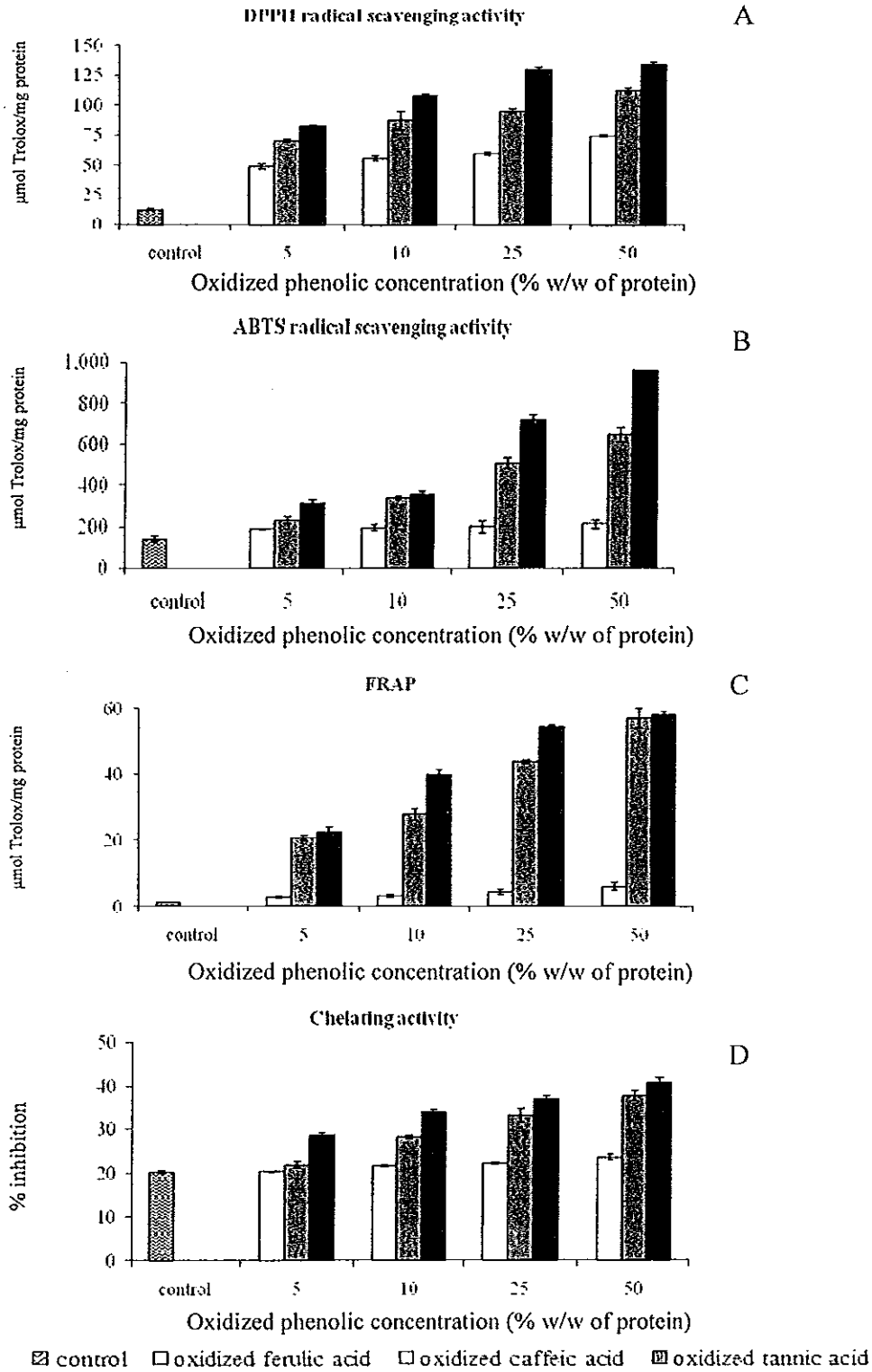


Figure 24. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), ferric reducing antioxidant power (FRAP) (C) and chelating activity (D) of cuttlefish skin gelatin modified with different oxidized phenolic compounds at various levels. Bars represent the standard deviation from triplicate determinations.

3.4.5.2 ABTS radical scavenging activity

Gelatin modified with different oxidized phenolic compounds showed different ABTS radical scavenging activities (Figure 24B). Similar result was found when comparing with DPPH radical scavenging activity (Figure 24A). The gelatin modified with OTA and OCA had the increase in ABTS radical scavenging activities with increasing concentration used. This result suggested that tannic acid and caffeic acid attached with gelatin could scavenge the radicals, forming more stable product, thereby terminating the radical chain reaction. Nevertheless, gelatin modified with OFA had no changes in ABTS radical scavenging activity even when higher level of OFA was used. The result suggested that ferulic acid attached to gelatin might not be able to quench ABTS radicals. ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Binsan *et al.*, 2008). It reconfirmed that radical scavenging activity of gelatin was determined by the type of phenolic compounds attached.

3.4.5.3 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP), generally measures the reducing capacity of ferric ion and has been correlated to the radical scavenging capacity (Moure *et al.*, 2006). FRAP of gelatin modified with different oxidized phenolic compounds at various levels is shown in Figure 24C. FRAP of gelatin modified with OFA, OCA and OTA at a level of 5% was 2.80, 20.67 and 22.27 μmol TE/mg protein, respectively, which was higher than that of the control gelatin (1.32 μmol TE/mg protein). As the concentration of oxidized phenolic compounds increased, FRAP activities increased ($p<0.05$). Marked increase in FRAP of gelatin was observed in gelatin modified with OCA and OTA, in comparison with the control gelatin, especially at high concentration used. It was noted that FRAP of gelatin modified with OFA slightly increased. The antioxidative activity of phenolic acids depends on the number and position of hydroxyl groups bound to the aromatic ring (Sroka and Cisowski, 2003). The result generally was in agreement with those of DPPH and ABTS radical scavenging activities (Figures 24A and 24B).

3.4.5.4 Chelating activity

The ability of gelatin modified with different oxidized phenolic compounds at different levels in metal chelating is depicted in Figure 24D. Generally,

the metal chelating activity of gelatin increased as the concentration of oxidized phenolic compounds increased ($p<0.05$). At the same level of oxidized compounds introduced, gelatin modified with OTA exhibited the highest metal chelating activity, followed by those modified by OCA and OFA, respectively. Ferrous ion (Fe^{2+}) is the most powerful prooxidant among various species of metal ions, which can catalyze the generation of reactive oxygen species, hydroxyl radical ($OH^{\bullet}$), by which the lipid peroxidation chain reaction is accelerated (Stohs and Bagchi, 1995). Thus gelatin attached with phenolic compounds had the enhanced ability in metal chelation, in which prooxidation metals can be sequestered, leading to the lowered oxidation.

3.4.6 Changes in FTIR spectra

Due to the high antioxidative activity of gelatin modified with 5% OTA without any change in EAI, gelatin modified with 5% OTA was characterized by FTIR. FTIR spectroscopy at 4,000 to 1,000 cm^{-1} was used to monitor the changes in the secondary structure of gelatin with and without the modification with 5% OTA (Figure 25A). A spectra of cuttlefish skin gelatin displayed the major bands at 3278 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), 1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^{-}), 1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching) and 1236 cm^{-1} (amide III, representative of NH bending). Similar spectra were observed between gelatin with and without modification with 5% OTA. However, the decrease in the amplitude of amide A, I, II and III bands was observed in gelatin modified with 5% OTA. These changes are indicative of greater disorder (Friess and Lee, 1996) in gelatin and are associated with loss of triple helix state (Muyonga *et al.*, 2004b). The shift to lower wavenumber of amide I, II and III peaks was observed from 1637, 1541 and 1238 cm^{-1} of the control gelatin to 1635, 1535 and 1236 cm^{-1} of gelatin modified with 5% OTA, respectively. A shift of peaks to lower wavenumbers is associated with a lower molecular order (Payne and Veis, 1988). Muyonga *et al.* (2004b) reported that the amide I and II peak of collagen extracted from adult Nile perch was at a higher frequency than the young fish skin collagen, due to more intermolecular cross-links in the adult fish collagen (Muyonga *et al.*, 2004b). On the other hand, the shift to higher wavenumber of amide A peaks from 3278 cm^{-1}

to 3296 cm^{-1} was observed when gelatin was modified with 5% OTA. The peaks at wavenumber of 3400-3300 cm^{-1} were attributable to the OH stretch of alcohols and phenols (Smith, 1999).

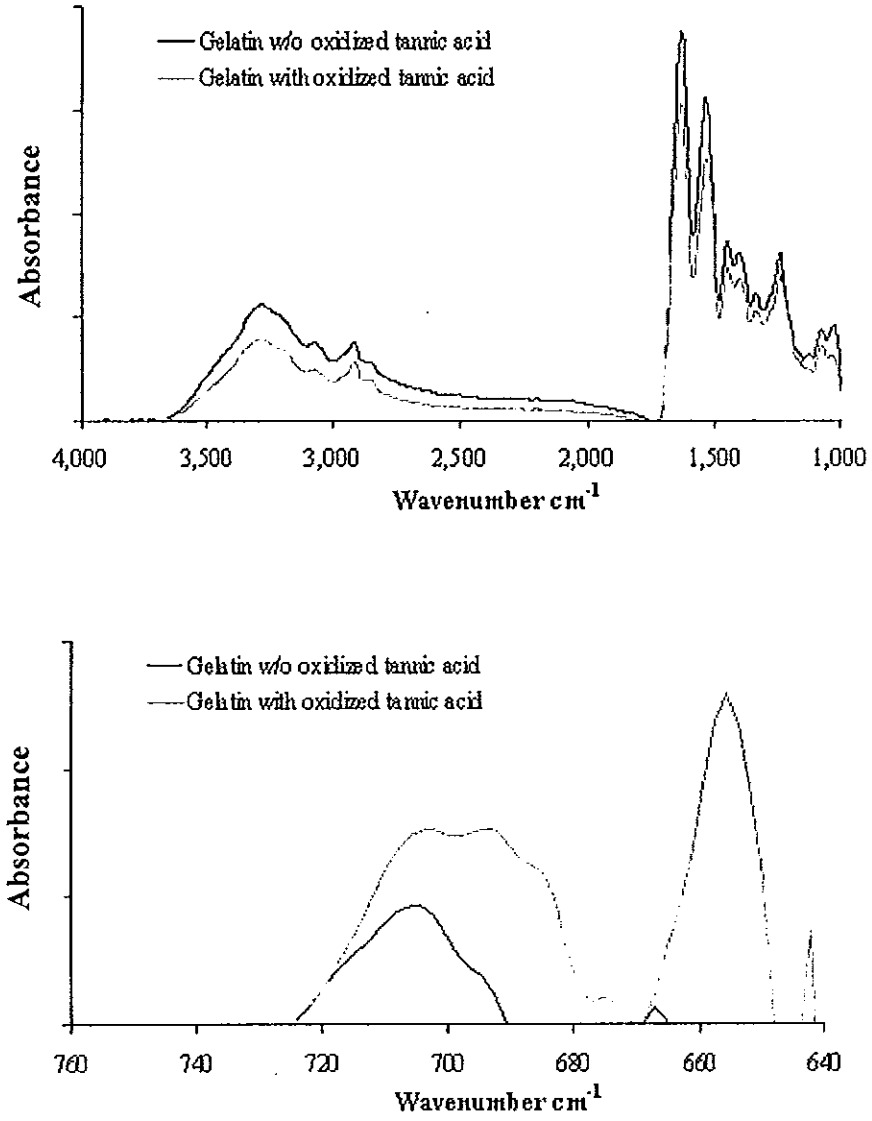


Figure 25. Fourier transform infrared (FTIR) spectra in wavenumber range of 4,000-1,000 cm^{-1} (A) and 760-640 cm^{-1} (B) of gelatin modified with 5% OTA.

The result indicated the incorporation of tannic acid with OH group into gelatin molecule. Moreover, the obvious increases in amplitude of peak at wavenumber 1080 cm^{-1} was found in gelatin modified with 5% OTA, suggesting the stretching of the C-O bonds in secondary and tertiary alcohols (Smith, 1999) in modified gelatin. These results suggested that OTA might induce the changes in secondary structure and functional groups of resulting gelatin.

The bands situated at 720 to 680 cm^{-1} and 655 cm^{-1} were observed in gelatin modified with 5% OTA, while the band with wavenumber of 720 to 690 cm^{-1} was found in the control gelatin. (Figure 25B). The wavenumber at ~690±10 and ~655 cm^{-1} were attributable to the bending of the C-C bonds in the aromatic and the out of plane bending of OH group in phenol, respectively (Smith, 1999). This result indicated that OTA could bind with the gelatin molecules and the unbound quinone groups were more likely reduced during dialysis as indicated by the additional band of OH group in phenol found in gelatin modified by 5% OTA. Thus the modification could introduce the aromatic ring and hydroxyl group in gelatin.

3.4.7 Effect of gelatin modified with 5% OTA on emulsion stability and lipid oxidation of menhaden oil-in-water emulsion

3.4.7.1 Zeta potential and particle size of emulsion

When tested at pH 7, zeta potential of menhaden oil emulsion droplets stabilized by control gelatin (without modification) and gelatin modified with 5% OTA was -38.98±0.77 and -32.16±1.2 mV, respectively (Table 13). The negative charge of the emulsion droplets might be due to the negatively charged amino acid in gelatin surrounding the oil droplet. At pH higher than pI, the carboxyl groups are negatively charged ($-COO^-$), whereas most of the amino groups are neutral ($-NH_2$) (Onsaard *et al.*, 2006). The emulsion droplets were covered by a biopolymer with an appreciable electrical charge. Electrostatic repulsion may play an important role in stabilizing them against droplet aggregation (McClements, 2005). The lower negative charge was observed in emulsion stabilized by gelatin modified with 5% OTA, when compared with that of emulsion stability by control gelatin. These differences might be due to the differences in the number of ionizable amino acids in the gelatin. It was suggested that the occupation of amino groups by OTA might introduce the positive

charge of phenolic compound to gelatin. As a consequence, the lower negatively charged complex was obtained. Moreover, hydroxyl group of tannic acid might interact with carboxyl groups of protein via hydrogen bond, leading to a loss of negative charge of the resulting complex.

Table 13. Particle size and zeta potential of menhaden oil-in-water emulsion stabilized by 0.5 and 1.0 % gelatin with and without the modification with 5% OTA at pH 7

Gelatin	Concentration (% protein)	Particle size (μm)	Zeta potential (mV)
Control (unmodified)	0.5	2.24 ± 0.19c*	-
	1.0	0.33 ± 0.02a	-38.98 ± 0.77a
Gelatin modified with 5% OTA	0.5	2.65 ± 0.09d	-
	1.0	0.37 ± 0.02b	-32.16 ± 1.2b

Mean±SD (n=3)

* Different letters in the same column indicate significant differences ($p<0.05$).

Particle diameter of oil droplets of 10% menhaden oil-in-water emulsion stabilized by 0.5 and 1.0% gelatin modified without and with 5% OTA is shown in Table 13. Particle size of emulsion stabilized by 0.5 and 1.0% control gelatin was 2.24±0.19 and 0.33±0.02 μm, respectively, whereas that of emulsion stabilized by gelatin modified with 5% OTA was 2.65±0.09 and 0.37±0.02 μm, respectively. Droplet size of emulsion decreased markedly when the higher concentration of gelatin either with or without modification was used. The sufficient amount of proteins at oil-water interface is required to cover oil droplet completely. This result was in agreement with Surh *et al.* (2005) who found that increase in fish gelatin concentration from 0.5 to 4.0% protein could increase the fraction of small droplets and decrease the fraction of large particles. When protein amount is limited, there is no longer sufficient protein to fully stabilize the droplet interface, and therefore larger particles may be formed as a result of coalescence or bridging flocculation. The increase in protein concentration enhanced protein adsorption and surface coverage of oil droplets, which effectively inhibited droplet aggregation or coalescence (Sun and Gunasekaran, 2009). The larger particle size was observed in

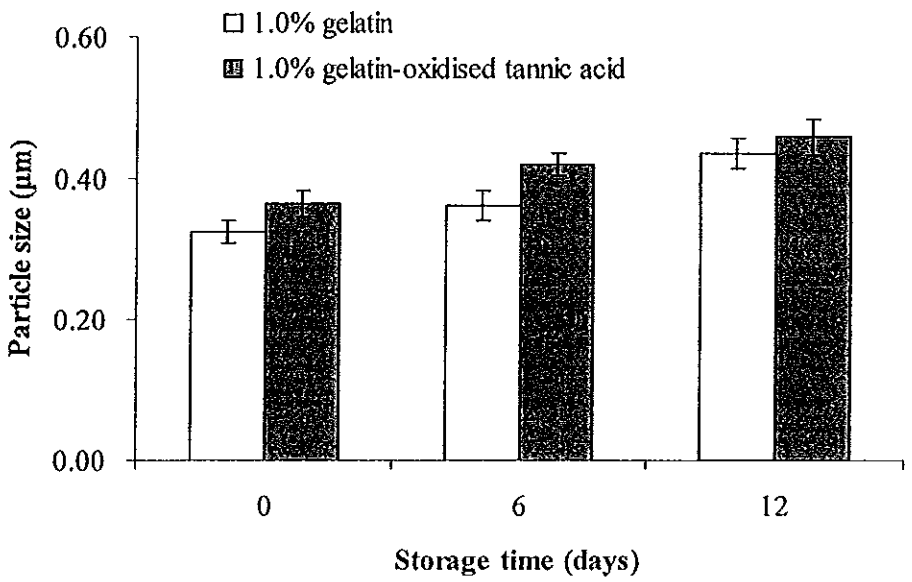
emulsion stabilized by gelatin modified with 5% OTA when compared with that found in emulsion stabilized by the control gelatin. The decreased surface hydrophobicity might not favor the migration of gelatin modified by 5% OTA to the oil droplets, which were lipophilic. Thus, the amount of modified gelatin occupied at oil droplet might be slightly lower. This led to the lower emulsifying property of modified gelatin.

3.4.7.2 Changes in particle size of emulsion during storage

Changes in particle size of emulsions stabilized by 1.0% gelatin and gelatin modified with 5% OTA during storage at room temperature for 12 days are shown in Figure 26A. Particle size of both emulsions gradually increased with increasing storage time. For emulsion stabilized by control gelatin, the particle size increased from 0.33±0.02 to 0.44±0.02 μm within 12 days of storage, whereas that of emulsion stabilized by gelatin modified with 5% OTA increased from 0.37±0.02 to 0.46±0.03 μm. This result might be due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during storage (Djordjevic *et al.*, 2008). At day 12 of storage, no difference in size of oil droplet was noticeable between both emulsions though the smaller size of droplet was found in emulsion stabilized by control gelatin at day 0. This indicated that gelatin modified by 5% OTA yielded the similar emulsion stability to gelatin without modification at the extended storage.

3.4.7.3 Changes in lipid oxidation of emulsion during storage

Lipid oxidation of menhaden oil-in-water emulsion stabilized with gelatin modified with and without 5% OTA was monitored and expressed as TBARS values during the storage of 12 days (Figure 26B). Generally, no TBARS were found in the oil-in-water emulsion within the first 2 days of storage. Thereafter, the marked increase in TBARS was observed, especially in emulsion stabilized by control gelatin ($p<0.05$). Emulsion stabilized by gelatin modified with 5% OTA had the lower TBARS ($p<0.05$). This result was in accordance with the antioxidative activity of gelatin modified with OTA (Figure 24).



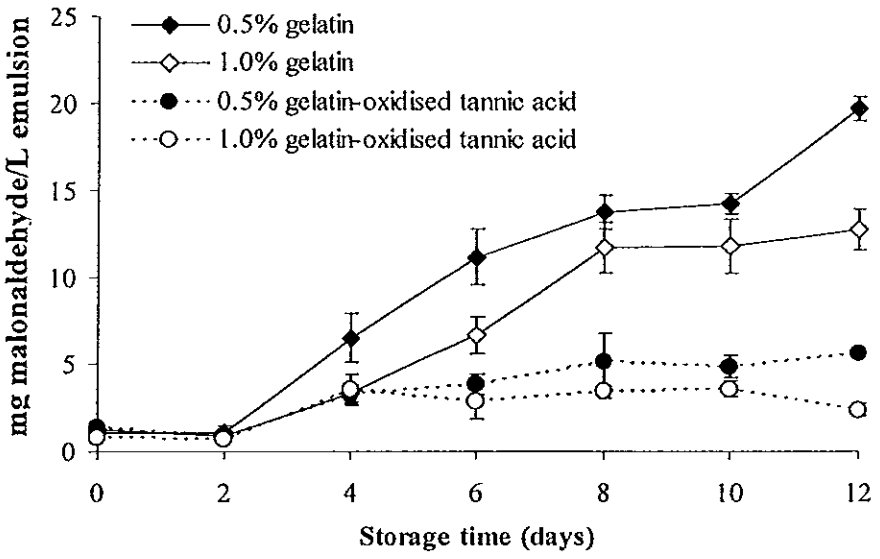


Figure 26. Particle size (A) and TBARS value (B) of menhaden oil-in-water emulsion stabilized by 0.5 and 1.0% gelatin with and without the modification with 5% OTA during storage at room temperature for 12 days. Bars represent the standard deviation from triplicate determinations.

Antioxidative activity of gelatin modified with or without 5% OTA increased with increasing concentration used. Additionally, preventive effect of gelatin at the higher level used on lipid oxidation might be associated with the thicker film around the oil droplet. This could prevent the penetration of oxygen into oil droplet more effectively. Therefore, gelatin modified with 5% OTA had ability to retard TBARS formation in oil-in-water emulsion system. This result was in agreement with Almajano and Gordon (2004) who reported that interaction of BSA and epigallocatechin gallate (EGCG) could induce the formation of a BSA-antioxidant adduct and cause a synergistic increase in the oxidative stability of sunflower oil-in-water emulsion effectively, when compared with BSA or EGCG alone.

3.5 Conclusion

Attachment of 5% oxidized tannic acid (OTA) to cuttlefish skin gelatin enhanced antioxidative activity with no detrimental effect on emulsifying properties of resulting gelatin. Use of gelatin modified with 5% OTA could inhibit lipid oxidation of menhaden oil-in-water emulsion effectively. The efficacy was dose dependent. Therefore, cuttlefish skin gelatin modified with 5% OTA could be used as emulsifier possessing antioxidative activity in emulsion systems.

CHAPTER 4

Antioxidative activity and emulsifying properties of cuttlefish skin gelatin-tannic acid complex as influenced by types of interaction

4.1 Abstract

The non-covalent interaction between cuttlefish skin gelatin and tannic acid was observed in gelatin modified with unoxidized tannic acid (TA) at pH 7, whereas covalent interaction was found in gelatin modified with oxidized tannic acid (OTA) at pH 9. Degree of tannic acid incorporation into gelatin via non-covalent interaction was more pronounced than that found via covalent interaction as evidenced by lowered free amino group content and increased total phenolic content and hydroxyl group and aromatic ring determined by FTIR. Gelatin modified with OTA had the slight decrease in surface hydrophobicity, with no changes in particle size distribution of the emulsions. Modification of gelatin with tannic acid, especially via non-covalent interaction, increased *in vitro* antioxidative activity, compared with the control gelatin. Gelatin modified with tannic acid via covalent interaction rendered the emulsion with high stability and could inhibit lipid oxidation of menhaden oil-in-water emulsion effectively throughout the storage of 12 days.

4.2 Introduction

Many food products containing lipids are commonly found in emulsion forms either as water-in-oil, for example, butter and margarine, or oil-in-water, for example, mayonnaise, milk and cream. Lipid oxidation negatively affects the quality of foods, especially emulsion type products by altering appearance, odor, flavor, shelf-life and nutritional value. This results in unacceptability by consumers (2006). To retard or prevent such changes, antioxidants are increasingly used to control lipid oxidation in emulsions. Due to the anxiety of possible toxicity of synthetic

antioxidants, the natural antioxidants have been paid increasing attention among the consumers. Polyphenol compounds have received attention as antioxidant agents in food products. The antioxidant properties of phenolics are mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al.*, 1997). Additionally, these phenolic compounds can bind metal ions and scavenge the radicals (Moure *et al.*, 2006). Nevertheless, phenolic compounds with higher antioxidative activity generally consist of the higher number of hydroxyl group in structure (Prasad *et al.*, 2005). These polar antioxidants are sparingly soluble in oil. In oil-in-water emulsions system, polar antioxidants readily partition into the aqueous phase, decreasing their concentration in the lipid phase and thus lowering their capability to prevent oxidation (Yuji *et al.*, 2007). However, the effectiveness of polar antioxidant in oil-in-water emulsions can be improved by increasing their surface activity and ability to accumulate at the oil-water interface where oxidative reactions take place (Yuji *et al.*, 2007).

Proteins are surface/interfacial active and are widely used as emulsifiers in foods to produce oil-in-water emulsions with desirable physicochemical properties and improved stability (McClements, 2004). They can adsorb on oil-water interfaces and various hydrophobic segments penetrate into the oil phase. To improve the amphiphilic property of protein, the appropriate modification by incorporating the phenolic compound could be a potential means to obtain the modified protein with the higher emulsifying property as well as antioxidant activity. A protein-phenolic complex is concentrated at the oil-water interface, yielding the surface active nature of the protein and antioxidative activity of phenolic compounds (Almajano and Gordon, 2004). The interaction between proteins and phenolic compound may occur through non-covalent interaction (such as hydrogen bonding and hydrophobic interaction) or covalent interaction (Kroll *et al.*, 2003).

Cuttlefish is one of major seafood products of Thailand. During processing, the skin was removed and become the by-product with the low market value. In general, it has been used as the animal feed. Recently, Aewsiri *et al.* (2009a) extracted the gelatin from cuttlefish. However, it showed the low bloom strength. To exploit the gelatin from cuttlefish shin, it has been used as the emulsifying agent after modification with some selected phenolic compounds (Aewsiri *et al.*, 2009b). The

incorporation of oxidized phenolic compounds especially tannic acid in gelatin from cuttlefish skin can increase antioxidative activity and emulsifying properties of resulting gelatin. Tannic acid is affirmed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) for the use as a direct additive in some food products such as baked goods and baking mixes, alcoholic and non-alcoholic beverages, frozen dairy desserts and mixes, hard candy and cough drop as well as meat products (21 CFR184. 1097, US Code of Federal Regulation, 2006). Tannin contains sufficient hydroxyls and other groups such as carboxyls to form strong complexes with the proteins and other macromolecules (Kroll *et al.*, 2003). Nevertheless, a little information regarding the type of interaction between gelatin and tannic acid and its impact on stability and lipid oxidation of emulsion system has been reported. Therefore, the objective of this study was to investigate the influence of the modification of cuttlefish skin gelatin with tannic acid via covalent and non-covalent interaction on antioxidative activity and emulsifying properties of resultant gelatin.

4.3 Materials and methods

4.3.1 Chemicals

Tannic acid, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyl-triazine (TPTZ), Trolox and menhaden (*Brevoortia tyrannus*) oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Folin-Ciocalteu's phenol reagent, sodium sulfite and ferric chloride ($FeCl_3$) were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

4.3.2 Preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from the dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla

University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20°C until use. Storage time was not longer than 2 months.

4.3.3 Extraction of gelatin from cuttlefish skin

Cuttlefish skin gelatin was prepared according to the method of Aewsiri *et al.* (2009b). Skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28°C). The alkaline solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5% H_2O_2 at 4°C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (ScanVac Model CoolSafe 55-4, Lynge, Denmark).

4.3.4 Modification of cuttlefish skin gelatin by tannic acid

Cuttlefish skin gelatin was modified with tannic acid using different conditions to obtain gelatin-tannic acid complexes via covalent or non-covalent interactions. For covalent interaction, the gelatin was modified by OTA at pH 9. The gelatin was dissolved in distilled water containing 0.02 % sodium azide (NaN_3) to obtain a final concentration of 1.2 % protein (w/v). The pH of gelatin solution was adjusted to 9 using 1 M NaOH. To prepare OTA, tannic acid was dissolved in distilled water to obtain the concentration of 2% (w/v), followed by pH adjustment to 9 with 1 M NaOH. Solution was then bubbled with oxygen at 40°C for 1 h to convert tannic acid into OTA, an oxidized form. To 75 ml of gelatin solution, the solution of OTA (2.25 mL) was added to obtain the final concentration of 5% (based on protein content). Final volume was raised to 90 mL using distilled water (pH 9), in which a final protein concentration of 1% (w/v) was obtained. The mixtures were stirred

continuously using a magnetic stirrer (IKAMAG® model RO 10 power, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 200 rpm at room temperature for 12 h. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 24 h at room temperature (26-28°C) against 20 volumes of water to remove free phenolic compounds (unbound to proteins).

For non-covalent interaction, the gelatin was modified by TA at different pHs (pH 7 and 9). The reaction solutions were prepared as previously described but tannic acid solution was not converted into OTA. Both gelatin and TA solutions were adjusted to pHs 7 or 9 prior to mixing together thoroughly as previously described. The control gelatin was prepared in the same manner except that either TA or OTA was excluded. Modified gelatin and the control gelatin were subjected to analyses.

4.3.5 Determination of incorporation of tannic acid into gelatin

Modified samples (1% of protein, 18 mL) either via covalent or non-covalent interaction were added with 2 ml of 10% sodium dodecyl sulfate (SDS) solution to obtain the final SDS concentration of 1%. The mixture was stirred gently for 1 h at room temperature to disrupt the non-covalent interaction of gelatin-tannic acid complexes. The untreated sample was prepared in the same manner except the distilled water was added instead of 10% SDS solution. Thereafter, the samples were dialyzed for 24 h against 20 volumes of water to remove unbound TA or OTA and SDS. Subsequently, gelatin attached with tannic acid via covalent bond was retained in the dialysate after treatment with SDS and dialysis. All samples treated without and with SDS were subjected to analyses.

4.3.5.1 Determination of free amino group content

Free amino group content of samples treated without and with SDS was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µL), 2.0 ml of 0.2125 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1

M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm using a UV–vis spectrophotometer (UV-160, Shimadzu, Kyoto, Japan) and free amino group content was expressed in terms of L-leucine.

4.3.5.2. Determination of total phenolic content

TA or OTA incorporated into gelatin was measured in terms of total phenolic content. Total phenolic content of samples treated without and with SDS was measured according to the method of Slinkard and Singleton (Slinkard and Singleton, 1977) with some modifications. Sample solution (1 mL) with an appropriate dilution was mixed with 200 μL of the freshly prepared Folin–Ciocalteu reagent using a vortex mixer (Vortex genie® 2, Scientific Industries, Bohema, NY, USA). After 3 min, 3 mL of sodium carbonate (15%, w/v) were added and the mixture was allowed to stand for 30 min at room temperature. The absorbance at 760 nm was measured using a spectrophotometer. Sample blank was prepared in the same manner except the deionized water was used instead of Folin-Ciocalteu reagent. Total phenolic content was expressed as A_{760} after blank substraction.

4.3.6 Study on some properties and antioxidative activity of modified gelatin

4.3.6.1 Determination of surface hydrophobicity

Surface hydrophobicity (S_0ANS) of gelatin modified with TA or OTA was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (4 mg/ml) was diluted in 10 mM phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5%. The diluted protein solution (2 ml) was mixed with 20 μl of 8 mM ANS in 0.1 M phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm using a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan). The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

4.3.6.2 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of gelatin modified with TA or OTA were obtained using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulfate (DLATGS) detector (Bruker, Ettlingen, Germany). The Horizontal Attenuated Total Reflectance Accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), which was made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–600 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25°C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

4.3.6.3 Determination of antioxidative activities

The antioxidative activities of the solutions of modified gelatins and the control gelatin (1% protein) were determined in comparison with tannic acid solution at a concentration of 0.05% (w/v) using different assays.

4.3.6.3.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan *et al.* (2008) with a slight modification. To the diluted sample (1.5 mL), 1.5 mL of 0.10 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol was added. The mixture was then mixed vigorously using a vortex mixer and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that ethanol was used instead of DPPH solution. The standard curve of Trolox (60-600 µM) was prepared in the same manner. The activity was expressed as µmol Trolox equivalent (TE)/ g protein.

4.3.6.3.2 ABTS radical scavenging activity

ABTS free radical scavenging assay was determined according to the method of Binsan *et al.* (2008) with a slight modification. 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical was generated by reacting 7.4 mM ABTS and potassium persulfate (2.6 mM) at a ratio of 1:1 (v/v) and leaving

at room temperature in the dark for 12 h. ABTS free radical solution was diluted by mixing 1 mL of ABTS solution with 50 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. To 150 µL of sample, 2850 µL of ABTS•+ solution was added and mixed thoroughly using a vortex mixer. The extent of quenching of the ABTS•+ was measured at 734 nm after 2 h incubation at room temperature in the dark. Sample blank was prepared in the same manner except that methanol was used instead of ABTS solution. The standard curve of Trolox (60 – 600 µM) was prepared in the same manner. The activity was expressed as µmol TE/ g protein.

4.3.6.3.3 Ferric reducing antioxidant power (FRAP)

FRAP was determined as described by Binsan *et al.* (2008) with a slight modification. A 2.85 mL of freshly prepared FRAP solution (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution and 25 ml of 300 mM acetate buffer, pH 3.6) was incubated at 37°C for 30 min before mixing with 150 µL of sample. The mixture was allowed to react in the dark at room temperature. Absorbance at 593 nm was measured after 30 min of reaction. Sample blank was prepared by omitting $FeCl_3$ from the FRAP solution and distilled water was used instead. The standard curve of Trolox (60 – 600 µM) was prepared in the same manner. FRAP was expressed as µmol TE/ g protein.

4.3.6.3.4 Determination of Fe^{2+} chelating activity

Chelating activity toward Fe^{2+} was measured by the method of Maqsood and Benjakul (2009) with a slight modification. Diluted sample (4.7 mL) was mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = [(B-A) / B] \times 100$$

where A is A_{562} of sample and B is A_{562} of the blank.

4.3.7 Effect of modified gelatin with different types of interaction on emulsion stability and lipid oxidation of fish oil-in-water emulsion with and without ferric chloride

Fish oil-in-water emulsions were prepared by homogenizing the mixture of menhaden oil and 1% (w/v) modified gelatin solution (1:9, v/v) at a speed of 10,000 rpm for 2 min. These coarse emulsions were then passed through a two-stage high-pressure valve homogenizer (M-110P, Microfluidizer, Newton, MA, USA) at 10,000 psi for 2 times. NaN_3 (0.02 %) was added to the emulsions as an antimicrobial agent. The control oil-in-water emulsion was prepared in the same manner and the control gelatin (without modification) was used. Emulsion containing 0.05% tannic acid (w/v) and 1% tween 40 was also prepared. All samples were then stored in the absence and presence of 50 μM ferric chloride ($FeCl_3$) at room temperature (28-30°C) for 12 days. The samples were taken every two days for analyses.

4.3.7.1. Measurement of zeta potential and particle size

Zeta potential and particle size of emulsions were determined with ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) at room temperature. The instrument used electrophoretic light scattering and the laser doppler velocimetry method to measure the zeta potential and number average distribution of particle sizes. Prior to analysis, the samples were adjusted to pH 7 and 1,000-fold diluted with 10 mM KCl solution to avoid multiple scattering effects. The particle size was reported as surface-volume mean particle diameter, $d_{43} (= \sum n_i d_i^4 / \sum n_i d_i^3)$, where d_i is the diameter and n_i is the number of particles in the i^{th} size class.

4.3.7.2 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Two ml of emulsion sample were dispersed in 10 ml of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 x g for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The

standard curve was prepared using malonaldehyde (2-10 ppm) and TBARS were expressed as mg malonaldehyde/ L of emulsion.

4.3.8 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

4.3.9 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

4.4 Results and discussion

4.4.1 Incorporation of tannic acid via covalent and non-covalent interaction

4.4.1.1 Changes in free amino group content of gelatin

Free amino group contents of cuttlefish skin gelatin modified with TA and OTA after the treatments without and with SDS are shown in Table 14. In general, modified gelatin had a lower free amino group content than the control gelatin ($p<0.05$). The result indicated that both TA and OTA could interact with gelatin, mainly via amino groups. A reaction of protein with phenolic compounds led to a decrease in free amino group content (Kroll *et al.*, 2003). Without SDS treatment, a lower free amino group content was observed in gelatin modified with TA at pH 7 when compared with gelatin modified with OTA or TA at pH 9 ($p<0.05$). However, after treatment with SDS, free amino group content of gelatin modified with TA at both pH 7 and 9 markedly increased. SDS is a denaturant, which destroys non-covalent protein interaction such as hydrogen bond and some hydrophobic interaction (Hamada, 1992). The result suggested that non-covalent interaction seemed to play an important role in modification of gelatin with TA at both pH 7 and 9. The non-covalent interaction might occur between TA and different functional groups of

gelatin by hydrogen bond and by hydrophobic interaction. The hydroxyl groups of tannic acid are hydrogen donors, which form hydrogen bond with the carbonyl groups in the protein as hydrogen acceptors (Simon *et al.*, 2003). Moreover, hydrophobic interactions could take place between the hydrophobic domains of aromatic rings of tannic acid and the hydrophobic side chains of amino acids in the protein (Charlton *et al.*, 2002).

A larger difference in free amino group content was observed in gelatin modified with TA at pH 7 before and after treatment with SDS in comparison with that modified with TA at pH 9. This suggested that the non-covalent interaction might be the major contributor in gelatin modified with TA at pH 7, whereas both covalent and non-covalent interaction were more likely involved in gelatin modified with TA at pH 9. Phenolic compound was easily oxidized to its corresponding quinone in an alkaline solution (Kroll *et al.*, 2003). Although TA was not subjected to oxidation by oxygen bubbling, it might undergo oxidation under alkaline condition (pH 9) used for gelatin modification. Nevertheless, no change in free amino acid content was observed in gelatin modified with OTA at pH 9 before and after SDS treatment. This result suggested that OTA or quinone, an electrophilic agent, might cross-link with nucleophilic amino groups of lysine through covalent interaction. Covalent bonds were reported to involve in the formation of SDS stable protein-phenolic complexes (Chen and Hagerman, 2004). The degree of loss in free amino group of gelatin depended on type of interaction between gelatin and tannic acid. Gelatin modified via non covalent interactions generally showed a higher loss in free amino groups than that modified through covalent interactions ($p<0.05$). This suggested that hydroxyl group or aromatic ring of TA might be more reactive toward gelatin molecules than electrophilic quinone of OTA as evidenced by the higher loss in amino group of the former.

Table 14. Free amino group content and total phenolic content of cuttlefish skin gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9

Gelatin	Free amino group content(%)		Total phenolic content	
	Without SDS	With SDS[a]	Without SDS	With SDS
Control (unmodified)	100.00 ± 0.00c*A**	100.00 ± 0.00cA	0.12 ± 0.01aA	0.12 ± 0.01aA
Modified with TA at pH 7	81.16 ± 1.99aA	98.82 ± 2.11bcB	0.38 ± 0.00dB	0.17 ± 0.02bA
Modified with TA at pH 9	89.86 ± 3.65bA	97.10 ± 0.42bB	0.33 ± 0.00cB	0.21 ± 0.03cA
Modified with OTA at pH 9	92.69 ± 1.39bA	92.92 ± 2.11aA	0.25 ± 0.00bA	0.25 ± 0.01cA

Mean±SD (n=3).

[a] Sample was treated with 1% SDS, followed by dialysis.

* Different letters in the same column indicate significant differences ($p<0.05$).

**Different capital letters in the same row within the same parameter indicated significant differences ($p<0.05$).

4.4.1.2 Changes in total phenolic content

Total phenolic contents of cuttlefish skin gelatin modified with TA and OTA before and after SDS treatment determined by Folin–Ciocalteu method are expressed as A_{760} (Table 14). Modified gelatin exhibited higher A_{760} than the control gelatin ($p<0.05$). This result indicated that both TA and OTA could incorporate into gelatin to some extent. Without SDS treatment, the gelatin modified with TA had the higher total phenolic content than that modified with OTA ($p<0.05$). The highest total phenolic content was observed in gelatin modified with TA at pH 7. This result reconfirmed that hydroxyl group or aromatic ring of TA might be able to interact with gelatin molecule more effectively than quinone which reacted mainly with nucleophilic groups of gelatin. For gelatin modified with TA at pH 9, the phenolic content was slightly lower than that modified by TA at pH 7 ($p<0.05$).

After SDS treatment, the decrease in total phenolic content was noticeable in gelatin modified with TA at both pH 7 and 9. SDS might disrupt non-covalent interaction of gelatin modified with TA, resulting in the loss in tannic acid previously incorporated. However, no change in total phenolic content was observed in gelatin modified with OTA. This result reconfirmed that gelatin modified with OTA was governed by covalent interaction. A_{760} observed in the control gelatin was more likely due to the presence of amino acid residues including tyrosine, tryptophan, cysteine, histidine and asparagines (Lowry *et al.*, 1951). Those amino acids were able to reduce Folin-Ciocalteu reagent. Thus A_{760} could represent tannic acid, either oxidized (OTA) or unoxidized (TA) form, incorporated into gelatin.

4.4.2 Changes in some properties and antioxidative activity of modified gelatin

4.4.2.1 Changes in surface hydrophobicity

Surface hydrophobicity of cuttlefish skin gelatin modified with TA and OTA is shown in Figure 27. A marked decrease in surface hydrophobicity was observed in both gelatins modified with TA and OTA, compared with the control gelatin ($p<0.05$). Tannic acid incorporated more likely induced the conformational changes of cuttlefish skin gelatin to some extent. ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as

phenylalanine and tryptophan, and can be used to indicate the surface hydrophobicity of proteins (Benjakul *et al.*, 1997). The decrease in surface hydrophobicity of the resulting gelatin indicated that tannic acid bound with gelatin most likely contributed to the increased hydrophilicity at the surface of gelatin molecules, possibly caused by hydroxyl and carboxyl groups of phenolic compounds attached to gelatin. This result was in agreement with Rawel *et al.* (2002) who reported that the surface hydrophobicity of soy protein decreased when reacted with phenolic compounds. The surface hydrophobicity was noticeably lower in gelatin modified by TA at both pH 7 and 9, in comparison with gelatin modified by OTA (pH 9). It indicated that non-covalent interaction played an important role in the reduction of surface hydrophobicity of gelatin. Hydrophobic region or aromatic nuclei of tannic acid in unoxidized form might react with hydrophobic amino acids of gelatin such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues through hydrophobic interaction. This led to the higher loss in hydrophobic group on surface of protein.

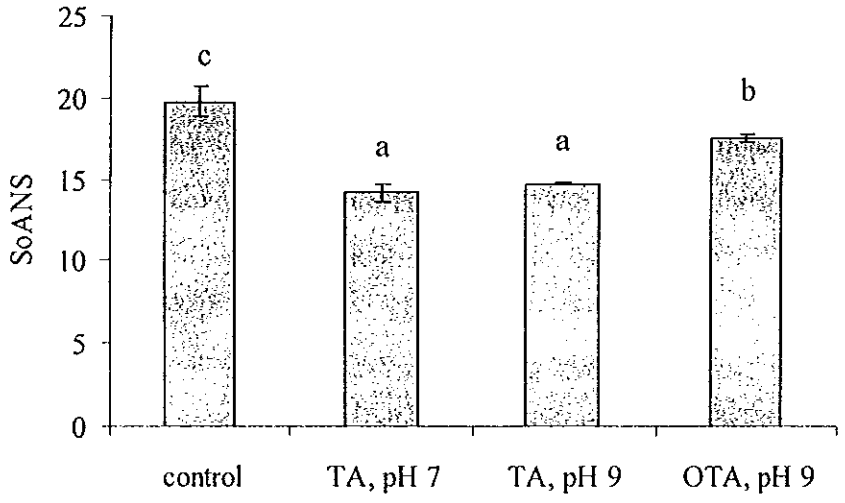


Figure 27. Surface hydrophobicity of cuttlefish skin gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9. Bars represent the standard deviation (n=3). Different letters on the bars denote the significant differences ($p<0.05$).

4.4.2.2 Changes in antioxidative activities

4.4.2.2.1 DPPH radical scavenging activity

DPPH radical scavenging activities of gelatin modified with TA and OTA are depicted in Figure 28A. Generally, DPPH radical scavenging activity of modified gelatin was higher than that of the control gelatin ($p<0.05$). The antioxidative activity of the control gelatin was 10.52 μmol TE/ g protein. When gelatin was modified with OTA (pH 9) or TA at pH 7 and 9, the activity was increased to 84, 94 and 88 μmol TE/g protein, respectively. It indicated that the incorporation of tannic acid, especially TA, into gelatin contribute to the increased antioxidative activity of the resulting gelatin. This result was in agreement with Almajano *et al.* (2007) who reported that radical scavenging activity of BSA, α-lactalbumin and β-lactoglobulin was increased when epigallocatechin gallate (EGCG) was incorporated. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability (Maqsood and Benjakul, 2009). The DPPH radical scavenging activity of modified gelatin was governed by the form of tannic acid used. Gelatin modified with TA exhibited higher DPPH radical scavenging activity than those modified by OTA. This result was associated with the highest degree of incorporation of tannic acid in gelatin modified with TA. Furthermore, hydroxyl groups of tannic acid incorporated could donate hydrogen to DPPH radicals. At the same concentration of tannic acid used, tannic acid in free form exhibited a higher antioxidative activity, compared with gelatin-tannic acid complexes. This was in accordance with Arts *et al.* (2002) who found that the addition of catechin to β-casein increased the antioxidant capacity of the β-casein solution, but the increase was smaller than the antioxidant capacity of catechin itself. The formation of gelatin-tannic acid complexes through covalent and non covalent interaction might lead to the loss of phenolic hydroxyl groups of tannic acid, thereby losing hydrogen donor ability, compared with free tannic acid.

