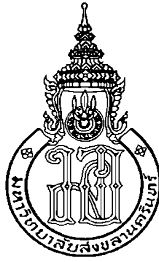

Development of Gelatin Hydrolysate from Skin of Unicorn Leatherjacket with Enhanced Bioactivities

Supatra Karnjanapratum

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

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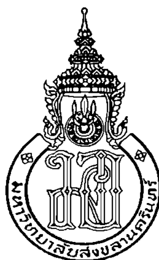

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ชื่อวิทยานิพนธ์ การพัฒนาเจลาตินไฮโดรไลเสตจากหนังปลาวัวที่มีฤทธิ์ทางชีวภาพสูง
ผู้เขียน นางสาวสุพัตรา กาญจนประทุม
สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา 2558

บทคัดย่อ

จากการใช้กระบวนการย่อยสลายตัวเองโดยโปรตีเอสที่พบในหนังร่วมกับการย่อยสลายด้วยความร้อนและปาเปนเพื่อผลิตเจลาตินไฮโดรไลเสตจากหนังปลาวัว พบว่าการใช้กระบวนการย่อยสลายตัวเองร่วมกับการย่อยสลายด้วยความร้อนหรือปาเปนร้อยละ 2 สามารถเพิ่มผลผลิตและฤทธิ์การต้านออกซิเดชั่นของเจลาตินไฮโดรไลเสตได้ โดยเฉพาะอย่างยิ่ง เมื่อทำการย่อยต่อด้วยเอนไซม์ปาเปนที่ความเข้มข้นร้อยละ 2 เจลาตินไฮโดรไลเสตที่ได้มีฤทธิ์ในการเข้าจับอนุมูล ABTS และไฮโดรเจนเปอร์ออกไซด์ รวมทั้งสามารถชะลอปฏิกิริยาออกซิเดชั่นในระบบเลซิติน-ลิโปโซม

เมื่อใช้ระบบการแยกชนิดสองเฟส (Aqueous two-phase, ATPS) ร่วมกับการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวสำหรับแยกส่วน ไกลซิลเอนโดเปปติเดส (GE) จากยางมะละกอสายพันธุ์เรดเลดี้และแขกดำ พบว่า GE จากยางมะละกอสายพันธุ์เรดเลดี้ที่แยกอยู่ในเฟสเกลือของ ATPS ซึ่งประกอบด้วย PEG6000 (ร้อยละ 10, น้ำหนัก/น้ำหนัก) และแอมโมเนียมซัลเฟต (ร้อยละ 10, น้ำหนัก/น้ำหนัก) และทำบริสุทธิ์ต่อโดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวร้อยละ 40-60 มีความบริสุทธิ์สูงสุด (2.1 เท่า) และให้ผลผลิตร้อยละ 80.23 ภายหลังการแยกส่วน สารประกอบที่เป็นต้นเหตุของกลิ่นไม่พึงประสงค์ใน GE โดยเฉพาะ benzyl isothiocyanate ถูกกำจัด เจลาตินไฮโดรไลเสตที่เตรียมโดยใช้ GE มีฤทธิ์ในการจับอนุมูล ABTS สูงขึ้นและไม่มีกลิ่นไม่พึงประสงค์จากยางมะละกอ

เมื่อนำ GE ที่ได้จากการแยกส่วนจากยางมะละกอสายพันธุ์เรดเลดี้ไปใช้ในการผลิตเจลาตินไฮโดรไลเสตร่วมกับกระบวนการย่อยสลายตัวเองของหนังปลาวัวที่ผ่านและไม่ผ่านการปฏิบัติเบื้องต้นด้วยกรด พบว่าฤทธิ์ในการจับอนุมูล ABTS และความสามารถในการรีดิวซ์เฟอร์ริค (FRAP) ของเจลาตินไฮโดรไลเสตเพิ่มขึ้นตามความเข้มข้นของ GE ที่ใช้ในการเตรียมเจลาตินไฮโดรไลเสต โดยเจลาตินไฮโดรไลเสตที่ได้จากกระบวนการย่อยสลายตัวเองของหนังปลาวัวที่ผ่าน (SS-8GE) และไม่ผ่าน (NS-8GE) การปฏิบัติเบื้องต้นด้วยกรดร่วมกับ GE ที่ร้อยละ 8 (น้ำหนัก/น้ำหนัก) มีกลไกการทำงานต่างกัน โดย NS-8GE แสดงฤทธิ์ในการจับอนุมูล ABTS สูงสุดและ SS-