4.4.2.2.2 ABTS radical scavenging activity

ABTS radical scavenging activities of gelatin modified with TA and OTA are shown in Figure 28B. ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS-radical complex (Binsan *et al.*, 2008).

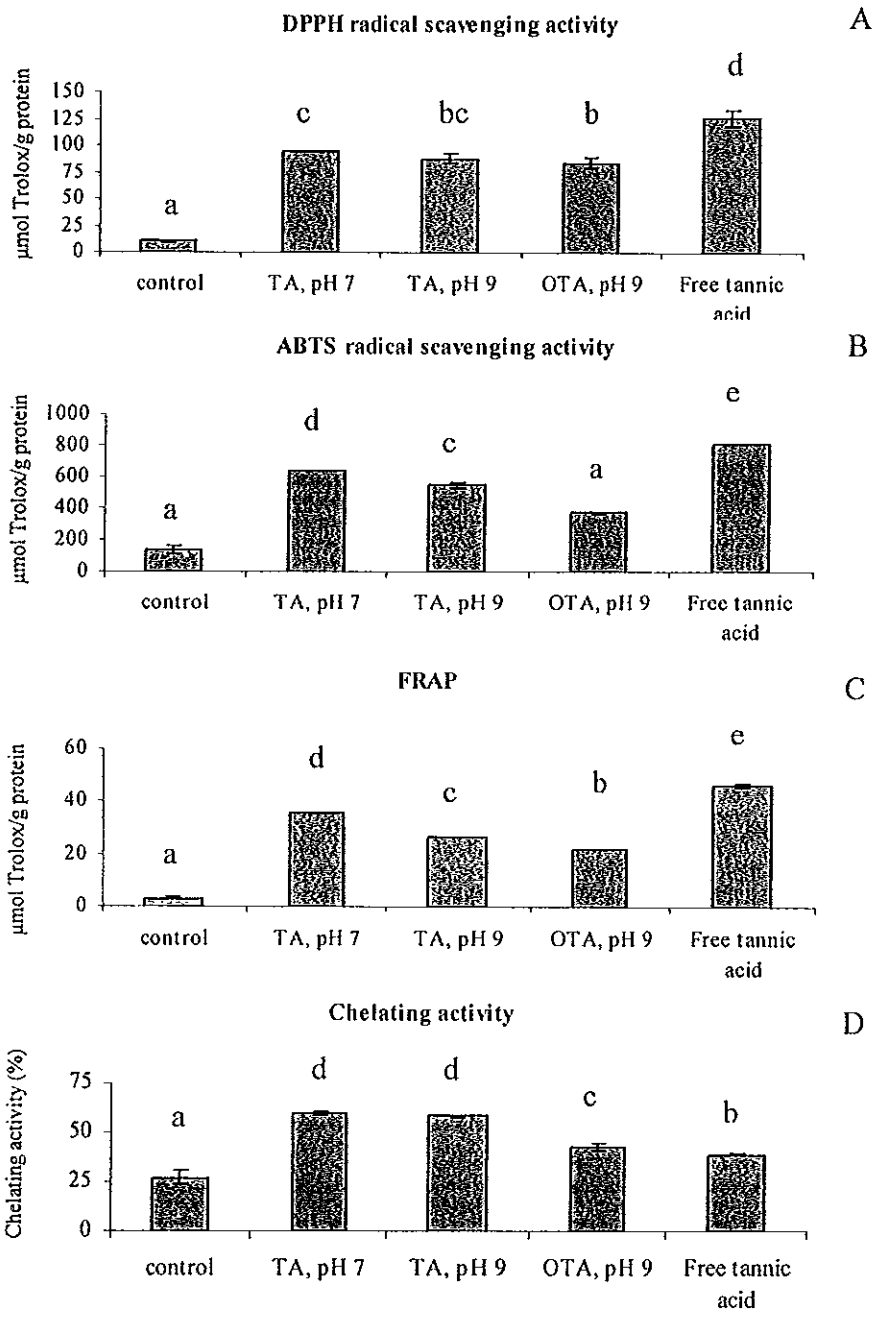


Figure 28. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), ferric reducing antioxidant power (FRAP) (C) and chelating activity (D) of cuttlefish skin gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9. Bars represent the standard deviation (n=3). Different letters on the bars within the same assay denote the significant differences ($p<0.05$).

ABTS radical scavenging activity of samples was generally in accordance with DPPH radical scavenging activity (Figure 28A). Generally, the modified gelatin had a higher ABTS radical scavenging activity than did the control gelatin but the activity was lower than that of free tannic acid. Tannic acid attached with gelatin could scavenge the radicals, forming more stable products, thereby terminating the radical chain reaction. However, hydroxyl group of tannic acid most likely decreased as it was incorporated with gelatin. This resulted in the loss in antioxidative activity, in comparison with free tannic acid. The lowered ABTS radical scavenging activity of gelatin modified with OTA was observed, compared with other gelatin samples. OTA, oxidized form, incorporated with gelatin might lose its capacity of quenching the radicals.

4.4.2.2.3 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP), generally measures the reducing capacity of ferric ion and has been correlated to the radical scavenging capacity (Maqsood and Benjakul, 2009; Moure *et al.*, 2006). FRAP of gelatin modified with TA and OTA is illustrated in Figure 28C. Modified gelatin exhibited higher FRAP, compared to the control gelatin. FRAP of the control gelatin was 3.02 μmol TE/g protein. After modification with TA at pH 7 and 9, FRAP increased to 35.63 and 26.54 μmol TE/g protein, respectively. Nevertheless, gelatin modified with OTA had FRAP of 22.06 μmol TE/g protein. This result reconfirmed that the modification of gelatin with either OTA or TA could improve antioxidative activity of gelatin. However, it was noted that non-covalent attachment of tannic acid to gelatin resulted in the higher antioxidative activity, when compared with covalent interaction. However, the FRAP values observed for all the modified gelatins were lower than those of free tannic acid at the same concentration used. The result generally was in agreement with those of DPPH and ABTS radical scavenging activities (Figures 28A and 24B).

4.4.2.2.4 Chelating activity

The ability of gelatin modified with TA and OTA in metal chelating is depicted in Figure 28D. The highest metal chelating activity was found in gelatin modified with TA at pH 7, followed by those modified with TA at pH 9 and those with OTA, respectively. Ferrous ion (Fe^{2+}) is one of the most important prooxidants

among various species of metal ions, which can catalyze the generation of reactive oxygen species, hydroxyl radical ($OH^{\bullet}$), by which the lipid peroxidation chain reaction is accelerated (Stohs and Bagchi, 1995). Thus gelatin-tannic acid complex had the enhanced ability in metal chelation, in which prooxidation metals can be sequestered, leading to the lowered oxidation. Additionally, gelatin-tannic acid complex also exhibited a higher chelating activity than free tannic acid. The complexes might form the complexation with Fe^{2+} to a higher extent than the free tannic acid. Negative carboxyl group of gelatin also contributed to the chelation of Fe^{2+} via electrostatic interaction, resulting in the higher capacity of chelation for gelatin-tannic acid complex.

4.4.2.3 Changes in FTIR spectra

The FTIR spectra of gelatin modified with 5% of TA and OTA are depicted in Figure 29. Generally, the spectra of cuttlefish skin gelatin without modification exhibited the major bands at 3278 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), 1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^{-}) and 1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching). Modification of gelatin with tannic acid resulted in the decreases in the amplitude of amide A, I, II bands of resulting gelatins. These changes were indicative of greater disorder (Friess and Lee, 1996) in gelatin and were probably associated with the loss of triple helix state (Muyonga *et al.*, 2004b).

The infrared spectra of the modified gelatin revealed the notable changes occurring in the 1500–1000 cm^{-1} regions, as compared to the control gelatin. The obvious increases in amplitude of peak at wavenumber ~1350 and ~1080 cm^{-1} was found in gelatin modified with tannic acid. The wavenumber at 1350±50 and ~1080 cm^{-1} were attributable to the bending of the O-H bonds and the stretching of the C-O bonds in secondary and tertiary alcohols (Smith, 1999). It reconfirmed that both TA and OTA could bind with the gelatin molecules as indicated by the additional band of OH group in phenol found in modified gelatin.

A similar spectra was observed in gelatin modified with TA and OTA. However, a higher amplitude of the peaks at ~1250 cm^{-1} (representative of the C-N stretching on secondary amide) was observed in gelatin modified with OTA,

compared with gelatin modified with TA. This result indicated that electrophilic region of quinone was less reactive in incorporating with NH_3-group, as indicated by higher amplitude of that peak. This was in agreement with the higher free amino group content of gelatin retained after the modification with OTA.

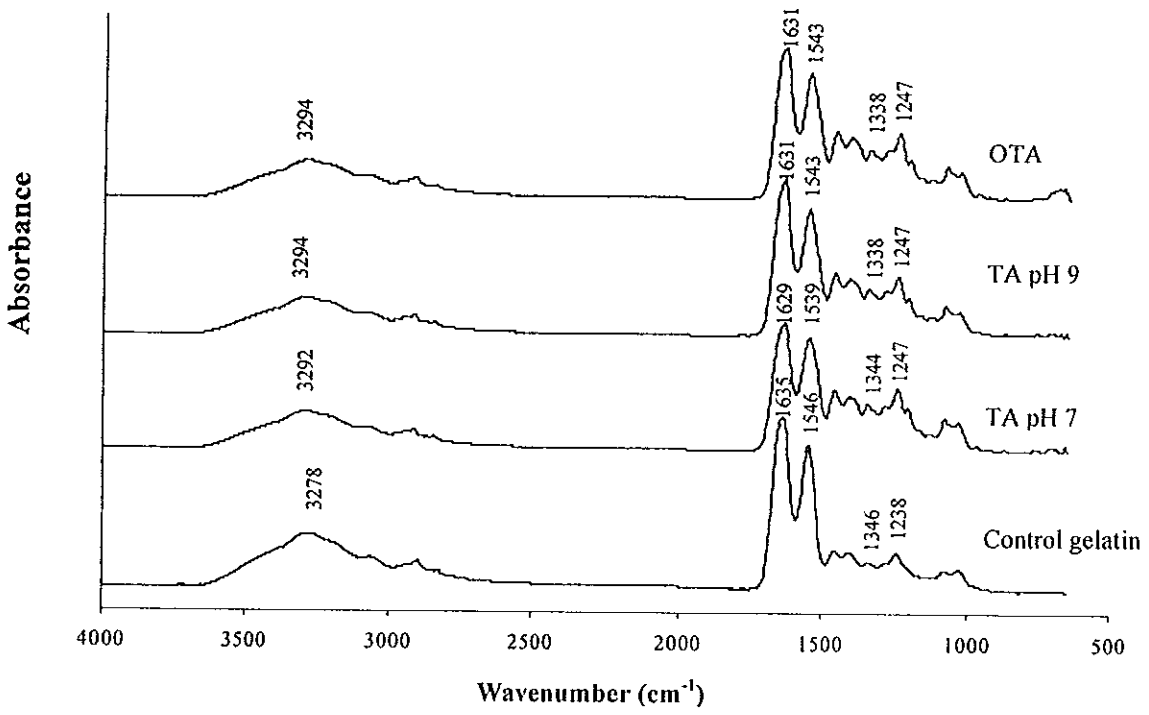


Figure 29. Fourier transform infrared (FTIR) spectra of cuttlefish skin gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9.

4.4.3 Effect of gelatin modified with OTA and TA on emulsion stability and lipid oxidation of menhaden oil-in-water emulsion

4.4.3.1 Zeta potential

The zeta potential of menhaden oil-in-water emulsion stabilized by the control gelatin and gelatin modified with TA and OTA is shown in Table 15. At pH 7, the net charge of the emulsion stabilized by the control gelatin was -38.7±0.6 mV, whereas that of emulsion stabilized by gelatin modified by OTA was -35.3±0.8 mV. Lower negative charge was obtained in gelatin modified by TA at pH 7 and 9

(-30.2±0.6 and -31.4±1.1 mV, respectively). The negative charge of the emulsion might be due to the negatively charged amino acid in gelatin surrounding the oil droplet. At pH 7, which higher than isoelectric point (pI) of cuttlefish skin gelatin (~4.5), carboxyl groups are negatively charged ($-COO^-$), whereas most of the amino groups were neutral ($-NH_2$). The lower negative charge was observed in emulsion stabilized by modified gelatin when compared with that of emulsion stabilized by the control gelatin. This result indicated that the incorporation of tannic acid into gelatin might lead to the loss in negative charge of gelatin. The lowest negative charge was observed in gelatin modified with TA. This result suggested that the introduction of hydroxyl group of tannic acid lowered the negative charge of the resulting complex. Gelatin, a steric stabilizer, more likely stabilized the emulsion by the formation of gel-like hydrophilic layers. Additionally, the electrostatic repulsion mediated by the charged groups of modified gelatin might also contribute to the higher stability of emulsion (McClements, 2005).

Table 15. Zeta potential and particle size (d_{43}) of menhaden oil-in-water emulsion stabilized by 1.0 % gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9

Gelatin	Zeta potential (mV)	Particle size (μm)	
		Day 0	Day 12
Control (unmodified)	-38.7±0.6c*	0.345±0.004aA**	1.150±0.065aB
Modified with TA at pH 7	-30.2±0.6a	0.380±0.005cA	1.925±0.186cB
Modified with TA at pH 9	-31.4±1.1a	0.363±0.005bA	1.689±0.142bB
Modified with OTA at pH 9	-35.3±0.8b	0.346±0.004aA	1.230±0.108aB

Mean±SD (n=3).

* Different letters in the same column indicate significant differences ($p<0.05$).

**Different capital letters in the same row indicated significant differences ($p<0.05$).

4.4.3.2 Changes in particle size of emulsion during storage

Changes in particle size of emulsions stabilized by 1% control gelatin or 1% gelatin modified with TA and OTA during storage at room temperature for 12 days are shown in Table 15. At day 0, the larger particle size was observed in

emulsion stabilized by modified gelatin, compared with that stabilized by the control gelatin (0.345 μm) ($p<0.05$). The loss in surface hydrophobicity of modified gelatin might cause the decrease in migration of gelatin to the oil droplets, which were lipophilic. Therefore, the amount of modified gelatin occupied at oil droplet might be slightly lower, leading to the lowered emulsifying property of modified gelatin. The larger particle size (d_{43}) of oil droplet was observed in emulsion stabilized by gelatin modified by TA, compared with that modified with OTA ($p<0.05$). This was possibly associated with the lowest surface hydrophobicity of gelatin modified with TA (Figure 27). Thus, the resulting gelatin could not migrate and adsorb at oil-water interface effectively. The particle size of emulsion stabilized by gelatin modified with OTA was not different from that stabilized by the control gelatin. Gelatin without and with OTA modification might cover the oil droplets and form sufficiently dense adsorption layer during emulsification. As a result, the smaller particles might be formed to a high degree as evidenced by the lower d_{43}.

After storage for 12 days, the increases in particle size of oil droplet were observed in all samples ($p<0.05$). This result might be due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during storage (Djabourov *et al.*, 1993). Marked increase in particle size was observed in emulsion stabilized by gelatin modified by TA, compared with gelatin modified by OTA and the control gelatin. The highest rate of increase in particle size was observed in emulsion stabilized by gelatin modified with TA at pH 7 ($p<0.05$). However, no differences in size of oil droplet were found in emulsions stabilized by gelatin modified by OTA, compared with emulsion stabilized by the control gelatin after storage for 12 days ($p<0.05$). This indicated that gelatin modified by OTA could stabilize oil-in-water emulsion similarly to the control gelatin (without modification) at the extended time.

4.4.3.3 Changes in lipid oxidation of emulsion during storage

Lipid oxidation of menhaden oil-in-water emulsion stabilized by gelatin modified with TA and OTA in the absence and presence of $FeCl_3$ was monitored and expressed as TBARS values during the storage of 12 days (Figure 30). In the absence of $FeCl_3$, TBARS value in the emulsion increased as the storage time increased ($p<0.05$). The highest increase was found in emulsion stabilized by the

control gelatin (Figure 30A). Modification of gelatin with tannic acid could improve the ability to inhibit the TBARS formation in fish oil-in-water emulsion system. Both gelatins modified with tannic acid via covalent and non-covalent interaction could retard lipid oxidation of emulsion more effectively than free tannic acid ($p<0.05$).

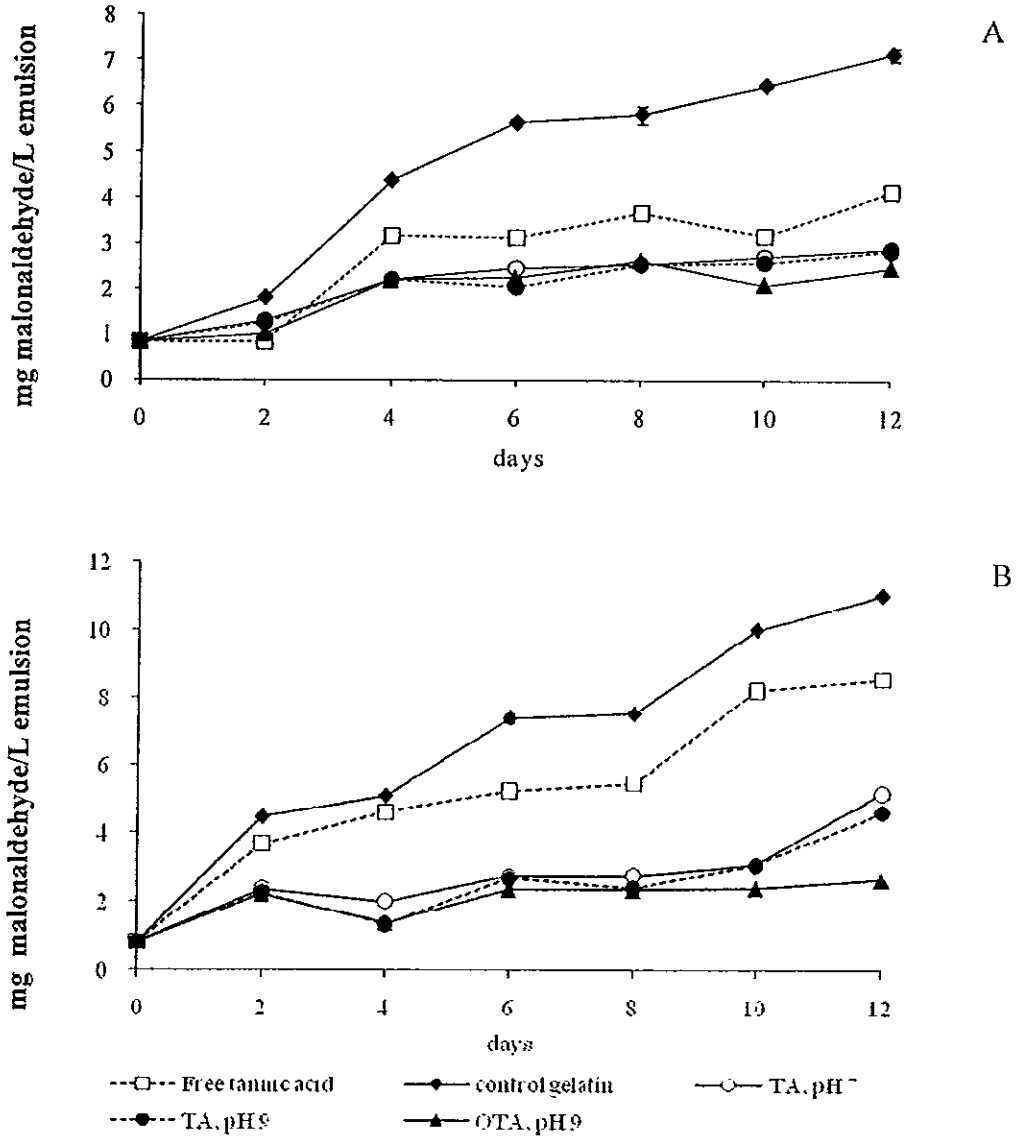


Figure 30. TBARS value of menhaden oil-in-water emulsion stabilized by 1.0% gelatin modified with 5% (w/w based on protein content) of OTA at pH 9 and TA at pH 7 and 9 without (A) and with (B) 50 μM ferric chloride during storage at room temperature for 12 days. Bars represent the standard deviation (n=3).

This result was in accordance with Almajano and Gordon (2004) who reported that interaction of BSA and epigallocatechin gallate (EGCG) could induce the formation of a BSA-antioxidant adduct and cause a synergistic increase in the oxidative stability of sunflower oil-in-water emulsion, compared with BSA or EGCG alone. Protein-antioxidant adduct might be concentrated at the oil-water interface due to the surface-active nature of the protein (Almajano and Gordon, 2004). Lipid hydroperoxides are surface-active compounds and are thus able to accumulate at the lipid-water interface of emulsion. Lipid oxidation in oil-in-water emulsions primarily occurs at the emulsion droplet interface (Kellerby *et al.*, 2006). This result suggested that gelatin modified with tannic acid could transport to the oil-water interface and functioned at the interface, whereas free tannic acid which was polar substance, was mainly localized in the aqueous phase.

The antioxidative activity of modified gelatin in emulsion depended on the type of interaction between gelatin and tannic acid. The highest activity was found in gelatin modified with OTA, when compared with gelatin modified with TA as shown by the lower TBARS formation throughout the storage of 12 days. This result suggested that the higher emulsifying properties of gelatin modified with OTA might cause the gelatin-tannic acid complex to localize and adsorb at oil-water interface. On the other hand, gelatin modified with TA with the lower emulsifying properties could not align at the interface effectively. It was noted that TBARS value was similar when gelatin modified with OTA and TA conducted at pH 9 were used. At pH 9, tannic acid might undergo oxidation to some degree, which might lower the hydrophilicity of gelatin and favored emulsification.

In the presence of $FeCl_3$, higher TBARS values were observed in all emulsion samples (Figure 30B) in comparison with those found in emulsion containing no $FeCl_3$ (Figure 30A). Generally, similar results were noticeable, compared to the systems without $FeCl_3$ addition. After 12 days of storage, the lowest TBARS value was observed in emulsion stabilized by gelatin modified with OTA ($p<0.05$). Fe^{3+} could accelerate the lipid oxidation in fish oil-in-water emulsion system. Due to metal reducing properties of phenolic compound, Fe^{3+} might be reduced into Fe^{2+} which increased the oxidative reaction (Paiva-Martins and Gordon, 2005). This was indicated by the marked increase in TBARS value in emulsion

containing free tannic acid. Nevertheless, only slight difference in TBARS value was observed in emulsion stabilized by gelatin modified with tannic acid between those without and with $FeCl_3$ during incubation for 12 days. This result showed that the modification of gelatin with tannic acid, especially through covalent interaction, increased the stability of fish oil-in-water emulsion, regardless of $FeCl_3$ incorporated.

4.5 Conclusion

Modification of cuttlefish skin gelatin with 5% tannic acid via non-covalent and covalent interaction enhanced antioxidative activity but had different impact on the emulsifying properties of the resulting gelatin, depending on the types of interaction. Gelatin modified with tannic acid via non-covalent interactions showed a higher *in vitro* antioxidative activity than that modified through covalent interactions. However, gelatin modified with tannic acid via covalent rendered the emulsion with high stability and could inhibit lipid oxidation of fish oil-in-water emulsion effectively during the extended storage.

CHAPTER 5

Improvement of foaming properties of cuttlefish skin gelatin by modification with *N*-hydroxysuccinimide esters of fatty acid

5.1 Abstract

Conformation and foaming properties of cuttlefish skin gelatin modified by *N*-hydroxysuccinimide esters of different saturated fatty acids including capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) at different molar ratios (0.25, 0.50, 1.00 and 2.00) were investigated. Covalent attachment of fatty acids into gelatin was observed as evidenced by the decrease in amino groups. Fourier transform infrared spectroscopic study indicated the presence of alkyl group of modified gelatin. The higher increase in surface activity with coincidental increase in surface hydrophobicity was observed in gelatin modified with fatty acid ester having a longer chain, especially at the higher molar ratio. The increase in foam expansion was related with the improved surface activity mediated by the modification by *N*-hydroxysuccinimide esters of fatty acid.

5.2 Introduction

Foams are known as thermodynamically instable colloidal systems comprising a consistently dispersed gas phase in a liquid matrix (Dickinson, 1992). Generally, foams are formed by incorporating a high volume fraction of gas in a surfactant solution, which stabilizes the bubbles (Wang and Narsimhan, 2004). Proteins are natural high molecular weight surfactants, widely used as foaming agent in food, pharmaceutical, medical, and technical applications. During foam formation, proteins diffuse from the aqueous phase and adsorb at the air–water interface with polar groups exposed towards water phase and the non-polar groups towards the air phase (Zayas, 1997). The main driving force is the entropy increase, resulting from

dehydration of the hydrophobic interface and of hydrophobic regions of the protein surface (Dickinson and McClements, 1995). Adsorption of protein at air-water interface results in the decrease in interfacial tension, which facilitates the formation of a larger interface area in foams (Pezennec *et al.*, 2000). In addition proteins can form extensive intermolecular interactions at the interface, forming cohesive films which are resistant to further deformation.

Gelatin, the denatured form of collagen, is one of protein used as surfactants in industry. Generally, gelatin is commercially made from skins and skeletons of bovine and porcine (Derkatch *et al.*, 1999). However, the current trends necessitate the studies on new gelatin sources for replacement of mammalian gelatins, mainly due to the bovine spongiform encephalopathy (BSE) crisis or religious and social reasons. There is the growing interest on new gelatin sources to replace mammalian gelatin. Gelatin from skin of cuttlefish could be such an alternative, and its extraction was recently described by Aewsiri *et al.* (2009a). However, gelatin from cuttlefish skin had the poorer surface active properties, compared with commercial gelatin (bovine gelatin), due to the lower hydrophobic amino acids content (proline and leucine) and a large portion of hydrophilic amino acids (lysine, serine, arginine, hydroxyproline, aspartic and glutamic acids). Gelatin with a higher hydrophilic region has limited ability to function as a surface active agent (Toledano and Magdassi, 1997).

The chemical modification of protein may lead to changes in its hydrophobicity, causing the changes in the surface activity and functional properties of protein (Kato and Nakai, 1980; Toledano and Magdassi, 1997). Covalent attachment of fatty acids into protein by reaction between *N*-hydroxysuccinimide esters of fatty acids with free amino groups of proteins is an effective tool for improving surface activity of protein (Magdassi *et al.*, 1996). Wierenga *et al.* (2003) reported that attachment of C10 alkyl chain to ovalbumin caused an increase in surface activity as determined by the kinetics of protein adsorption to the air-water interface. Toledano and Magdassi (1998) showed that bovine gelatin modified with fatty acid ester had the higher foaming and emulsifying properties in comparison with native gelatin. Nevertheless, a little information regarding the effect of modification of gelatin with fatty acid ester on conformation of resulting gelatin has been reported.

Therefore, the objectives of this study were to improve surface activity and foaming properties of cuttlefish skin gelatin by interaction with *N*-hydroxysuccinimide esters of various saturated fatty acids with different chain lengths at various molar ratios to obtain the foam properties as well as that of commercial gelatin (bovine gelatin) and to investigate the conformation of resulting gelatin.

5.3 Materials and methods

5.3.1 Chemicals

Capric acid, lauric acid, myristic acid, *N*-hydroxysuccinimide, anhydrous tetrahydroxyfuran, 1,3-dicyclohexylcarbodiimide, *o*-phthalic dialdehyde (OPA), 2-(dimethylamino)ethanethiol hydrochloride (DMA), 1-anilinonaphthalene-8-sulphonic acid (ANS) and bovine skin gelatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), sodium hydroxide, ethanol, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), sodium chloride, sodium hydroxide, sodium sulfite and boric acid were procured from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

5.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from the dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bag and stored at -20°C until use. Storage time was not longer than 2 months.

5.3.3 Extraction of gelatin from cuttlefish skin

Cuttlefish skin gelatin was prepared according to the method of Aewsiri et al. (2009b). Skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28 °C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching

with 10 volumes of 5 % H_2O_2 at 4°C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using the distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min at 25°C using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ μP/Dura Dry™ μP, FTS® System, Inc., Stone Ridge, NY, USA).

5.3.4 Preparation of N-hydroxysuccinimide ester of fatty acids

N-hydroxysuccinimide esters of three saturated fatty acids (capric acid, C10:0; lauric acid, C12:0; myristic acid, C14:0) were prepared according to the method of Wierenga *et al.* (2003). Esterification was performed using an equimolar amount (1 M) of fatty acid and *N*-hydroxysuccinimide in 100 mL of anhydrous tetrahydroxyfuran in the presence of 1,3-dicyclohexylcarbodiimide for 36 h with a gentle stirring at room temperature. The precipitated product (dicyclohexylurea) was removed by filtration through a filter paper No. 595½ (Schleicher & Schuell MicroScience GmbH, Dassel, Germany), and the filtrate was dried in a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4001, Viertrieb, Germany). The succinimidic ester (1 g) was redissolved in 5 mL of 60 % ethanol at 60°C and recrystallized by cooling to room temperature to remove other impurities. The crystals were recovered by filtration using a filter paper, air dried and used as hydroxysuccinimidic ester.

5.3.5 Modification of cuttlefish skin gelatin

The covalent attachments of fatty acids were carried out by the reaction of *N*-hydroxysuccinimide ester of fatty acid with amino groups of cuttlefish skin gelatin according to the method of Wierenga *et al.* (2003) with a slight modification. Cuttlefish skin gelatin was dissolved in 100 mM sodium carbonate buffer (pH 8.5) to obtain a final concentration of 1% protein determined by the Biuret method (Robinson and Hodgen, 1940). *N*-hydroxysuccinimidic ester of fatty acids was dissolved in DMSO to obtain the different concentrations. To 20 mL of gelatin

solution, *N*-hydroxysuccinimidic ester of fatty acid solution (80 mL) was added to obtain the different final molar ratios of *N*-hydroxysuccinimide esters of fatty acid to gelatin (0.25, 0.50, 1.00 and 2.00). The mixtures were stirred continuously for 18 h at room temperature. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 24 h at room temperature against 20 volumes of water to remove free fatty acids and *N*-hydroxysuccinimide (unbound to proteins). The dialysate was freeze-dried. The resulting gelatin powder was stored at -20°C until use. The control was prepared in the same manner except that *N*-hydroxysuccinimide ester of each fatty acid was excluded and the distilled water was used instead. Gelatin-fatty acid complexes were subjected to analyses.

5.3.6 Determination of free amino group content

Free amino group content in native and modified cuttlefish skin gelatin were determined using *o*-phthalicdialdehyde (OPA) as described by Church *et al.* (1983). The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium borate, 200 mg of 2-(dimethylamino)ethanethiol hydrochloride (DMA), and 5 mL of 10 % SDS. The total volume was adjusted to 50 mL with H_2O. To determine free amino group content, 65 µL of sample (1 % protein) was mixed with 3 mL of reagent solution. After 2 min, the absorbance was read at 340 nm to measure alkylisoindole derivatives formed after reaction of OPA with free amino groups. Free amino group content was calculated from a standard curve of L-leucine (0 – 10 mM).

5.3.7 Determination of the secondary structure

Samples (0.05 mg protein/mL) were dissolved in 10 mM sodium phosphate buffer (pH 7.0) according to the method of Wierenga *et al.* (2003). Far-UV CD spectra were measured at 20°C, with a scan speed of 100 nm/min from 190 to 260 nm, a data interval of 0.2 nm, a band width of 1.0 nm, and a response time of 0.125 s using a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan). All spectra were corrected for the corresponding protein-free sample. The secondary structure was estimated using a nonlinear least-square fitting procedure with reference spectra as described by De Jongh *et al.* (1994).

5.3.8 Fourier transform infrared (FTIR) spectroscopy

Spectra of native and modified gelatin were obtained using a BioRad FTS-60A FTIR spectrometer (Biorad, Cambridge, MA, USA) equipped with a Attenuated Total Reflectance Accessory (ATR) using a ZnSe ATR crystal. MID-IR spectra were recorded between 1200 and 4000 cm^{-1} at a resolution of 2 cm^{-1} in the ATR mode on thin films at room temperature which were purged with dry nitrogen to remove spectral interference resulting from water vapor and carbon dioxide. Sixty-four interferograms were coadded for a high signal-to-noise ratio. All experiments were performed in duplicate. Prior to data analysis, the spectra were baseline corrected and normalized (mean normalization option).

5.3.9 Determination of surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (4 mg/mL) was diluted in 10 mM sodium phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5 % (w/v). The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

5.3.10 Measurement of mean particle size

The particle size of gelatin samples dispersed in 10 mM sodium phosphate buffer (pH 7.0) with the final concentration of 1 mg protein/mL was measured by dynamic light scattering at room temperature. The measurements were performed by a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). A refractive index of 1.42 was used for dispersed phase of all protein solutions.

5.3.11 Surface activity

Gelatin solutions (1 mg protein/mL) were prepared freshly before measurement. The surface pressure and elastic modulus of gelatin samples were

measured as a function of time (0–5000 s) on an automated drop tensiometer (ADT; I. T. Concept, Longessaigne, France), using the condition suggested by Wierenga *et al.* (2005). An air bubble was formed at the tip of a syringe needle placed in a cuvette containing the protein solution. Both the cuvette and the syringe were temperature controlled (20 ± 0.1 °C). Bubble volume was kept constant at 4 μL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. The surface elastic modulus was measured by inducing sinusoidal changes in the interfacial area with a period of 10 s and amplitude of 10 %. The modulus was calculated from the measured changes in surface pressure and surface area averaged over a sequence of five sinuses; every 500 s such a sequence was performed. These measurements of the modulus did not affect the development of surface pressure in time.

5.3.12 Determination of foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi *et al.* (1995) with a slight modification. Ten mL of gelatin solution (0.5 % protein, w/v) were transferred into 100 mL-cylinders (PYREX®, Corning, NY, USA). The mixtures were homogenized for 1 min using a homogenizer model T25 basic (IKA LABORTECNIK, Staufen, Germany) at a speed of at 13,400 rpm for 1 min at room temperature. The foam volume was recorded at t= 0, 15 and 30 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_0/V_1) \times 100$$
$$FS\ (\%) = (V_t/V_1) \times 100$$

where V_0 is total volume after whipping (0 min); V_1 is the original liquid volume before whipping and V_t is total volume after leaving at room temperature for different times (15 and 30 min).

5.3.13 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard

5.3.14 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

5.4 Results and Discussion

The complexes between cuttlefish skin gelatin and fatty acid were synthesized by incubating cuttlefish skin gelatin with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. Conformational and functional changes of modified gelatin were quantified by determination of the free amino group content, secondary structure, FTIR spectra, surface hydrophobicity, particle size, adsorption kinetics and foaming properties.

5.4.1 Free amino group content

Free amino group content and the degree of modification of gelatin by *N*-hydroxysuccinimide esters of various fatty acids using different mole ratios are shown in Table 16. The cuttlefish skin gelatin contained 0.38 mmol NH_2 groups/g gelatin. Gelatin modified by fatty acid esters had the lower free amino group content than the control gelatin (without modification) ($p<0.05$). With the same fatty acid ester used, free amino acid content decreased with increasing molar ratio ($p<0.05$). At the same molar ratio used, the lower free amino content was obtained with gelatin modified with fatty acid ester having the shorter chain length. The result indicated that *N*-hydroxysuccinimide esters of fatty acid could interact with gelatin via amino groups of N-termini or ε-amino group of lysine. The incorporation of fatty acid into gelatin led to a decrease in free amino group content and might alter the reactivity and functional properties of modified gelatin.

Table 16. Free amino group content, degree of modification and surface hydrophobicity of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios

Treatment	Molar fatty acid ester/ gelatin ratio	Free amino content (mM)	Degree of modification	Surface hydrophobicity
Control (unmodified)		3.75 ± 0.05j*	0.00	16.68 ± 0.25a
Gelatin-C10:0	0.25	2.71 ± 0.01g	26.34 ± 0.19c	19.31 ± 0.78b
	0.50	2.08 ± 0.08d	43.38 ± 2.11f	19.91 ± 0.63b
	1.00	1.20 ± 0.11b	67.40 ± 2.89h	19.97 ± 0.52b
	2.00	0.56 ± 0.07a	84.88 ± 1.89i	22.10 ± 0.40cd
Gelatin-C12:0	0.25	3.05 ± 0.09h	17.05 ± 2.54b	20.02 ± 0.47b
	0.50	2.78 ± 0.06g	24.24 ± 1.66c	22.25 ± 0.78cd
	1.00	2.42 ± 0.04e	34.20 ± 1.15e	23.26 ± 0.37cd
	2.00	1.78 ± 0.01c	51.57 ± 0.38g	24.38 ± 0.77d
Gelatin-C14:0	0.25	3.38 ± 0.08i	7.97 ± 2.30a	26.91 ± 0.72e
	0.50	3.10 ± 0.05h	15.72 ± 1.34b	36.21 ± 1.10f
	1.00	2.76 ± 0.07g	24.90 ± 1.85c	66.08 ± 4.85g
	2.00	2.56 ± 0.07f	30.44 ± 1.83d	95.38 ± 3.03h

Mean±SD (n=3)

Different letters in the same column indicate significant differences ($p<0.05$).

The covalent attachment of fatty acids to gelatin was quantified by determining the degree of modification as expressed in the number of modified NH_2 groups divided by the total number of free amino groups in the control gelatin. Increase in degree of modification was more pronounced as the mole ratio of active ester increased ($p<0.05$). The degree of modification of gelatin with fatty acid ester of C10:0 was in the range of 26-84 %, whereas that of gelatin modified with C12:0 and C14:0 possessed the degree of modification in the range of 17-51 % and 8-30 %, respectively. At the same molar ratio, a higher degree of modification was observed for gelatin modified with fatty acid ester of C10:0 ($p<0.05$), followed by those modified with fatty acid esters of C12:0 and C14:0, respectively. This result indicated that the increase in chain length more likely lowered reactivity of the fatty acid ester. This might be due to the lower ability in incorporation of fatty acid ester with longer chain length into the gelatin molecules. On the other hand, the shorter chain fatty acid ester (C10:0) might disperse and interact with amino group of gelatin in aqueous phase more effectively. However, Magdassi *et al.* (1996) reported that antibodies

(IgG) modified by *N*-hydroxysuccinimide esters of fatty acid in aqueous system with the lower chain length (C_8) yielded the lower degrees of attachment in comparison with those possessing longer chain length (C_{16} and C_{18}). Toledano and Magdassi (1997) found no difference in the degree of modification in gelatin modified with fatty acid ester having different chain lengths in DMSO. For cuttlefish skin gelatin modified with *N*-hydroxysuccinimide ester of fatty acids in a mixture of DMSO: sodium carbonate buffer (80:20), degree of modification depended on both chain length of fatty acid in the ester as well as molar ratio.

5.4.2 Far-UV CD

Far UV circular dichroic (CD) spectra of protein solutions provide information about the secondary structure of proteins (Li *et al.*, 2008). Secondary structure of cuttlefish skin gelatin consisted of 21.18 % of α-helix, 71.17 % of random coil and 8.65 % of β-sheet as estimated by spectral analysis. Modification of various fatty acids esters at the molar ratio of 0.50 and 2.00 affected the secondary structure of resulting gelatin differently as shown by the differences in CD spectra (Figure 31). Generally, CD spectra of collagen reveal the peak at around 220-230 nm and a negative peak at around 200 nm. Gelatin is a protein obtained by breaking the triple-helix structure of collagen (Kuijpers *et al.*, 1999). When the triple-helix in collagen disappears completely, only the negative peak at 200 nm of gelatin remains (Wu *et al.*, 2007). The negative peak at 200 nm corresponds to the random conformation (Wu *et al.*, 2007).

After modification, the higher amplitude of the negative peak at 200 nm was observed. Gelatin modified with fatty acid esters having a longer chain length showed the higher amplitude. The amplitude of negative peak at 200 nm increased with increasing molar ratios used. Gelatin modified with fatty acid esters having a longer chain length showed the higher amplitude. Wu *et al.* (2007) reported that the addition of germini surfactant ($C_{12}C_2C_{12}$) to gelatin solution led to a more random structure, as evidenced by the decrease in CD spectra at 200 nm. After modification of gelatin with fatty acid ester of C14:0 at the molar ratio of 2.00, random coil content increased to 75.60 %, whereas α-helix and β-sheet contents decreased to 18.78 % and

5.62 %, respectively. This result indicated that the attachment of fatty acid to gelatin caused the changes in the secondary structure of resulting gelatin.

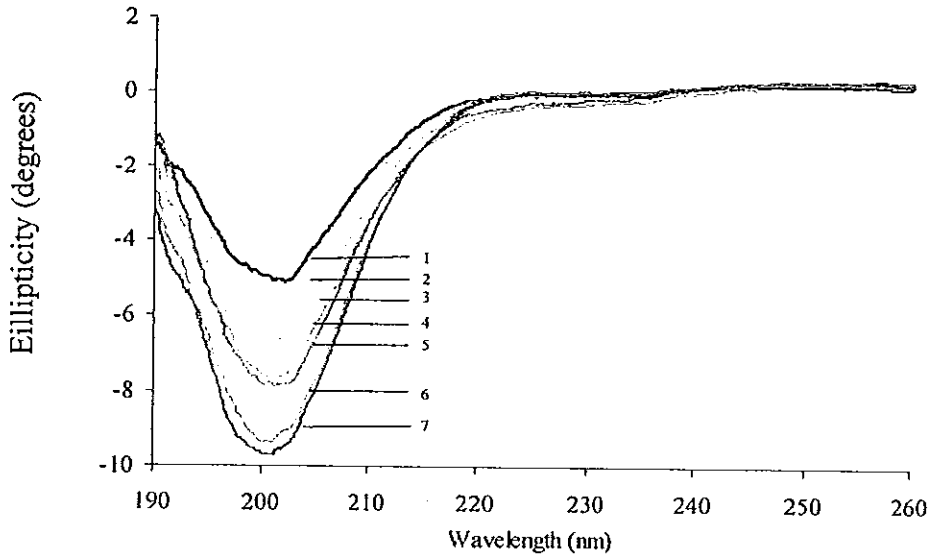


Figure 28. Far UV circular dichroism (CD) spectra of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. 1: unmodified gelatin; 2 and 3: gelatin modified with C10:0 at molar ratios of 0.50 and 2.00; 4 and 5: gelatin modified with C12:0 at molar ratios of 0.50 and 2.00; 6 and 7: gelatin modified with C14:0 at molar ratios of 0.50 and 2.00.

5.4.3 FTIR

FTIR spectra of gelatin modified gelatin modified by various fatty acid esters at the molar ratios of 0.50 and 2.00 are depicted in Figure 29. Generally, the spectra of cuttlefish skin gelatin without modification exhibited the major bands at ~3280 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), ~1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-) and ~1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching). Similar spectra were observed in gelatin modified by all fatty acid esters. However, the modified gelatin had the lower amplitude of amide A bands than the control gelatin. The result indicated the loss in H-bond of resulting gelatin. Moreover, the increase in amplitude of amide I and II band was observed in gelatin

after modification in comparison with the control gelatin. This result suggested that the formation of amide bond between fatty acid ester and gelatin lead to increasing C=O stretching of Amide I band and CN stretching of Amide II band.

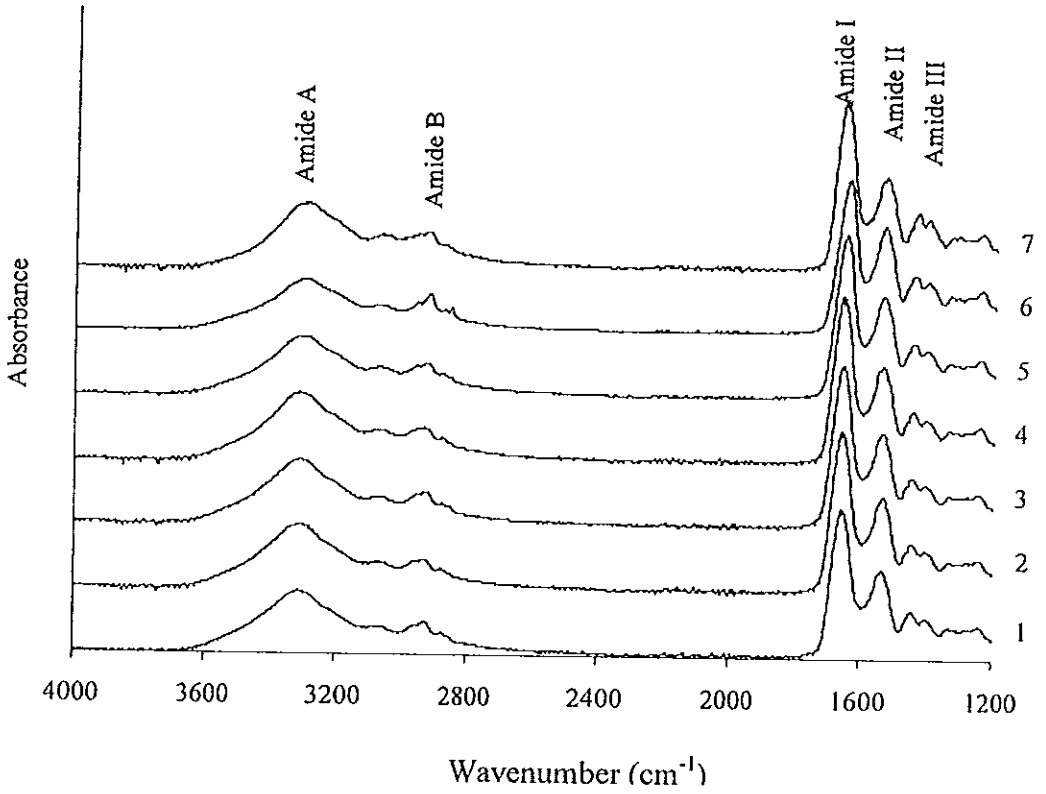


Figure 32. Fourier transform infrared (FTIR) spectra of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. 1: unmodified gelatin; 2 and 3: gelatin modified with C10:0 at molar ratios of 0.50 and 2.00; 4 and 5: gelatin modified with C12:0 at molar ratios of 0.50 and 2.00; 6 and 7: gelatin modified with C14:0 at molar ratios of 0.50 and 2.00.

Modification of gelatin with fatty acid ester resulted in the slight increase in the amplitude at wavenumber 2936 and 2867 cm^{-1} of resulting gelatin, compared with gelatin without modification. The wavenumber at ~2936 and ~2867 cm^{-1} were attributable to the asymmetric and symmetric stretching of CH_2, respectively (Smith, 1999). This result was in agreement with Lin and Chen (2006)

who reported that attachment of C_{12} alkyl chains to the gelatin hydrolysate caused an increase of the peaks, indicating the symmetric and asymmetric of CH_2. When the fatty acid ester with the same chain length was incorporated, the resulting gelatin had the higher peak amplitude as the higher molar ratio was used. It indicated that fatty acid could bind with the gelatin molecules as indicated by the additional band of alkyl group in modified gelatin.

5.4.4 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of gelatin modified by various fatty acid esters at different molar ratios is shown in Table 16. Generally, gelatin modified with fatty acid esters had the increases in surface hydrophobicity, compared with the control gelatin. The rate of increase in surface hydrophobicity of modified gelatin increased with increasing molar ratio used. Fatty acid ester could attach to gelatin, resulting in the increase in hydrophobicity of resulting gelatin. This result was in agreement with Wierenga *et al.* (2003) who reported that surface hydrophobicity of ovalabumin increased when attached with fatty acid. When the same molar ratio was used, the higher surface hydrophobicity was observed in gelatin modified with fatty acid ester of C14:0, compared with gelatin modified with fatty acid esters of C10:0 and C12:0 ($p<0.05$), except for those modified with fatty acid esters of C10:0 and C12:0 with a molar ratio of 0.25, where the same hydrophobicity was obtained ($p>0.05$). In general, all hydrophobic groups are not buried in the interior domain of the molecules due to the balance between hydrophobic and hydrophilic residues in protein (Djagny *et al.*, 2001). Toledano and Magdassi (1997) found that the increasing chain length of fatty acid caused a significant increase in hydrophobicity of modified gelatin. Additionally, gelatin modified with fatty acid ester of C14:0 showed the higher surface hydrophobicity than others, when the same molar ratio was used ($p<0.05$). It was noted that even though those gelatin has the lower degree of modification, it exhibited the higher surface hydrophobicity.

Table 17. Mean particle size of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios

Treatment	Molar fatty acid ester/ gelatin ratio	Mean particle size (nm)
Control (unmodified)		163.4 ± 7.6a
Gelatin-C10:0	0.25	203.6 ± 10.7c
	0.50	217.0 ± 14.5cd
	1.00	228.0 ± 6.9d
	2.00	255.7 ± 8.8e
Gelatin-C12:0	0.25	180.3 ± 7.1b
	0.50	184.8 ± 0.9b
	1.00	201.4 ± 18.4bc
	2.00	232.6 ± 8.3d
Gelatin-C14:0	0.25	220.7 ± 6.9c
	0.50	226.2 ±14.5d
	1.00	321.9 ± 6.1f
	2.00	443.4 ± 24.8g

Mean±SD (n=3)

* Different letters in the same column indicate significant differences ($p<0.05$).

5.4.5 Particle size

The mean particle size of gelatin modified with various fatty acid esters at different molar ratios was determined using dynamic light scattering (Table 17). The mean particle size of the control gelatin was 163.4 nm. After modification, the larger mean particle size ranging from 180.3 to 443.4 nm was observed in resulting gelatin. Marked increase in mean particle size was observed in gelatin modified with fatty acid ester of C14:0, followed by gelatin modified with fatty acid ester of C10:0 and C12:0, respectively. This result was in agreement with Toledano and Magdassi (1997) who found that the mean particle size of bovine gelatin modified with fatty acid ester increased, depending on the degree of modification and chain length. This result suggested that the increases in surface hydrophobicity gelatin modified with fatty acid ester probably contributed to the cross-linking of gelatin molecules with higher hydrophobicity by hydrophobic interaction. Since gelatin modified with fatty acid ester of C14:0 had the highest surface hydrophobicity, those gelatin molecules more likely underwent interaction via hydrophobic interaction. This probably resulted in the formation of large aggregate.

5.4.6 Surface activity

Surface pressure and elastic modulus of bubble in gelatin solution without and with modification during adsorption from a bulk solution to air-water interface are shown in Figure 33. The change in surface pressure at air-water interface is used to indicate the amount of adsorbed protein at interface or the interactions between the adsorbed molecules. From Figure 33A, surface pressure of all samples increased as the storage time increased. After the first 3000 sec, surface pressure in all samples had a slight increase. When compared between cuttlefish skin gelatin (the control gelatin) and bovine gelatin (commercial gelatin), surface pressure of cuttlefish skin gelatin was lower than that of bovine gelatin. This result suggested that cuttlefish skin gelatin has a lower hydrophobic region than bovine gelatin, resulted in the surface activity of cuttlefish gelatin was lower than bovine gelatin. Modification of cuttlefish skin gelatin with fatty acid ester could improve the surface activity of resulting gelatin. Surface pressure of gelatin modified with fatty acid ester increased with increasing molar ratio used. The highest surface pressure was observed in gelatin modified with fatty acid ester of C14:0, follow by fatty acid esters of C12:0 and C10:0, respectively. Cuttlefish skin gelatin modified with fatty acid ester of C14:0 and C12:0 at molar ratio of 0.50 and 2.00 have a higher surface pressure than bovine gelatin. The incorporation of fatty acid into gelatin more likely led to the increased surface hydrophobicity of resulting gelatin (Figure 34). As a result, gelatin could diffusionally transport and absorb to air-water interface effectively by aligning hydrophobic domain toward air bubble. Moreover, absorbed gelatin might undergo aggregation by hydrophobic interaction. This might result in the increased surface pressure.

The elastic modulus at air-water interface of different gelatins without and with modification is shown in Figure 33B. The result generally was in agreement with change in surface pressure. The higher elastic modulus was observed in gelatin modified with fatty acid ester with higher chain length. For the same fatty acid ester used, elastic modulus was higher for gelatin modified with the higher molar ratios. Elastic modulus is often an important parameter indicating the stability of foams. Low values for this modulus imply that the interfaces are very mobile and exhibit viscous behavior, whereas high values mean that the interfaces behave more like an elastic

solid (Sagis *et al.*, 2008). From this result, it indicated that incorporation of fatty acid into gelatin could enhance the foam stability by forming elastic film of gelatin at interface.

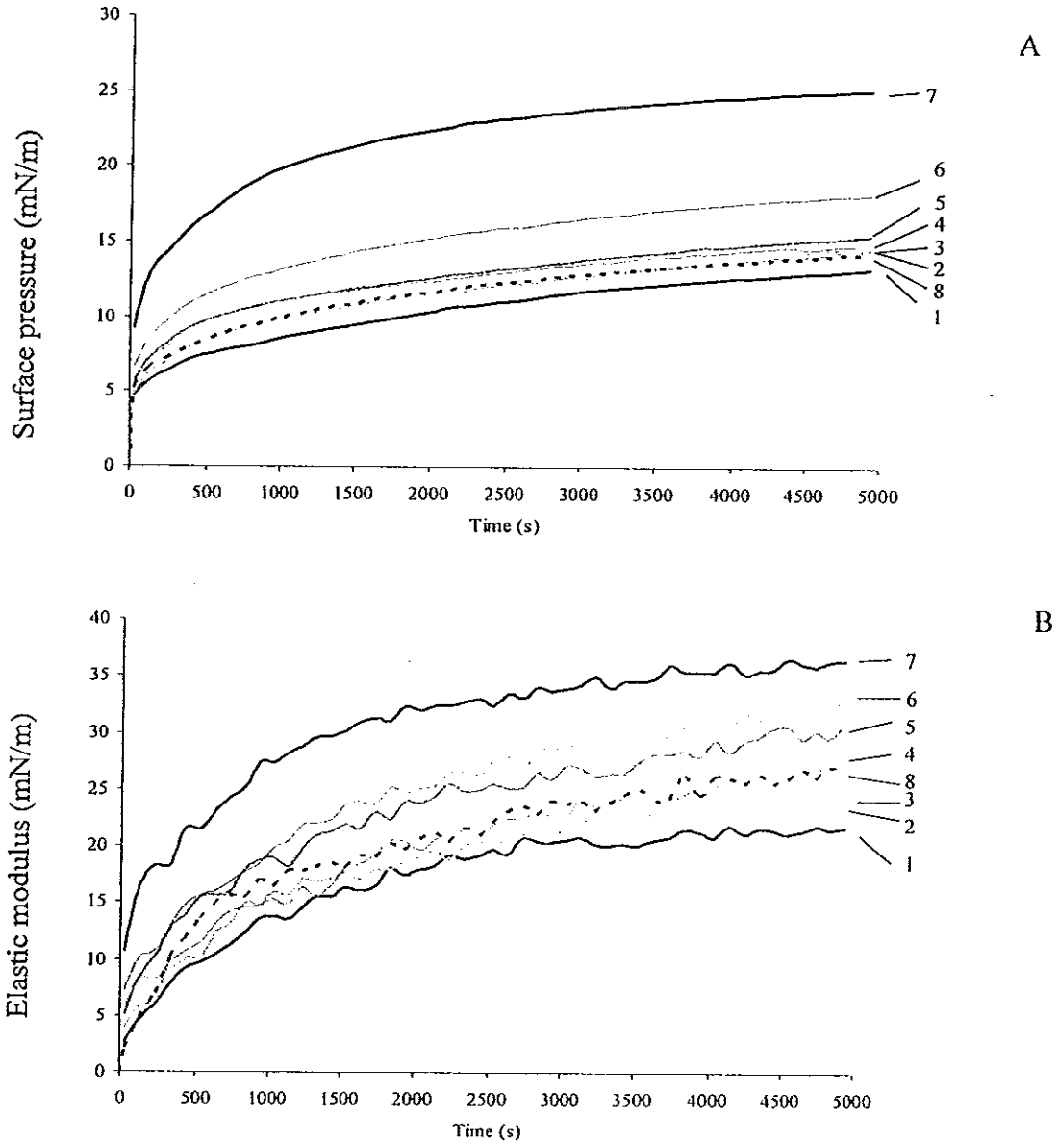


Figure 33. Surface pressure (A) and elastic modulus (B) of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. 1: unmodified gelatin; 2 and 3: gelatin modified with C10:0 at molar ratios of 0.50 and 2.00; 4 and 5: gelatin modified with C12:0 at molar ratios of 0.50 and 2.00; 6 and 7: gelatin modified with C14:0 at molar ratios of 0.50 and 2.00; 8: bovine gelatin.

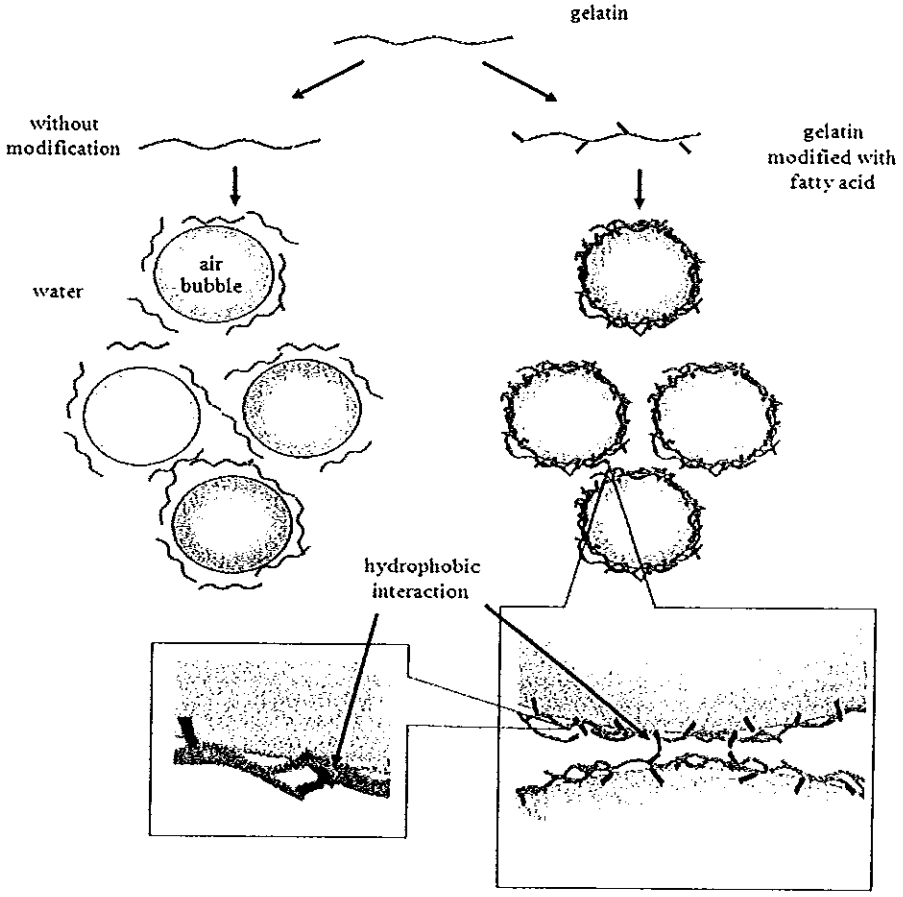


Figure 34. Schematic representation of the adsorption to the air/water interface, and inter- and intra-molecular hydrophobic interaction of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters in solution during foam formation.

5.4.7 Foaming properties

Foam expansion (FE) and foam stability (FS) of cuttlefish skin gelatin modified with various fatty acid esters at different molar ratios are shown in Figure 35. Generally, cuttlefish skin gelatin (control gelatin) have foaming ability inferior that of bovine gelatin ($p<0.05$). Incorporation of fatty acid into gelatin can improved foam ability of cuttlefish skin gelatin as indicated by increasing in FE of resulting gelatin ($p<0.05$). When the same fatty acid ester was used, the increase in FE was obtained as the molar ratio increased ($p<0.05$). At the same molar ratio used for modification, gelatin modified with fatty acid ester of C14:0 had the higher FE, compared with gelatin modified by fatty acid esters of C12:0 and C10:0, respectively.

Cuttlefish skin gelatin modified with fatty acid ester of C14:0 or fatty acid esters of C12:0 and C10:0 at molar ratio of 2.00 have a higher FE than bovine gelatin ($p<0.05$). The foaming ability of proteins is correlated with their film-forming ability at the air-water interface. Proteins that rapidly adsorb at the newly created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foam ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). When fatty acids were incorporated into gelatin, the increases in surface hydrophobicity of resulting gelatin were obtained. The increase in hydrophobic region at surface of modified gelatin facilitated gelatin molecules to localize at the air-water interface and reduce surface tension more effectively. This resulted in the improved FE of gelatin after modification with fatty acid ester.

Foam stability depends principally on the nature of the film and reflects the extent of protein–protein interaction within the matrix (Mutilangi *et al.*, 1996). Foam height of all samples decreased throughout the storage, suggesting the instability of all foam samples. This result might be due to gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via interbubble gas diffusion (Yu and Damodaran, 1991). Moreover, the coalescence of bubbles occurs because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). When the relative decrease in foam height versus time of all samples was plotted (data not shown), it was observed that the foam stability of cuttlefish skin gelatin was lower than that of bovine gelatin. Modification of gelatin with fatty acid ester esters of C10:0 or C12:0 at molar ratio of 2 or C14:0 at molar ratio higher than 0.5 could improve the foam stability of resulting gelatin having the foam stability as well as bovine gelatin but higher foam ability. From this result, it showed that increasing hydrophobic exposure of cuttlefish gelatin can indeed improve its foam ability, without negative effect on foam stability.

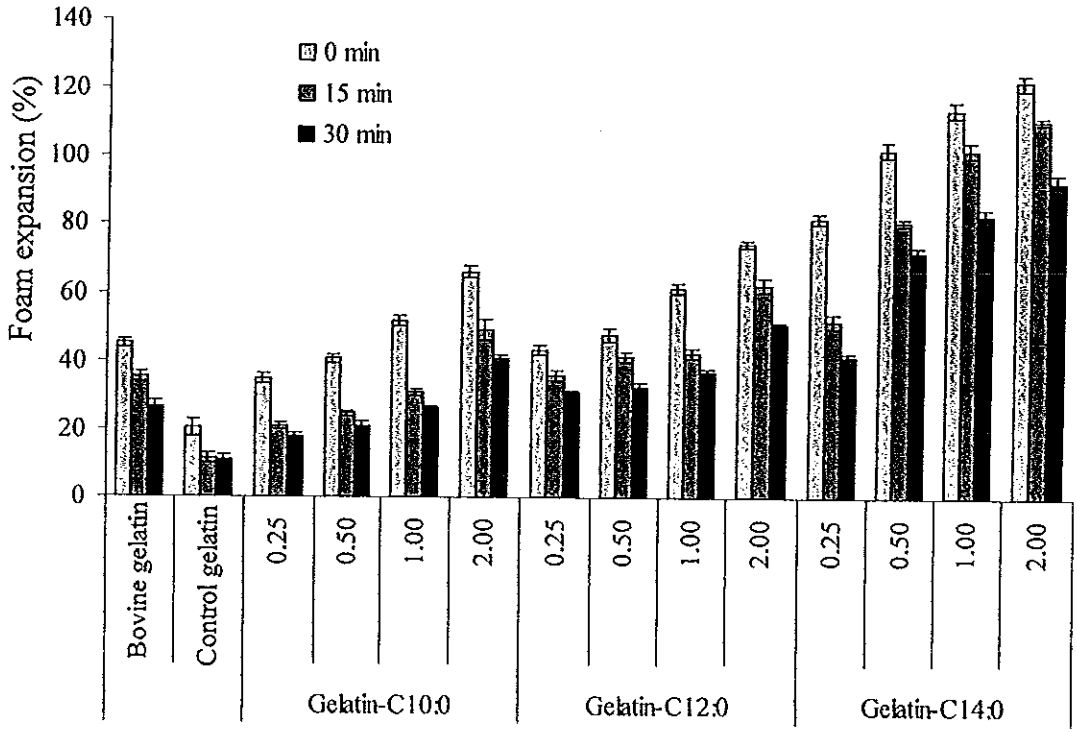


Figure 35. Foam properties of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. Bars represent the standard deviation from triplicate determinations.

5.5 Conclusion

Attachment of fatty acid into cuttlefish skin gelatin could improve surface activity of resulting gelatin as evidenced by increase in foaming properties and surface pressure at air-water interface. The increases in hydrophobic region at surface of gelatin play a key role in enhancing foaming ability of modified gelatin. Gelatin modified with fatty acid ester, especially C14:0 showed the higher surface activity and foam properties than that of bovine gelatin. Therefore, modified cuttlefish skin gelatin might be used as surface active agent or foaming agent to replace bovine gelatin in food industry.

CHAPTER 6

Enhancement of emulsifying properties of cuttlefish skin gelatin by modification with *N*-hydroxysuccinimide esters of fatty acid

6.1 Abstract

Cuttlefish (*Sepia pharaonis*) skin gelatin modified with *N*-hydroxysuccinimide esters of various fatty acids including capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0) at different molar ratios was characterized and determined for emulsifying property. Fatty acid esters were incorporated to gelatin as indicated by the decrease in amino groups. Gelatin modified with fatty acid ester had the increased surface hydrophobicity and emulsifying property with coincidental decrease in surface tension. Gelatin modified with fatty acid ester (C14:0) showed the highest surface activity, especially with the high degree of modification. Emulsion stabilized by gelatin modified with fatty acid ester of C14:0 had the smaller mean particle diameter with higher stability, compared with that stabilized by the control gelatin (without modification). Emulsion stabilized by modified gelatin remained stable at various pH (3-8) and salt concentrations (NaCl 0-500 mM). Emulsion was also stable after being heated at 50-90°C for 30 min.

6.2 Introduction

Gelatin, the denatured form of collagen, is used widely in food, medical and pharmaceutical applications due to its multiple functional properties. Bovine and porcine gelatin are currently also used as an emulsifier in oil-in-water emulsions (Karim and Bhat, 2009). They can adsorb on oil-water interfaces and various hydrophobic segments of oil phase. Generally, gelatin is obtained from skin and skeleton of cows and pigs (Derkatch *et al.*, 1999). However, the current trends necessitate the studies on new gelatin sources for replacement of mammalian gelatins,

mainly due to the bovine spongiform encephalopathy (BSE) crisis and religious or social reasons. There is a growing interest on new sources of gelatin to replace mammalian gelatin (Kittiphattanabawon *et al.*, 2010). As a consequence, gelatin from skin and bone from different fish species such as Nile perch, bigeye snapper and blacktip shark has been intensively studied (Kittiphattanabawon *et al.*, 2010; Muyonga *et al.*, 2004a; Nalinanon *et al.*, 2008).

Cuttlefish is one of major seafood products of Thailand. During processing, the skin is removed and becomes the by-product with the low market value. In general, it is used as animal feed. Recently, Aewsiri *et al.* (2009a) successfully extracted gelatin from cuttlefish. However, gelatin from cuttlefish skin had poorer surface activity due to the lower hydrophobic amino acids content (proline and leucine) and a large portion of hydrophilic amino acids (lysine, serine, arginine, hydroxyproline, aspartic acid and glutamic acid) (Aewsiri *et al.*, 2009b; Hoque *et al.*, 2010). Gelatin with a higher hydrophilic region generally has limited ability to function as a surface active agent (Toledano and Magdassi, 1997).

Covalent coupling of hydrophobic groups to the protein will lead to an increase of the hydrophobicity, resulting in increased surface activity and improved functional properties of protein (Toledano and Magdassi, 1997; Wierenga *et al.*, 2003). Covalent attachment of fatty acids into protein by reaction between *N*-hydroxysuccinimide esters of fatty acids with free amino groups of proteins is an effective tool for improving surface activity of protein (Magdassi *et al.*, 1996; Wierenga *et al.*, 2003). Lin and Chen (2006) reported that attachment of hydrophobic group to bovine gelatin caused an increase in surface activity of resulting gelatin. Additionally, Toledano and Magdassi (1998) found that bovine gelatin modified with fatty acid ester had higher foaming and emulsifying properties in comparison with native gelatin. Nevertheless, a little information regarding the characteristics and emulsifying property of gelatin from cuttlefish skin modified using fatty acid ester has been reported. Therefore, the objectives of this work were to study the improvement of surface activity and emulsifying properties of cuttlefish skin gelatin via the modification with *N*-hydroxysuccinimide esters of various saturated fatty acids with different chain lengths at various molar ratios and to investigate the effect of modified cuttlefish skin gelatin on the stability of emulsion under different harsh conditions.

6.3 Materials and methods

6.3.1 Chemicals

Capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), *N*-hydroxysuccinimide, anhydrous tetrahydroxyfuran, 1,3-dicyclohexylcarbodiimide, *o*-phthalic dialdehyde (OPA), 2-(dimethylamino)ethanethiol hydrochloride (DMA) and 1-anilinonaphthalene-8-sulphonic acid (ANS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), sodium hydroxide, ethanol, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), sodium chloride, sodium hydroxide, sodium sulfite and boric acid were procured from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

6.3.2 Extraction of gelatin from cuttlefish skin

Cuttlefish skin gelatin was prepared according to the method of Aewsiri *et al.* (2009b). Ventral skin of cuttlefish was washed with tap water and cut into small pieces (1 x 1 cm^2). Skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28°C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5 % H_2O_2 at 4°C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using the distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min at 25°C using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ µP/Dura Dry™ µP, FTS® System, Inc., Stone Ridge, NY, USA).

6.3.3 Preparation and characterization of gelatin modified with *N*-hydroxysuccinimide ester of fatty acid

6.3.3.1 Preparation of *N*-hydroxysuccinimide ester of fatty acid

N-hydroxysuccinimide esters of three saturated fatty acids (capric acid, C 10:0; lauric acid, C 12:0; myristic acid, C 14:0) were prepared according to the method of Wierenga *et al.* (2003). Esterification was performed using an equimolar amount of fatty acid (1 M) and *N*-hydroxysuccinimide (1 M) in 100 mL of anhydrous tetrahydroxyfuran, in the presence of 1,3-dicyclohexylcarbodiimide for 36 h with a gentle stirring at room temperature. The precipitated product (dicyclohexylurea) was removed by filtration through a filter paper No. 595½ (Schleicher & Schuell, Dassel, Germany) and the filtrate was dried in a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4001, Viertrieb, Germany). After drying, the succinimidic ester (1 g) was redissolved in 5 mL of 60% ethanol at 60°C and recrystallized by cooling to room temperature to remove other impurities. The crystals were recovered by filtration using a filter paper, air dried and used as *N*-hydroxysuccinimide ester of fatty acids.

6.3.3.2 Modification of cuttlefish skin gelatin

The covalent attachment of fatty acids was carried out by reaction of *N*-hydroxysuccinimide ester of fatty acid with amino groups of cuttlefish skin gelatin. Cuttlefish skin gelatin was dissolved in 100 mM sodium carbonate buffer (pH 8.5) to obtain a final protein concentration of 1% determined by the Biuret method (Robinson and Hodgen, 1940). *N*-hydroxysuccinimide ester of fatty acid was dissolved in DMSO to obtain a concentration of 1 M. To 20 mL of gelatin solution, *N*-hydroxysuccinimide ester of fatty acid solution (80 mL) was added to obtain with different final molar ratios of *N*-hydroxysuccinimide esters of fatty acid-to-gelatin (0.25, 0.5, 1 and 2). The mixture was stirred continuously for 18 h at room temperature. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 24 h at room temperature against 20 volumes of water to remove *N*-hydroxysuccinimide ester of fatty acid (unbound to proteins) and free *N*-hydroxysuccinimide. The dialysate was freeze-dried and the resulting modified gelatin powder was stored at -20°C until use. The control was prepared in the same manner except that *N*-hydroxysuccinimide ester of fatty acid was

excluded and the distilled water was used instead. Gelatin-fatty acid complexes were subjected to analyses.

6.3.4 Determination of free amino group content and degree of modification

Free amino group content cuttlefish skin gelatin without and with modification was determined using *o*-phthalic dialdehyde (OPA) as described by Church *et al.* (1983). The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium borate, 200 mg of 2-(dimethylamino)ethanethiol hydrochloride (DMA), and 5 mL of 10% SDS. The total volume was adjusted to 50 mL with distilled water. To determine free amino group content, 65 µL of sample (1% protein) was mixed with 3 mL of reagent solution. After 2 min, the absorbance was read at 340 nm using a UV–vis spectrophotometer (UVmini-1204, Shimadzu, Duisburg, Germany). to measure alkylisoindole derivatives formed after the reaction of OPA with free amino group. Free amino group content was calculated from a standard curve of L-leucine (0 – 10 mM) and expressed as mmol/g protein. Degree of modification was expressed as the percentage of amino group attached with fatty acid esters relative to total amino group.

6.3.5. Determination of surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (10 mg/mL) was diluted in 10 mM sodium phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5%. The diluted protein solution (2 mL) was mixed with 20 µL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0). The fluorescence intensity was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

6.3.6 Determination of zeta potential

Zeta potential of the gelatin was determined using a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). Gelatin was dissolved in distilled water to obtain the final concentration of 0-1 mg/mL and adjusted to pH 3-9 using either 1.0 M nitric acid or 1.0 M KOH. Electrophoretic mobility was monitored at 150 V applied voltage using a He–Ne laser at 632 nm.

6.3.7 Determination of surface tension

Gelatin solutions (1 mg protein/mL) were prepared freshly before measurement. The surface tension of gelatin samples was measured as a function of time (0–5000 s) on an automated drop tensiometer (ADT; I. T. Concept, Longessaigne, France), as described by Wierenga *et al.* (2005). An air bubble was formed at the tip of a syringe needle placed in a cuvette containing the sample solution. Both the cuvette and the syringe were temperature controlled (20 ± 0.1°C). Bubble volume was kept constant at 4 μL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. Surface tension was determined by bubble shape analysis. (Windrop software, I. T. Concept, Longessaigne, France).

6.3.8 Determination of emulsifying properties

Emulsion activity index (EAI), a rough estimate of the particle size of the emulsion based on the interfacial area per unit of protein, was calculated by turbidity EAI of gelatin samples was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and modified gelatin solution (1 % protein, 6 mL) were homogenized using a homogenizer (model T25 basic, IKA LABORTECHNIK, Staufen, Germany) at a speed of 19,000 rpm for 1 min. Emulsions were pipetted out (100 μL) at 0 and 10 min after homogenization and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer. EAI at 0 and 10 min after homogenization were calculated by the following formula (Pearce and Kinsella, 1978).

$$EAI\ (m^2/g) = (2\times 2.303\ A)DF/l\phi C$$

where A is the absorbance measured at 500 nm, l = path length of cuvette (m), DF= the dilution factor (100), ϕ = oil volume fraction (0.25) and C = protein concentration in aqueous phase (g/m^3).

6.3.9 Effect of gelatin modified with *N*-hydroxysuccinimide ester of fatty acid on stability of emulsion

6.3.9.1 Preparation of emulsion

Gelatins without and with modification were dissolved in 10 mM sodium phosphate buffer solution (pH 7.0) to obtain a final concentration of 2 % (w/v). Oil-in-water emulsions were prepared by homogenizing the mixture of sunflower oil and modified gelatin solution (1:9, v/v) at a speed of 13,000 rpm for 2 min using a homogenizer. These coarse emulsions were then passed through a laboratory-scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands) at 75 bar for 10 times. The pH of the emulsion (40 mL) was adjusted to pH 7.0 using 1 M NaOH or 1 M HCl. The final volume of all emulsions was adjusted to 50 mL using 10 mM sodium phosphate at pH 7. NaN_3 (0.02 %) was added to the emulsions as an antimicrobial agent. All emulsion samples were stored at room temperature for 10 days. At day 0 and 10 of storage, the emulsion samples were analyzed.

6.3.9.2 Measurement of zeta potential and mean particle diameter

Zeta potential and mean particle diameter of oil droplet in emulsion were determined using Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK) at 25°C. Prior to analysis, the samples were 100-fold diluted with the buffer solution having the same pH and NaCl concentration with the sample. The particle diameter was reported as volume-surface mean particle diameter, d_{32} ($=\sum n_i d^3_i/\sum n_i d^2_i$) where d_i is the diameter and n_i is the number of particles in the i^{th} size class. Volume-weighted mean diameter, d_{43} ($=\sum n_i d^4_i/\sum n_i d^3_i$), was also calculated.

6.3.9.3 Emulsion stability under different conditions

The effect of different environmental conditions (pH, ionic strength, and temperature) on emulsion stability was investigated. An emulsion was prepared as described above (10 wt% sunflower oil, 90 wt% of 2% (w/v) gelatin solution, pH 7.0). To study the effect of pH on emulsion stability, the pH of the emulsions (20 mL) was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 using 1 M HCl or 1 M NaOH. The final volume of all emulsions was adjusted to 25 mL using 10 mM sodium phosphate at the same pH. To test the effect of ionic strength on emulsion stability, 0, 0.5, 1.25 and 2.5 mL of 4 M NaCl solutions were added to 20 mL of emulsions. The final volume of all emulsions was adjusted to 25 mL using distilled water to obtain a final NaCl concentration of 0, 100, 250 and 500 mM. To determine the impact of heating temperature on emulsion stability, emulsion samples in 10 mM sodium phosphate buffer (pH 7) (10 mL) were added with 2.5 mL of distilled water. The emulsion samples were transferred into screw cap test tubes and then incubated in a water bath for 30 min at 50, 70 and 90°C. After incubation, the emulsion samples were cooled under running tap water. All emulsion samples (10 mL) in glass test tubes (internal diameter 15 mm, height 125 mm) were stored at room temperature for 1 day. For all emulsion samples, the zeta potential and mean particle diameter of oil droplet were measured as described above.

6.3.10 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

6.4 Results and Discussion

6.4.1 Degree of modification

The covalent attachment of fatty acids to gelatin was estimated by determining the degree of modification, which was expressed as the extent of NH_2 groups of gelatin attached with fatty acids. The degrees of modification of gelatin modified with *N*-hydroxysuccinimide esters of various fatty acids using different molar ratios are shown in Figure 36. Generally, the degree of modification increased with increasing molar ratio of fatty acid ester-to-gelatin used ($p<0.05$). This result indicated that *N*-hydroxysuccinimide esters of fatty acid could interact with gelatin via amino groups, either N-terminal or ε-amino group of lysine, resulting in a decrease in free amino group content of modified gelatin.

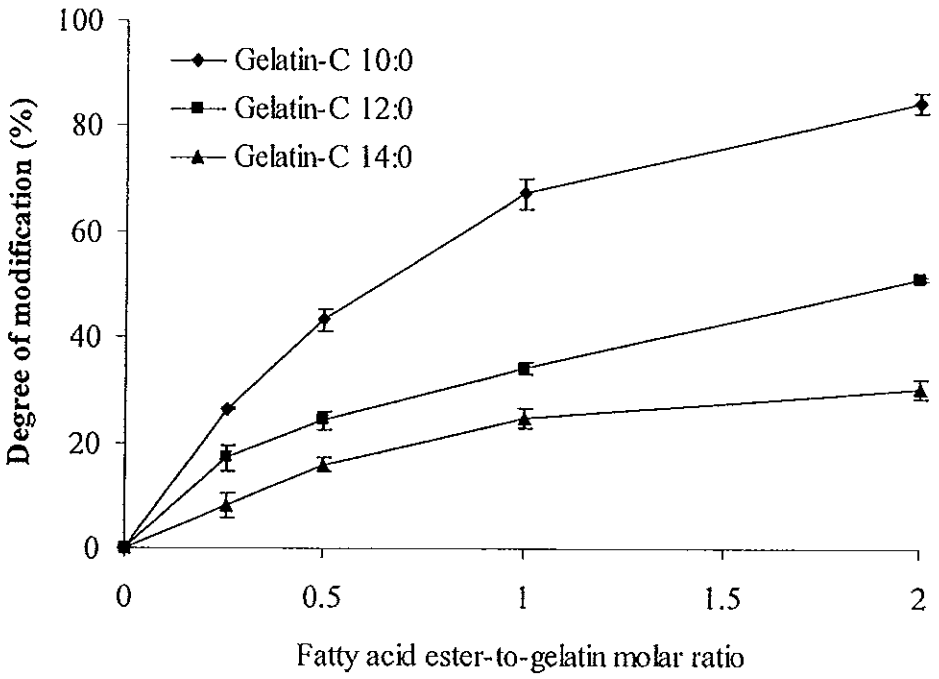


Figure 36. The degree of modification of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. Bars represent the standard deviation (n=3).

At the same molar ratio, the highest degree of modification was observed in gelatin modified with fatty acid ester of C10:0 ($p<0.05$), followed by those modified with fatty acid esters of C12:0 and C14:0, respectively. This result suggested that the increase in chain length of fatty acids more likely lowered reactivity of fatty acid ester in attachment with gelatin. However, gelatin modified with fatty acid esters with longer chain such as C16:0 had the low solubility and could not act as the potential emulsifier (data not shown). Fatty acids with the longer chain length might not align properly, whereby the covalent interaction between fatty acid and amino group was prevented, more likely due to steric hindrance. On the other hand, the shorter chain fatty acid ester (C10:0) might disperse and interact with amino group of gelatin in aqueous phase more effectively.

6.4.2 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of gelatin modified with various fatty acid esters generally increased as the degree of modification increased (Figure 37). Attachment of fatty acids could therefore increase the surface hydrophobicity of resulting gelatin. Fatty acids, which are hydrophobic in nature, contributed to the hydrophobic domain of modified gelatin. The higher increase in surface hydrophobicity was observed in gelatin modified with fatty acid ester of C14:0, followed by gelatin modified with fatty acid esters of C12:0 and C10:0, respectively. At the same degree of modification, gelatin modified with fatty acid ester of C14:0 showed the higher surface hydrophobicity than others ($p<0.05$). Although the higher degree of modification was observed for gelatin modified with fatty acid ester having a shorter chain, gelatin modified with fatty acid ester with a longer chain showed a higher increase in surface hydrophobicity. This result was in agreement with Toledano and Magdassi (1997) who reported that the increasing chain length of fatty acid caused a greater increase in hydrophobicity of modified bovine gelatin. Therefore, surface hydrophobicity of gelatin modified with fatty acid ester was governed by both fatty acid chain length and degree of modification.

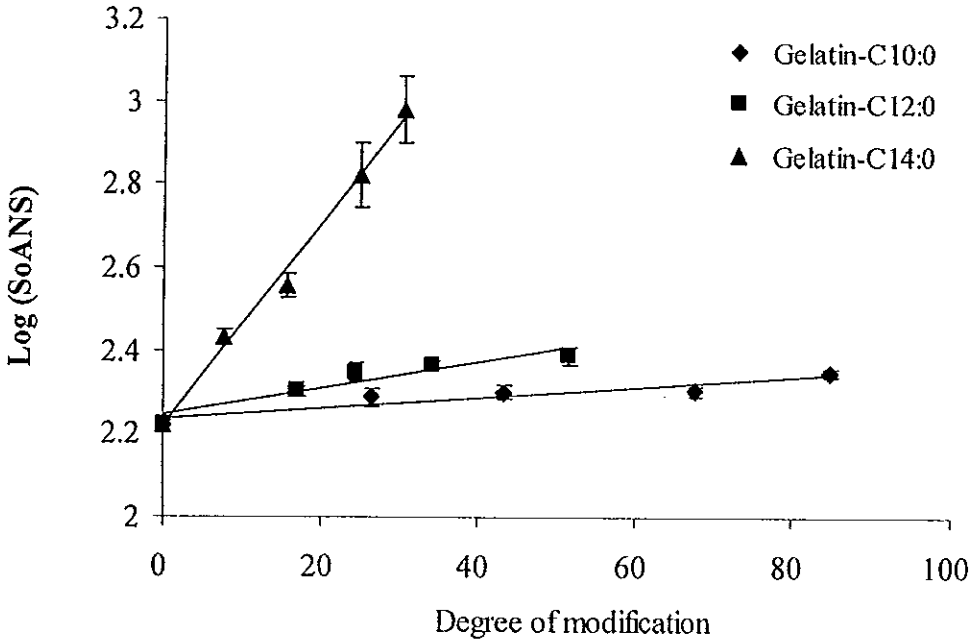


Figure 37. Log of surface hydrophobicity (S_0ANS) vs the degree of modification of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids. Bars represent the standard deviation (n=3).

6.4.3 Zeta potential

The surface charges of gelatin modified with various fatty acid esters at a molar ratio of 2 at different pHs as measured by zeta potential analyzer are shown in Figure 38. The surface charges of all samples varied with pH used. At low pH, the amino and carboxyl groups are protonated. Most of amino groups are positively charged ($-NH_3^+$) but most of carboxyl groups are neutral (-COOH), resulting in the net positive charge. When the pH is increased, both carboxyl and amino groups are deprotonated. Carboxyl groups become the negatively charged ($-COO^-$), whereas the amino groups are neutralized ($-NH_2$), leading to the net negative charge (Onsaard *et al.*, 2006). The pH at which the positive charges on a protein equal the negative charges or the pH at which the net charge of the protein is zero is defined as isoelectric point (pI). Generally, pI of gelatin modified with fatty acid esters decreased when compared with that of control gelatin. The pI of control gelatin was estimated to be 4.5, whereas pI of gelatin modified with fatty acid ester of C10:0, C12:0 and

C14:0 were estimated to be 3.9, 4.0 and 4.3, respectively. The lowest pI was observed in gelatin modified with fatty acid ester of C10:0.

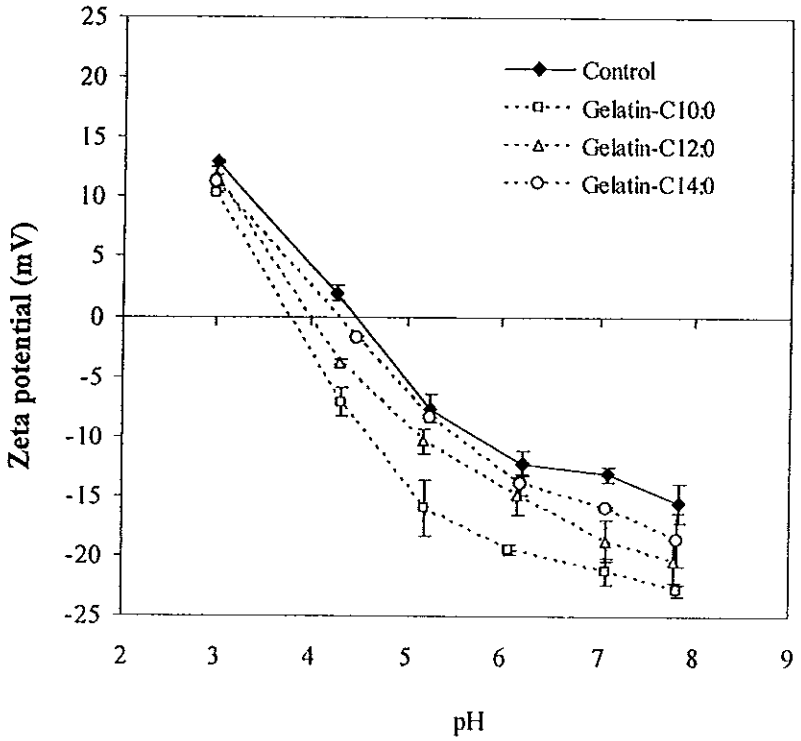


Figure 38. Zeta potential of gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at a molar ratio of 2. Bars represent the standard deviation (n=3).

Kamyshny and Magdassi (1997) reported that the attachment of *N*-hydroxysuccinimide ester of fatty acids led to changes in both the amplitude of charge and the pI of human IgG. At the same pH, it was noted that modified gelatin had the higher amplitude of negative charge, compared with the control gelatin (unmodified) ($p<0.05$). This was probably due to the attachment of fatty acids with amino groups, which were associated with the less protonation of these groups. This led to the lowered positively charged amino groups. When comparing the charge amplitude of modified gelatin with different fatty acid esters, the one modified with longer chain fatty acid showed lower negative charge than those with shorter chain fatty acids. Fatty acid ester with the shorter chain could attach to amino groups of gelatin to a higher extent. As a consequence, available amino groups became less, and protonation of these groups took place at a lower extent. This resulted in the less negatively

charged amino groups. Interaction between fatty acid ester and gelatin might block the available amino residue of gelatin via formation of amide bond, causing the loss in number of positive charge and might alter the net charge of modified gelatin (Kamyshny and Magdassi, 1997).

6.4.4 Surface tension

Surface tension of solutions of gelatin without and with modification is shown in Figure 39. Generally, surface tension of sample solutions decreased as the storage time increased. The degree of reduction of surface tension could indicate the ability of gelatin to localize or accumulate at air-water interface. Gelatin modified with fatty acid esters showed a rapid decrease in surface tension, compared with the control gelatin. This indicated that the incorporation of fatty acid into gelatin could yield more surface active gelatin. The decrease in surface tension of modified gelatin depended on the degree of modification and chain length of fatty acid used. Surface tension of modified gelatin decreased with increasing chain length.