8GE ให้ FRAP สูง โดยเจลาตินไฮโดรไลเสตทั้งสองสามารถยับยั้งการเกิดออกซิเดชั่นในระบบเลซิติน-ลิโปโซม และระบบจำลองทางเดินอาหาร นอกจากนี้ SS-8GE และ NS-8GE ยังแสดงฤทธิ์ในการป้องกันการเกิดผลึกน้ำแข็ง โดย NS-8GE มีฤทธิ์สูงกว่า SS-8GE โดยสามารถลดเอนทาลปี ที่ใช้สำหรับละลาย eutectic และผลึกน้ำแข็ง การเติม NS-8GE ลงในซูริมิสามารถชะลอการเปลี่ยนแปลงสมบัติทางเคมี-กายภาพของแอกโตไมโอซินธรรมชาติของระบบเนื้อปลาล้าง โดยสามารถลดการเปลี่ยนแปลงกิจกรรมของ Ca^{2+}-ATPase ไฮโดรโฟบิกซิตีบริเวณผิวหน้า และการเกิดพันธะไดซัลไฟด์ได้มากว่าชุดควบคุม และสามารถลดการเกิดออกซิเดชั่นของไขมันในระบบเนื้อปลาล้าง โดยลดปริมาณ TBARS, hexanal, heptanal และ 1-pentaten-3-ol

ปฏิกิริยาเมลลาร์ดถูกนำมาใช้เพื่อเพิ่มฤทธิ์การต้านออกซิเดชั่นของ NS-8GE ภายใต้สภาวะแห้ง พบว่า ผลิตภัณฑ์จากปฏิกิริยาเมลลาร์ด (MRP) ที่เตรียมโดยการให้ความร้อนผงผสมระหว่าง NS-8GE และกาแลกโตสแสดงฤทธิ์การต้านออกซิเดชั่นสูงสุดเมื่อเปรียบเทียบกับ MRP ที่เตรียมโดยใช้กลูโคสและฟรุคโตส เมื่อศึกษาสภาวะการให้ความร้อนที่อุณหภูมิ (50, 60 และ 70 °C) และความชื้นสัมพัทธ์ (ร้อยละ 55, 65 และ 75) สำหรับเตรียม MRP พบว่า MRP ที่เตรียมโดยการให้ความร้อนผงผสมระหว่าง NS-8GE และกาแลกโตส (2:1, น้ำหนัก/น้ำหนัก) ที่อุณหภูมิ 70 °ซ ความชื้นร้อยละ 55 เป็นเวลา 36 ชั่วโมง มีฤทธิ์การต้านออกซิเดชั่นสูงสุด

จากการศึกษาฤทธิ์การต้านออกซิเดชั่น ผลต่อภูมิคุ้มกัน และฤทธิ์การต้านมะเร็งของ NS-8GE และ MRP ในระบบจำลองเซลล์เพาะเลี้ยง พบว่า NS-8GE และ MRP สามารถป้องกันการทำลาย DNA ของเซลล์ต่อมน้ำเหลือง U937 ที่ถูกเหนี่ยวนำโดยไฮโดรเจนเปอร์ออกไซด์ สามารถลดการทำลายซุปเปอร์ออกไซด์ดิสมูเตสและคะตาเลสภายในเซลล์ตับ HepG2 และลดการผลิตไซโตไคน์ที่กระตุ้นให้เกิดการอักเสบ (interleukin-6 (IL-6), IL-1β) และไนตริกออกไซด์ในเซลล์เม็ดเลือดขาว RAW264.7 ที่ถูกกระตุ้นโดยไลโปโพลีแซคคาไรด์ นอกจากนี้สามารถยับยั้งการแพร่ขยายของเซลล์มะเร็งลำไส้ใหญ่ Caco-2 ตามปริมาณความเข้มข้น ของ NS-8GE (0.0-1.0 มิลลิกรัม/มิลลิลิตร) และ MRPs (0.0-5.0 มิลลิกรัม/มิลลิลิตร) ที่ใช้

จากการเติม MRP ในการแฟสำเร็จรูปที่ระดับความเข้มข้นต่างๆ (0, 0.5, 1.0 มิลลิกรัม/มิลลิลิตร) และทำการศึกษาฤทธิ์การต้านออกซิเดชั่น และสมบัติทางประสาทสัมผัสของผลิตภัณฑ์กาแฟ พบว่าการเติม MRP (0.5-1.0 มิลลิกรัม/มิลลิลิตร) ไม่มีผลต่อค่าดัชนีการเกิดสี

น้ำตาล L^* a^* และ b^* ของผลิตภัณฑ์กาแฟ ($P>0.05$) ฤทธิ์ในการจับอนุมูล ABTS และ FRAP ของผลิตภัณฑ์กาแฟที่ได้เพิ่มขึ้นตามปริมาณ MRP ที่เติมลงไป ($P<0.05$) ผลิตภัณฑ์กาแฟที่ผ่านการเติม MRP มีปริมาณสารระเหยทั้งหมดจาก MRP มากกว่าในตัวอย่างกาแฟชุดควบคุม ดังนั้น MRP สามารถใช้เป็นส่วนเพิ่มฤทธิ์ต้านออกซิเดชั่นในกาแฟสำเร็จรูป จากการทดสอบทางประสาทสัมผัสพบว่าผลิตภัณฑ์กาแฟที่ผ่านการเติม MRP ทุกความเข้มข้นได้คะแนนการยอมรับสำหรับทุกคุณลักษณะไม่แตกต่างจากชุดควบคุมที่ไม่ผ่านการเติม MRP