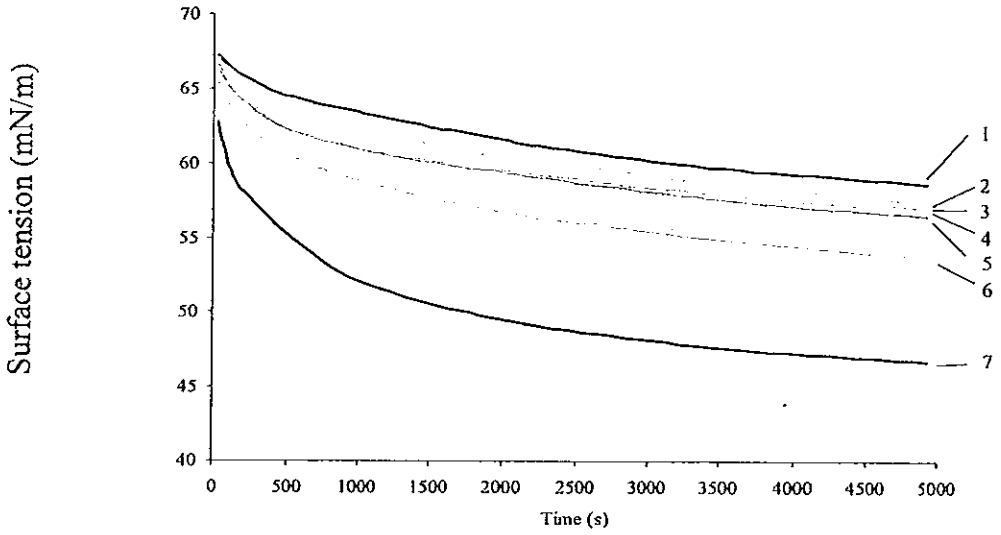


Figure 39. Surface tension of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios. 1: unmodified gelatin; 2 and 3: gelatin modified with C10:0 at molar ratios of 0.5 and 2; 4 and 5: gelatin modified with C12:0 at molar ratios of 0.5 and 2; 6 and 7: gelatin modified with C14:0 at molar ratios of 0.5 and 2.

Among three types of modified gelatin, that modified with fatty acid ester of C14:0 was more surface active than others, when the same molar ratio was used ($p<0.05$). The increase in surface activity was in accordance with the increase in surface hydrophobicity of modified gelatin (Figure 37). When comparing the decrease in surface tension between gelatin modified with the same fatty acid ester at different molar ratios, it was found that the higher ratio (2) rendered the gelatin with more surface active behavior, compared with the lower ratio (0.5). The higher fatty acids incorporated into gelatin most likely provided the hydrophobic domain to the gelatin. As a result, modified gelatin could diffusionally transport and absorb at interface more effectively.

6.4.5 Emulsifying properties

6.4.5.1 Emulsifying activity of gelatin

Emulsion activity index (EAI) of cuttlefish skin modified with various fatty acid esters at different molar ratios is shown in Figure 40. Modification of gelatin with fatty acid esters resulted in the increase in EAI, both at 0 and 10 min after emulsification, when compared with the control gelatin. EAI of modified gelatin increased with increasing fatty acid ester-to-gelatin molar ratio used. At the same molar ratio, gelatins modified with fatty acid ester of C14:0 had the higher EAI, compared with gelatin modified with fatty acid esters of C12:0 and C10:0, respectively ($p<0.05$). The increase in EAI was coincidental with the increases in surface hydrophobicity of modified gelatin (Figure 37). Surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved (Kato and Nakai, 1980). When the same fatty acid ester was incorporated into gelatin, EAI was increased as the fatty acid ester-to-gelatin molar ratio increased ($p<0.05$). However, for gelatin modified with C10:0 or C12:0, no increase in EAI was found when the ratio of higher than 0.5 was used ($p>0.05$). Thus, the type of fatty acid used also had the impact on EAI of resulting gelatin. The increase in hydrophobic region at surface of modified gelatin facilitated gelatin molecules to localize at the oil-water interface and reduce surface tension more effectively. This resulted in the improved emulsifying properties of gelatin after modification with fatty acid ester.

For stability of emulsion, the relative decrease of EAI at 10 min was determined. It was observed that emulsion stabilized by modified gelatin, especially gelatin modified with fatty acid ester of C14:0, was more stable than that of the control gelatin ($p<0.05$). When the same fatty acid ester was used, stability of emulsion tended to be higher as the higher fatty acid-to-gelatin molar ratio was used ($p<0.05$). The result indicated that increasing hydrophobic region of gelatin could indeed improve its emulsifying ability and emulsion stability.

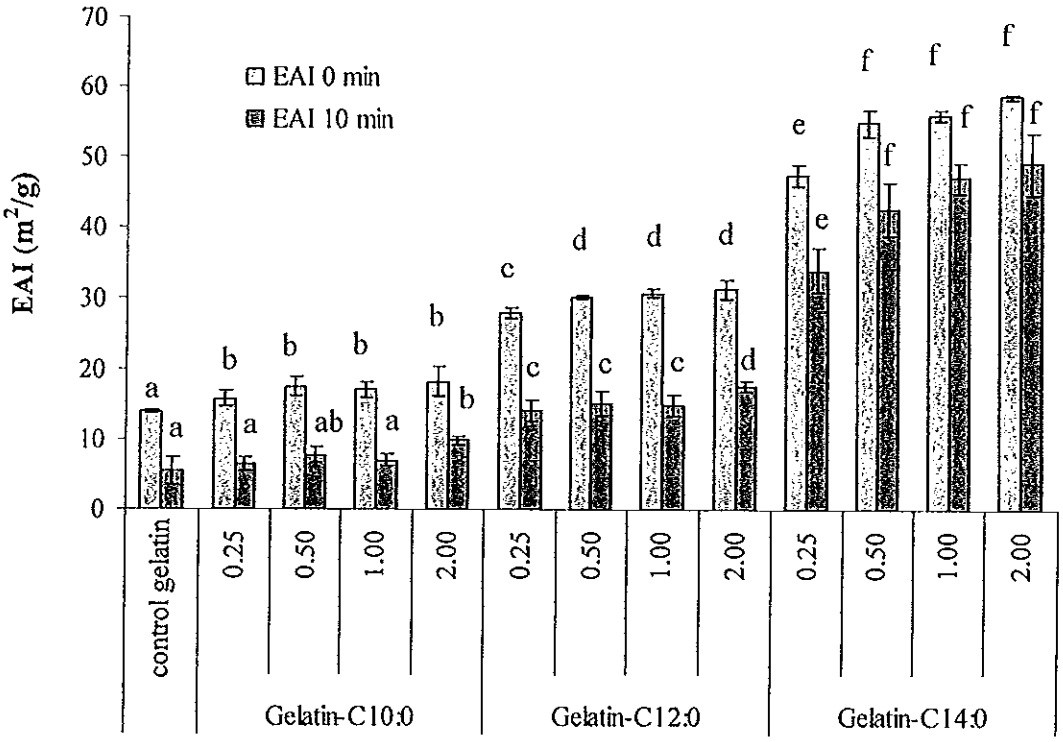


Figure 40. Emulsion activity index (EAI) of cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of different fatty acids at various molar ratios at 0 and 10 min after emulsification. Bars represent the standard deviation (n=3). Different letters in the same time after emulsification indicate significant differences ($p<0.05$).

6.4.5.2 Zeta potential and mean particle diameter of oil droplet in emulsion

The zeta potential and mean particle diameter of oil-in-water emulsion stabilized by the control gelatin and gelatin modified with fatty acid esters at a molar ratio of 2 are shown in Table 18. From zeta potential analysis, oil droplet in all samples had the negative charge. Since the emulsion was prepared at neutral pH, which was higher than pI of each gelatin (Figure 38), gelatin covering the oil droplet became negatively charged. The measurement of zeta potential values in emulsion provides the useful information on the electrostatic repulsion between oil droplets, closely related with coalescence and stability phenomena (McClements, 2005). Emulsion stabilized by gelatin modified with fatty acid esters, especially shorter chain fatty acid, had higher negative charge than emulsion stabilized by gelatin modified with the longer chain fatty acid and the control gelatin, respectively. Net charge of emulsion was in agreement with that of gelatin modified with different fatty acid ester (Figure 38). A high zeta potential implies the resistance to cohesion or coalescence of emulsion because of the electrostatic repulsion of adjacent emulsion droplets (McClements, 2005). Nevertheless, not only charge of gelatin surrounding oil droplet but also the hydrophobicity as well as the steric hindrance mediated by fatty acid could contribute to stability of emulsion.

Mean particle diameters of oil droplet in emulsions stabilized by gelatin modified with fatty acid ester at a molar ratio of 2 at day 0 and 10 of storage at room temperature are shown in Table 18. The mean particle diameter of oil droplet was calculated and expressed as a volume-surface mean particle diameter (d_{32}) and volume-weighted mean particle diameter (d_{43}) (Surh *et al.*, 2006). Generally, the diameter size of oil droplet in emulsion stabilized by modified gelatin after emulsification was smaller than that found in emulsion stabilized by the control gelatin ($p<0.05$). At day 0, the d_{32} and d_{43} of oil droplet in emulsion stabilized by control gelatin was 1.10 ± 0.07 and 1.34 ± 0.09 μm, respectively. The lowest d_{32} and d_{43} of oil droplets were observed in emulsion stabilized by gelatin modified with fatty acid ester of C14:0 (0.72 ± 0.02 and 0.84 ± 0.02 μm), followed by C12:0 (0.85 ± 0.04 and 1.03 ± 0.13 μm) and C10:0 (0.97 ± 0.02 and 1.30 ± 0.07 μm), respectively.

Table 18. Zeta potential and mean particle diameter of oil droplet in emulsion stabilized by 2.0 % gelatin modified without and with fatty acid ester at a molar ratio of 2 at pH 7.

Gelatin	Zeta potential (mV)	Mean particle diameter size (μm)			
		Day 0		Day 10	
		d_{32}	d_{43}	d_{32}	d_{43}
Control	-19.05 ± 1.18a*	1.10 ± 0.07dA#	1.34 ± 0.09cA	1.85 ± 0.08dB	2.12 ± 0.23dB
Gelatin-C10:0	-28.31 ± 1.12d	0.97 ± 0.02cA	1.30 ± 0.07cA	1.41 ± 0.08cB	1.85 ± 0.07cB
Gelatin-C12:0	-24.97 ± 0.66c	0.85 ± 0.04bA	1.03 ± 0.13bA	1.13 ± 0.03bB	1.40 ± 0.03bB
Gelatin-C14:0	-22.53 ± 0.74 b	0.72 ± 0.02aA	0.84 ± 0.02aA	0.85 ± 0.07aB	1.06 ± 0.09aB

Mean±SD (n=3).

* Different letters in the same column indicate significant differences ($p<0.05$).

Different capital letters in the same sample for the same mean diameter indicated significant differences ($p<0.05$).

The result suggested that alkyl group of fatty acid with the longer chain more likely migrated to the interface at a faster rate after emulsification. As a result, the gelatin could adsorb and align as a film surrounding the oil droplet more effectively. This result was in agreement with Lin and Chen (2006) who reported that bovine gelatin attached with C_{12} had the lower mean particle diameter than that of gelatin attached with C_9. Modified gelatin might cover the oil droplets and simultaneously lower the surface tension. Thus, sufficiently dense adsorption layer was formed during emulsification. As a consequence, the smaller oil droplets could be produced as evidenced by the lower d_{32} and d_{43}.

After storage for 10 days at the room temperature, the increases in mean particle diameter (d_{32} and d_{43}) of oil droplets were obtained in all samples ($p<0.05$). This result might be due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during storage (Djordjevic *et al.*, 2008). The higher increase in mean particle diameter was observed in emulsion stabilized by control gelatin, compared with modified gelatin ($p<0.05$). This reconfirmed that modified gelatin could stabilize oil-in-water emulsion more effectively. Among all emulsion samples, that stabilized by gelatin modified with fatty acid ester of C14:0 had the lowest increase in d_{32} and d_{43}, when compared with others ($p<0.05$). Formation of small oil droplet along with the thick film of gelatin increased emulsion stability. The small droplet emulsion had much better stability to gravitational separation and aggregation than the large droplet emulsion (Tadros *et al.*, 2004). Therefore, gelatin modified with fatty acid ester of C14:0, especially at fatty acid ester-to-gelatin molar ration of 2, showed the highest emulsion activity as well as rendered the emulsion with the highest stability.

6.4.6 Effect of environmental conditions on stability of emulsion stabilized by modified gelatin

6.4.6.1 Effect of pH

The effect of various pHs (3-8) on the zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified with fatty acid ester of C14:0 with a fatty acid ester-to-gelatin ratio of 2 is shown in Figure 41. Generally, surface charge of oil droplet switched from positive charge to negative charge when

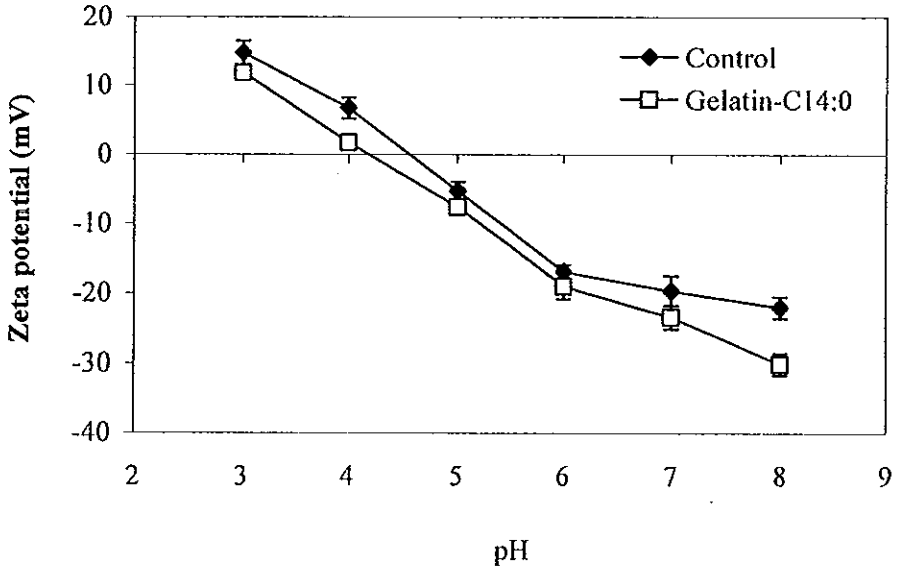
pH was increased (Figure 41A). With increasing pH from 3 to 8, the zeta potential of oil droplet decreased from +14.75 to -21.89 mV for emulsion stabilized by the control gelatin and from +11.85 to -29.29 mV for emulsion stabilized by modified gelatin. The result of zeta potential of emulsion was in accordance with that of gelatin (Figure 38). This reconfirmed that gelatin occupied at the oil droplet surface mainly contributed to the charge of emulsion. The zero net charge of oil droplets in emulsion stabilized by the control gelatin and modified gelatin was observed at pH 4.5 and 4.2, respectively. At the pH having the net charge of zero, the emulsion might not be stable due to the lowering of repulsive force.

No differences in mean particle diameters of oil droplet in emulsion stabilized by both control gelatin and modified gelatin were observed at different pHs ($p>0.05$). This result indicated that both emulsions stabilized by the control gelatin and modified gelatin remained relatively stable to droplet aggregation at all pH tested, even at pH close to that giving the net charge of zero. The result suggested that gelatin film might be strong enough to prevent the flocculation or coalescence of oil droplets.

6.4.6.2 Effect of salt concentration

The effect of different salt concentrations on stability of emulsion stabilized by gelatin modified without and with fatty acid ester of C14:0 is shown in Table 19. Generally, the amplitude of negative charge of emulsion stabilized by both control gelatin and modified gelatin decreased with increasing NaCl concentrations used. In the presence of NaCl at concentrations of 100, 250 and 500 mM, the negative charge of emulsion stabilized by the control gelatin decreased from -19.53 mV to -9.85, -8.39 and -4.24 mV, respectively, whereas that of emulsion stabilized by modified gelatin decreased from -23.3 mV to -9.24, -9.10 and -6.24 mV, respectively. In general, the increase in ionic strength affects the electrostatic repulsion between the droplets of emulsion (Onsaard *et al.*, 2006). In the presence of counter ions, the charge on an emulsion is reduced by electrostatic screening, in which the repulsion between the droplets is no longer sufficiently strong to overcome the attractive force (e.g., vander Waals and hydrophobic) acting between the droplets (Gu *et al.*, 2005; Onsaard *et al.*, 2006).

A



B

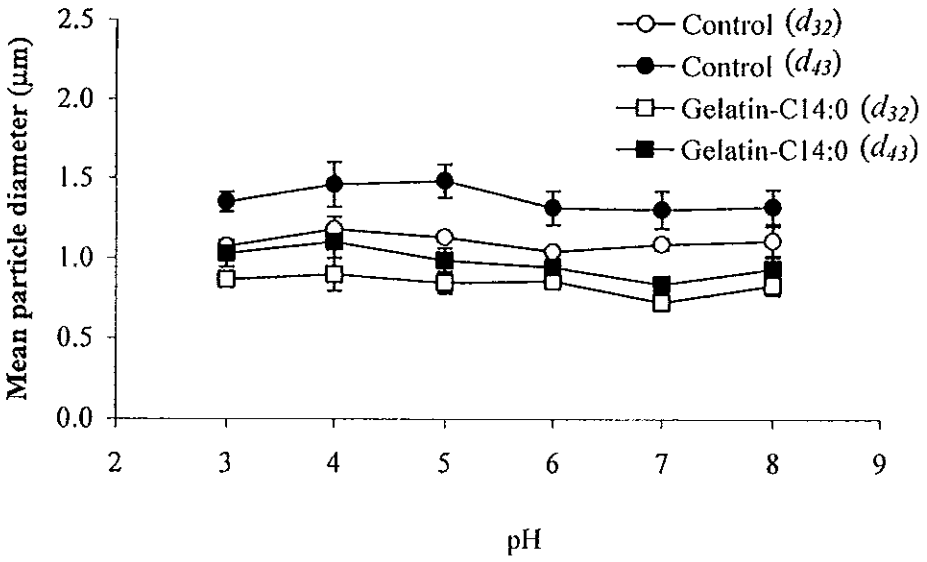


Figure 41. Effect of pH on zeta potential (A) and mean particle diameter (B) of emulsion stabilized by the cuttlefish skin gelatin modified without and with fatty acid ester of C14:0. Bars represent the standard deviation (n=3).

Table 19. Effect of NaCl concentration on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified without and with fatty acid ester of C14:0 at pH 7.

Gelatin	NaCl (mM)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Control	0	-19.53 ± 0.92a*	1.02 ± 0.02a	1.27 ± 0.10a
	100	-9.85 ± 0.81b	1.04 ± 0.05a	1.30 ± 0.01a
	250	-8.38 ± 0.23b	1.04 ± 0.02a	1.43 ± 0.03b
	500	-4.24 ± 0.42c	1.17 ± 0.09b	1.52 ± 0.08b
Gelatin-C14:0	0	-23.31 ± 0.71a	0.76 ± 0.01a	0.84 ± 0.03a
	100	-9.24 ± 0.76b	0.80 ± 0.01b	0.93 ± 0.03b
	250	-9.10 ± 0.01b	0.88 ± 0.01c	0.97 ± 0.01b
	500	-6.24 ± 0.23c	0.88 ± 0.06bc	1.04 ± 0.04c

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

For the particle size of oil droplet, the slight increase in mean particle size (both d_{32} and d_{43}) of oil droplet was found in emulsion stabilized by both the control gelatin and modified gelatin when NaCl concentrations increased ($p<0.05$). When the NaCl concentration was increased from 0 to 500 mM, the mean particle size of emulsion stabilized by the control gelatin increased from 1.02 ± 0.02 to 1.17 ± 0.09 μm for d_{32} and from 1.27 ± 0.10 to 1.52 ± 0.18 μm for d_{43}, whereas that of emulsion stabilized by modified gelatin increased from 0.76 ± 0.00 to 0.88 ± 0.06 μm for d_{32} and from 0.84 ± 0.03 to 1.04 ± 0.04 μm for d_{43}. The increase in emulsion droplet size was in agreement with the decrease in negative charge amplitude, suggesting that the decrease in repulsion between oil droplets mainly caused the coalescence of oil droplets. Therefore, stability of emulsion containing gelatins, without and with modification, was affected to same degree by high ionic strength. Surh *et al.* (2006) found that fish gelatin stabilized emulsions remained relatively stable to droplet aggregation at high ionic strength (500 mM). It was suggested that not only electrostatic repulsion played a major role in preventing the droplets from aggregation, but also steric repulsion, which could stabilize emulsion.

6.4.6.3 Effect of heating

The effect of heating at different temperatures (50, 70 and 90°C) on the stability of emulsions stabilized by gelatin modified without and with fatty acid ester of C14:0 is shown in Table 20. Generally, no significant effect of heating on surface charge of oil droplet was observed in both emulsions stabilized by the control gelatin and modified gelatin ($p<0.05$). Negative charge of oil droplet in emulsion stabilized by the control gelatin was lower than that of emulsion stabilized by modified gelatin.

For mean particle diameter, there was no change in d_{32} and d_{43} of oil droplet in all emulsions heated at 50, 70 and 90°C, except the emulsion stabilized by the control gelatin and heated at 90°C ($p<0.05$). The slight increase in mean particle diameter of d_{32} and d_{43} was found in emulsion stabilized by control gelatin after being heated at 90°C for 30 min. It was suggested that some partial desorption of gelatin molecules from the droplet surfaces at elevanted temperatures might lead to flocculation and/or coalescence (Surh *et al.*, 2006). However, no changes in droplet size of oil were found in emulsion stabilize by modified gelatin after heating at 90°C. The result suggested that modified gelatin yielded the emulsion with higher stability, especially when heated at high temperature. This might be due to the higher stability of hydrophobic film of gelatin modified with fatty acid to heat treatment. As a result, the emulsion could be retained after heating.

Table 20. Effect of heating at different temperatures on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified without and with fatty acid ester of C14:0 at pH 7.

Gelatin	Temperature (°C)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Control	50	-18.80 ± 0.14a*	1.04± 0.01a	1.29 ± 0.07a
	70	-19.09 ± 0.92a	1.07 ± 0.01a	1.30 ± 0.01a
	90	-18.82 ± 0.24a	1.14 ± 0.11b	1.43 ± 0.03b
Gelatin-C14:0	50	-23.92 ± 0.11a	0.71 ± 0.02a	0.84 ± 0.02a
	70	-23.43 ± 0.57a	0.75 ± 0.03a	0.82 ± 0.02a
	90	-24.86 ± 0.18b	0.74 ± 0.02a	0.84 ± 0.01a

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

6.5 Conclusion

Modification of gelatin with fatty acid ester could improve surface activity of obtained gelatin as indicated by the decrease in surface tension and the increase in emulsifying properties. Gelatin modified with fatty acid ester of C14:0, especially at the high degree of modification, rendered the emulsion with small oil droplet size and high stability, compared with the control gelatin. Generally, the enhanced emulsifying properties of gelatin modified with fatty acid ester of C14:0 were obtained, though the degree of modification was relatively low. The emulsion stabilized by gelatin modified with fatty acid ester of C14:0 was stable in the wide pH range (3 to 8), and at NaCl concentration up to 500 mM. The emulsion was also stable after being heated up to 90°C. Therefore, the modified gelatin could be used as a promising emulsifier in both food and non-food systems.

CHAPTER 7

Surface active properties and molecular characteristics of cuttlefish skin gelatin modified by oxidized linoleic acid

7.1 Abstract

Surface activity and molecular changes of cuttlefish skin gelatin modified with oxidized linoleic acid (OLA) prepared at 60, 70 and 80°C at different times were investigated. Modification of gelatin with OLA could improve surface activity of resulting gelatin as evidenced by the decreased surface tension and the increased foaming and emulsifying properties. Interaction between OLA and gelatin led to the generation of carbonyl groups, loss of free amino content and the increase in particle size of resulting gelatin. Emulsion stabilized by modified gelatin had the smaller mean particle diameter with higher stability, compared with that stabilized by gelatin without modification.

7.2 Introduction

Gelatin, the denatured form of collagen, is used as foaming, emulsifying and wetting agents in food, pharmaceutical, medical and technical applications due to its surface active properties (Karim and Bhat, 2009). In emulsion, gelatin can adsorb at interfaces between oil and water and form a continuous viscoelastic membrane-like film around oil droplets, resulting in the improved stability of oil-in-water emulsion (Damodaran, 2005). Gelatin is commercially made from skins and skeletons of bovine and porcine by alkaline or acidic extraction (Gilsenan and Ross-Murphy, 2000). However, gelatin from aquatic sources (fish skins, bones, and fins) can be an alternative to mammalian counterpart (Kim *et al.*, 1996). Fish gelatin is advantageous due to the lack of BSE outbreaks. It is acceptable for Islam and can be used with minimal restrictions in Judaism and Hinduism.

Moreover, the production of gelatin from by-product of the fish-processing industry could reduce waste and pollution.

Cuttlefish is one of major seafood products of Thailand. During processing, the skin is removed and becomes the by-product with the low market value. Recently, Aewsiri *et al.* (2009a) successfully extracted the gelatin from cuttlefish skin. However, gelatin from cuttlefish skin had the poorer surface activity due to the lower hydrophobic amino acids content (proline and leucine) and a large portion of hydrophilic amino acids (lysine, serine, arginine, hydroxyproline, aspartic acid and glutamic acid) (Aewsiri *et al.*, 2009a; Hoque *et al.*, 2010). Gelatin with a higher hydrophilic region generally has the limited ability to function as a surface active agent (Toledano and Magdassi, 1997).

Attachment of hydrophobic domain to protein may lead to the increase in its hydrophobicity, causing the changes in the surface activity and functional properties of protein (Kato and Nakai, 1980; Toledano and Magdassi, 1997). Lipid oxidation products, including lipid hydroperoxides and reactive aldehyde derivatives, could modify amino acids of proteins (Stadtman and Berlett, 1997). Alkyl and peroxyl radical decomposed from hydroperoxide can directly interact with side chains of proteins (Kato *et al.*, 1992). The secondary lipid oxidation products, such as aldehydes, react mainly with amino acids via condensation reaction to from Schiff's bases or by Michael addition reactions (Liu *et al.*, 2003; Stadtman and Berlett, 1997). Therefore, the introduction of lipid oxidation products to gelatin might provide the hydrophobic domain for gelatin, thereby facilitating the migration of gelatin to the interface. Therefore, the objectives of this work were to study the improvement of surface activity of cuttlefish skin gelatin via the modification with oxidized linoleic acid as affected by reaction time and temperature and to investigate the effect of modified cuttlefish skin gelatin on the stability of emulsion under different harsh conditions.

7.3 Materials and methods

7.3.1 Chemicals

Linoleic acid, *o*-phthalic dialdehyde (OPA), 2-(dimethylamino)ethanethiol hydrochloride (DMA), cumene hydroperoxide and 1-anilinonaphthalene-8-sulphonic acid (ANS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), sodium hydroxide, ethanol, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), sodium chloride, sodium hydroxide, sodium sulfite, ammonium thiocyanate (NH_4SCN), barium chloride ($BaCl_2$), ferrous sulfate ($FeSO_4$) and boric acid were procured from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

7.3.2 Extraction of gelatin from cuttlefish skin

Cuttlefish (*Sepia pharaonis*) skin gelatin was prepared according to the method of Aewsiri *et al.* (2009b). Ventral skin of cuttlefish was washed with tap water and cut into small pieces (1 x 1 cm^2). Skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28°C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5 % H_2O_2 at 4 °C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using the distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min at 25°C using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ µP/Dura Dry™ µP, FTS® System, Inc., Stone Ridge, NY, USA).

7.3.3 Preparation of oxidized linoleic acid

Prior to the preparation of the oxidized linoleic acid (OLA), the mixture of linoleic acid/Milli-Q water (a ratio of 1:1) was homogenized at a speed of

9,500 rpm for 2 min using a homogenizer (model T25 basic, IKA LABORTECHNIK, Staufen, Germany) to remove soluble lipid oxidation products. The linoleic acid was recovered by centrifugation at 3,000 x g for 15 min at room temperature using a centrifuge (model z323k, Hermle Labor Technik, Wehingen, Germany). Ten mL of linoleic acid were placed in 15 mL-glass test tube. The linoleic acid was flushed with air continuously and incubated at different temperatures (60, 70 and 80°C) for various times (3, 6, 9 and 12 h). All samples obtained were subjected to analyses.

7.3.3.1 Peroxide value

Peroxide value was determined using a method of Shantha and Decker (1994) with a slight modification. Sample (0.3 mL) was added to 1.5 mL of a mixture of isooctane/2-propanol (3:1, v/v), vortexed 3 times for 10 s each, followed by centrifuging for 2 min at 2000 x g. The organic phase (approximately 0.2 mL) was added to a mixture of methanol/butanol (2:1, v/v), followed by addition of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ at a ratio of 1:1, v/v). The solution was vortexed and allowed to stand for 20 min. The absorbance was then measured at 510 nm. The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve (0-500 nmol/mL) and expressed as nmol hydroperoxide/ kg sample.

7.3.3.2 Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). One mL of sample was dispersed in 4 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 x g for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The standard curve was prepared using malonaldehyde (2-10 ppm) and TBARS were expressed as mg malonaldehyde/ kg sample.

7.3.4 Modification of cuttlefish skin gelatin

Cuttlefish skin gelatin was dissolved in distilled water to obtain a final concentration of 1.1 % protein determined by the Biuret method (Robinson and

Hodgen, 1940). The linoleic acid oxidized at various conditions was dissolved in ethanol and used as the stock solution. To 90 mL of gelatin solution, 1 mL of OLA was added. The final volume was adjusted to 100 mL with water to obtain the final protein concentration of 1% and the OLA-to-free amino group molar ratio of 5:1 and 10:1. The mixtures were stirred continuously for 3 h at room temperature. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 12 h at room temperature against 20 volumes of 1% ethanol to remove OLA (unbound to proteins) and 12 h against 20 volumes of distilled water to remove ethanol. The dialysate was filtered through a filter paper No. 595½ (Schleicher & Schuell, Dassel, Germany) to remove precipitated product and the filtrate was freeze-dried. The gelatin-linoleic acid complexes were stored at -20°C until used. The control was prepared in the same manner except that oxidized linoleic acid was excluded and the distilled water was used instead. Gelatin-linoleic acid complexes were subjected to analyses.

7.3.4.1 Determination of free amino group content

Free amino group content in native and modified cuttlefish skin gelatin was determined using *o*-phthalicdialdehyde (OPA) as described by Church *et al.* (1983). The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium borate, 200 mg of 2-(dimethylamino)ethanethiol hydrochloride (DMA), and 5 mL of 10 % SDS. The total volume was adjusted to 50 mL with distilled water. To determine free amino group content, 65 µL of sample (1 % protein) was mixed with 3 mL of OPA reagent solution. After 2 min, the absorbance was read at 340 nm to measure alkylisoindole derivatives formed after reaction of OPA with free amino groups using a spectrophotometer (UVmini-1204, Shimadzu, Duisburg, Germany). Free amino group content was calculated from a standard curve of L-leucine (0 – 10 mM).

7.3.4.2 Determination of carbonyl content

Carbonyl content of gelatin without and with modification was determined according to the method of Liu *et al.* (2000). Sample solution (0.5 mL, 4 mg protein/mL) was added with 2.0 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl. The mixture was allowed to stand for 1 h at room temperature. Thereafter, 2 mL of 20% (w/v) trichloroacetic acid (TCA) were added to precipitate protein. The pellet was washed twice with 4 mL of ethanol:ethylacetate (1:1, v/v)

mixture to remove unreacted DNPH, blow-dried, and dissolved in 1.5 mL of 0.6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). The absorbance of solution was measured at 370 nm using a spectrophotometer. A molar absorptivity of 22,400 M^{-1} cm^{-1} was used to calculate carbonyl content (Levine *et al.*, 1990).

7.3.4.3 Measurement of mean particle size

The particle size of gelatin samples dispersed in 10 mM sodium phosphate buffer (pH 7.0) with the final concentration of 1 mg protein/mL was measured by dynamic light scattering at room temperature. The measurements were performed by a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). A refractive index of 1.42 was used for dispersed phase of all protein solutions.

7.3.4.4 Determination of surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Gelatin solution (4 mg/mL) was diluted in 10 mM sodium phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5 % (w/v). The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

7.3.4.5 Fourier transform infrared (FTIR) spectroscopy

Spectra of gelatin without and with modification were obtained using a BioRad FTS-60A FTIR spectrometer (Biorad, Cambridge, MA, USA) equipped with a Attenuated Total Reflectance Accessory (ATR) using a ZnSe ATR crystal. MID-IR spectra were recorded at room temperature between 800 and 4000 cm^{-1} at a resolution of 2 cm^{-1} in the ATR mode on thin films which were purged with dry nitrogen to remove spectral interference resulting from water vapor and carbon dioxide. Sixty-four interferograms were coadded for a high signal-to-noise ratio. Prior to data analysis, the spectra were baseline corrected and normalized (mean normalization option).

7.3.5 Surface active properties of gelatin modified by oxidized linoleic acid

Gelatins without and with modification by linoleic acid oxidized under different conditions were subjected to determination of surface active properties.

7.3.5.1 Surface tension

Gelatin solutions (1 mg protein/mL) were prepared freshly before measurement. The surface tension of gelatin samples was measured as a function of time (0–5000 s) on an automated drop tensiometer (ADT; I. T. Concept, Longessaigne, France), as described by Wierenga *et al.* (2005). An air bubble was formed at the tip of a syringe needle placed in a cuvette containing the sample solution. Both cuvette and syringe were temperature controlled (20 ± 0.1°C). Bubble volume was kept constant at 4 μL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. Surface tension was determined by bubble shape analysis.

7.3.5.2 Foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi *et al.* (1995) with a slight modification. Ten mL of gelatin solution (1 % protein, w/v) were transferred into 100 mL-cylinders (PYREX®, Corning, NY, USA). The mixtures were homogenized for 1 min using a homogenizer at a speed of at 13,400 rpm for 1 min at room temperature. The foam volume was recorded at t= 0 and 30 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_0/V_l) \times 100$$

$$FS\ (\%) = (V_t/V_i) \times 100$$

where V_0 is total volume after whipping (0 min); V_l is the original liquid volume before whipping and V_t is total volume after leaving at room temperature for 30 min.

7.3.5.3 Emulsifying properties

Emulsion activity index (EAI) of gelatin samples was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and gelatin solution (1 % protein, 6 mL) were homogenized using a homogenizer at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0

and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer. EAI at 0 and 10 min were calculated by the following formula:

$$EAI\ (m^2/g) = (2\times 2.303\ A)DF/l\phi C$$

where A is the absorbance measured at 500 nm, l = path length of cuvette (m), DF= the dilution factor (100), ϕ = oil volume fraction (0.25) and C = protein concentration in aqueous phase (g/m^3).

7.3.5.4 Mean particle diameter and the stability of emulsion

Oil-in-water emulsions were prepared by homogenizing the mixture of sunflower oil and gelatin solution (2.25% protein, w/v) at a ratio of 1:9 (v/v) at a speed of 9,500 rpm for 2 min by a homogenizer. These coarse emulsions (pH 7.0) were then passed through a laboratory-scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands) at 75 bar for 10 times. NaN_3 (0.02 %) was added to the emulsions as an antimicrobial agent. The mean particle diameter of oil droplet in emulsion was determined with Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK) at 25°C. Electrophoretic light scattering technique was used to analyze the velocity distribution of particle movement by measuring dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particle. Data was calculated via the Stokes-Einstein equation and expressed as number average distribution of particle diameter. Prior to analysis, the samples were 100-fold diluted with 10 mM sodium phosphate buffer solution. The particle diameter was reported as volume-surfaced mean particle diameter, d_{32} ($=\sum n_i d^3_i/\sum n_i d^2_i$) and volume-weighted mean diameter, d_{43} ($=\sum n_i d^4_i/\sum n_i d^3_i$), where d_i is the diameter and n_i is the number of particles in the i^{th} size class.

To study the stability of emulsion under different conditions, the emulsion was prepared as described above (10 wt% sunflower oil, 2.0 wt% fish gelatin, pH 7.0) and then diluted to a droplet concentration of 2.0 wt% using 10 mM sodium phosphate buffer. The emulsion was incubated in a beaker overnight to allow any large droplets or non-emulsified oil to rise to the top, and then only the lower portion of emulsion was collected.

For pH stability study, the pH of the emulsions (20 mL) was adjusted to 3, 4, 5, 6, 7 and 8 using 0.5 M HCl of 0.5 M NaOH solutions and the final volume of all emulsions was adjusted to the same volume (25 mL) using distilled water previously adjusted to corresponding pH. The emulsions were then stored at room temperature for 24 h before being analyzed. To study the impact of NaCl on emulsion stability, emulsion samples (20 mL) were added with 0, 0.25, 0.5, 1.25 and 2.5 mL of 4 M NaCl solution and adjusted to 25 mL using distilled water to obtain a final NaCl concentrations of 0, 50, 100, 250 and 500 mM. Emulsion samples (10 mL) were then transferred into glass test tubes (internal diameter 15 mm, height 125 mm) and stored at room temperature for 24 h before measurement.

For thermal stability, emulsion samples in 10 mM sodium phosphate buffer (pH 7) (10 mL) were added with 2.5 mL of distilled water. The emulsion samples were transferred into screw cap test tubes and then incubated in a temperature controlled water bath for 30 min at 50, 70 and 90 °C. After incubation, the emulsion samples were cooled under running tap water. All emulsion samples (10 mL) in glass test tubes (internal diameter 15 mm, height 125 mm) were stored at room temperature for 1 day. The zeta potential and mean particle diameter of oil droplet were then measured as described above.

7.3.6 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard

7.3.7 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

7.4 Results and Discussion

7.4.1 Oxidation of linoleic acid as affected by temperature and time

The oxidation of linoleic acid during incubation at different temperatures for various times was monitored as shown in Figure 42. Generally, PV and TBARS of linoleic acid increased with increasing incubation times, except PV of samples incubated for 9 and 12 h, which had the similar value ($p>0.05$) (Figure 42A). At the same incubation time, the higher temperature yielded the higher PV and TBARS ($p<0.05$). The result suggested that the oxidation of linoleic acid proceeded to a higher extent at higher temperature, especially with the longer time. Lipid oxidation is a complex process, in which unsaturated fatty acids react with molecular oxygen, usually via a free radical mechanism, to form hydroperoxides, the primary oxidation products. Thereafter, unstable hydroperoxides are decomposed with the subsequent formation of the secondary lipid oxidation products. PV has been used to indicate the formation of the primary lipid oxidation products, whereas TBARS value represents the formation of secondary lipid oxidation products. Different types and amounts of lipid oxidation products formed under different conditions in OLA might affect the degree of interaction with gelatin differently.

7.4.2 Modification of cuttlefish skin gelatin with linoleic acid oxidized under different conditions

7.4.2.1 Free amino group content

Free amino group content of gelatin modified with linoleic acids oxidized under various conditions with a ratio of OLA-to-free amino group of 5:1 and 10:1 is shown in Table 21. Cuttlefish skin gelatin contained around 0.375 mmol NH_2 groups/ g gelatin. Modification of gelatin with OLA caused the loss in free amino group content of resulting gelatin, compared with the control gelatin (without modification) ($p<0.05$).

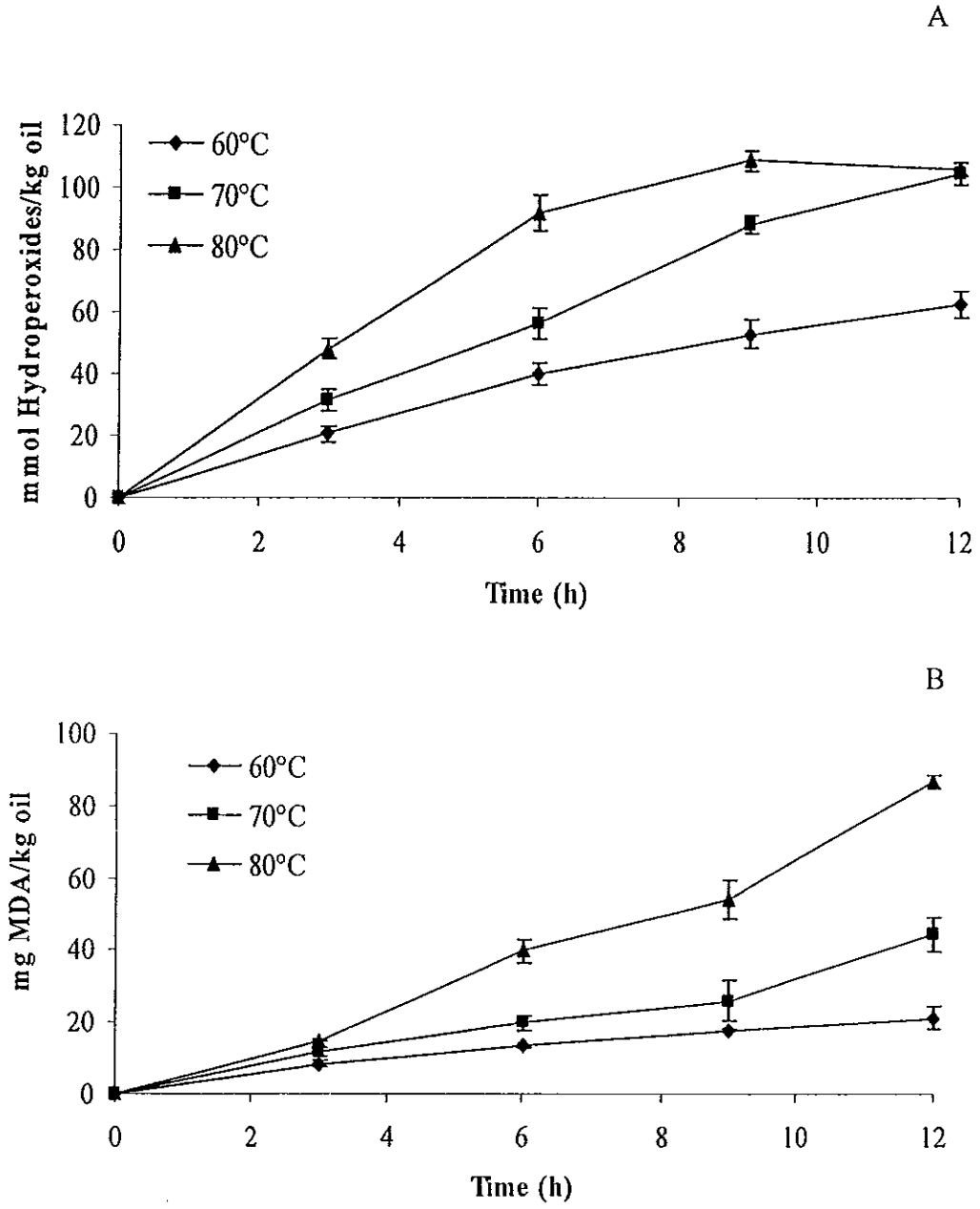


Figure 42. Peroxide value (A) and TBARS value (B) of OLA prepared at different temperatures for various times. Bars represent the standard deviation (n=3).

Table 21. Free amino group content, carbonyl content, particle size and surface hydrophobicity of cuttlefish skin gelatin modified with OLA prepared at different temperatures for various times using different molar ratio.

Incubation temperature	Time (h)	Free amino content (mM)	Carbonyl content(nmole/mg protein)	Particle size (nm)	Surface hydrophobicity
Control		3.75 ± 0.05i*	6.53 ± 0.14a	164 ± 15a	17.39 ± 0.18a
At molar ratio of 5:1					
60°C	3	3.66 ± 0.02f	6.75 ± 0.20ab	169 ± 14ab	17.70 ± 0.22a
	6	3.63 ± 0.04f	7.01 ± 0.11abc	182 ± 2cd	19.30 ± 0.20b
	9	3.54 ± 0.06e	7.77 ± 0.20de	188 ± 2ef	19.20 ± 0.29b
	12	3.47 ± 0.05e	8.30 ± 0.21ef	207 ± 5h	19.59 ± 0.22b
70°C	3	3.52 ± 0.06e	7.11 ± 0.12abc	177 ± 6bc	21.42 ± 0.07c
	6	3.38 ± 0.05d	7.49 ± 0.22cd	197 ± 2fg	23.24 ± 0.16e
	9	3.32 ± 0.05d	8.53 ± 0.41f	202 ± 1gh	26.61 ± 0.11h
	12	3.17 ± 0.05c	10.57 ± 0.44g	208 ± 2h	24.56 ± 0.28g
80°C	3	3.34 ± 0.04d	7.37 ± 0.22bcd	194 ± 5fg	23.42 ± 0.53e
	6	3.18 ± 0.04c	10.14 ± 0.56g	219 ± 2i	24.48 ± 0.10g
	9	3.04 ± 0.05b	14.00 ± 0.50h	243 ± 10j	22.29 ± 0.14d
	12	2.92 ± 0.02a	16.86 ± 0.62i	293 ± 4k	23.99 ± 0.21f
At molar ratio of 10:1					
60°C	3	3.58 ± 0.02h	7.42 ± 0.22b	169 ± 5a	20.64 ± 0.43b
	6	3.51 ± 0.00h	8.41 ± 0.13c	195 ± 4cd	23.50 ± 0.15c
	9	3.27 ± 0.02g	8.48 ± 0.21c	211 ±1 0cde	23.12 ± 0.27c
	12	3.12 ± 0.02f	9.96 ± 0.25d	236 ±1 4fg	24.88 ± 0.82d
70°C	3	3.30 ± 0.10g	8.54 ± 0.14c	187 ± 3ab	23.00 ± 0.91c
	6	3.19 ± 0.05f	8.99 ± 0.26c	219 ± 6df	29.67 ± 0.20g
	9	3.03 ± 0.01e	10.23 ± 0.49d	236 ± 20fg	32.38 ± 0.10h
	12	2.86 ± 0.04d	12.68 ± 0.53e	251 ± 19g	29.23 ± 0.41g
80°C	3	2.96 ± 0.05e	10.55 ± 0.15d	213 ± 10edf	25.89 ± 0.28e
	6	2.74 ± 0.02c	14.19 ± 0.78f	288 ± 24h	26.51 ± 0.13e
	9	2.66 ± 0.02b	19.60 ± 0.70h	330 ± 31i	29.14 ± 0.39g
	12	2.52 ± 0.07a	25.97 ± 0.96h	360 ± 18j	28.27 ± 0.97f

Mean±SD (n=3)

Different letters in the same column within the same molar ration indicate significant differences ($p<0.05$).

It suggested that OLA more likely interacted with gelatin molecules via amino groups of N-termini or ε-amino group of lysine, leading to the decrease in free amino group content. The degree of decrease in free amino group of modified gelatin depended on incubation time and temperature used for OLA preparation as well as the molar ratio of OLA-to-free amino group. When the same OLA was used, free amino

group content decreased with increasing molar ratio ($p<0.05$). At the same molar ratio used, gelatin modified with OLA prepared at 80°C had the higher loss in free amino group content, compared with OLA prepared at 70 and 60°C, in the descending order. OLA prepared with the longer incubation time generally caused the decrease in free amino group content to a higher extent than those with the shorter time ($p<0.05$). Refsgaard *et al.* (2000) reported that lipid oxidation products, both primary (hydroperoxide) and secondary product (reactive aldehydes), can react with protein and lead to the loss in lysine residues. In general, the loss in free amino group of modified gelatin correlated with the degree of oxidation of linoleic acid. OLA with higher degree of oxidation might interact with free amino groups of protein more effectively than OLA having the lower degree of oxidation. The secondary lipid oxidation products showed high reactivity with ε-amino group of lysine, imidazole group of histidine and sulfhydryl group of cysteine(Wu *et al.*, 2010). A number of breakdown products, especially reactive aldehyde such as malondialdehyde, 4-hydroxynonenal (HNE), and 4-hydroxyhexenal (HHE), could interact with amino groups of protein (Liu *et al.*, 2003). Although hydroperoxides could decompose to reactive free radical, those radicals had low stability, thereby having the lower interaction with proteins (Girotti, 1998). Therefore, the degree of interaction of OLA with gelatin via amino groups was governed by the degree of oxidation. Also, the molar ratio was another factor affecting the modification.

7.4.2.2 Carbonyl content

Carbonyl content of gelatin modified with various OLA at different molar ratios is shown in Table 21. Carbonyl content is one of the most reliable measures of protein oxidation (Levine *et al.*, 1990). Gelatins modified with OLA showed the marked increase in the carbonyl content, compared with the control gelatin ($p<0.05$). The result suggested that OLA might induce the oxidation of protein, leading to the formation of carbonyl in resulting protein. Additionally, carbonyl groups of OLA attached to gelatin directly contributed to the increased carbonyl content of modified gelatin. For the same OLA, carbonyl content in modified gelatin increased with increasing the molar ratio used ($p<0.05$). Among all samples, gelatin modified with OLA prepared at 80°C had higher carbonyl content than those modified with OLA prepared at lower temperatures (70 and 60°C).

Carbonyl content of modified gelatin increased when OLA was prepared with the longer incubation time ($p<0.05$). Thus, the degree of oxidation of linoleic acid used was coincidental with the increase in carbonyl content as well as the decrease in free amino group content of modified gelatins. This result was in agreement with Wu *et al.* (2010) who reported that modification of soy protein with reactive aldehyde such as acrolein resulted in the loss in histidine, lysine and cysteine residues and led to protein carbonylation. Zamora *et al.* (1999) reported that lipid oxidation product can interact with amino acids, inducing the oxidation of protein. The most sensitive amino acids toward oxidation are heterocyclic amino acid (such as tryptophan, histidine and proline) and amino acid with OH-, S- or N-containing groups (tyrosine, methionine, cysteine and lysine) (Doorn and Petersen, 2002). Oxidation of protein is associated with the alteration of protein structure, peptide chain scission, formation of amino acid derivatives and polymers, the decreases in solubility, and changes in the functional properties (Decker *et al.*, 1993). Therefore the incorporation of OLA into gelatin contributed to some alterations of modified gelatin.

7.4.2.3 Particle size

Mean particle size of gelatin modified with various OLA with a ratio of OLA-to-free amino group of 5:1 and 10:1 is shown in Table 21. Generally, mean particle size of the control gelatin (without modification) was 164 ± 15 nm. After modification, gelatin modified with OLA had the larger mean particle size than the control gelatin. This result suggested that OLA might induce inter- and intra-molecular cross-linking of gelatin, resulting in the increased particle size of modified gelatin. When OLA prepared under the same condition was used, mean particle size of gelatin increased with increasing molar ratio ($p<0.05$). Marked increase in mean particle size was observed in gelatin modified with OLA prepared at 80°C, especially with longer incubation time. The lowest mean particle size was found in gelatin modified with OLA prepared at 60°C, particularly with the shorter incubation time. It was noted that the increasing rate of mean particle size of gelatin was coincidental with the increase in TBARS value in OLA used (Figure 42B). This result suggested that secondary lipid oxidation products more likely induced the formation of larger gelatin. Stadman and Berlett (1997) reported that secondary lipid oxidation product, such as aldelydes, react mainly with amino acids via condensation reaction to from

Schiff's bases and by Michael addition reactions. The reaction occurred between carbon atom of the aldehyde and the nucleophilic amino acid residue (ε-amino group of lysine, the imidazole moiety of histidine or the sulfhydryls group of cysteine) (Stadtman and Berlett, 1997). The Schiff's bases or Michael adducts formed might subsequently be involved in inter- and inter-molecular cross-linking with amino acid residues such as lysine, cysteine, or histidine residues (Liu *et al.*, 2003; Stadtman and Berlett, 1997). Therefore, the modification of gelatin with OLA with higher degree of oxidation promoted the aggregation and precipitation of modified gelatin.

7.4.2.4 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of gelatin modified with OLA with a ratio of OLA-to-free amino group of 5:1 and 10:1 is shown in Table 21. Modification of gelatin with OLA increased surface hydrophobicity of resulting gelatin ($p<0.05$). With the same OLA used, the higher increase in surface hydrophobicity of modified gelatin was obtained when molar ratio increased ($p<0.05$). OLA, which was hydrophobic in nature attached to gelatin, more likely served as the hydrophobic domain of modified gelatin. At the same molar ratio, the considerable increase in surface hydrophobicity was observed in gelatin modified with OLA prepared at 70 and 80°C, whereas gelatin modified with OLA prepared at 60°C had the lower increase in surface hydrophobicity. The highest surface hydrophobicity was found in gelatin modified with OLA prepared at 70°C for 9 h, when the same molar ratio was used ($p<0.05$). It was noted that even though gelatin modified with OLA prepared at 70°C for 9 h had the lower degree of interaction when compared with gelatin modified with OLA prepared at 70°C for 12 h or at 80°C for 6, 9 and 12 h, the less polarity of OLA prepared at 70°C for 9 h led to the higher surface hydrophobicity. This result suggested that the primary and secondary lipid oxidation products in OLA varied, depending upon the conditions used. Oxidation of lipid most likely contributed to the decreased non-polar lipids, resulting from the decomposition of lipid molecule to shorter chain length product, or the presence of polar groups such as aldehyde or hydroperoxide groups (Porter *et al.*, 1995). Hydroperoxide having longer chain length might promote the increase in hydrophobic group more effectively than the secondary product with shorter chain length. Moreover, modification of gelatin with OLA with

higher secondary lipid oxidation product could increase the carbonyl compound, resulting in the increase in hydrophilic group in the modified gelatin.

Wu *et al.* (2010) reported that soy protein modified by lipid peroxidation product acrolein had the decrease in surface hydrophobicity. Oxidized lipids might bind to hydrophobic amino acids of protein, resulting in the decrease in surface hydrophobicity of modified protein (Liang, 1999). Oxidative modification of protein could simultaneously results in the exposure of hydrophobic groups due to protein partial unfolding, formation of hydrophilic group (protein carbonyls groups) and aggregation via hydrophobic interactions. Those phenomena lead to the decreased surface hydrophobicity of protein (Wu *et al.*, 2010).

7.4.2.5 FTIR

FTIR spectra of gelatin modified by various OLA with a ratio of OLA-to-free amino group of 10:1 are illustrated in Figure 43. Generally, the spectra of cuttlefish skin gelatin consisted of the major bands at ~3280 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), ~1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-) and ~1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching). Similar spectra were observed between modified gelatin and the control gelatin. However, gelatin modified with OLA had the decrease in intensity of amide A, I and II bands, compared with the control gelatin. These changes were indicative of greater disorder (Friess and Lee, 1996) in gelatin. The amide I is the most useful peak for infrared analysis of the secondary structure of protein including gelatin (Surewicz and Mantsch, 1988). Yakimets *et al.* (2005) reported that the absorption peak at 1633 cm^{-1} was the characteristic for the coil structure of gelatin. The lowering in amide I band of gelatin suggested that OLA might affect the helix coil structure of gelatin. Modification of gelatin with OLA resulted in the slight increase in the amplitude at wavenumber ~2935 and ~2870 cm^{-1} of resulting gelatin, compared with gelatin without modification. The wavenumber at ~2936 and ~2867 cm^{-1} were attributable to the asymmetric and symmetric stretching of CH_2, respectively (Smith, 1999).

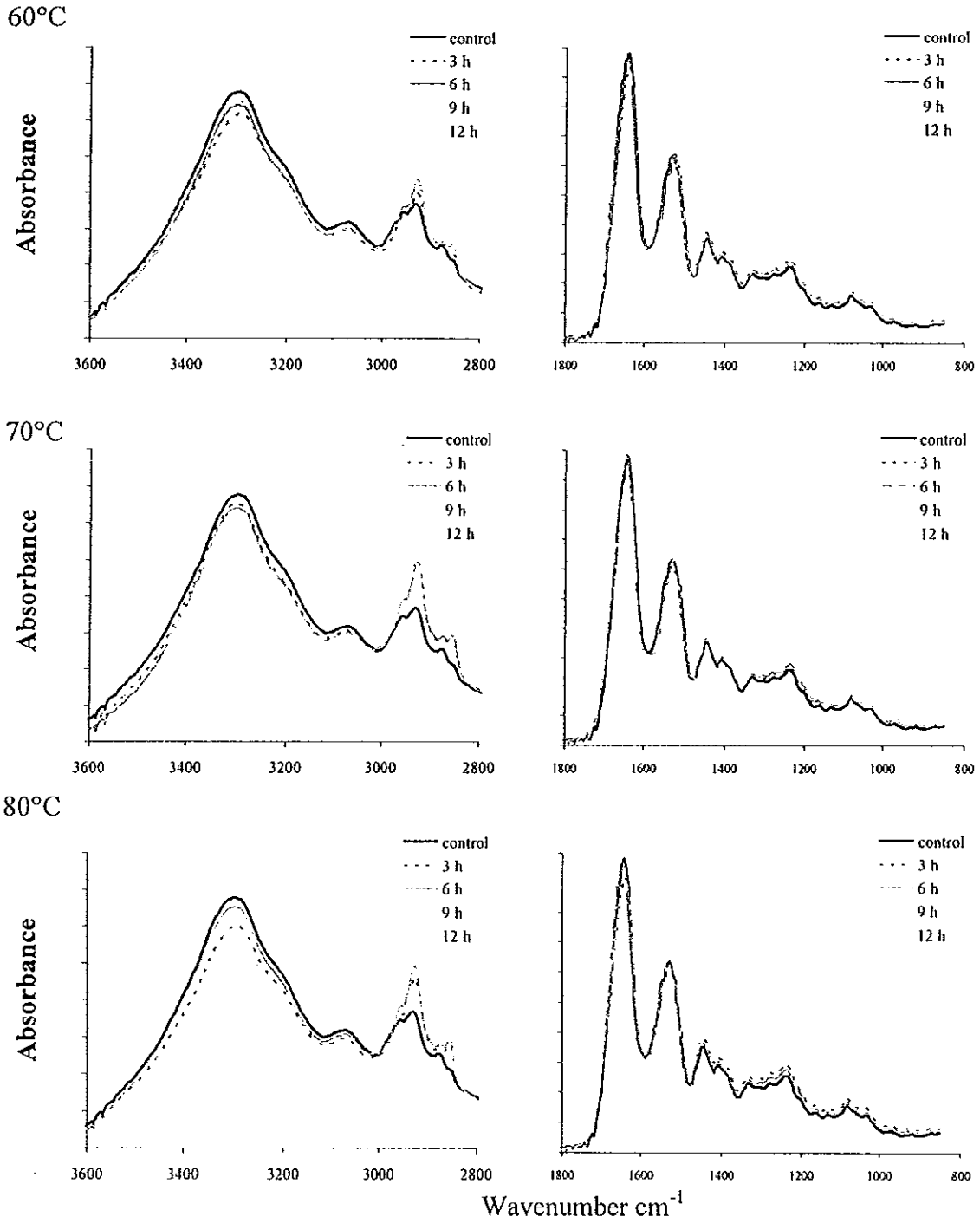


Figure 43. Fourier transform infrared (FTIR) spectra of cuttlefish skin gelatin modified with OLA prepared at different temperatures for various times using a ratio of OLA-to-free amino group of 10:1.

Lin and Chen (2006) reported that attachment of C_{12} alkyl chains to the gelatin hydrolysate caused an increase of these peaks, indicating the symmetric and asymmetric of CH_2. This result indicated that OLA could bind with the gelatin molecules as indicated by the additional band of alkyl group in modified gelatin. Moreover, the marked increase in amplitude of peak in the wavenumber range of 1450 – 1250 cm^{-1}, representative of stretching of C-N, was observed in modified gelatin when compared with the control gelatin. The interaction of OLA and gelatin might be mediated via bonding between carbon atom of OLA and amino group of molecular gelatin, resulting in increasing of C-N bond. It was coincidental with the decrease in free amino group content of modified gelatin (Table 21).

From FTIR spectra, temperature and time used for OLA preparation were the crucial factors affecting the properties and characteristic of modified gelatin. Modification of gelatin with OLA prepared at 80°C, especially with longer incubation time, had the higher degree of interaction between OLA and gelatin. This was revealed by the higher changes in secondary structure of gelatin molecule as evidenced by the remarkable increase in C-N bonding and higher decreases in Amide A, I and II brands. Nevertheless, the modification of gelatin with OLA prepared at 70°C, especially with incubation time of 9 h, rendered gelatin with the higher hydrophobic domain, indicated by the higher amplitude of peak representing alkyl group incorporated.

7.4.3 Functional properties of modified gelatin

7.4.3.1 Surface tension

Surface tension of solutions of gelatin without and with modification is shown in Figure 44. The degree of reduction of surface tension is used to indicate the ability of gelatin to localize or accumulate at air-water interface. Adsorption of protein at air-water interface results in the decrease in interfacial tension (Pezennec *et al.*, 2000). Generally, surface tension of all sample solutions decreased as the running time increased from 0 to 5000 s. Gelatin modified with OLA showed a faster decrease in surface tension, compared with the control gelatin. This indicated that the modification of gelatin with OLA could yield more surface active gelatin.

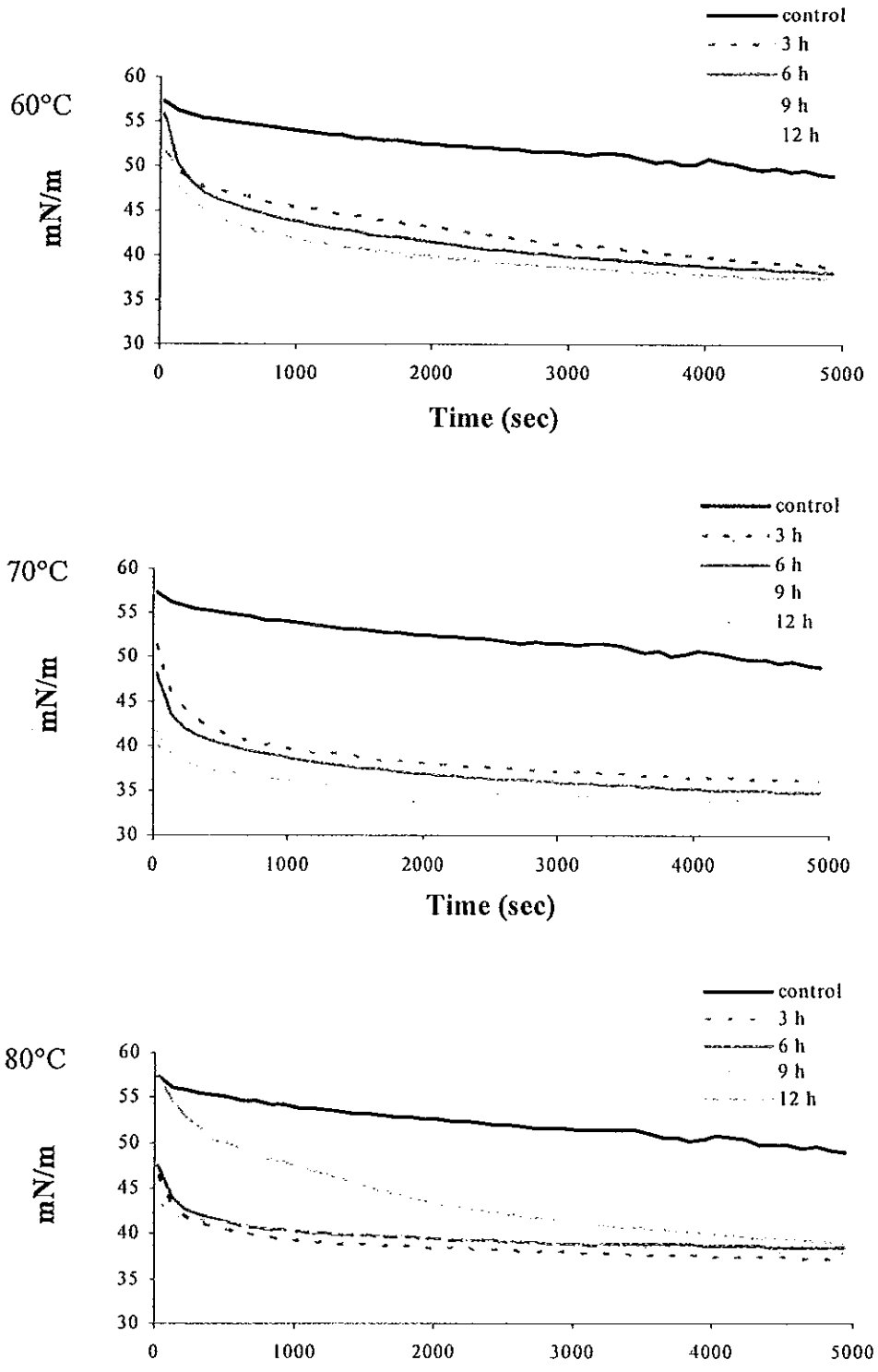


Figure 44. Surface tension of cuttlefish skin gelatin modified with OLA prepared at different temperatures for various times using a ratio of OLA-to-free amino group of 10:1. Bars represent the standard deviation (n=3).

The degree of decrease in surface tension of modified gelatin depended on incubation time and temperature to generate OLA. When OLA prepared for 3 h was used, the lowered surface tension was observed in gelatin modified with OLA prepared at 80°C, followed by those modified with OLA prepared at 70 and 60°C, respectively. Surface tension of modified gelatin decreased when incubation time for OLA preparation increased. However, modification of gelatin with OLA prepared at 70°C for 12 h and at 80°C with longer incubation time led to the higher increased surface tension of resulting gelatin, compared with using OLA with the shorter incubation time. Change in surface tension correlated with change in surface hydrophobicity of modified gelatin (Table 21). This result suggested that the different amount and type of primary and secondary lipid oxidation products in OLA played a key rule in enhancing surface hydrophobicity and surface activity of modified gelatin. Increased surface hydrophobicity of modified gelatin facilitated gelatin to transport and accumulate at air-water interface effectively by aligning hydrophobic domain toward air bubble, thereby lowering surface tension.

7.4.3.2 Foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin modified with various OLA at a ratio of OLA-to-free amino group of 5:1 and 10:1 are shown in Figure 45. Generally, gelatin modified with OLA had the higher foaming ability than the control gelatin. Modification of gelatin with OLA was able to improve foam properties of resulting gelatin. FE of gelatin modified with OLA at the lower molar ratio (5:1) increased non-significantly when compared with the control gelatin ($p>0.05$), whereas the use of OLA at the higher molar ratio (10:1) increased FE markedly ($p<0.05$). When OLA reacted with gelatin, surface hydrophobicity of resulting gelatin was increased. The increased hydrophobicity at surface of modified gelatin facilitated the gelatin molecules to localize at the air-water interface and reduce surface tension more effectively. This resulted in the improved FE of gelatin after modification with OLA. Foaming ability of modified gelatin depended on OLA used. With incubation time of 3 h, OLA prepared at 80°C yielded gelatin with higher foaming ability than those prepared at 70 and 60°C ($p<0.05$). However, modification of gelatin with OLA prepared at 80°C with longer incubation time caused the decrease in foaming ability of resulting gelatin. When the incubation temperatures of

70 and 60°C were used, OLA with increasing incubation time was able to improve foaming ability of resulting gelatin more potentially. This result was coincidental with change in surface hydrophobicity and conformation of gelatin after modification. The foaming ability of proteins is correlated with their film-forming ability at the air-water interface. Proteins that rapidly adsorb at the newly created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foam ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). Gelatin modified with OLA prepared at 70°C for 9 h with the highest surface hydrophobicity was more likely localized at the air-water interface, thereby reducing surface tension more effectively. On the other hand, OLA prepared at 80°C with longer incubation time caused the decrease in surface hydrophobicity and conformation change of resulting gelatin. As a result, the decrease in foaming ability was obtained.

After storage for 30 min, foam height of all samples decreased. The instability of foam is caused by gravitational drainage of liquid from the lamella and disproportionation of gas bubbles via interbubble gas diffusion (Yu and Damodaran, 1991). The coalescence of bubbles generally occurs because of liquid drainage from the lamella film as two gas bubbles approach each other, leading to film thinning and rupture (Damodaran, 2005). The foam height of all samples decreased, suggesting the instability of foam previously formed. Nevertheless, the higher foam height was found in foam stabilized by gelatin modified with OLA prepared at 70°C for 9 and 12 h. Therefore, the condition for OLA preparation directly affected the efficacy in improvement of foaming ability of gelatin.

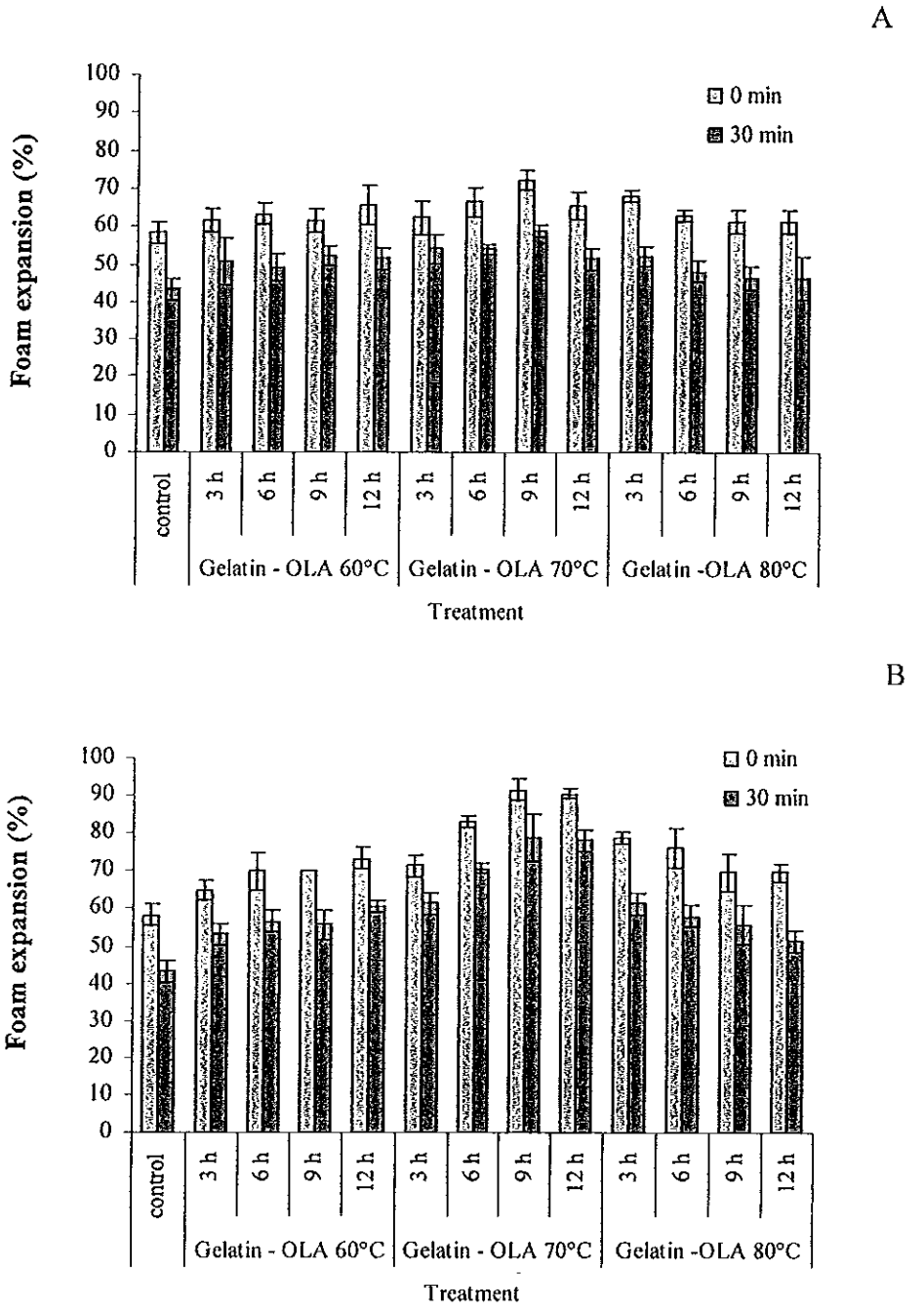


Figure 45. Foam properties of cuttlefish skin gelatin modified with OLA prepared at different temperatures for various times using a ratio of OLA-to-free amino group of 5:1 (A) and 10:1 (B). Bars represent the standard deviation (n=3).

7.4.3.3 Emulsifying properties

7.4.3.3.1 Emulsifying activity of gelatin

Emulsion activity index (EAI) of gelatin modified with various OLA is shown in Figure 46. Modification of gelatin with OLA improved emulsifying properties of resulting gelatin as evidenced by the increases in EAI, both at 0 and 10 min, compared with the control gelatin. Improved emulsifying activity might be due to the increased surface hydrophobicity of modified gelatin caused by OLA incorporated. Surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved (Kato and Nakai, 1980). This resulted in the improved emulsifying properties of gelatin modified with OLA. EAI of modified gelatin increased as the molar of OLA-to-free amino group ratio increased ($p<0.05$). At the same molar ratio used, OLA prepared under different conditions had different impact on EAI of modified gelatin. Generally, OLA prepared at higher temperature and time rendered gelatin with greater emulsifying properties. However, OLA prepared at 80°C with longer incubation time negatively affected the emulsifying properties of resulting gelatin as evidenced by the lowered EAI. The gelatin modified with OLA prepared at 70°C for 9 h had the highest EAI ($p<0.05$), when the same molar ratio used. This was coincidental with the change in foaming property (Figure 45).

For stability of emulsion, the relative decreases of EAI at 10 min were determined, compared with EAI at 0 min (Figure 46). It was observed that emulsion stabilized by modified gelatin was more stable than that of the control gelatin. With the same OLA used, stability of emulsion tended to be higher with increasing OLA - to-free amino group ratio used. However, modification of gelatin with OLA prepared at 80°C, especially at longer incubation time and higher molar ratio used, showed the detrimental impact on stability of emulsion. This might be due to the pronounced aggregation of gelatin molecules after modification. As a result, gelatin modified with OLA prepared at 80°C could not migrate and unfold at the oil-water interface properly. As a consequence, the film surrounding the oil droplet could not be formed. This led to instability of emulsion.

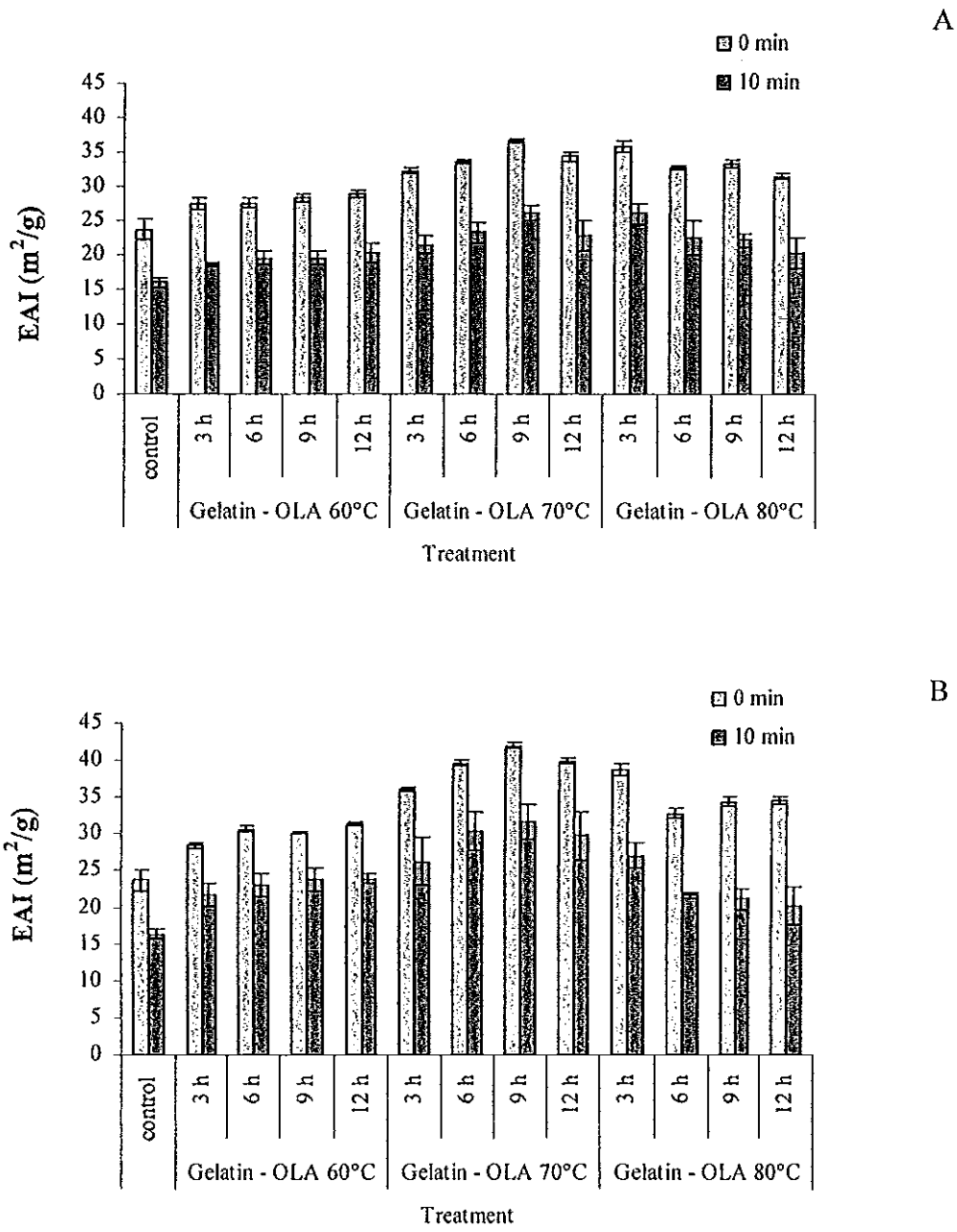


Figure 46. Emulsion properties of cuttlefish skin gelatin modified with OLA prepared at different temperatures for various times using a ratio of OLA-to-free amino group of 5:1 (A) and 10:1 (B). Bars represent the standard deviation (n=3).

7.4.3.3.2 Mean particle diameter of oil droplet in emulsion

Mean particle diameters of oil droplet in emulsions stabilized by gelatin modified with OLA a ratio of OLA-to-free amino group of 10:1 at day 0 and 10 of storage at room temperature are shown in Table 22. The mean particle diameter of oil droplets was calculated and expressed as a volume-surface mean particle diameter (d_{32}) and volume-weighted mean particle diameter (d_{43}) (Surh *et al.*, 2006). Generally, the diameter size of oil droplet in emulsion stabilized by modified gelatin after emulsification was smaller than that found in emulsion stabilized by the control gelatin. At day 0, the d_{32} and d_{43} of oil droplet in emulsion stabilized by the control gelatin was 1.01 ± 0.05 and 1.25 ± 0.06 μm, respectively. The lower d_{32} and d_{43} of oil droplets were observed in emulsion stabilized by modified gelatin. Emulsion stabilized by gelatin modified with OLA prepared at 70°C for 9 h had d_{32} and d_{43} of 0.84 ± 0.04 and 0.97 ± 0.04 μm, respectively, which were the smallest oil droplet size when compared with others ($p<0.05$). This result was associated with increasing surface hydrophobicity of modified gelatin. During emulsification, modified gelatin could migrate to the interface at a faster rate, align and form as a film surrounding the oil droplet more effectively. As a consequence, the smaller oil droplets could be formed as evidenced by the lower d_{32} and d_{43}. However, gelatin modified with OLA prepared at 80°C with longer incubation time showed the higher oil droplet size. This result might be due to cross-linking or aggregation of gelatin molecules after modification. As a result, gelatin might not localize and rearrange at the oil-water interface properly.

After 10 days of storage, the increases in mean particle diameter (both d_{32} and d_{43}) of oil droplets were found in all emulsion samples ($p<0.05$). This was probably due to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during storage (Surh *et al.*, 2006). The degree of increase in mean particle diameter in emulsion stabilized by control gelatin was higher than emulsion stabilized by modified gelatin. Therefore, modified gelatin could stabilize oil-in-water emulsion more effectively than control gelatin. Among all emulsions, those stabilized by gelatin modified with OLA prepared at 70°C for 9 h had the lowest increase in d_{32} and d_{43}, when compared with others ($p<0.05$). Formation of small oil droplet along with the thick film of gelatin increased emulsion

stability. The small droplet emulsion had much better stability to gravitational separation and aggregation than the large droplet emulsion (Tadros *et al.*, 2004). Therefore, gelatin modified with OLA prepared at 70°C for 9 h showed the highest emulsion activity as well as emulsion stability. From this reason, gelatin modified with OLA prepared at 70°C for 9 h was selected for further study.

Table 22. Mean particle diameter of oil droplet in emulsion stabilized by 2.0 % gelatin modified without and with OLA prepared at different temperatures for various times using a ratio of OLA-to-/free amino group of 10:1 at pH 7.

Incubation temperature	Time (h)	Mean particle diameter size (μm)			
		Day 0		Day 10	
		d_{32}	d_{43}	d_{32}	d_{43}
Control		1.01 ± 0.05	1.25 ± 0.06	1.70 ± 0.06	2.05 ± 0.09
60°C	3	0.95 ± 0.02	1.10 ± 0.03	1.61 ± 0.06	2.00 ± 0.12
	6	0.94 ± 0.03	1.08 ± 0.03	1.47 ± 0.03	1.90 ± 0.10
	9	0.94 ± 0.03	1.08 ± 0.03	1.43 ± 0.05	1.80 ± 0.09
	12	0.91 ± 0.02	1.05 ± 0.02	1.34 ± 0.07	1.59 ± 0.04
70°C	3	0.91 ± 0.04	1.05 ± 0.04	1.18 ± 0.03	1.50 ± 0.05
	6	0.87 ± 0.02	1.01 ± 0.02	1.14 ± 0.04	1.44 ± 0.03
	9	0.84 ± 0.04	0.97 ± 0.04	1.07 ± 0.05	1.38 ± 0.05
	12	0.92 ± 0.05	1.06 ± 0.02	1.17 ± 0.02	1.49 ± 0.05
80°C	3	0.87 ± 0.03	1.00 ± 0.02	1.11 ± 0.01	1.39 ± 0.01
	6	0.88 ± 0.05	1.01 ± 0.02	1.23 ± 0.02	1.51 ± 0.02
	9	0.90 ± 0.01	1.04 ± 0.06	1.32 ± 0.04	1.63 ± 0.05
	12	0.94 ± 0.02	1.09 ± 0.03	1.28 ± 0.02	1.57 ± 0.03

Mean±SD (n=3).

7.4.4 Effect of environmental conditions on stability of emulsion stabilized by modified gelatin

7.4.4.1. Effect of pH

The effect of various pHs (3-8) on the zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified with OLA prepared at 70°C for 9 h is shown in Figure 47. Generally, surface charge of oil droplet switched from positive charge to negative charge when pH was increased (Figure 47A). When pH increased from 3 to 8, the zeta potential of oil droplet decreased from

+18.29 to -22.10 mV for emulsion stabilized by the control gelatin and from +15.82 to -24.77 mV for emulsion stabilized by modified gelatin.

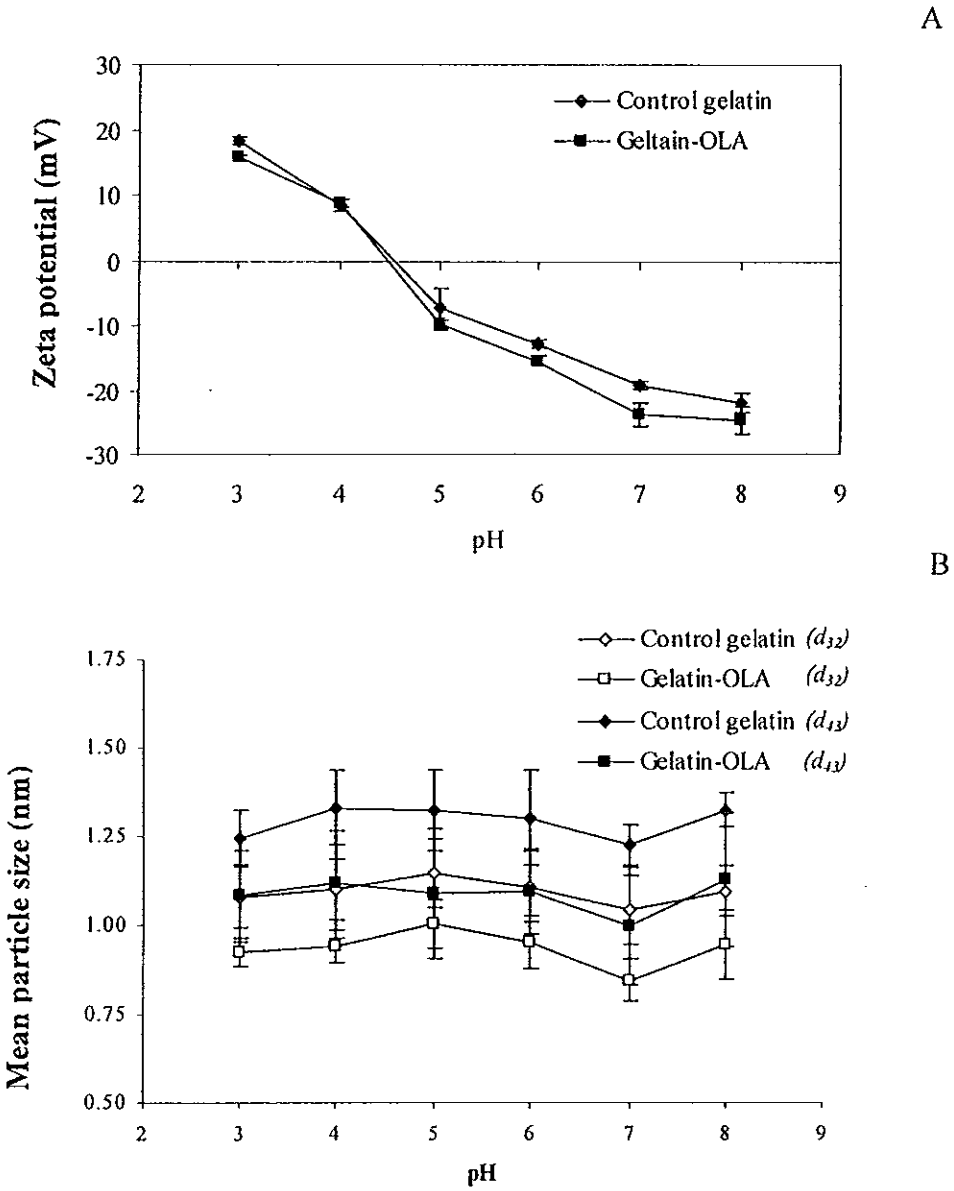


Figure 47. Effect of pH on zeta potential (A) and mean particle diameter (B) of emulsion stabilized by gelatin modified without and with OLA prepared at 70°C for 9 h. Bars represent the standard deviation (n=3).

At the same pH, it was noted that emulsion stabilized by modified gelatin had the higher amplitude of negative charge, compared with that with control gelatin. The charge of droplet was most likely governed by charge of gelatin surrounding the droplet. The zero net charge of oil droplets in emulsion stabilized by the control gelatin and modified gelatin was observed at pH 4.5 and 4.4, respectively. At the pH having the net charge of zero, the emulsion might be less stable due to the lowering of repulsive force.

For mean particle diameter, there was no change in both d_{32} and d_{43} of emulsion stabilized by control gelatin and modified gelatin when different pHs were used ($p>0.05$) (Figure 47B). This result showed that emulsions stabilized by the control gelatin and modified gelatin were relatively stable at pH tested, even at pH close to that giving the net charge of zero. The result suggested that gelatin film might be strong enough to prevent the flocculation or coalescence of oil droplets at all pHs tested.

7.4.4.4.2 Effect of salt concentration

The effect of different salt concentrations on the zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified with OLA prepared at 70°C for 9 h is shown in Table 23. As NaCl concentration increased, the amplitude of negative charge of oil droplet in all emulsion samples decreased. Similar trend was found in both emulsions stabilized by modified gelatin and the control gelatin. Increasing ionic strength affects the electrostatic repulsion between the droplets of emulsion (Onsaard *et al.*, 2006). In the presence of counter ions, the charge on an emulsion is reduced by electrostatic screening, in which the repulsion between the droplets is no longer sufficiently strong to overcome the attractive force (e.g., van der Waals and hydrophobic) acting between the droplets (Onsaard *et al.*, 2006).

The slight increase in mean particle size (both d_{32} and d_{43}) of oil droplet was observed in emulsion stabilized by both control gelatin and modified gelatin when NaCl concentrations increased ($p<0.05$). As NaCl concentration increased from 0 to 500 mM, the mean particle size of emulsion stabilized by the control gelatin increased from 1.01 ± 0.05 to 1.39 ± 0.08 μm for d_{32} and from 1.25 ± 0.06 to 1.64± 0.15 μm for d_{43}, whereas that of emulsion stabilized by modified gelatin

increased from 0.84 ± 0.04 to 1.17 ± 0.17 μm for d_{32} and from 0.97 ± 0.16 to 1.25 ± 0.18 μm for d_{43}. The increase in emulsion droplet size was coincidental with the decrease in negative charge amplitude. This result suggested that the decrease in repulsion between oil droplets caused the flocculation and/or coalescence of oil droplets. Although increasing ionic strength, especially with high NaCl concentration (500 mM), affected oil droplet size of emulsion, emulsions stabilized by both modified gelatin and the control gelatin were quite stable as evidenced by the negligible increases in mean particle size. This result was in agreement with Surh *et al.* (2006) who reported that emulsion stabilized by fish gelatin was stable to droplet aggregation under high ionic strength condition (500 mM NaCl). Not only electrostatic repulsion, but also steric repulsion played a major role in preventing the droplets from coalescence.

Table 23. Effect of NaCl concentration on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified without and with OLA prepared at 70°C for 9 h at pH 7.

Gelatin	NaCl (mM)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Control	0	-19.33 ±0 .57a*	1.01 ± 0.05a	1.25 ± 0.06a
	50	-8.49 ± 1.22b	1.07 ± 0.20a	1.29 ± 0.15ab
	100	-7.63 ± 1.53b	1.11 ± 0.17a	1.31 ± 0.10ab
	250	-4.32 ± 1.38c	1.14 ± 0.12a	1.40 ± 0.18bc
	500	-2.93 ± 1.39c	1.39 ± 0.08b	1.64 ± 0.15c
Gelatin-OLA	0	-23.83 ± 1.86a	0.84 ± 0.04a	0.97 ± 0.04a
	50	-9.18 ± 1.02b	0.89 ± 0.10ab	1.06 ± 0.13ab
	100	-8.75 ± 1.25b	0.89 ± 0.12ab	1.09 ± 0.14ab
	250	-4.38 ± 1.07c	1.01 ± 0.13bc	1.16 ± 0.19ab
	500	-3.48 ± 1.31c	1.17 ± 0.10c	1.25 ± 0.18b

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

7.4.4.4.3 Effect of heating

The effect of heating at different temperatures (50, 70 and 90°C) on the stability of emulsions stabilized by gelatin modified with OLA prepared at 70 for 9 h

is shown in Table 24. Generally, heating had no effect on surface charge of oil droplet in both emulsions stabilized by the control gelatin and modified gelatin. The amplitude of negative charge of oil droplet in emulsion stabilized by the control gelatin was lower than that of emulsion stabilized by modified gelatin.

For mean particle diameter, slight increase in d_{32} and d_{43} of oil droplet was observed in both emulsions stabilized by the control gelatin and modified gelatin after heating at 50, 70 and 90°C for 30 min ($p>0.05$). It suggested that some partial desorption of gelatin molecules from the droplet surfaces at elevated temperatures might lead to flocculation and/or coalescence (Surh *et al.*, 2006). However, lowered degree of increase in oil droplet size was found in emulsion stabilized by gelatin modified gelatin, compared with that of emulsion stabilized by the control gelatin. The result suggested that modified gelatin yielded the emulsion with higher stability, especially when heated at high temperature. The higher stability of hydrophobic film of gelatin modified with OLA to heat treatment was presumed. As a result, the emulsion could be retained after heating.

Table 24. Effect of heating at different temperature on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin modified without and with OLA prepared at 70°C for 9 h at pH 7.

Gelatin	Temperature (°C)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Control	50	-19.57 ± 0.88a*	1.01 ± 0.11a	1.28 ± 0.15a
	70	-19.89 ± 0.55a	1.12 ± 0.25a	1.33 ± 0.07a
	90	-19.61 ± 0.54a	1.17 ± 0.16a	1.42 ± 0.15a
Gelatin-OLA	50	-23.47 ± 1.52a	0.82 ± 0.15a	1.01 ± 0.15a
	70	-24.00 ± 0.66a	0.95 ± 0.18a	1.13 ± 0.12a
	90	-24.23 ± 0.56a	0.97 ± 0.08a	1.19 ± 0.21a

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

7.5 Conclusion

Modification of gelatin with OLA could improve surface activity of obtained gelatin as indicated by the decrease in surface tension and the increase in foaming and emulsifying properties. Gelatin modified with OLA prepared at 70°C for 9 h showed the highest surface activity, especially at high molar ratio used. The increases in hydrophobic region at surface of gelatin play a key role in enhancing surface activity, foam and emulsifying properties of modified gelatin. Gelatin modified with OLA could yield the emulsion with small oil droplet size and high stability toward different harsh environments (pH, heat and salt), compared with the control gelatin.

CHAPTER 8

Emulsifying property and antioxidative activity of cuttlefish skin gelatin modified with oxidized linoleic acid and oxidized tannic acid

8.1 Abstract

Oxidized linoleic acid (OLA) and oxidized tannic acid (OTA) were incorporated to cuttlefish skin gelatin to improve its emulsifying property and antioxidative activity. Attachment of OLA into gelatin increased surface hydrophobicity and emulsifying property with coincidental decrease in surface tension. Incorporation of OTA to gelatin increased the total phenolic content and antioxidative activity but decreased surface hydrophobicity. Attachment of both OLA and OTA at a concentration of 5% increased antioxidative activity but had no impact on emulsifying property. The emulsion stabilized by gelatin modified with OLA and OTA was more stable to lipid oxidation and phase separation than the commercial bovine gelatin.

8.2 Introduction

Lipid oxidation negatively affects the quality of foods, especially emulsion type products by altering appearance, odor, flavor, shelf-life and nutritional value. This results in unacceptability by consumers (Kellerby *et al.*, 2006). To prevent those changes, antioxidants are used to control lipid oxidation in food emulsions. Phenolics have received attention as natural antioxidants in food products. Antioxidative properties of phenolics are mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al.*, 1997). Generally, phenolic compounds with high antioxidative activity consist of high number of hydroxyl group in structure (Prasad *et al.*, 2005). These polar antioxidants are sparingly soluble in oil. In oil-in-water

emulsions system, polar antioxidants readily partition into the aqueous phase, thereby decreasing their concentration in the lipid phase, and lowering their capability to prevent oxidation (Yuji *et al.*, 2007). However, the effectiveness of polar antioxidant in oil-in-water emulsions can be improved by increasing their surface activity and ability to accumulate at the oil-water interface where oxidative reactions take place (Yuji *et al.*, 2007).

Proteins are surface/interfacial active and are widely used as emulsifiers in foods to produce oil-in-water emulsions with desirable physicochemical properties and improved stability (McClements, 2004). They can adsorb on oil-water interfaces and various hydrophobic segments penetrate into the oil phase. However, some proteins had the poor emulsifying property. The appropriate modification by incorporating the phenolic compound into protein could therefore be a potential means to obtain the modified protein with the higher emulsifying property as well as antioxidative activity. A protein-phenolic complex is concentrated at the oil-water interface, stabilizing oil droplet and simultaneously providing antioxidative activity at interface (Almajano and Gordon, 2004).

Recently, Aewsiri *et al.* (2009b) reported that modification of gelatin with oxidized tannic acid (OTA) could enhance antioxidative activity but had negative effect on its emulsifying property. Attachment of oxidized linoleic acid (OLA) along with OTA into cuttlefish gelatin can be an approach to improve the surface activity of cuttlefish skin gelatin by yielding the hydrophobic domains and improving antioxidative activity. The objective of this study was to improve emulsifying property and antioxidative activity of cuttlefish skin gelatin by modification with OLA together with OTA.

8.3 Materials and methods

8.3.1 Chemicals

Linoleic acid, tannic acid, *o*-phthalic dialdehyde (OPA), 2-(dimethylamino)ethanethiol hydrochloride (DMA), cumene hydroperoxide, 1-anilinonaphthalene-8-sulphonic acid (ANS), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-

triazine (TPTZ), 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), Trolox and menhaden oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), sodium hydroxide, ethanol, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), sodium chloride, sodium hydroxide, sodium sulfite, ammonium thiocyanate (NH_4SCN), barium chloride ($BaCl_2$), ferrous sulfate ($FeSO_4$) and boric acid were procured from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

8.3.2 Extraction of gelatin from cuttlefish skin

Cuttlefish (*Sepia pharaonis*) skin gelatin was prepared according to the method of Aewsiri *et al.* (2009b). Ventral skin of cuttlefish was washed with tap water and cut into small pieces (1 x 1 cm^2). Skin was treated with 10 volumes of 0.05 M NaOH for 6 h with a gentle stirring at room temperature (26-28°C). The solution was changed every 1 h for up to 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching with 10 volumes of 5 % H_2O_2 at 4°C for 48 h at room temperature and then washed with 10 volumes of water for 3 times. Gelatin was extracted from bleached skin using the distilled water (60°C) for 12 h with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously. The extract was centrifuged at 8,000 x g for 30 min at 25°C using a refrigerated centrifuge (Sorvall Model RC-B Plus, Newtown, CT, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a freeze dryer (Model Dura-Top™ µP/Dura Dry™ µP, FTS® System, Inc., Stone Ridge, NY, USA). Gelatin extracted from cuttlefish skin consisted of 87.97 % protein, 11.17 % moisture, 0.94 % fat and 0.53 % ash as determined by the method of AOAC (2000). The molecular weight of major proteins in cuttlefish skin gelatin was estimated to be 97 kDa as analyzed by SDS-PAGE using 4 % stacking gel and 10 % separating gel (Laemmli, 1970).

8.3.3 Preparation of oxidized linoleic acid (OLA)

To prepare OLA, the mixture of linoleic acid and Milli-Q water (a ratio of 1:1) was homogenized at a speed of 9,500 rpm for 2 min using a homogenizer

(model T25 basic, IKA LABORTECHNIK, Staufen, Germany) to remove soluble lipid oxidation products. Linoleic acid was recovered by centrifugation at 3,000 x g for 15 min at room temperature using a centrifuge (model z323k, Hermle Labor Technik, Wehingen, Germany). Ten mL of linoleic acid were placed in 15 mL-glass test tube. The linoleic acid was flushed with air continuously and incubated at 70°C for 9 h. OLA obtained was used for further study.

8.3.4 Preparation of gelatin-OLA complex

Cuttlefish skin gelatin was dissolved in distilled water to obtain a final concentration of 1.1 % protein determined by the Biuret method (Robinson and Hodgen, 1940). OLA was dissolved in ethanol and used as the stock solution. To 90 mL of gelatin solution, 1 mL of OLA was added. The final volume was adjusted to 100 mL with water to obtain the final protein concentration of 1% and the OLA-to-free amino group molar ratio of 10:1. The mixture was stirred continuously for 3 h at room temperature. Thereafter, the sample was dialyzed (MW cut-off = 14000 Da) for 12 h at room temperature against 20 volumes of 1% ethanol to remove OLA (unbound to proteins) and 12 h against 20 volumes of distilled water to remove ethanol. The gelatin-OLA complex was filtered through a filter paper No. 595½ (Schleicher & Schuell, Dassel, Germany) to remove precipitated product and the filtrate was freeze-dried.

8.3.5 Modification of gelatin-OLA complex with OTA

To prepare OTA, tannic acid was dissolved in distilled water to obtain the concentration of 2% (w/v) and pH was adjusted to 9 with 1 M NaOH. Solution was then bubbled with oxygen at 40°C for 1 h to convert tannic acid into OTA, an oxidized form. Gelatin-OLA complex was incorporated with OTA to yield "Gelatin-OLA-OTA complex". Firstly, gelatin-OLA complex was dissolved in distilled water to obtain a final concentration of 1.2 % protein (w/v). The pH of sample solution was adjusted to 9 using 1 M NaOH. To 75 mL of gelatin-OLA solution, OTA solution was added to obtain different final concentrations (2.5, 5 and 10 %, based on protein content). Final volume was raised to 90 mL using distilled water (pH 9) to obtain a final concentration of 1% protein. The mixture was stirred continuously at room

temperature for 12 h. Thereafter, the samples were dialyzed (MW cut-off = 14000 Da) for 24 h at room temperature against 20 volumes of water to remove free OTA (unbound to proteins). The control was prepared in the same manner except that OTA was excluded. Gelatin-OLA-OTA complexes were subjected to analyses.

8.3.6 Analyses

8.3.6.1 Determination of free amino group content

Free amino group content in cuttlefish skin gelatin without and with modification was determined using *o*-phthalicdialdehyde (OPA) as described by Church *et al.* (1983). The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium borate, 200 mg of 2-(dimethylamino)ethanethiol hydrochloride (DMA), and 5 mL of 10 % SDS. The total volume was adjusted to 50 mL with distilled water. To determine free amino group content, 65 µL of sample solution (1 % protein) was mixed with 3 mL of OPA reagent solution. After 2 min, the absorbance was read at 340 nm to measure alkylisoindole derivatives formed after reaction of OPA with free amino groups using a spectrophotometer (UVmini-1204, Shimadzu, Duisburg, Germany). Free amino group content was calculated from a standard curve of L-leucine (0 – 10 mM).

8.3.6.2 Determination of total phenolic content

Total phenolic content of gelatin without and with modification was measured according to the method of Slinkard and Singleton (1977) with some modifications. Sample solution (1 mL) with an appropriate dilution was mixed with 200 µL of the freshly prepared Folin–Ciocalteu reagent. After 3 min, 3 mL of sodium carbonate (15%, w/v) were added and the mixture was allowed to stand for 30 min at room temperature. The absorbance at 760 nm was measured using a spectrophotometer. Sample blank was prepared in the same manner except the deionized water was used instead of Folin-Ciocalteu reagent. Total phenolic content was expressed as A_{760} after blank substraction.

8.3.6.3 Determination of surface hydrophobicity

Surface hydrophobicity was determined as described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. Sample

solution (4 mg/mL) was diluted in 10 mM sodium phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3 and 0.5 % (w/v). The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0). The fluorescence intensity of ANS-conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

8.3.6.4 Measurement of mean particle size

The particle size of gelatin samples dispersed in 10 mM sodium phosphate buffer (pH 7.0) with the final concentration of 1 mg protein/mL was measured by dynamic light scattering at room temperature. The measurements were performed by a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). A refractive index of 1.42 was used for dispersed phase of all solutions.

8.3.6.5 Fourier transform infrared (FTIR) spectroscopy

Spectra of gelatin without and with modification were obtained using a BioRad FTS-60A FTIR spectrometer (Biorad, Cambridge, MA, USA) equipped with a Attenuated Total Reflectance Accessory (ATR) using a ZnSe ATR crystal. MID-IR spectra were recorded at room temperature between 800 and 4000 cm^{-1} at a resolution of 2 cm^{-1} in the ATR mode on thin films, which were purged with dry nitrogen to remove spectral interference resulting from water vapor and carbon dioxide. Sixty-four interferograms were coadded for a high signal-to-noise ratio. Prior to data analysis, the spectra were baseline corrected and normalized (mean normalization option).

8.3.6.6 Determination of antioxidative activities

The antioxidative activities of the solutions of gelatin without and with modification (1% protein) were determined in comparison with OTA solution at a concentration of 0.05% (w/v) using different assays.

8.3.6.6.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan *et al.* (2008) with a slight modification. To the diluted sample (1.5 mL), 1.5 mL of 0.10 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol was added. The mixture was then mixed vigorously using a vortex mixer and allowed to stand at

room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that ethanol was used instead of DPPH solution. The standard curve of Trolox (60-600 μM) was prepared in the same manner. The activity was expressed as μmol Trolox equivalent (TE)/ g protein.

8.3.6.6.2. ABTS radical scavenging activity

ABTS free radical scavenging assay was determined according to the method of Binsan *et al.* (2008) with a slight modification. 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical was generated by reacting 7.4 mM ABTS and potassium persulfate (2.6 mM) at a ratio of 1:1 (v/v) and leaving at room temperature in the dark for 12 h. ABTS free radical solution was diluted by mixing 1 mL of ABTS solution with 50 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. To 150 μL of sample, 2850 μL of ABTS•+ solution was added and mixed thoroughly using a vortex mixer. The extent of quenching of the ABTS•+ was measured at 734 nm after 2 h incubation at room temperature in the dark. Sample blank was prepared in the same manner except that methanol was used instead of ABTS solution. The standard curve of Trolox (60 – 600 μM) was prepared in the same manner. The activity was expressed as μmol TE/ g protein.

8.3.6.6.3 Ferric reducing antioxidant power (FRAP)

FRAP was determined as described by Binsan *et al.* (2008) with a slight modification. A 2.85 mL of freshly prepared FRAP solution (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution and 25 mL of 300 mM acetate buffer, pH 3.6) was incubated at 37°C for 30 min before mixing with 150 μL of sample. The mixture was allowed to react in the dark at room temperature. Absorbance at 593 nm was measured after 30 min of reaction. Sample blank was prepared by omitting $FeCl_3$ from the FRAP solution and distilled water was used instead. The standard curve of Trolox (60 – 600 μM) was prepared in the same manner. FRAP was expressed as μmol TE/ g protein.

8.3.6.7 Surface activity of gelatin without and with modification by OLA and OTA

8.3.6.7.1 Surface tension

Sample solutions (1 mg protein/mL) were prepared freshly before measurement. The surface tension of gelatin samples was measured as a function of time (0–5000 s) on an automated drop tensiometer (ADT; I. T. Concept, Longessaigne, France) as described by Wierenga *et al.* (2005). An air bubble was formed at the tip of a syringe needle placed in a cuvette containing the sample solution. Both cuvette and syringe were temperature controlled (20 ± 0.1°C). Bubble volume was kept constant at 4 mL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. Surface tension was determined by bubble shape analysis.

8.3.6.7.2 Emulsifying property

Emulsion activity index (EAI) of gelatin samples was determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 mL) and gelatin solution (1 % protein, 6 mL) were homogenized using a homogenizer at a speed of 20,000 rpm for 1 min. Emulsions were pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer. A_{500} of the resulting dispersion was measured using a spectrophotometer. EAI at 0 and 10 min were calculated by the following formula:

$$EAI\ (m^2/g) = (2\times 2.303\ A)DF/l\phi C$$

where A is the absorbance measured at 500 nm, l = path length of cuvette (m), DF= the dilution factor (100), ϕ = oil volume fraction (0.25) and C = protein concentration in aqueous phase (g/m^3).

8.3.8 Effect of gelatin modified with OLA and OTA on emulsion stability and lipid oxidation of menhaden oil-in-water emulsion

8.3.6.8 Emulsion stability and lipid oxidation during the storage

Menhaden oil-in-water emulsions were prepared by homogenizing the mixture of sunflower oil and solution of gelatin modified with OLA and OTA (2.25% protein, w/v) at a ratio of 1:9 (v/v) at a speed of 9,500 rpm for 2 min using a homogenizer. These coarse emulsions (pH 7.0) were then passed through a laboratory-scale high-pressure homogenizer (Delta Instruments, Drachten, The Netherlands) at 75 bar for 10 times. NaN_3 (0.02 %) was added to the emulsions as an antimicrobial agent. The fish oil emulsions were then stored at 30°C for 10 days. The samples were taken every two days for analyses.

8.3.6.8.1 Measurement of mean particle size of oil droplet

The mean particle diameter of oil droplet in emulsion was determined with Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK) at 25°C. Electrophoretic light scattering technique was used to analyze the velocity distribution of particle movement by measuring dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particle. Data was calculated via the Stokes-Einstein equation and expressed as number average distribution of particle diameter. Prior to analysis, the samples were 100-fold diluted with 10 mM sodium phosphate buffer solution (pH 7). The particle diameter was reported as volume-surface mean particle diameter, d_{32} ($=\sum n_i d^3_i / \sum n_i d^2_i$) and volume-weighted mean diameter, d_{43} ($=\sum n_i d^4_i / \sum n_i d^3_i$), where d_i is the diameter and n_i is the number of particles in the i^{th} size class.

8.3.6.8.2 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Two mL of fish oil emulsion sample were dispersed in 10 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 x g for 20 min at room temperature. The absorbance of the supernatant was measured at

532 nm. The standard curve was prepared using malonaldehyde (2-10 ppm) and TBARS were expressed as mg malonaldehyde/L emulsion.

8.3.6.8.3 Emulsion stability under different conditions

To study the stability of emulsion under different conditions, the emulsion was prepared as described above (10 wt% sunflower oil, 2.0 wt% fish gelatin, pH 7.0) and then diluted to a droplet concentration of 2.0 wt% using 10 mM sodium phosphate buffer (pH 7). The emulsion was incubated in a beaker overnight to allow any large droplets or non-emulsified oil to rise to the top, and then only the lower portion of emulsion was collected.

For pH stability study, the pH of the emulsions (20 mL) was adjusted to 3, 4, 5, 6, 7 and 8 using 0.5 M HCl of 0.5 M NaOH solutions and the final volume of all emulsions was adjusted to the same volume (25 mL) using distilled water previously adjusted to corresponding pH. The emulsions were then stored at room temperature for 24 h before being analyzed.

To study the impact of NaCl on emulsion stability, emulsion samples (20 mL) were added with 0, 0.25, 0.5, 1.25 and 2.5 mL of 4 M NaCl solution and were adjusted to 25 mL using distilled water to obtain a final NaCl concentrations of 0, 50, 100, 250 and 500 mM, respectively. Emulsion samples (10 mL) were then transferred into glass test tubes (internal diameter 15 mm, height 125 mm) and stored at room temperature for 24 h before measurement.

For thermal stability study, emulsion samples in 10 mM sodium phosphate buffer (pH 7) were added with 2.5 mL of distilled water. The emulsion samples were transferred into screw cap test tubes and then incubated in a temperature controlled water bath for 30 min at 50, 70 and 90°C. After incubation, the emulsion samples were cooled under running tap water. All emulsion samples (10 mL) in glass test tubes (internal diameter 15 mm, height 125 mm) were stored at room temperature for 1 day. The zeta potential and mean particle diameter of oil droplets were then measured using a Zetasizer nano ZS as described above.

8.3.6.9 Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard

8.3.6.10 Statistical analysis

The experiments were run in triplicate. All data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). The SPSS statistic program (Version 10.0) (SPSS Inc., Chicago, IL, USA) was used for data analysis.

8.4 Results and Discussion

8.4.1 Characteristics of gelatin modified with OLA and OTA

8.4.1.1 Free amino group content

Free amino group content of gelatin without and with modification by OLA and OTA is shown in Table 25. Cuttlefish skin gelatin contained 0.375 mmol NH_2 groups/ g gelatin. Attachment of OLA into gelatin was monitored by the decrease in free amino group of resulting gelatin. Free amino group content of gelatin-OLA complex was 0.309 mmol NH_2 groups/ g gelatin. After gelatin-OLA complex was further modified with 2.5, 5 and 10% OTA, free amino group contents of 0.294, 0.275 and 0.261 mmol NH_2 groups/ g gelatin were obtained, respectively. When gelatin was incorporated with OTA, free amino group content of resulting gelatin was decreased to 0.340 mmol NH_2 groups/ g gelatin. It was suggested that OLA and OTA more likely incorporated to gelatin via amino groups, either α-amino group or ε-amino group of lysine. Refsgaard *et al.* (2000) reported that lipid oxidation products can react with protein, leading to the loss in lysine residues. Furthermore, Rawel *et al.* (2001) found that oxidized phenolic compound could covalently interact with nucleophilic group of lysine. Therefore, both OLA and OTA were able to interact with gelatin from cuttlefish skin.

When considering the a number of free amino groups in gelatin and gelatin-OLA complex after modification with 5% OTA, the lower decrease in free amino group was found the latter, compared with the former. The result indicated that higher degree of incorporation of OTA was obtained in gelatin, compared with gelatin-OLA complex. After being incorporated with OLA, less amino groups were available for OTA to be interacted. Additionally, OLA attached to gelatin might cause

the steric hindrance for OTA to undergo interaction with amino group of gelatin-OLA complex. Moreover, gelatin-OLA complex with higher surface hydrophobicity showed the less reactivity with OTA, which was mainly localized in aqueous phase.

Table 25. Free amino group content, total phenolic content, surface hydrophobicity and particle size of cuttlefish skin gelatin without and with modification by OLA and OTA

Samples	Free amino content (mM)	A_{760}**	Surface hydrophobicity	Particle size (nm)
Gelatin	3.75 ± 0.05f*	0.33 ± 0.03a	17.39 ± 0.18c	163 ± 15a
Gelatin-OLA	3.09 ± 0.03d	0.31 ± 0.02a	32.38 ± 0.10f	236 ± 20c
Gelatin-OLA-2.5% OTA	2.94 ± 0.07c	0.58 ± 0.01b	30.12 ± 1.00e	251 ± 15cd
Gelatin-OLA-5% OTA	2.85 ± 0.05b	0.82 ± 0.03c	25.15 ± 0.35d	276 ± 13de
Gelatin-OLA-10% OTA	2.71 ± 0.07a	1.04 ± 0.03e	16.80 ± 0.10b	303 ± 23e
Gelatin-5% OTA	3.40 ± 0.03e	0.87 ± 0.01d	10.12 ±0.56a	200 ± 14b

Mean±SD (n=3)

*Different letters in the same column indicate significant differences ($p<0.05$).

**A_{760} represent total phenolic content.

8.4.1.2 Total phenolic content

Total phenolic contents of gelatin without and with modification by OLA and OTA determined by Folin–Ciocalteu method are expressed as A_{760} (Table 25). Generally, A_{760} observed in the control gelatin solution was more likely due to the presence of amino acids including tyrosine, tryptophan, cysteine, histidine and asparagine (Lowry *et al.*, 1951). Those amino acids were able to reduce Folin-Ciocalteu reagent as evidenced by the increase in A_{760}. After incorporation with OTA, gelatin had A_{760} of 0.87. No difference in A_{760} was found between gelatin and gelatin-OLA complex. This result suggested that the attachment of OLA into gelatin had no effect on the reducing ability of modified gelatin. The increases in A_{760} were observed in gelatin and gelatin-OLA complex modified with OTA ($p<0.05$). It reconfirmed that OTA could incorporate into gelatin and gelatin-OLA complex. Total phenolic content of modified gelatin increased with increasing concentration of OTA used. Gelatin-

OLA modified with 10% OTA had the highest A_{760} (1.04), followed by those modified with 5% OTA (0.82) and 2.5% OTA (0.58), respectively. When the same level of OTA (5%) was used, the modified gelatin had the higher A_{760} than gelatin-OLA complex modified with OTA. This indicated the lowered degree of incorporation of OTA into gelatin-OLA complex, compared with gelatin. OLA attached with gelatin more likely hampered the incorporation of OTA with gelatin.

8.4.1.3 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of gelatin-OLA complex modified with OTA is shown in Table 25. Gelatin-OLA complex exhibited the higher surface hydrophobicity than the control gelatin ($p<0.05$). OLA attached provided the hydrophobic domain to gelatin-OLA complex. Surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved (Kato and Nakai, 1980). Increase in surface hydrophobicity of gelatin modified with OLA reflected the improved surface activity of gelatin-OLA complex. When the modification of gelatin and gelatin-OLA complex with OTA was carried out, the decreased surface hydrophobicity of resulting gelatin was observed. Surface hydrophobicity of gelatin-OLA complex modified with OTA decreased as the concentration of OTA increased. OTA bound with gelatin and gelatin-OLA complex most likely contributed to the increased hydrophilicity at the surface of gelatin. This result was in agreement with Rawel *et al.* (2002) who reported that the surface hydrophobicity of soy protein decreased when reacted with phenolic compounds. Aewsiri *et al.* (2009b) also found that attachment of oxidized phenolic compound on cuttlefish skin gelatin resulted in the decrease in surface hydrophobicity of resulting gelatin. The decrease in surface hydrophobicity of gelatin and gelatin-OLA complex was possibly caused by phenolic hydroxyl groups of OTA attached to gelatin. However, gelatin-OLA modified with 2.5 and 5% OTA still had higher surface hydrophobicity, when compared with the control gelatin. Therefore, OLA and OTA incorporated into gelatin had the impact on surface hydrophobicity of resulting gelatin.

8.4.1.4 Particle size

Mean particle size of gelatin without and with modification by OLA and OTA is shown in Table 25. Generally, modified gelatin had the larger mean

particle size, compared with the control gelatin ($p<0.05$). Control gelatin (without modification) had mean particle size of 163 ± 15 nm. Mean particle size of gelatin modified with OLA (gelatin-OLA complex) and gelatin modified with 5% OTA was 236 ± 20 and 200 ± 14 nm, respectively. Attachment of OLA or OTA into gelatin resulted in the larger size of resulting gelatin. OLA and OTA might induce inter- and intra- molecular cross-linking of gelatin, resulting in the increased particle size of modified gelatin. Stadman and Berlett (1997) reported that the lipid oxidation products were able to induce the oxidation of amino acid side chains and involved in inter- and inter-molecular cross-linking between protein molecules. Similarly, oxidized phenolic compounds could interact with protein and induce cross-linking of protein (Kroll *et al.*, 2003).

Modification of gelatin with OLA together with OTA increased the mean particle size of resulting gelatin. Gelatin-OLA complex modified with OTA had the larger particle size, compared with gelatin modified with only OLA or OTA ($p<0.05$). The largest particle size was found in gelatin-OLA complex modified with 10% OTA, followed by the complex modified with 5 and 2.5% OTA, respectively. The increase in mean particle size of gelatin-OLA complex modified with OTA was a result of OTA attached and the higher cross-linking of gelatin modified by OTA.

8.4.1.5 FTIR

FTIR spectra of gelatin without and with modification by OLA and OTA are depicted in Figure 48. Spectra of cuttlefish skin gelatin without modification exhibited the major bands at ~3280 cm^{-1} (Amide A, representative of NH-stretching, coupled with hydrogen bonding), ~1635 cm^{-1} (amide I, representative of C=O stretching/hydrogen bonding coupled with COO^-) and ~1535 cm^{-1} (amide II, representative of NH bending coupled with CN stretching). Modification of gelatin with OLA and OTA caused the change in FTIR spectra of resulting gelatin.

Gelatin modified with OLA and OTA had the decreased amplitude of amide A, I and II bands, compared with the control gelatin. These changes were indicative of greater disorder (Friess and Lee, 1996) in gelatin. The amide I is the most useful peak to characterize the coil structure of gelatin (Yakimets *et al.*, 2005). The decrease in amide I band of modified gelatin suggested that the incorporation of OLA and OTA into gelatin more likely affected the helix coil structure of gelatin.

OLA and OTA incorporated might induce the conformational changes of gelatin, thereby affecting the property of resulting gelatin.

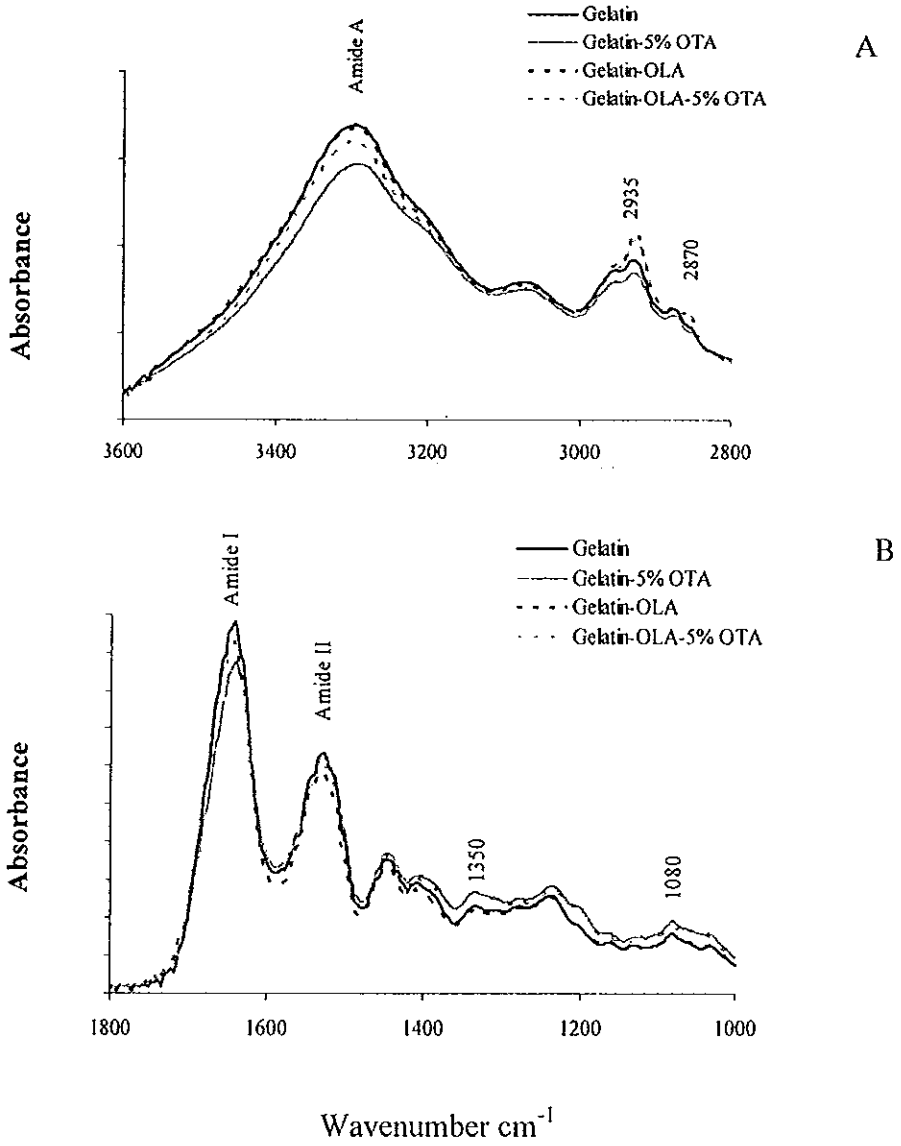


Figure 48. Fourier transform infrared (FTIR) spectra in wavenumber range of 3600-2800 cm^{-1}(A) and 1800-1000 cm^{-1} (B) of cuttlefish skin gelatin without and with modification by OLA and OTA

In gelatin-OLA complex, the higher amplitudes at wavenumber ~2935 and ~2870 cm^{-1} (representative of asymmetric and symmetric stretching of CH_2) were observed in resulting gelatin (Figure 48A), compared with the control gelatin. This result indicated that OLA could bind with the gelatin molecules as indicated by the increase in alkyl group in resulting gelatin. For gelatin modified with 5% OTA, the increase in amplitude of peak at wavenumber ~1350 and ~1080 cm^{-1} (representative of O-H bonds and the stretching of the C-O bonds in secondary and tertiary alcohols) was obtained (Figure 48B). This result indicated the incorporation of OTA into gelatin molecules as evidenced by the additional band of OH group in phenol found in modified gelatin. Modification of gelatin with OLA together with OTA resulted in the presence of alkyl group of OLA and phenolic hydroxyl group of OTA in resulting gelatin as indicated by the increase in amplitude of peak at ~2935 and ~2870 cm^{-1} and the increase in amplitude of peak ~1350 and ~1080 cm^{-1}, respectively.

8.4.1.6 Antioxidative activity

Antioxidative activities including DPPH and ABTS radical scavenging activities, and ferric reducing antioxidant power (FRAP) of gelatin without and with modification by OLA and OTA are shown in Figure 49. Generally, control gelatin (without modification) and gelatin-OLA complex showed a weak antioxidative activity. Amino acids such as histidine, tyrosine and methionine in cuttlefish skin gelatin might contribute to antioxidative activity. After modification with OTA, gelatin and gelatin-OLA complex had the increased antioxidative activity as indicated by the increases in DPPH and ABTS radical scavenging activities and FRAP ($p<0.05$). The result indicated that OTA introduced to gelatin contributed to the increased antioxidative activity of gelatin. This result was in agreement with Almajano *et al.* (2007) who reported that antioxidative activity of BSA, α-lactalbumin and β-lactoglobulin was increased when epigallocatechin gallate (EGCG) was incorporated. This suggested that phenolic hydroxyl groups of OTA incorporated might play a key rule in donating hydrogen and electron, or scavenging with the radicals, thereby terminating the radical chain reaction.

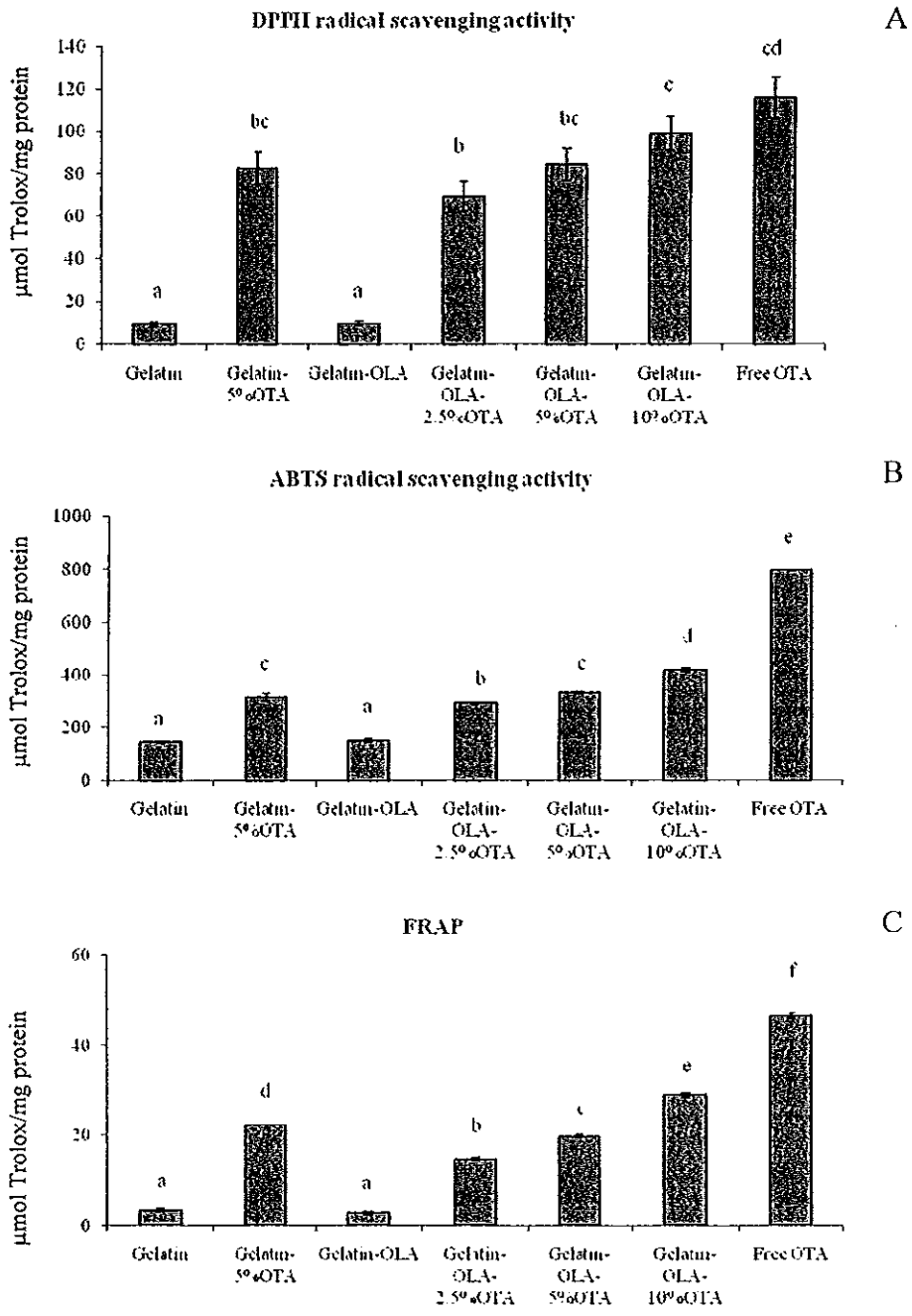


Figure 49. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B) and ferric reducing antioxidant power (FRAP) (C) of cuttlefish skin gelatin without and with modification by OLA and OTA. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant differences ($p<0.05$).

Although OTA, an oxidized form, was prepared, not all hydroxyl groups were converted to quinone (Balange and Benjakul, 2009). Also, the quinone of OTA attached to gelatin might be reduced to some extent by some amino acids in gelatin. Remaining hydroxyl groups more likely contributed to antioxidative activity. The degree of increase in antioxidative activity of modified gelatin was related with the degree of incorporation of OTA into the gelatin. For gelatin-OLA complex modified with OTA, the highest antioxidative activity was found in the complex modified with 10% OTA, followed by the complex modified with 5 and 2.5% OTA, respectively. At the same level of OTA (5%) used, the higher antioxidative activity was observed in modified gelatin, compared with modified gelatin-OLA complex ($p<0.05$). This was in accordance with the lower degree of incorporation of OTA in gelatin-OLA complex (Table 25)

Furthermore, gelatin-OLA-OTA complex with larger size and higher surface hydrophobicity might not be well solubilized in aqueous system, thereby lowering antioxidative activity in the aqueous system. When antioxidative activity between OTA in free form and different gelatins modified with OTA at the same concentration was compared, OTA exhibited a higher activity than gelatin and gelatin-OLA and gelatin-OLA modified with OTA ($p<0.05$). This was in accordance with Rohn *et al.* (2004) who reported that the antioxidant activity of the quinone-protein complexes is less than the antioxidant activity of free phenolic compounds. When OTA was incorporated, free hydroxyl group content in gelatin or gelatin-OLA was decreased.

8.4.1.7 Surface tension

Surface tension of solution of gelatin without and with modification using OLA and OTA is shown in Figure 50. The degree of reduction of surface tension is used to indicate the ability of gelatin to localize or accumulate at air-water interface. Adsorption of protein at air-water interface results in the decrease in interfacial tension (Pezennec *et al.*, 2000). The faster and lower decreases in surface tension were observed in cuttlefish skin gelatin, compared with bovine gelatin. This result indicated that bovine gelatin could move and adsorb at air-water interface more effectively than cuttlefish skin gelatin. It might be due to the lower hydrophobic amino acids content (proline and leucine) and a large portion of hydrophilic amino

acids (lysine, serine, arginine, hydroxyproline, aspartic and glutamic acids) of cuttlefish skin gelatin (Aewsiri *et al.*, 2009a; Hoque *et al.*, 2010).

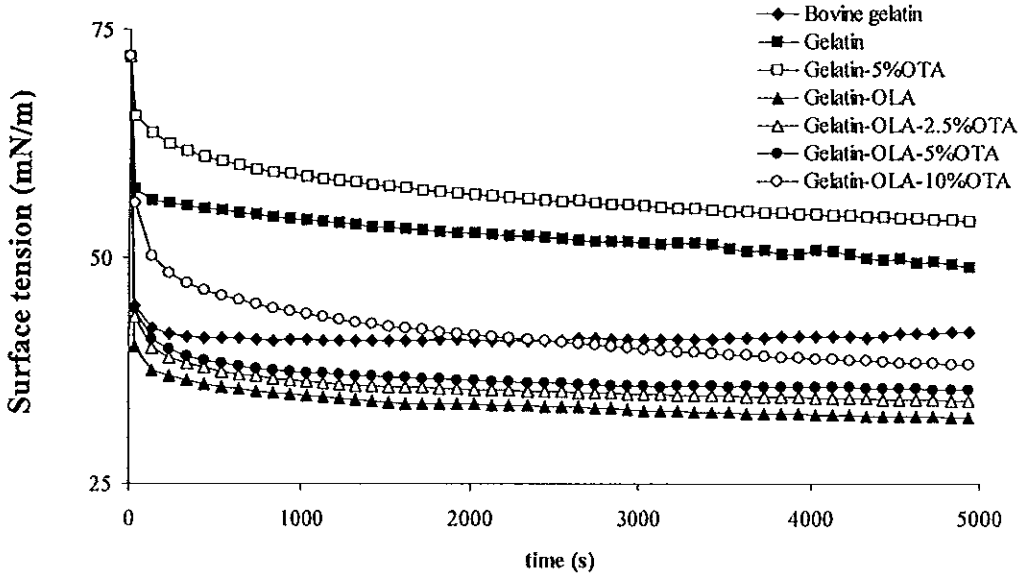


Figure 50. Surface tension of cuttlefish skin gelatin without and with modification by OLA and OTA.

Gelatin-OLA complex solution had the lowered surface tension, compared with bovine gelatin solution. This suggested that increased surface hydrophobicity of gelatin-OLA complex facilitated gelatin to transport and accumulate at air-water interface effectively. On the other hand, the modification of gelatin and gelatin-OLA complex with OTA decreased surface activity of resulting gelatin. Surface tension of gelatin modified with OTA was generally higher than that of the control gelatin. Surface tension of gelatin-OLA complex modified with OTA increased as the concentration of OTA increased. OTA incorporated more likely contributed to the increased hydrophilicity of modified gelatin. As a consequence, gelatin could not localize and accumulate at air-water interface potentially. However, gelatin-OLA complex modified with 2.5 and 5% OTA still had the faster decreases in surface tension than bovine gelatin, whereas that modified with 10% OTA had slower decrease in surface tension. Thus, the modification of gelatin using OLA and OTA

affected the surface activity of resulting gelatin. This was governed by the nature and amount of OLA and OTA attached.

8.4.1.8 Emulsifying property

Emulsion activity index (EAI) of gelatin without and with modification by OLA and OTA is shown in Figure 51. Generally, the control gelatin had the poorer emulsifying property than bovine gelatin as evidenced by the lower EAI ($p<0.05$). However, the incorporation of OLA into gelatin could improve emulsifying property of resulting gelatin. Gelatin-OLA complex exhibited the higher EAI, both at 0 and 10 min, compared with bovine gelatin ($p<0.05$). Improved emulsifying activity might be due to the increased surface hydrophobicity of modified gelatin caused by OLA incorporated. This result was in agreement with Lin and Chen (2006) who reported that attachment of hydrophobic group to gelatin resulted in the increase in emulsifying property. Conversely, the incorporation of OTA into gelatin and gelatin-OLA complex led to the decreased EAI of modified gelatin. The higher decrease in EAI of gelatin-OLA complex was observed when the concentration of OTA increased ($p<0.05$). This was possibly associated with the decreases in surface hydrophobicity after modification with OTA. Kato and Nakai (1980) reported that surface hydrophobicity of protein is generally associated with a better surface activity, in which the reduction in interfacial tension and the increase in emulsifying activity are achieved. The decrease in surface hydrophobicity of sample modified with OTA might lower the ability of resulting gelatin to localize at the oil-water interface. Moreover, interactions of OTA with gelatin led to the aggregation of gelatin as indicated by the increased mean particle size (Table 25). As a result, gelatin might not unfold at the oil-water interface and form the film around the oil droplet effectively. Nevertheless, gelatin-OLA complex modified with 2.5 and 5% OTA still had the higher EAI than bovine gelatin, whereas EAI of gelatin-OLA complex modified with 10% OTA was lower than that of bovine gelatin.

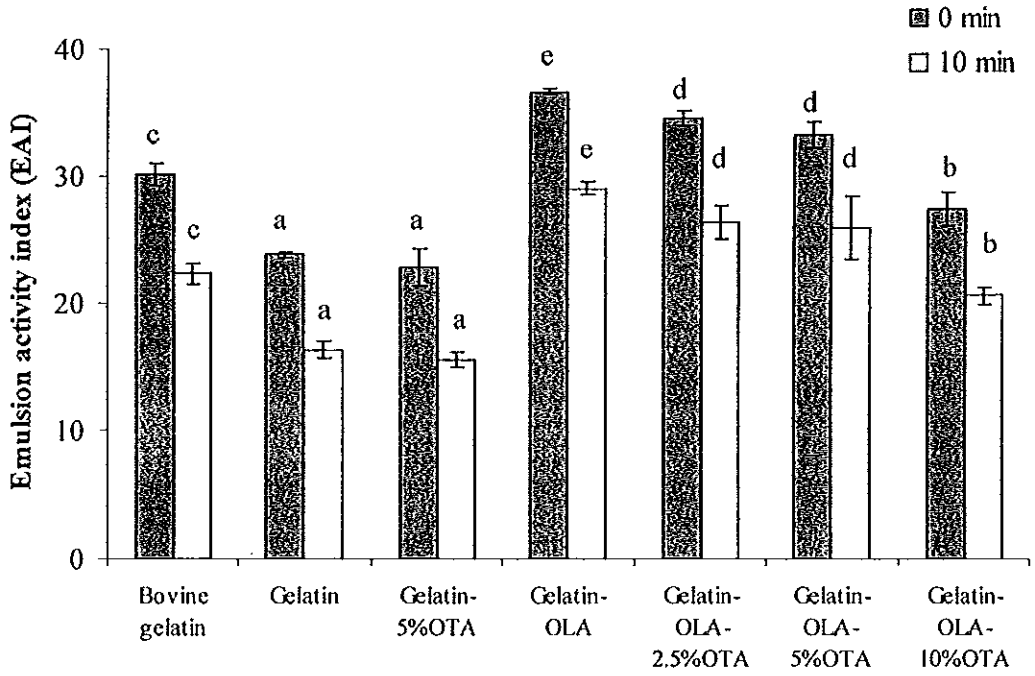


Figure 51. Emulsion activity of cuttlefish skin gelatin without and with modification by OLA and OTA. Bars represent the standard deviation (n=3). Different letters within the same time after emulsification indicate significant differences ($p<0.05$).

8.4.2 Effect of gelatin-OLA complex modified with OTA on emulsion stability and lipid oxidation of menhaden oil-in-water emulsion

8.4.2.1 Changes in particle size of emulsion during storage

Mean particle diameters of oil droplet in emulsions stabilized by gelatin and various modified gelatins at day 0 and 10 of storage at 30°C are shown in Table 26. The mean particle diameter of oil droplets was calculated and expressed as a volume-surface mean particle diameter (d_{32}) and volume-weighted mean particle diameter (d_{43}) (Surh *et al.*, 2006). Diameter size of oil droplet in emulsion stabilized by the cuttlefish skin gelatin (control gelatin) was similar to that of emulsion stabilized by bovine gelatin ($p>0.05$). The incorporation of OLA into gelatin could increase surface activity of gelatin. The oil could be reduced into smaller droplet easily. Simultaneously, modified gelatin could migrate into interface and form the film surrounding the droplet after emulsification. However, the modification of

gelatin and gelatin-OLA complex with OTA caused the increase in oil droplet size of resulting emulsion. The oil droplet size of emulsion stabilized by gelatin-OLA complex modified with OTA increased with increasing the concentration of OTA incorporated into gelatin. This result was in agreement with the decrease in EAI of the sample modified with OTA (Figure 51).

Table 26. Mean particle diameter of oil droplet in emulsion stabilized by gelatin without and with modification by OLA and OTA at day 0 and 10 at pH 7.

Samples	Mean particle diameter size (μm)			
	Day 0		Day 10	
	d_{32}	d_{43}	d_{32}	d_{43}
Gelatin	0.98±0.06b*A[#]	1.23±0.07bA	1.82±0.24dB	2.13±0.18cdB
Gelatin-5%OTA	1.00±0.06bA	1.33±0.06cA	2.06±0.20dB	2.38±0.26dB
Gelatin-OLA	0.79±0.02aA	0.82±0.03aA	1.03±0.10aB	1.30±0.15aB
Gelatin-OLA-2.5%OTA	0.83±0.03aA	0.85±0.05aA	1.19±0.16abB	1.63±0.25abB
Gelatin-OLA-5%OTA	0.88±0.04abA	0.90±0.06aA	1.39±0.13bcB	1.72±0.24bcB
Gelatin-OLA-10%OTA	0.93±0.04bA	1.18±0.05bA	1.74±0.24cdB	1.98±0.35bcB
Bovine gelatin	0.93±0.09abA	0.97±0.17abA	1.57±0.12cdB	1.74±0.19bB

Mean±SD (n=3).

* Different letters in the same column indicate significant differences ($p<0.05$).

[#] Different capital letters in the same sample row within the same mean diameter indicated significant differences ($p<0.05$).

Decrease in surface hydrophobicity of modified gelatin impeded its localization and arrangement at the oil-water interface, resulting in the formation of the larger oil droplet in the emulsion. It was noted that gelatin-OLA complex modified with 2.5 and 5% OTA, except gelatin-OLA complex modified with 10% OLA yielded the emulsion with the smaller oil droplet size, when compared with emulsion stabilized by bovine gelatin. At day 10 of storage, the increases in both d_{32} and d_{43} of oil droplets were found in all emulsion samples. This might be owing to individual droplet growth (Ostwald ripening) or droplet aggregation (flocculation or coalescence) during extended storage (Surh *et al.*, 2006). Among all emulsions, those

stabilized by gelatin-OLA complex had the lowest increase in d_{32} and d_{43}, compared with others.

The incorporation of OTA into gelatin-OLA complex resulted in the decreased emulsion stability, especially when the higher concentration of OTA was used for gelatin modification. The lower stability was reflected by the larger droplet size of oil in the emulsion. Emulsion with larger droplets had much poorer stability to gravitational separation and aggregation than that with smaller droplet (Tadros *et al.*, 2004). Gelatin-OLA complex modified with 2.5 and 5% OTA could stabilize oil-in-water emulsion more effectively than bovine gelatin as evidenced by the lower increase in both d_{32} and d_{43} after 10 days of storage.

8.4.2.2 Changes in lipid oxidation of emulsion during storage

Lipid oxidation of menhaden oil-in-water emulsion stabilized by gelatin with and without modification using OLA and OTA was monitored and expressed as TBARS values during the storage of 10 days (Figure 52). Generally, TBARS value in the emulsion increased as the storage time increased ($p<0.05$). Emulsion stabilized by gelatin had the lower formation of TBARS in emulsion, compared with that stabilized by 1% Tween 20 (control emulsion). Gelatin might be able to donate hydrogen or electron to free radicals, resulting in the inhibition of the oxidation of emulsion. Moreover, gelatin can form thicker film around the oil droplet, thereby preventing the penetration of oxygen into oil droplet more effectively. The emulsion stabilized by bovine gelatin had more oxidative stability than that stabilized by cuttlefish skin gelatin as evidenced by the lower TBARS value. This possibly resulted from the differences in the intrinsic properties, composition and conformation of protein between both gelatins.

Gelatin modified with OLA and OTA had the impact on oxidative stability of emulsion. Gelatin modified with OLA yielded the slightly increased oxidative stability of emulsion. This was possibly due to the increased accumulated gelatin around oil droplet, where modified gelatin could scavenge free radical effectively. Incorporation of OTA into gelatin and gelatin-OLA complex more likely improved the ability to inhibit the TBARS formation in oil-in-water emulsion system. This result was in accordance with the increased antioxidative activity of resulting gelatin after modification with OTA (Figure 49). No differences in TBARS value

were observed in emulsion stabilized by gelatin-OLA complex modified with 5 and 10% OTA after 10 days of storage. Therefore, OTA at concentration of 5% was the optimum level for modification of gelatin-OLA complex, in which the obtained gelatin had both antioxidative activity as well as emulsifying property.

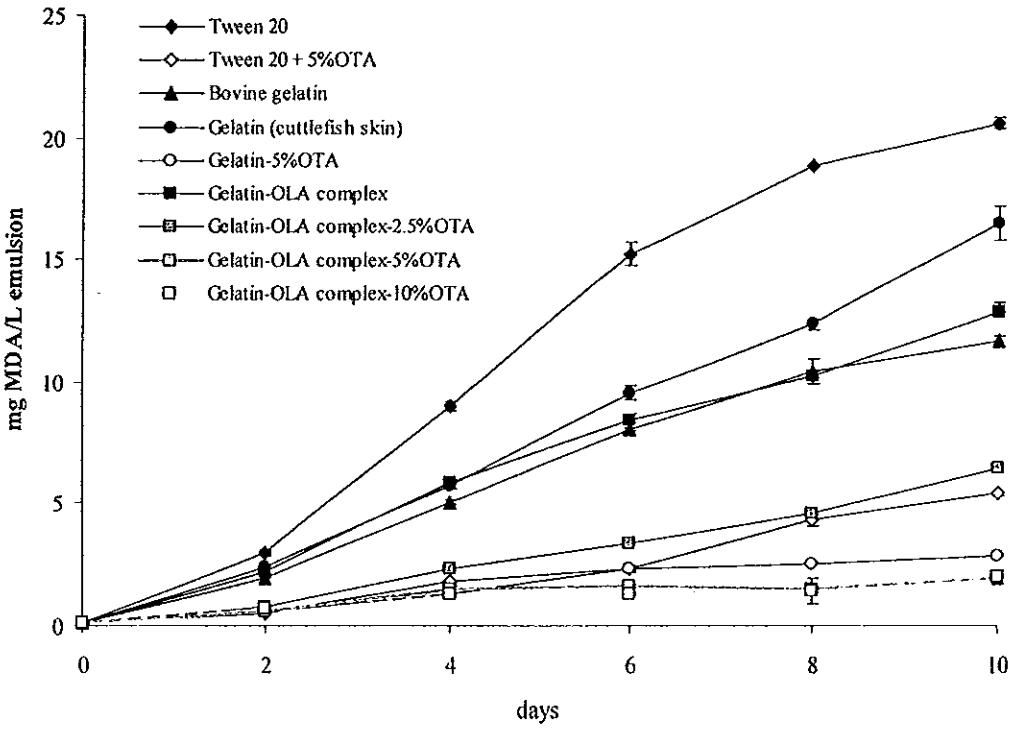


Figure 52. TBARS value of menhaden oil-in-water emulsion stabilized by cuttlefish skin gelatin without and with modification by OLA and OTA during storage at 30 °C for 10 days. Bars represent the standard deviation (n=3).

OTA bound to gelatin-OLA complex or gelatin could act as antioxidant in emulsion more effectively than OTA in free form, especially when the storage time increased ($p<0.05$). Free OTA was more likely localized in the aqueous phase and less amount was located at the interface. Almajano and Gordon (2004) reported that interaction of BSA and epigallocatechin gallate (EGCG) could induce the formation of a BSA-antioxidant adduct and cause a synergistic increase in the oxidative stability of sunflower oil-in-water emulsion, compared with BSA or EGCG alone. Lipid oxidation in oil-in-water emulsions primarily occurs at the emulsion

droplet interface (Kellerby *et al.*, 2006). Protein-antioxidant complex might be concentrated at the oil-water interface and exhibited the antioxidant activity at the interface (Almajano *et al.*, 2006). Gelatin and gelatin-OLA complex modified with OTA could therefore transport to the oil-water interface and functioned at the interface. As a result, lipid oxidation was prevented.

8.4.2.3 Stability of emulsion stabilized by modified gelatin under different conditions

8.4.2.3.1. Effect of pH

Zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin-OLA complex without and with modification by 5% OLA at various pHs (3-8) are shown in Figure 53. As pH increased from 3 to 8, the zeta potential in emulsion stabilized by the control gelatin decreased from +18.29 to -22.10 mV, whereas that of emulsion stabilized by gelatin-OLA complex without and with modification by 5% OTA decreased from +15.82 to -24.77 mV and +13.76 to -14.61 mV, respectively (Figure 53A). The zero net charge of oil droplets in emulsion stabilized by the control gelatin, gelatin-OLA complex and gelatin-OLA complex modified with 5% OTA was observed at pH 4.5, 4.4 and 4.3, respectively. At the same pH, the lower zeta potential was found in emulsion stabilized by gelatin-OLA complex when compared with that of emulsion stabilized by the control gelatin.

This result was in agreement with Kamyshny and Magdassi (1997) who reported that the attachment of hydrophobic chain led to changes in both amplitude of charge and pI of human IgG. Attachment of OLA and OTA via amino group during modification would neutralize the positively charged group, thereby rendering the higher negative charge in the modified gelatin (Kamyshny and Magdassi, 1997). No difference in both d_{32} and d_{43} was observed in all emulsion at all pHs tested ($p>0.05$). This result revealed that emulsions stabilized by the control gelatin and gelatin-OLA complex without and with modification by 5% OTA were relatively stable at all pHs, even at pH giving the net charge of zero. Steric repulsion played a major role in preventing the droplets from flocculation or coalescence at all pHs tested.

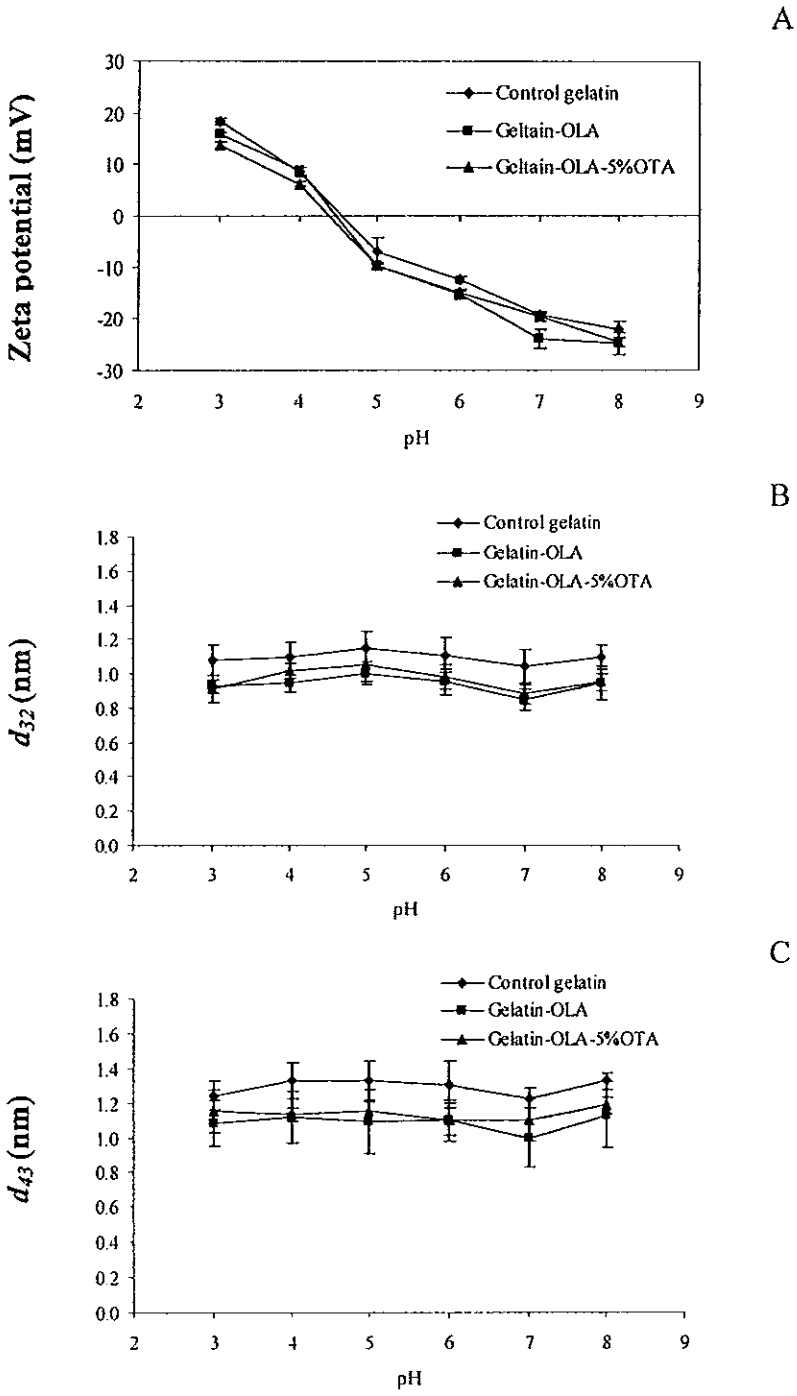


Figure 53. Effect of pH on zeta potential (A) and mean particle diameter including d_{32} (B) and d_{43} (C) of emulsion stabilized by cuttlefish skin gelatin and gelatin-OLA complex without and with modification by 5% OTA. Bars represent the standard deviation (n=3).

8.4.2.3.2 Effect of salt concentration

Zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin and gelatin-OLA complex without and with modification by 5% OLA at different salt concentrations (0-500 mM NaCl) are presented in Table 27. Generally, the amplitude of negative charge of oil droplet in all emulsion samples decreased with increasing NaCl concentrations ($p<0.05$). At 500 mM NaCl, zeta potential in emulsion stabilized by the control gelatin was reduced to -2.93 mV, whereas that of emulsion stabilized by gelatin-OLA complex without and with modification by OTA was -3.48 and -3.91 mV, respectively.

Table 27. Effect of NaCl concentration on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin without and with modification by OLA and OTA at pH 7.

Samples	NaCl (mM)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Gelatin	0	-19.33 ± 0.57a*	1.01 ± 0.05a	1.25 ± 0.06a
	50	-8.49 ± 1.22b	1.07 ± 0.20a	1.29 ± 0.15ab
	100	-7.63 ± 1.53b	1.11 ± 0.17a	1.31 ± 0.10ab
	250	-4.32 ± 1.38c	1.14 ± 0.12a	1.40 ± 0.18bc
	500	-2.93 ± 1.39c	1.39 ± 0.08b	1.64 ± 0.15c
Gelatin-OLA	0	-23.83 ± 1.86a	0.84 ± 0.04a	0.97 ± 0.04a
	50	-9.18 ± 1.02b	0.89 ± 0.10ab	1.06 ± 0.13ab
	100	-8.75 ± 1.25b	0.89 ± 0.12ab	1.09 ± 0.14ab
	250	-4.38 ± 1.07c	1.01 ± 0.13bc	1.16 ± 0.19ab
	500	-3.48 ± 1.31c	1.17 ± 0.10c	1.25 ± 0.18b
Gelatin-OLA-5%OTA	0	-19.59 ± 0.82a	0.90 ± 0.02a	1.06 ± 0.06a
	50	-8.69 ± 1.00b	0.92 ± 0.23a	1.06 ± 0.20a
	100	-7.53 ± 1.15b	1.04 ± 0.15b	1.09 ± 0.19a
	250	-4.45 ± 0.97c	1.13 ± 0.16bc	1.38 ± 0.14ab
	500	-3.91 ± 1.29c	1.27 ± 0.07c	1.50 ± 0.15b

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

Increasing ionic strength affects the electrostatic repulsion between the droplets of emulsion (Onsaard *et al.*, 2006). In the presence of counter ions, the charge of emulsion is reduced by electrostatic screening, in which the repulsion

between the droplets is no longer sufficiently strong to overcome the attractive force (e.g., vander Waals and hydrophobic) acting between the droplets (Onsaard *et al.*, 2006). As a result, the flocculation and/or coalescence of oil droplets took place. In general, slight increases in d_{32} and d_{43} of oil droplet were observed in all emulsion samples when NaCl concentrations increased ($p<0.05$). Nevertheless, Surh *et al.* (2006) reported that emulsion stabilized by fish gelatin was stable to droplet aggregation under high ionic strength condition (500 mM NaCl). This result suggested that not only electrostatic repulsion, but also steric repulsion played a major role in preventing the droplets from coalescence.

8.4.2.3.3 Effect of heating

Zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin and gelatin-OLA complex without and with modification by 5% OLA after heating at different temperatures (50, 70 and 90°C) are shown in Table 28. Generally, heating had not effect on the zeta potential of oil droplet in emulsions stabilized by modified gelatin and the control gelatin. However, heating increased oil droplet size (both d_{32} and d_{43}) in those emulsions, especially at the higher temperature ($p<0.05$). Some partial desorption of gelatin from the droplet surfaces at elevated temperatures might lead to flocculation and/or coalescence (Surh *et al.*, 2006). Among all emulsions, the emulsion stabilized by gelatin-OLA complex had higher thermal stability as evidenced by the lowered increase in both d_{32} and d_{43}, followed by that stabilized by gelatin-OLA complex modified with 5% OTA and the control gelatin, respectively. Incorporation of OTA into gelatin-OLA complex affected the emulsion stability of resulting gelatin as described previously. Nevertheless, gelatin-OLA complex modified with 5% OTA still stabilized the emulsion subjected to heating more effectively than the control gelatin. Strong film of modified gelatin around oil droplet could prevent the flocculation and/or coalescence of oil droplet from heat treatment.

Table 28. Effect of heating at different temperatures on zeta potential and mean particle diameter of oil droplet in emulsion stabilized by gelatin without and with modification by OLA and OTA at pH 7.

Samples	Temperature (°C)	Zeta potential (mV)	Particle size (μm)	
			d_{32}	d_{43}
Gelatin	25	-19.33 ±0 .57a*	1.01 ± 0.05a	1.25 ± 0.06a
	50	-19.57 ± 0.88a	1.01 ± 0.11a	1.28 ± 0.15a
	70	-19.89 ± 0.55a	1.12 ± 0.25a	1.33 ± 0.07a
	90	-19.61 ± 0.54a	1.17 ± 0.16a	1.42 ± 0.15a
Gelatin-OLA	25	-23.83 ± 1.86a	0.84 ± 0.04a	0.97 ± 0.04a
	50	-23.47 ± 1.52a	0.82 ± 0.15a	1.01 ± 0.15a
	70	-24.00 ± 0.66a	0.95 ± 0.18a	1.13 ± 0.12a
	90	-24.23 ± 0.56a	0.97 ± 0.08a	1.19 ± 0.21a
Gelatin-OLA-5%OTA	25	-19.59 ± 0.82a	0.90 ±0.02a	1.06 ± 0.06a
	50	-20.01 ± 0.59a	1.01 ± 0.12a	1.11 ± 0.20a
	70	-19.72 ± 1.01a	0.97 ± 0.13a	1.21 ± 0.23a
	90	-20.55 ± 0.62a	1.05 ± 0.20a	1.25 ± 0.19a

Mean±SD (n=3).

* Different letters in the same column within the same sample indicate significant differences ($p<0.05$).

8.5 Conclusion

Modification of gelatin-OLA complex with 5% OTA could enhance antioxidative activity and still retained the high emulsion property. Gelatin-OLA complex modified with 5% OTA could prevent oil-in-water emulsion from lipid oxidation and the flocculation or coalescence of oil droplet effectively, compared with bovine gelatin. The emulsion stabilized by gelatin modified with OLA together with OTA was stable in wide pH range and in the presence of NaCl up to 500 mM. The emulsion was also stable after being heated up to 90°C.

CHAPTER 9

SUMMARY AND FUTURE WORKS

9.1 Summary

1. Bleaching of cuttlefish skin with H_2O_2 not only improved the color of resulting gelatin but also increased the yield of gelatin. Cuttlefish skin gelatin bleached with 5% H_2O_2 for 48 h gave the highest yield. Furthermore, bleaching also improved bloom strength, emulsifying and foaming properties of resulting gelatin, mostly via the oxidation of gelatin molecule.

2. The incorporation of 5% oxidized phenolic compounds into cuttlefish skin gelatin could enhance antioxidative activity with no detrimental effect on emulsifying properties of resulting gelatin. Gelatin modified with 5% oxidized tannic acid (OTA) could prevent lipid oxidation of menhaden oil-in-water emulsion during the extended storage effectively.

3. Gelatin modified with tannic acid via non-covalent interactions showed a higher antioxidative activity than that modified through covalent interactions. However, gelatin modified with OLA via covalent interaction rendered the emulsion with high stability and could inhibit lipid oxidation of menhaden oil-in-water emulsion more effectively than gelatin modified with tannic acid via non-covalent interaction during the extended storage.

4. Attachment of fatty acid into cuttlefish skin gelatin could improve the surface activity of resulting gelatin. The increases in hydrophobic region at surface of gelatin play a key role in enhancing surface activity including foaming and emulsifying properties of modified gelatin. Gelatin modified with *N*-hydroxysuccinimide esters of C14:0, especially at the high degree of modification, showed the higher foam ability and emulsifying properties, when compared with the control gelatin.

5. Modification of gelatin with oxidized linoleic acid (OLA) could improve surface activity of obtained gelatin, depending on the degree of oxidation of linoleic acid used. Gelatin modified with OLA prepared at 70°C for 9 h showed the highest surface activity, especially at high molar ratio used. Gelatin modified with OLA could yield the emulsion with small oil droplet size and high stability toward different harsh environments, compared with the control gelatin.

6. Modification of gelatin with both OLA and OTA could enhance antioxidative activity and emulsion property, compared with the control gelatin. Modified gelatin could prevent menhaden oil-in-water emulsion from lipid oxidation and the flocculation or coalescence of oil droplet effectively during the extended storage, compared with bovine gelatin. The emulsion stabilized by gelatin modified with OLA together with OTA was stable in varying environments.

9.2 Future works

1. Gelatin of other mollusks should be further extracted and characterized.

2. Different chemical and enzymatic modifications should be performed to provide gelatin with desirable emulsifying properties and antioxidative activity.

3. The modified gelatin with high emulsifying properties or antioxidative activity should be isolated and characterized.

4. Mode of action of lipid oxidation products as well as selected phenolic compounds on the modification of gelatin should be further investigated.

References

Abdille, M.H., Singh, R.P., Jayaprakasha, G.K. and Jena, B.S. 2005. Antioxidant activity of the extracts from *Dillenia indica* fruits. Food Chem. 90: 891-896.

Aewsiri, T., Benjakul, S., Visessanguan, W. and Tanaka, M. 2008. Chemical compositions and functional properties of gelatin from pre-cooked tuna fin. Int. J. Food Sci. Technol. 43: 685-693.

Aewsiri, T., Benjakul, S. and Visessanguan, W. 2009a. Functional properties of gelatin from cuttlefish (*Sepia pharaonis*) skin as affected by bleaching using hydrogen peroxide. Food Chem. 115: 243-249.

Aewsiri, T., Benjakul, S., Visessanguan, W., Eun, J.B., Wierenga, P.A. and Gruppen, H. 2009b. Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by oxidised phenolic compounds. Food Chem. 117: 160-168.

Ahmad, I., Alaiz, M., Zamora, R. and Hidalgo, F.J. 1996. Antioxidative activity of lysine/13-hydroperoxy-9(Z),11(E)-octadecadienoic acid reaction products. J. Agric. Food Chem. 44: 3946-3949.

Ahmad, M. and Benjakul, S. 2011. Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. Food Hydrocolloids. 25: 381-388.

Alaiz, M., Zamora, R. and Hidalgo, F.J. 1995. Addition of oxidized lipid/amino acid reaction products delays the peroxidation initiated in a soybean oil. J. Agric. Food Chem. 43: 2698-2701.

Alaiz, M., Zamora, R. and Hidalgo, F.J. 1996. Contribution of the formation of oxidized lipid/amino acid reaction products to the protective role of amino acids in oils and fats. J. Agric. Food Chem. 44: 1890-1895.

Alaiz, M., Hidalgo, F.J. and Zamora, R. 1997. Antioxidative activity of nonenzymatically browned proteins produced in oxidized lipid/protein reactions. J. Agric. Food Chem. 45: 1365-1369.

Almajano, M.P. and Gordon, M.H. 2004. Synergistic effect of BSA on antioxidant activities in model food emulsions. J. Am. Oil Chem. Soc. 81: 275-280.

Almajano, M.P., Delgado, M.E. and Gordon, M.H. 2006. Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate. Food Chem. 101: 126-130.

Almajano, M.P., Delgado, M.E. and Gordon, M.H. 2007. Albumin causes a synergistic increase in the antioxidant activity of green tea catechins in oil-in-water emulsions. Food Chem. 102: 1375-1382.

Alzagtat, A.A. and Alli, I. 2002. Protein-lipid interactions in food systems: A review. Int. J. Food Sci. Technol. Nutr. 53: 249-260.

Amarnath, V., Valentine, W.M., Montine, T.J., Patterson, W.H., Amarnath, K., Bassett, C.N. and Graham, D.G. 1998. Reactions of 4-hydroxy-2(E)-nonenal and related aldehydes with proteins studied by carbon-13 nuclear magnetic resonance spectroscopy. Chem. Res. Toxicol. 11: 317-328.

Amitai, G., Adani, R., Sod-Moriah, G., Rabinovitz, I., Vincze, A., Leader, H., Chefetz, B., Leibovitz-Persky, L., Friesem, D. and Hadar, Y. 1998. Oxidative biodegradation of phosphorothiolates by fungal laccase. FEBS Lett. 438: 195-200.

AOAC. 2000. Official Methods of Analysis. Association of Official Analytical Chemists Inc. Arlington, VA, USA.

Arnesen, J.A. and Gildberg, A. 2007. Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. Biores. Technol. 98: 53-57.

Arts, M.J.T.J., Haenen, G.R.M.M., Wilms, L.C., Beetstra, S.A.J.N., Heijnen, C.G.M., Voss, H.P. and Bast, A. 2002. Interactions between flavonoids and proteins: Effect on the total antioxidant capacity. J. Agric. Food Chem. 50: 1184-1187.

Asamarai, A.M., Addis, P.B., Epley, R.J. and Krick, T.P. 1996. Wild rice hull antioxidants. J. Agric. Food Chem. 44: 126-130.

Aubourg, S.P. 1999. Recent advances in assessment of marine lipid oxidation by using fluorescence. J. Am. Oil Chem. Soc. 76: 409-419.

Avena-Bustillos, R.J., Olsen, C.W., Olson, D.A., Chiou, B., Yee, E., Bechtel, P.J. and McHugh, T.H. 2006. Water vapor permeability of mammalian and fish gelatin films. J. Food Sci. 71: E202-E207.

Aynié, S., Le Meste, M., Colas, B. and Lorient, D. 1992. Interactions between lipids and milk proteins in emulsion. J. Food Sci. 57: 883-886.

Bacon, J.R. and Rhodes, M.J.C. 1998. Development of a competition assay for the evaluation of the binding of human parotid salivary proteins to dietary complex phenols and tannins using a peroxidase-labeled tannin. J. Agric. Food Chem. 46: 5083-5088.

Badii, F. and Howell, N.K. 2006. Fish gelatin: Structure, gelling properties and interaction with egg albumen proteins. Food Hydrocolloids. 20: 630-640.

Balange, A.K. and Benjakul, S. 2009. Effect of oxidised tannic acid on the gel properties of mackerel (*Rastrelliger kanagurta*) mince and surimi prepared by different washing processes. Food Hydrocolloids. 23: 1693-1701.

Baxter, N.J., Lilley, T.H., Haslam, E. and Williamson, M.P. 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry. 36: 5566-5577.

Beckwith, A.C. 1984. Interaction of phosphatidylcholine vesicles with soybean 7S and 11S globulin proteins J. Agric. Food Chem. 32: 1397-1402.

Benjakul, S. and Morrissey, M.T. 1997. Protein hydrolysates from Pacific whiting solid wastes. J. Agric. Food Chem. 45: 3423-3430.

Benjakul, S., Seymour, T.A., Morrissey, M.T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. J. Food Sci. 62: 729-733.

Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': The FRAP assay. Anal. Biochem. 239: 70-76.

Bergman, I. and Loxley, R. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal. Chem. 35: 1961-1965.

Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M. and Kishimura, H. 2008. Antioxidative activity of mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). Food Chem. 106: 185-193.

Boyer, R.F. and McCleary, C.J. 1987. Superoxide ion as a primary reductant in ascorbate-mediated ferritin iron release. Free Rad. Biol. Med. 3: 389-395.

Bruenner, B.A., Jones, A.D. and German, J.B. 1995. Direct characterization of protein adducts of the lipid peroxidation product 4-hydroxy-2-nonenal using electrospray mass spectrometry. Chem. Res. Toxicol. 8: 552-559.

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation. Methods Enzymol. 52: 302-310.

Burcham, P.C. and Kuhan, Y.T. 1996. Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. Biochem. Biophys. Res. Commun. 220: 996-1001.

Burghagen, M. 1999. Meat. *In* Food Chemistry. 2nd Ed. (Belitz, H. D. and Grosch, W., eds.). p. 527-580. Springer-Verlag. Berlin, Germany.

Butterfield, D.A. and Stadtman, E.R. 1997. Chapter 7 Protein Oxidation Processes in Aging Brain. Advances in Cell Aging and Gerontology. 2: 161-191.

Carbonaro, M., Virgili, F. and Carnovale, E. 1996. Evidence for protein-tannin interaction in legumes: Implications in the antioxidant properties of faba bean tannins. LWT-Food Sci. Technol. 29: 743-750.

Carvalho, E., Mateus, N. and De Freitas, V. 2004. Flow nephelometric analysis of protein-tannin interactions. Anal. Chim. Acta. 513: 97-101.

Castle, J., Dickinson, E., Murray, A., Murray, B.S. and Stainsby, G. 1986. Surface behavior of adsorbed films of food proteins. *In* Gums and Stabilizers for the Food Industry. (Phillips, G. O. *et al.*, eds.). p. 409-417. Esevier Applied Sci. London, UK.

Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J.G., Haslam, E., Davies, A.P. and Williamson, M.P. 2002. Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. 50: 1593-1601.

Chen, Y. and Hagerman, A.E. 2004. Quantitative examination of oxidized polyphenol-protein complexes. J. Agric. Food Chem. 52: 6061-6067.

Cheow, C.S., Norizah, M.S., Kyaw, Z.Y. and Howell, N.K. 2006. Preparation and characterisation of gelatins from the skins of sin croaker (*Johnius dussumieri*) and shortfin scad (*Decapterus macrosoma*). Food Chem. 101: 386-391.

Chiou, B.S., Avena-Bustillos, R.J., Shey, J., Yee, E., Bechtel, P.J., Imam, S.H., Glenn, G.M. and Orts, W.J. 2006. Rheological and mechanical properties of cross-linked fish gelatins. Polymer. 47: 6379-6386.

Cho, S.H., Jahncke, M.L., Chin, K.B. and Eun, J.B. 2006. The effect of processing conditions on the properties of gelatin from skate (*Raja Kenojei*) skins. Food Hydrocolloids. 20: 810-816.

Cho, S.M., Kwak, K.S., Park, D.C., Gu, Y.S., Ji, C.I., Jang, D.H., Lee, Y.B. and Kim, S.B. 2004. Processing optimization and functional properties of gelatin from shark (*Isurus oxyrinchus*) cartilage. Food Hydrocolloids. 18: 573-579.

Cho, S.M., Gu, Y.S. and Kim, S.B. 2005. Extracting optimization and physical properties of yellowfin tuna (*Thunnus albacares*) skin gelatin compared to mammalian gelatins. Food Hydrocolloids. 19: 221-229.

Chobert, J.M. and Haertle, T. 1997. Protein-lipid and protein-flavour interactions *In* Food Proteins and Their Applications. (Damodaran, S. and Paraf, A., eds.). p. 143-170. Marcel Dekker. New York, USA.

Choi, S.S. and Regenstein, J.M. 2000. Physicochemical and sensory characteristics of fish gelatin. J. Food Sci. 65: 194-199.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66: 1219-1227.

Cole, B. 2000. Gelatin. Encyclopedia of Food Science and Technology. 1183-1188.

Cornell, D.G. 1991. Surface activity of bovine whey proteins at the phospholipid-water interface. *In* Interactions of Food Proteins. (Parris, N. and Barford, R., eds.). p. 123-136. American Chemical Society. Washington, DC, USA.

Courts, A. 1961. Structural changes in collagen. 2. Enhanced solubility of bovine collagen: reactions with hydrogen peroxide and some properties of soluble collagen. Biochem. J. 81: 356-365.

Damodaran, S. 1997. Protein-stabilized foams and emulsions. *In* Food Proteins and Their Applications. (Damodaran, S. and A., P., eds.). p. 57-110. Marcel Dekker, Inc. New York, USA.

Damodaran, S. 2005. Protein stabilization of emulsions and foams. J. Food Sci. 70: R54-R66.

De Freitas, V. and Mateus, N. 2001. Structural features of procyanidin interactions with salivary proteins. J. Agric. Food Chem. 49: 940-945.

De Jongh, H.H.J., Goormaghtigh, E. and Killian, J.A. 1994. Analysis of circular dichroism spectra of oriented protein-lipid complexes: Toward a general application. Biochemistry. 33: 14521-14528.

de Wolf, F.A. 2003. Collagen and gelatin. Progress Biotechnol. 23: 133-218.

Decker, A.E. 1998. Antioxidant mechanism. *In* Food Lipid: Chemistry, Nutrition, and Biotechnology. (Akoh, C. C. and Min, D. B., eds.). p. 397-448. Marcel Decker. New York, USA.

Decker, E.A., Xiong, Y.L., Calvert, J.T., Crum, A.D. and Blanchard, S.P. 1993. Chemical, physical, and functional properties of oxidized turkey white muscle myofibrillar protein. J. Agric. Food Chem. 41: 186-189.

Decker, E.A., Ivanov, V., Zhu, B.Z. and Frei, B. 2001. Inhibition of low-density lipoprotein oxidation by carnosine and histidine. J. Agric. Food Chem. 49: 511-516.

Department of Fisheries. 2006. Rainbow Cuttlefish (Online). Available http://www.ku.ac.th/AgrInfo/thaifish/aquatic/aq271.html (2006.June 1).

Derkatch, S.R., Petrova, L.A., Izmailova, V.N. and Tarasevitch, B.N. 1999. Properties of emulsion films made from binary aqueous mixtures of gelatin-surfactant: The effect of concentration and pH. Colloids Surf., A. 152: 189-197.

Dickinson, E., Rolfe, S.E. and Dalgleish, D.G. 1988. Competitive adsorption of α_{s1}-casein and β-casein in oil-in-water emulsions. Food Hydrocolloids. 2: 397-405.

Dickinson, E. 1992. Foams. *In* An Introduction to Food Colloids. (Dickinson, E., ed.). p. 123-139. Oxford University Press. Oxford, UK.

Dickinson, E. and Hong, S.T. 1994. Surface coverage of β-lactoglobulin at the oil - water interface: Influence of protein heat treatment and various emulsifiers. J. Agric. Food Chem. 42: 1602-1606.

Dickinson, E. and McClements, D.J. 1995. Advances in Food Colloids. *In* (Dickinson, E., ed.). Blackie Academic & Professiona. London, UK.

Dickinson, E. and Yamamoto, Y. 1996. Viscoelastic properties of heat-set whey protein-stabilized emulsion gels with added lecithin. J. Food Sci. 61: 811-816.

Dickinson, E. and Lopez, G. 2001. Comparison of the emulsifying properties of fish gelatin and commercial milk proteins. J. Food Sci. 66: 118-123.

Dickinson, E. 2003. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. Food Hydrocolloids. 17: 25-39.

Dickinson, E. 2010. Food emulsions and foams: Stabilization by particles. Curr. Opin. Colloid Interf. Sci. 15: 40-49.

Djabourov, M., Lechaire, J.P. and Gaill, F. 1993. Structure and rheology of gelatin and collagen gels. Biorheology. 30: 191-205.

Djagny, K.B., Wang, Z. and Xu, S. 2001. Chemical modification of pigskin gelatin: Factors affecting the esterification of gelatin with fatty acid. J. Food Sci. 66: 1326-1330.

Djordjevic, D., Cercaci, L., Alamed, J., McClements, D.J. and Decker, E.A. 2008. Stability of citral in protein- and gum arabic-stabilized oil-in-water emulsions. Food Chem. 106: 698-705.

Donnelly, T.H. and McGinnis, R.S. 1977. Gelatin manufacture; peroxide liquefaction process. U.S. Patent 4,043,996.

Doorn, J.A. and Petersen, D.R. 2002. Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. Chem. Res. Toxicol. 15: 1445-1450.

Douillard, R. 1994. Adsorption of serum albumin at the oil/water interface. Colloids Surf., A. 91: 113-119.

Eastoe, J.E. and Leach, A.A. 1977. Chemical constitution of gelatin. *In* The Science and Technology of Gelatin. (Ward, A. G. and Courts, A., eds.). p. 73-107. Academic Press. New York, USA.

Ericsson, B. 1990. Lipid-protein interactions. *In* Food Emulsions. (Larsson, K. and Friberg, S. E., eds.). p. 181-201. Macel Dekker. New York, USA.

Esterbauer, H., Schaur, R.J. and Zollner, H. 1991. Chemistry and Biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Rad. Biol. Med. 11: 81-128.

Evans, E.W. 1986. Interactions of milk components in food systems *In* Interactions of Food Components. (Birch, G. G. and Lindley, M. G., eds.). p. 241-275. Elsevier Applied Science Publishers. London, UK.

Farnum, C., Stanley, D.W. and Gray, J.I. 1976. Protein-lipid interactions in soy films. Can. Inst. Food Sci. Technol. J. 9: 201-206.

Farouk, M.M. and Swan, J.E. 1998. Effect of muscle condition before freezing and simulated chemical changes during frozen storage on protein functionality in beef. Meat Sci. 50: 235-243.

Fernández-Díaz, M.D., Montero, P. and Gómez-Guillén, M.C. 2001. Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by the coenhancers magnesium sulphate, glycerol and transglutaminase. Food Chem. 74: 161-167.

Fernández-Díaz, M.D., Montero, P. and Gómez-Guillén, M.C. 2003. Effect of freezing fish skins on molecular and rheological properties of extracted gelatin. Food Hydrocolloids. 17: 281-286.

Fernández-López, J., Fernández-Ginés, J.M., Aleson-Carbonell, L., Sendra, E., Sayas-Barberá, E. and Pérez-Alvarez, J.A. 2004. Application of functional citrus by-products to meat products. Trends Food Sci. Technol. 15: 176-185.

Finney, K.F. 1971. Fractionating and reconstituting techniques to relate functional (bread making) to biochemical properties of wheat flour components. Cereal Sci. Today. 16: 342-356.

Foegeding, E.A., Lanier, T.C. and Hultin, H.O. 1996. Characteristics of edible muscle tissue. *In* Food Chemistry 3rd Ed. (Fennema, O. R., ed.). p. 879-942. Marcel Dekker. New York, USA.

Fratzl, P. 2008. Structure and Mechanics, an Introduction. *In* Collagen, Structure and Mechanics. (Fratzl, P., ed.). p. 1-12. Springer. New York, USA.

Frazier, R.A., Papadopoulou, A., Mueller-Harvey, I., Kissoon, D. and Green, R.J. 2003. Probing protein-tannin interactions by isothermal titration microcalorimetry. J. Agric. Food Chem. 51: 5189-5195.

Friess, W. and Lee, G. 1996a. Basic thermoanalytical studies of insoluble collagen matrices. Biomaterials. 17: 2289-2294.

Galazka, V.B., Dickinson, E. and Ledward, D.A. 1999. Emulsifying behaviour of 11S globulin *Vicia faba* in mixtures with sulphated polysaccharides: Comparison of thermal and high-pressure treatments. Food Hydrocolloids. 13: 425-435.

García, P., Romero, C., Brenes, M. and Garrido, A. 1996. Effect of metal cations on the chemical oxidation of olive *o*-diphenols in model systems. J. Agric. Food Chem. 44: 2101-2105.

Gerrard, J.A., Brown, P.K. and Fayle, S.E. 2002. Maillard crosslinking of food proteins I: The reaction of glutaraldehyde, formaldehyde and glyceraldehyde with ribonuclease. Food Chem. 79: 343-349.

Gilsenan, P.M. and Ross-Murphy, S.B. 2000. Rheological characterisation of gelatins from mammalian and marine sources. Food Hydrocolloids. 14: 191-195.

Giménez, B., Gómez-Guillén, M.C. and Montero, P. 2005a. The role of salt washing of fish skins in chemical and rheological properties of gelatin extracted. Food Hydrocolloids. 19: 951-957.

Giménez, B., Turnay, J., Lizarbe, M.A., Montero, P. and Gómez-Guillén, M.C. 2005b. Use of lactic acid for extraction of fish skin gelatin. Food Hydrocolloids. 19: 941-950.

Giménez, B., Gómez-Estaca, J., Alemán, A., Gómez-Guillén, M.C. and Montero, M.P. 2009. Physico-chemical and film forming properties of giant squid (*Dosidicus gigas*) gelatin. Food Hydrocolloids. 23: 585-592.

Girotti, A.W. 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J. Lipid Res. 39: 1529-1542.

Goli, A.H., Barzegar, M. and Sahari, M.A. 2005. Antioxidant activity and total phenolic compounds of pistachio (*Pistachia vera*) hull extracts. Food Chem. 92: 521-525.

Gómez-Guillén, M.C. and Montero, P. 2001. Extraction of gelatin from megrim (*Lepidorhombus boscii*) skins with several organic acids. J. Food Sci. 66: 213-216.

Gómez-Guillén, M.C., Turnay, J., Fernández-Díaz, M.D., Ulmo, N., Lizarbe, M.A. and Montero, P. 2002. Structural and physical properties of gelatin extracted from different marine species: A comparative study. Food Hydrocolloids. 16: 25-34.

Gómez-Guillén, M.C., Giménez, B. and Montero, P. 2005. Extraction of gelatin from fish skins by high pressure treatment. Food Hydrocolloids. 19: 923-928.

Gómez-Guillén, M.C., Ihl, M., Bifani, V., Silva, A. and Montero, P. 2007. Edible films made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (*Ugni molinae Turcz*). Food Hydrocolloids. 21: 1133-1143.

Gordon, M.H. 2001. The development of oxidative rancidity in foods. *In* Antioxidants in Food: Practical Applications. (Pokorny, J., ed.). Woodhead Publishing Ltd. Cambridge, UK.

Grossman, S. and Bergman, M. 1992. Process for the production of gelatin from fish skin. US Patent 5093474.

Gu, Y.S., Regnier, L. and McClements, D.J. 2005. Influence of environmental stresses on stability of oil-in-water emulsions containing droplets stabilized by β-lactoglobulin-*ι*-carrageenan membranes. J. Colloid Interf. Sci. 286: 551-558.

Hagerman, A.E. and Butler, L.G. 1981. The specificity of proanthocyanidin-protein interactions. J. Biol. Chem. 256: 4494-4497.

Hagerman, A.E., Rice, M.E. and Ritchard, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin16 (4→8) catechin (procyanidin). J. Agric. Food Chem. 46: 2590-2595.

Hagerman, A.E., Dean, R.T. and Davies, M.J. 2003. Radical chemistry of epigallocatechin gallate and its relevance to protein damage. Arch. Biochem. Biophys. 414: 115-120.

Hamada, M. 1992. Mechanism, behavior and cross linkages of heat-induced myosin gel. Nippon Suisan Gakkaishi. 58: 89-93.

Hawkins, C.L. and Davies, M.J. 1997. Oxidative damage to collagen and related substrates by metal ion/hydrogen peroxide systems: Random attack or site-specific damage? Biochim. Biophys. Acta, Mol. Basis Dis. 1360: 84-96.

Hidalgo, F.J., Alaiz, M. and Zamora, R. 2001. Pyrrolization and antioxidant function of proteins following oxidative stress. Chem. Res. Toxicol. 14: 582-588.

Hidalgo, F.J., Nogales, F. and Zamora, R. 2003. Effect of the pyrrole polymerization mechanism on the antioxidative activity of nonenzymatic browning reactions. J. Agric. Food Chem. 51: 5703-5708.

Holzer, D. 1996. Gelatin production. US patent 5,484,888.

Hoque, M.S., Benjakul, S. and Prodpran, T. 2010. Effect of heat treatment of film-forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. J. Food Eng. 96: 66-73.

Hoque, M.S., Benjakul, S. and Prodpran, T. 2011. Effects of partial hydrolysis and plasticizer content on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. Food Hydrocolloids. 25: 82-90.

Howell, K.N. 1991. Protein-protein interactions. *In* Developments in Food Protein. Vol. 7. (Hudson, B. F., ed.). p. 231-270. Elsevier Applied Science. London, UK.

Howell, N.K., Herman, H. and Li-Chan, E.C.Y. 2001. Elucidation of protein-lipid interactions in a lysozyme-corn oil system by fourier transform raman spectroscopy. J. Agric. Food Chem. 49: 1529-1533.

Hu, M., McClements, D.J. and Decker, E.A. 2003. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. J. Agric. Food Chem. 51: 1696-1700.

Huang, S.W. and Frankel, E.N. 1997. Antioxiciant activity of tea catechins in different lipid systems. J. Agric. Food Chem. 45: 3033-3038.

Hulmes, D.J.S. 2008. Collagen Diversity, Synthesis and Assembly. *In* Collagen, Structure and Mechanics. (Fratzl, P., ed.). p. 15-47. Springer. New York, USA.

Hurrell, R.F., Finot, P.A. and Cuq, J.L. 1982. Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. Br. J. Nutr. 47: 191-211.

Intarasirisawat, R., Benjakul, S., Visessanguan, W., Prodpran, T., Tanaka, M. and Howell, N.K. 2007. Autolysis study of bigeye snapper (*Priacanthus*

macracanthus) skin and its effect on gelatin. Food Hydrocolloids. 21: 537-544.

Israelachvili, J.N. 1992. Intermolecular and Surface Forces. Academic Press. London, UK.

Jadhav, S.J., Nimbalker, S.S., Kulkami, A.D. and Madhavi, D.L. 1996. Lipid oxidation in biological and food systems. *In* Food Antioxidants. (Madhavi, D. L. *et al.*, eds.). p. 5–64. Marcel Dekker. New York, USA.

Jamilah, B. and Harvinder, K.G. 2002. Properties of gelatins from skins of fish - Black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*). Food Chem. 77: 81-84.

Jangaard, P.M. and Ackman, R.G. 1965. Lipids and component fatty acids of the New Foundland squid, *Illex illecobrosus*. J. Fish. Res. Can. 2: 131-137.

Jayaprakasha, G.K., Singh, R.P. and Sakariah, K.K. 2001. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. Food Chem. 73: 285-290.

Johnston-Banks, F.A. 1990. Gelatin. *In* Food Gels. (Harris, P., ed.). p. 233-289. Elsevier Applied Science. London, UK.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chem. 93: 475-484.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Prodpran, T. and Tanaka, M. 2006a. Characterization of edible films from skin gelatin of brownstripe red snapper and bigeye snapper. Food Hydrocolloids. 20: 492-501.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2006b. Effects of plasticizers on the properties of edible films from skin gelatin of bigeye snapper and brownstripe red snapper. Eur. Food Res. Technol. 222: 229-235.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2006c. Skin gelatin from bigeye snapper and brownstripe red snapper: Chemical compositions and effect of microbial transglutaminase on gel properties. Food Hydrocolloids. 20: 1216-1222.

Kamat, V.B., Graham, G.E. and Davis, M.A.F. 1978. Vegetable protein: Lipid interactions. Cereal Chem. 55: 295-307.

Kamyshny, A. and Magdassi, S. 1997. Hydrophobically modified human IgG: Surface and biological activities. Colloids Surf., B. 9: 147-155.

Kantaria, S., Rees, G.D. and Lawrence, M.J. 1999. Gelatin-stabilised microemulsion-based organogels: Rheology and application in iontophoretic transdermal drug delivery. J. Controlled Release. 60: 355-365.

Karel, M. 1973. Symposium: Protein interactions in biosystems. Protein-lipid interactions. J. Food Sci. 38: 756-763.

Karim, A.A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloids. 23: 563-576.

Kasankala, L.M., Xue, Y., Weilong, Y., Hong, S.D. and He, Q. 2007. Optimization of gelatine extraction from grass carp (*Catenopharyngodon idella*) fish skin by response surface methodology. Biores. Technol. 98: 3338-3343.

Kato, A. and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochim. Biophys. Acta. 624: 13-20.

Kato, A., Osako, Y., Matsudomi, N. and Kobayashi, K. 1983. Changes in the emulsifying and foaming properties of proteins during heat denaturation. Agric. Biol. Chem. 47: 33-37.

Kato, Y., Uchida, K. and Kawakishi, S. 1992. Oxidative fragmentation of collagen and prolyl peptide by $Cu(II)/H_2O_2$. Conversion of proline residue to 2-pyrrolidone. J. Biol. Chem. 267: 23646-23651.

Kawamoto, H., Nakatsubo, F. and Murakami, K. 1996. Stoichiometric studies of tannin-protein co-precipitation. Phytochemistry. 41: 1427-1431.

Kawamoto, H. and Nakatsubo, F. 1997. Effects of environmental factors on two-stage tannin-protein co- precipitation. Phytochemistry. 46: 479-483.

Kellerby, S.S., Yeun, S.G., McClements, D.J. and Decker, E.A. 2006. Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. J. Agric. Food Chem. 54: 10222-10227.

Kikugawa, K., Kato, T. and Hayasaka, A. 1991. Formation of dityrosine and other fluorescent amino acids by reaction of amino acids with lipid hydroperoxides. Lipids. 26: 922-929.

Kim, S.K., Jeon, Y.J., Lee, B.J. and Lee, C.K. 1996. Purification and characterization of the gelatin from the bone of cod, *Gadus macrocephalus*. Korean J. Life Sci. 6: 14-26.

Kim, S.K. and Mendis, E. 2006. Bioactive compounds from marine processing byproducts - A review. Food Res. Int. 39: 383-393.

King, A.J., Ball, H.R., Catignani, G.L. and Swaisgood, H.E. 1984. Modification of egg white proteins with oleic acid. J. Food Sci. 49: 1240-1244.

Kinsella, J.E. 1984. Milk proteins: physicochemical and functional properties. Crit. Rev. Food Sci. Nutr. 21: 197-262.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chem. 89: 363-372.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010a. Comparative study on characteristics of gelatin from the skins of brownbanded bamboo shark and blacktip shark as affected by extraction conditions. Food Hydrocolloids. 24: 164-171.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010b. Effect of extraction temperature on functional properties and antioxidative activities of gelatin from shark skin. Food Bioprocess Technol. *In Press.* .

Kläui, H.M., Hausheer, W. and Huschke, G. 1970. Technological aspects of the use of fat-soluble vitamins and carotenoids and of the development of stabilized marketable forms. *In* Fat-Soluble Vitamins. Vol. 9. (R.A., M., ed.). p. 113-159. Pergaman. Oxford, UK.

Kołodziejska, I., Sikorski, Z.E. and Niecikowska, C. 1999. Parameters affecting the isolation of collagen from squid (*Illex argentinus*) skins. Food Chem. 66: 153-157.

Kołodziejska, I., Skierka, E., Sadowska, M., Kołodziejski, W. and Niecikowska, C. 2008. Effect of extracting time and temperature on yield of gelatin from different fish offal. Food Chem. 107: 700-706.

Kreuzer, R. 1984. Cephalopods: handling, processing and products. FAO Fish Technol. Pap. 154: 1-108.

Kroll, J., Rawel, H.M. and Rohn, S. 2003. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. Food Sci. Tech. Res. 9: 205-218.

Kuijpers, A.J., Engbers, G.H.M., Feijen, J., De Smedt, S.C., Meyvis, T.K.L., Demeester, J., Krijgsveld, J., Zaat, S.A.J. and Dankert, J. 1999. Characterization of the network structure of carbodiimide cross-linked gelatin gels. Macromolecules. 32: 3325-3333.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lapidot, Y., Rappoport, S. and Wolman, Y. 1967. Use of esters of *N*-hydroxysuccinimide in the synthesis of *N*-acylamino acids. J. Lipid Res. 8: 142-145.

Lasztity, R. 1996. Wheat protein. *In* The Chemistry of Cereal Proteins. 2nd Ed. p. 42-111. CRC Press. Boca Raton, FL, USA.

Lau, D.W. and King, A.J. 2003. Pre- and post-mortem use of grape seed extract in dark poultry meat to inhibit development of thiobarbituric acid reactive substances. J. Agric. Food Chem. 51: 1602-1607.

Ledward, D.A. 1986. Gelation of gelatin. *In* Functional Properties of Food Macromolecules. (J.R., M. and D.A, L., eds.). p. 171-201. Elsevier Applied Science Publishers. London, UK.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S. and Stadtman, E.R. 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186: 464-478.

Levine, R.L., Mosoni, L., Berlett, B.S. and Stadtman, E.R. 1996. Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. U. S. A. 93: 15036-15040.

Li, Y., Li, Y., Du, Z. and Li, G. 2008. Comparison of dynamic denaturation temperature of collagen with its static denaturation temperature and the configuration characteristics in collagen denaturation processes. Thermochim. Acta. 469: 71-76.

Liang, J.H. 1999. Fluorescence due to interactions of oxidizing soybean oil and soy proteins. Food Chem. 66: 103-108.

Liao, S.Y. and Mangino, M.E. 1987. Characterization of the composition, physicochemical and functional properties of acid whey protein concentrates. J. Food Sci. 52: 1033-1037.

Lin, L.H. and Chen, K.M. 2006. Preparation and surface activity of gelatin derivative surfactants. Colloids Surf., A. 272: 8-14.

Liu, G., Xiong, Y.L. and Butterfield, D.A. 2000. Chemical, physical, and gel-forming properties of oxidized myofibrils and whey- and soy-protein isolates. J. Food Sci. 65: 811-818.

Liu, H.Y., Li, D. and Guo, S.D. 2008. Extraction and properties of gelatin from channel catfish (*Ietalurus punetaus*) skin. LWT-Food Sci. Technol. 41: 414-419.

Liu, Q. and Yao, H. 2007. Antioxidant activities of barley seeds extracts. Food Chem. 102: 732-737.

Liu, Z., Minkler, P.E. and Sayre, L.M. 2003. Mass spectroscopic characterization of protein modification by 4-hydroxy-2-(E)-nonenal and 4-oxo-2-(E)-nonenal. Chem. Res. Toxicol. 16: 901-911.

Lobo, L. 2002. Coalescence during emulsification: 3. Effect of gelatin on rupture and coalescence. J. Colloid Interf. Sci. 254: 165-174.

Lobo, L. and Svereika, A. 2003. Coalescence during emulsification: 2. Role of small molecule surfactants. J. Colloid Interf. Sci. 261: 498-507.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lu, Y. and Bennick, A. 1998. Interaction of tannin with human salivary proline-rich proteins. Arch. Oral Biol. 43: 717-728.

Luck, G., Liao, H., Murray, N.J., Grimmer, H.R., Warminski, E.E., Williamson, M.P., Lilley, T.H. and Haslam, E. 1994. Polyphenols, astringency and proline-rich proteins. Phytochemistry. 37: 357-371.

Magdassi, S., Toledano, O. and Zakay-Rones, Z. 1996. Solubilization in colloidal immunoclusters. J. Colloid Interf. Sci. 184: 360-364.

Maqsood, S. and Benjakul, S. 2009. Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. Food Chem. 119: 123-132.

Mateus, N., Pinto, R., Ruão, P. and De Freitas, V. 2004. Influence of the addition of grape seed procyanidins to Port wines in the resulting reactivity with human salivary proteins. Food Chem. 84: 195-200.

Matheis, G. and Whitaker, J.R. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. J. Food Biochem. 8: 137-162.

Mayer, A.M. 1986. Polyphenol oxidases in plants-recent progress. Phytochemistry. 26: 11-20.

Mayer, A.M. and Staples, R.C. 2002. Laccase: New functions for an old enzyme. Phytochemistry. 60: 551-565.

McCarthy, T.L., Kerry, J.P., Kerry, J.F., Lynch, P.B. and Buckley, D.J. 2001. Evaluation of the antioxidant potential of natural food/plant extracts as compared with synthetic antioxidants and vitamin E in raw and cooked pork patties. Meat Sci. 58: 45-52.

McClements, D.J. 1999. Food Emulsions: Principles, Practice and Techniques. CRC Press. New York, USA.

McClements, D.J. 2004. Protein-stabilized emulsions. Curr. Opin. Colloid Interf. Sci. 9: 305-313.

McClements, D.J. 2005. Theoretical analysis of factors affecting the formation and stability of multilayered colloidal dispersions. Langmuir. 21: 9777-9785.

McWilliams, M. 1989. Foods: Experimental Perspectives. MacMillan Publ. Co. New York, USA.

Medina, I., Satué-Gracia, M.T., German, J.B. and Frankel, E.N. 1999. Comparison of natural polyphenol antioxidants from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. J. Agric. Food Chem. 47: 4873-4879.

Meng, G., Howell, K.N. and Li-Chan, E.C.Y. 2006. Investigation of protein-lipid Interactions by vibrational spectroscopy. *In* Lipid Analysis and Lipidomics: New Techniques and Applications. (Kramer, J. K. G. *et al.*, eds.). p. 355-376. AOCS Publishing. New York, USA.

Mine, Y. 2002. Recent advances in egg protein functionality in the food system. World's Poult. Sci. J. 58: 31-39.

Mizutani, R. and Nakamura, R. 1987. Emulsifying properties of a complex between apoprotein from hen's egg yolk lecithin. Agric. Biol. Chem. 51: 1115-1119.

Montero, P., Borderías, J., Turnay, J. and Leyzarbe, M.A. 1990. Characterization of hake (*Merluccius merluccius L.*) and trout (*Salmo irideus Gibb*) collagen. J. Agric. Food Chem. 38: 604-609.

Montero, P. and Gómez-Guillén, M.C. 2000. Extracting conditions for megrim (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting gelatin. J. Food Sci. 65: 434-438.

Moure, A., Domínguez, H. and Parajó, J.C. 2006. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. Process Biochem. 41: 447-456.

Müller, H.J. and Hermel, H. 1994. On the relation between the molecular mass distribution of gelatin and its ability to stabilize emulsions. Colloid Polym. Sci. 272: 433-439.

Murray, J.C., Buetow, K.H., Weber, J.L., Ludwigsen, S., Scherpbier-Heddema, T., Manion, F., Quillen, J., Sheffield, V.C., Sunden, S., Duyk, G.M., Weissenbach, J., Gyapay, G., Dib, C., Morrissette, J., Lathrop, G.M., Vignal, A., White, R., Matsunami, N., Gerken, S., Melis, R., Albertsen, H., Plaetke, R., Odelberg, S., Ward, D., Dausset, J., Cohen, D. and Cann, H. 1994. A comprehensive human linkage map with centimorgan density. Science. 265: 2049-2054.

Mutilangi, W.A.M., Panyam, D. and Kilara, A. 1996. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. J. Food Sci. 61: 270-303.

Muyonga, J.H., Cole, C.G.B. and Duodu, K.G. 2004a. Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. Food Hydrocolloids. 18: 581-592.

Muyonga, J.H., Cole, C.G.B. and Duodu, K.G. 2004b. Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). Food Chem. 86: 325-332.

Naczk, M., Amarowicz, R., Zadernowski, R. and Shahidi, F. 2001. Protein-precipitating capacity of crude condensed tannins of canola and rapeseed hulls. J. Am. Oil Chem. Soc. 78: 1173-1178.

Nadkarni, D.V. and Sayre, L.M. 1995. Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. Chem. Res. Toxicol. 8: 284-291.

Nagai, T. and Suzuki, N. 2000. Isolation of collagen from fish waste material - Skin, bone and fins. Food Chem. 68: 277-281.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N. and Suzuki, N. 2001. Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). Food Chem. 72: 425-429.

Nakayama, T., Osawa, T., Mendosa, E.N.T., Laurena, A.C. and Kawakishi, S. 1994. Search for new natural antioxidants in selected tropical plant food materials. *In* Postharvest Biochemistry of Plant Food Materials in the Tropics. (Uritani, I. *et al.*, eds.). p. 241-251. Japan Scientific Societies Press. Tokyo, Japan.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloids. 22: 615-622.

Namiki, M. 1990. Antioxidants/antimutagens in food. Crit. Rev. Food Sci. Nutr. 29: 273-300.

Neucere, N.J., Jacks, T.J. and Sumrell, G. 1978. Interactions of globular protein with simple polyphenols. J. Agric. Food Chem. 26: 214-216.

Niki, E. 1987. Antioxidants in relation to lipid peroxidation. Chem. Phys. Lipids. 44: 227-253.

Norland, R.E. 1990. Fish gelatin. *In* Advances in Fisheries Technology and Biotechnology for Increased Profitability. (M.N., V. and J.K., B., eds.). p. 325-333. Technomic Publishing Co. Lancaster.

O'Connell, J.E. and Fox, P.F. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: A review. Int. Dairy J. 11: 103-120.

Oh, H.I., Hoff, J.E., Armstrong, G.S. and Haff, L.A. 1980. Hydrophobic interaction in tannin-protein complexes. J. Agric. Food Chem. 28: 394-398.

Okuzumi, M. and Fujii, T., 2000. Nutritional and Functional Properties of Squid and Cuttlefish. National Cooperative Association of Squid Processor. Tokyo.

Olijve, J., Mori, F. and Toda, Y. 2001. Influence of the molecular-weight distribution of gelatin on emulsion stability. J. Colloid Interf. Sci. 243: 476-482.

Onsaard, E., Vittayanont, M., Srigam, S. and McClements, D.J. 2006. Comparison of properties of oil-in-water emulsions stabilized by coconut cream proteins with those stabilized by whey protein isolate. Food Res. Int. 39: 78-86.

Osawa, T. 1994. Novel natural antioxidants for utilization in food and biological systems. *In* Postharvest Biochemistry of Plant Food-Materials in the Tropics. (Uritani, I. *et al.*, eds.). p. 241-251. Japan Scientific Societies Press. Tokyo, Japan.

Otwell, W.S. and Hamann, D.B. 1979. Textural character of squid (*Loligo pealei*): instrumental and panel evaluations. J. Food Sci. 44: 1636-1643.

Paiva-Martins, F. and Gordon, M.H. 2005. Interactions of ferric ions with olive oil phenolic compounds. J. Agric. Food Chem. 53: 2704-2709.

Parr, A.J. and Bolwell, G.P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J. Sci. Food Agric. 80: 985-1012.

Payne, K.J. and Veis, A. 1988. Fourier transform IR spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies. Biopolymers. 27: 1749-1760.

Pazos, M., Gallardo, J.M., Torres, J.L. and Medina, I. 2005. Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. Food Chem. 92: 547-557.

Pearce, K.N. and Kinsella, J.E. 1978. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. J. Agric. Food Chem. 26: 716-723.

Perkins, W.S. 1996. Advances made in bleaching practice. Am. Text. Int. 25: 92-94.

Pezennec, S., Gauthier, F., Alonso, C., Graner, F., Croguennec, T., Brulé, G. and Renault, A. 2000. The protein net electric charge determines the surface rheological properties of ovalbumin adsorbed at the air-water interface. Food Hydrocolloids. 14: 463-472.

Phatcharat, S., Benjakul, S. and Visessanguan, W. 2006. Effects of washing with oxidising agents on the gel-forming ability and physicochemical properties of surimi produced from bigeye snapper (*Priacanthus tayenus*). Food Chem. 98: 431-439.

Phillips, L.G., Whitehead, D.M. and Kinsella, J. 1994. Structure-Function Properties of Food Proteins. Academic Press. San Diego, CA, USA.

Pokorny, J., Reblova, Z., Kourimska, L., Pudil, F. and Kwiatkowska, A. 1993. Effect of interactions with oxidized lipids on structure change and properties of food proteins. *In* Food Proteins: Structure and Functionality. (Schwenke, K. D. and Mothes, R., eds.). p. 232-235. VCH Publishers. Weinheim, Germany.

Pokorny, J. and Kolakowska, A. 2003. Lipid-protein and lipid-saccharide interaction. *In* Chemical and functional properties of food lipids. (Sikorski, Z. E., ed.). p. 345-363. CRC Press. Boca Raton, FL, USA.

Pomeranz, Y. 1973. Interaction between glycolipids and wheat flour macromolecules in breadmaking. Adv. Food Res. 20: 153-188.

Pomeranz, Y. 1991. Functional Properties of Food Components. 2nd Ed. Academic Press. New York, USA.

Poppe, J. 1997. Gelatin. *In* Thickening and Gelling Agents for Food. 2nd Ed. (Imerson, A., ed.). p. 144-168. Blackie Academic & Professional. London, UK.

Porter, N.A., Caldwell, S.E. and Mills, K.A. 1995. Mechanisms of free radical oxidation of unsaturated lipids. Lipids. 30: 277-290.

Potter, N.N. and Hotchkiss, J.H. 1998. Food Science. 2nd Ed. Aspen Publishers, Inc. Gaithersburg, Germany.

Prasad, K.N., Divakar, S., Shivamurthy, G.R. and Aradhya, S.M. 2005. Isolation of a free radical-scavenging antioxidant from water spinach (*Ipomoea aquatica* Forsk). J. Sci. Food Agric. 85: 1461-1468.

Pratt, D.E. and Hudson, B.J.F. 1990. Natural antioxidants not exploited commercially. *In* Food Antioxidants. (Hudson, B. J. F., ed.). p. 171-179. Elsevier. Amsterdam, The Netherlands.

Prigent, S.V.E., Gruppen, H., Visser, A.J.W.G., Van Koningsveld, G.A., De Jong, G.A.H. and Voragen, A.G.J. 2003. Effects of non-covalent interactions with 5-*o*-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. J. Agric. Food Chem. 51: 5088-5095.

Prigent, S.V.E., Voragen, A.G.J., Li, F., Visser, A.J.W.G., Van Koningsveld, G.A. and Gruppen, H. 2008. Covalent interactions between amino acid side chains and oxidation products of caffeoylquinic acid (chlorogenic acid). J. Sci. Food Agric. 88: 1748-1754.

Pugnaloni, L.A., Dickinson, E., Ettelaie, R., Mackie, A.R. and Wilde, P.J. 2004. Competitive adsorption of proteins and low-molecular-weight surfactants: Computer simulation and microscopic imaging. Adv. Colloid Interf. Sci. 107: 27-49.

Rajalakshmi, D. and Narasimhan, S. 1996. Food antioxidants: sources and method of evaluation. *In* Food Antioxidants. (MADHAVI, D. L. *et al.*, eds.). p. 65-158. Marcel Dekker. New York, USA.

Rawel, H.M., Kroll, J. and Rohn, S. 2001. Reactions of phenolic substances with lysozyme - Physicochemical characterisation and proteolytic digestion of the derivatives. Food Chem. 72: 59-71.

Rawel, H.M., Czajka, D., Rohn, S. and Kroll, J. 2002a. Interactions of different phenolic acids and flavonoids with soy proteins. Int. J. Biol. Macromol. 30: 137-150.

Rawel, H.M., Rohn, S., Kruse, H.P. and Kroll, J. 2002b. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. Food Chem. 78: 443-455.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26: 1231-1237.

Refsgaard, H.H.F., Tsai, L. and Stadtman, E.R. 2000. Modifications of proteins by polyunsaturated fatty acid peroxidation products. Proc. Natl. Acad. Sci. U. S. A. 97: 611-616.

Ricardo Da Silva, J.M., Darmon, N., Fernandez, Y. and Mitjavila, S. 1991. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J. Agric. Food Chem. 39: 1549-1552.

Rice-Evans, C.A., Miller, N.J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2: 152-159.

Rigby, B.J. 1968. Amino-acid composition and thermal stability of the skin collagen of the antarctic ice-fish. Nature. 219: 166-167.

Robinson, H.W. and Hodgen, C.G. 1940. The biuret reaction in the determination of serum protein. I. A study of the condition necessary for the production of the stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135: 707-725.

Rodríguez-López, J.N., Fenoll, L.G., Pealver, M.J., García-Ruiz, P.A., Varón, R., Martínez-Ortíz, F. and García-Cánovas, F. 2001. Tyrosinase action on monophenols: Evidence for direct enzymatic release of *o*-diphenol. Biochim. Biophys. Acta Protein Struct. Mol. Enzym. 1548: 238-256.

Rohn, S., Rawel, H.M. and Kroll, J. 2004. Antioxidant activity of protein-bound quercetin. J. Agric. Food Chem. 52: 4725-4729.

Rohn, S., Rawel, H.M., Rǎber, M. and Kroll, J. 2005. Reactions with phenolic substances can induce changes in some physico-chemical properties and activities of bromelain - The consequences for supplementary food products. Int. J. Food Sci. Technol. 40: 771-782.

Rubino, M.I., Arntfield, S.D., Nadon, C.A. and Bernatsky, A. 1996. Phenolic protein interactions in relation to the gelation properties of canola protein. Food Res. Int. 29: 653-659.

Sadowska, M., Kołodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257-262.

Sagis, L.M.C., Boeriu, C.G., Frissen, G.E., Schols, H.A. and Wierenga, P.A. 2008. Highly stable foams from block oligomers synthesized by enzymatic reactions. Langmuir. 24: 359-361.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L.R. 2004. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. Food Chem. 86: 99-103.

Salas, J.J., Williams, M., Harwood, J.L. and Sánchez, J. 1999. Lipoxygenase activity in olive (*Olea europaea*) fruit. J. Am. Oil Chem. Soc. 76: 1163-1168.

Sarabia, A.I., Gmumez-Guillsan, M.C. and Montero, P. 2000. The effect of added salts on the viscoelastic properties of fish skin gelatin. Food Chem. 70: 71-76.

Sarker, D.K., Wilde, P.J. and Clark, D.C. 1995. Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. J. Agric. Food Chem. 43: 295-300.

Sarma, J., Srikar, L.N. and Vidyasagar Reddy, G. 1998. Comparative effects of frozen storage on biochemical changes in pink perch (*Nemipterus japonicus*) and oil sardine (*Sardinella longiceps*). J. Food Sci. Technol. 35: 255-258.

Sarni-Manchado, P., Cheynier, V. and Moutounet, M. 1999. Interactions of grape seed tannins with salivary proteins. J. Agric. Food Chem. 47: 42-47.

Schrieber, R. and Gareis, H. 2007. Gelatine handbook. Wiley-VCH GmbH & Co. Weinhem, Germany.

Seal, R. 1980. Industrial soya protein technology. *In* Applied Protein Chemistry. (Grant, R. A., ed.). p. 87-112. Applied Science. London, UK.

Shahidi, F. and Wanasundara, P.K. 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. 32: 67-103.

Shahidi, F., Han, X.Q. and Symwiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem. 53: 285-293.

Shantha, N.C. and Decker, E.A. 1994. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. J. AOAC Int. 77: 421-424.

Sharma, R., Zakora, M. and Qvist, K.B. 2002. Characteristics of oil-water emulsions stabilised by an industrial α-lactalbumin concentrate, cross-linked before and after emulsification, by a microbial transglutaminase. Food Chem. 79: 493-500.

Shimizu, M., Kamiya, T. and Yamauchi, K. 1981. The adsorption of whey proteins on the surface of emulsified fat. Agric. Biol. Chem. 45: 2491-2496.

Shimizu, M., Ametani, A., Kaminogawa, S. and Yamauchi, K. 1986. The topography of α(s1)-casein adsorbed to an oil/water interface: An analytical approach using proteolysis. Biochim. Biophys. Acta Protein Struct. Mol. Enzym. 869: 259-264.

Siebert, K.J., Troukhanova, N.V. and Lynn, P.Y. 1996. Nature of polyphenol-protein interactions. J. Agric. Food Chem. 44: 80-85.

Siebert, K.J. 2006. Haze formation in beverages. LWT-Food Sci. Technol. 39: 987-994.

Sikorski, Z.E. 2001. Proteins. *In* Chemical and Functional Properties of Food Proteins. (Sikorski, Z. E., ed.). Technomic Publishing. Poland.

Simon, C., Barathieu, K., Laguerre, M., Schmitter, J.M., Fouquet, E., Pianet, I. and Dufourc, E.J. 2003. Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. Biochemistry. 42: 10385-10395.

Slinkard, K. and Singleton, V.L. 1977. Total phenol analysis: Automation and comparison with manual methods. Am. J. Enol. Vitic. 28: 49-55.

Smith, B. 1999. Infrared Spectral Interpretation. CRC Press. New York, USA.

Southgate, P.C. and Lou, D.C. 1995. Improving the n-3 PUFA composition of *Aretenmia* using microcapsules containing marine oils. Aquaculture. 134: 91-99.

Sroka, Z. and Cisowski, W. 2003. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. Food Chem. Toxicol. 41: 753-758.

Stadtman, E.R. and Berlett, B.S. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem. Res. Toxicol. 10: 485-494.

Stadtman, E.R. 1998. Free radical mediated oxidation of proteins. *In* Free Radicals, Oxidative Stress, and Antioxidants. (Ozben, T., ed.). p. 51-64. Plenum Press. New York, USA.

Stainsby, G. 1987. Gelatin gels. *In* Advances in meat research. Collagen as food. Vol. 4. (Pearson, A. M. *et al.*, eds.). p. 209-222. Van Nostrand. Teinhold Co. Inc. New York, USA.

Stauffe, C.E. 1999. Emulsifiers. Eagan Press. St. Paul, MN.

Steel, R.G.D. and Torrie, J.H. 1980. Principles and Procedures of Statistics. McGraw-Hill. New York, USA.

Stohs, S.J. and Bagchi, D. 1995. Oxidative mechanisms in the toxicity of metal ions. Free Rad.l Biol. Med. 18: 321-336.

Sugiyama, M., Kousu, S., Hanabe, M. and Okuda, Y. 1989. Utilization of squid. Balkeman. Rotterdam, The Netherlands.

Sun, C. and Gunasekaran, S. 2009. Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. Food Hydrocolloids. 23: 165-174.

Surewicz, W.K. and Mantsch, H.H. 1988. New insight into protein secondary structure from resolution-enhanced infrared spectra. Biochim. Biophys. Acta Protein Struct. Mol. Enzym. 952: 115-130.

Surh, J., Gu, Y.S., Decker, E.A. and McClements, D.J. 2005. Influence of environmental stresses on stability of O/W emulsions containing cationic droplets stabilized by SDS-fish gelatin membranes. J. Agric. Food Chem. 53: 4236-4244.

Surh, J., Decker, E.A. and McClements, D.J. 2006. Properties and stability of oil-in-water emulsions stabilized by fish gelatin. Food Hydrocolloids. 20: 596-606.

Suryaprakash, P., Kumar, R.P. and Prakash, V. 2000. Thermodynamics of interaction of caffeic acid and quinic acid with multisubunit proteins. Int. J. Biol. Macromol. 27: 219-228.

Tadros, T., Izquierdo, P., Esquena, J. and Solans, C. 2004. Formation and stability of nano-emulsions. Adv. Colloid Interf. Sci. 108-109: 303-318.

Tang, S., Kerry, J.P., Sheehan, D., Buckley, D.J. and Morrissey, P.A. 2001. Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. Food Res. Int. 34: 651-657.

Tcholakova, S., Denkov, N.D., Sidzhakova, D., Ivanov, I.B. and Campbell, B. 2003. Interrelation between drop size and protein adsorption at various emulsification conditions. Langmuir. 19: 5640-5649.

Te Nijenhuis, K. 1997. Thermoreversible Networks: Viscoelastic Properties and Structure of Gels. Adv. Polym. Sci. 130: 1-252.

Thanonkaew, A., Benjakul, S., Visessanguan, W. and Decker, E.A. 2008. The effect of antioxidants on the quality changes of cuttlefish (*Sepia pharaonis*) muscle during frozen storage. LWT-Food Sci. Technol. 41: 161-169.

Toledano, O. and Magdassi, S. 1997. Formation of surface active gelatin by covalent attachment of hydrophobic chains. J. Colloid Interf. Sci. 193: 172-177.

Toledano, O. and Magdassi, S. 1998. Emulsification and foaming properties of hydrophobically modified gelatin. J. Colloid Interf. Sci. 200: 235-240.

Tornberg, E., Olsson, A. and Persson, K. 1990. The structural and interfacial properties of food proteins in relation to their function in emulsions. *In* Food Emulsions. (Kare, L. and Stig, E. F., eds.). p. 247-326. Marcel Dekker. New York, USA.

US FDA. 1997. FDA Center of Biologics Evaluation and Research, Transmissible Spongiform Encephalopathies Adcisory Commitees. Transcript of Meeting 23 April 1997.

Van Der Meeren, P., El-Bakry, M., Neirynck, N. and Noppe, P. 2005. Influence of hydrolysed lecithin addition on protein adsorption and heat stability of a sterilised coffee cream simulant. Int. Dairy J. 15: 1235-1243.

van Vliet, T. 1988. Rheological properties of filled gels. Influence of filler matrix interaction. Colloid Polym. Sci. 266: 518-524.

Vergé, S., Richard, T., Moreau, S., Richelme-David, S., Vercauteren, J., Promé, J.C. and Monti, J.P. 2002. First observation of non-covalent complexes for a tannin-protein interaction model investigated by electrospray ionisation mass spectroscopy. Tetrahedron Lett. 43: 2363-2366.

Viljanen, K., Kivikari, R. and Heinonen, M. 2004. Protein-lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. J. Agric. Food Chem. 52: 1104-1111.

Viljanen, K., Halmos, A.L., Sinclair, A. and Heinonen, M. 2005. Effect of blackberry and raspberry juice on whey protein emulsion stability. Eur. Food Res. Technol. 221: 602-609.

Vinson, J.A., Dabbagh, Y.A., Serry, M.M. and Jang, J. 1995. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. J. Agric. Food Chem. 43: 2800-2802.

Walstra, P. 1996. Emulsion stability. *In* Encyclopedia of Emulsion Technology. (Becher, P., ed.). Marcel Dekker. New York, USA.

Walstra, P. 2003. Physical Chemistry of Foods. Marcel Decker. New York, USA.

Wang, Z. and Narsimhan, G. 2004. Evolution of liquid holdup profile in a standing protein stabilized foam. J. Colloid Interf. Sci. 280: 224-233.

Wierenga, P.A., Meinders, M.B.J., Egmond, M.R., Voragen, F.A.G.J. and De Jongh, H.H.J. 2003. Protein exposed hydrophobicity reduces the kinetic barrier for adsorption of ovalbumin to the air-water interface. Langmuir. 19: 8964-8970.

Wierenga, P.A., Meinders, M.B.J., Egmond, M.R., Voragen, A.G.J. and De Jongh, H.H.J. 2005. Quantitative description of the relation between protein net charge and protein adsorption to air-water interfaces. J. Phys. Chem. B. 109: 16946-16952.

Wong, D.W.S. 1989. Mechanism and Theory in Food Chemistry. Van Nostrand Reinhold. New York, USA.

Wróblewski, K., Muhandiram, R., Chakrabartty, A. and Bennick, A. 2001. The molecular interaction of human salivary histatins with polyphenolic compounds. Eur. J. Biochem. 268: 4384-4397.

Wu, D., Xu, G., Sun, Y., Zhang, H., Mao, H. and Feng, Y. 2007. Interaction between proteins and cationic gemini surfactant. Biomacromolecules. 8: 708-712.

Wu, W., Wu, X. and Hua, Y. 2010. Structural modification of soy protein by the lipid peroxidation product acrolein. LWT-Food Sci. Technol. 43: 133-140.

Yabuta, G., Koizumi, Y., Namiki, K., Hida, M. and Namiki, M. 2001. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. Biosci. Biotechnol. Biochem. 65: 2121-2130.

Yakimets, I., Wellner, N., Smith, A.C., Wilson, R.H., Farhat, I. and Mitchell, J. 2005. Mechanical properties with respect to water content of gelatin films in glassy state. Polymer. 46: 12577-12585.

Yang, H., Wang, Y., Jiang, M., Oh, J.H., Herring, J. and Zhou, P. 2007. 2-Step optimization of the extraction and subsequent physical properties of channel catfish (*Ictalurus punctatus*) skin gelatin. J. Food Sci. 72: C188-C195.

Yanishlieva, N.V. and Marinova, E.M. 2001. Stabilisation of edible oils with natural antioxidants. Eur. J. Lipid Sci. Technol. 103: 752-767.

Yen, G.C., Chang, Y.C. and Chen, J.P. 2002. Antioxidant activity of mycelia from *Aspergillus candidus*. J. Food Sci. 67: 567-572.

Yoshimura, Y., Iijima, T., Watanabe, T. and Nakazawa, H. 1997. Antioxidative effect of maillard reaction products using glucose-glycine model system. J. Agric. Food Chem. 45: 4106-4109.

Yu, L., Haley, S., Perret, J. and Harris, M. 2002. Antioxidant properties of hard winter wheat extracts. Food Chem. 78: 457-461.

Yu, M.A. and Damodaran, S. 1991. Kinetics of protein foam destabilization: evaluation of a method using bovine serum albumin. J. Agric. Food Chem. 39: 1555-1562.

Yuji, H., Weiss, J., Villeneuve, P., Giraldo, L.J.L., Figueroa-Espinoza, M.C. and Decker, E.A. 2007. Ability of surface-active antioxidants to inhibit lipid oxidation in oil-in-water emulsion. J. Agric. Food Chem. 55: 11052-11056.

Zamora, R., Alaiz, M. and Hidalgo, F.J. 1997. Feed-back inhibition of oxidative stress by oxidized lipid/amino acid reaction products. Biochemistry. 36: 15765-15771.

Zamora, R., Alaiz, M. and Hidalgo, F.J. 1999. Modification of histidine residues by 4,5-epoxy-2-alkenals. Chem. Res. Toxicol. 12: 654-660.

Zayas, J.F. 1997. Solubility of proteins. *In* Functionality of Proteins in Food. (Zayas, J. F., ed.). p. 6-67. Spinger-Verlag. New York, USA.

Zhang, J., Duan, R., Tian, Y. and Konno, K. 2009. Characterisation of acid-soluble collagen from skin of silver carp (*Hypophthalmichthys molitrix*). Food Chem. 116: 318-322.

Zhou, P. and Regenstein, J.M. 2005. Effects of alkaline and acid pretreatments on Alaska pollock skin gelatin extraction. J. Food Sci. 70: C392-C396.

Zhou, P., Mulvaney, S.J. and Regenstein, J.M. 2006. Properties of Alaska pollock skin gelatin: A comparison with tilapia and pork skin gelatins. J. Food Sci. 71: C313-C321.

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List of Publication and Proceedings

Publications

1. Aewsiri, T., Benjakul, S. and Visessanguan, W. H. 2009. Functional properties of gelatin from cuttlefish (*Sepia pharaonis*) skin as affected by bleaching using hydrogen peroxide. Food Chemistry. 115: 243-249.
2. Aewsiri, T., Benjakul, S., Visessanguan, W., Eun, J. –B., Wierenga, P.A. and Gruppen, H. 2009. Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by oxidised phenolic compounds. Food Chemistry. 117: 160-168.
3. Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2010. Antioxidative activity and emulsifying properties of cuttlefish skin gelatin-tannic acid complex as influenced by types of interaction. Innovative Food Science and Emerging Technologies. 11: 712-720.
4. Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2010. Improvement of foaming properties of cuttlefish skin gelatin by

modification with *N*-hydroxysuccinimide esters of fatty acid. Food Hydrocolloids. In Press.

5. Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2011. Enhancement of oil-in-water emulsion stability using cuttlefish skin gelatin modified with *N*-hydroxysuccinimide esters of fatty acid. Food and Bioprocess Technology. In Press.
6. Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2011. Surface active properties and molecular characteristics of cuttlefish skin gelatin modified by oxidized linoleic acid. Journal of Biological Macromolecules. Accepted.
7. Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P.A. and Gruppen, H. 2011. Emulsifying property and antioxidative activity of cuttlefish skin gelatin modified with oxidized linoleic acid and oxidized tannic acid. Food and Bioprocess Technology. In review.

Proceedings

1. Aewsiri, T. and Benjakul. S. 2008. Functional properties of gelatin from cuttlefish skin as affected by bleaching using hydrogen peroxide. The 9th International Hydrocolloids Conference (9th IHC), Rasa Sentosa Resort, Singapore. June 15-19, 2008 (Poster Presentation).
2. Aewsiri, T. and Benjakul. S. 2009. Effect of modification of cuttlefish skin gelatin using tannic acid on antioxidative activity and emulsifying properties. Food Colloids, Granada, Spain. March 21-24, 2010 (Poster Presentation).
3. Aewsiri, T. and Benjakul. S. 2010. Improvement of surface activity of cuttlefish skin gelatin by modification with oxidized linoleic acid. Pacific Fisheries Technologists Conference, Vancouver, Canada. February 13-16, 2010 (Poster Presentation).