ดังนั้น เจลาตินที่มีฤทธิ์ทางชีวภาพเพิ่มขึ้นสามารถเตรียมได้โดยการใช้กระบวนการย่อยสลายด้วยตัวเอง ตามด้วยการย่อยสลายโดยการใช้ไกลซิลเอนโดเปปติเดส การใช้ปฏิกิริยาเมลาลาร์ดสำหรับเจลาตินไฮโดรไลเสตที่สภาวะเหมาะสม สามารถเพิ่มฤทธิ์ของผลิตภัณฑ์ที่ได้ ซึ่งสามารถนำไปประยุกต์ใช้เป็นสารปรุงแต่งหรือสารเสริมอาหารได้

Thesis Title Development of gelatin hydrolysate from skin of unicorn leatherjacket with enhanced bioactivities

Author Ms. Supatra Karnjanapratum

Major Program Food Science and Technology

Academic Year 2015

ABSTRACT

Autolysis-assisted process mediated by indigenous protease in combination with thermal hydrolysis and hydrolysis using papain was used for production of gelatin hydrolysate from skin of unicorn leatherjacket (*Aluterus monoceros*). Prior autolysis could enhance the yield and antioxidative activity of gelatin hydrolysates, especially when subsequent hydrolysis by 2% papain was implemented. Hydrolysates possessed ABTS radical and H_2O_2 scavenging activities, metal chelating activity and could retard oxidation in lecithin liposome system.

An aqueous two-phase system (ATPS) in combination with ammonium sulphate ($(NH_4)_2SO_4$) precipitation was applied to fractionate glycyl endopeptidase (GE) from the papaya latex of Red Lady and Khack Dum cultivars. The partially purified GE from Red Lady cultivar obtained from the salt-rich bottom phase of ATPS with 10% PEG 6000-10% $(NH_4)_2SO_4$ followed by further precipitation with 40-60% saturation of $(NH_4)_2SO_4$ showed the highest purity fold (2.1-fold) with 80.23% yield. After fractionation, almost offensive odourous compounds, particularly benzyl isothiocyanate, were removed. Fish gelatin hydrolysate prepared using GE showed higher ABTS radical scavenging activity with negligible papaya latex associated odor.

When GE rich fraction from papaya latex of Red Lady was used for preparing gelatin hydrolysates from autolysed non-swollen and swollen unicorn leatherjacket skin, ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of resulting hydrolysates were increased with increasing levels of GE used. Antioxidative gelatin hydrolysate from autolysed skin, both non-swollen and

swollen skins, using 8% GE termed 'NS-8GE' and 'SS-8GE' exhibited different modes of action. NS-8GE had high ABTS radical scavenging activity, whereas SS-8GE showed high FRAP. Both hydrolysates also showed their antioxidative activities in lecithin liposome system and gastrointestinal tract model system (GIMs). Apart from antioxidative activity, both NS-8GE and SS-8GE also had cryoprotective effect. NS-8GE showed higher cryoprotective property in salt solution system than that from SS-8GE as indicated by lower enthalpy for melting of eutectic and ice crystal. NS-8GE retarded physicochemical changes of natural actomyosin from washed mince system, as evidenced by lower changes in Ca^{2+}-ATPase activity, surface hydrophobicity and disulfide bond formation, compared with the control. NS-8GE could prevent lipid oxidation in washed mince system as shown by lower TBARS value and less abundance of hexanal, heptanal and 1-pentene-3-ol.

To enhance antioxidative activity, NS-8GE was subjected to Maillard reaction under dry state. Maillard reaction products (MRPs) prepared using the mixture of NS-8GE and galactose showed the highest antioxidative activity, compared with those derived from other saccharides tested (glucose or fructose). When various incubation temperatures (50, 60, 70 °C) and RHs (55, 65, 75%) were used for MRPs preparation, that prepared by heating the mixture of GH and galactose (2:1) at 70 °C and 55% RH for 36 h showed the highest antioxidative activity.

Antioxidant activity, immunomodulatory properties and anticancer activity of both NS-8GE and its MRP were investigated in cell culture model systems. NS-8GE (750-1500 μg/mL) and its MRP (750-1500 μg/mL) protected against H_2O_2-induced DNA damage in U937 cells as well as H_2O_2-induced reduction in cellular antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), activities of HepG2 cells. NS-8GE and its MRP demonstrated immunomodulatory potential by reducing pro-inflammatory cytokine (interleukin-6 (IL-6) and IL-1β) production and nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The proliferation of colon carcinoma, Caco-2 cells, was significantly reduced in dose-dependent manner when both NS-8GE (0.0-1.0 mg/mL) and its MRP (0.0-5.0 mg/mL) were incorporated.

MRP was fortified in instant coffee at different levels (0, 0.5, 1.0 mg/mL) and antioxidative and sensory properties of the final products were examined. MRP addition had no effect on browning index, L^*, a^* and b^* values of coffee brew ($P>0.05$) ABTS radical scavenging activity and ferric reducing antioxidant power of coffee brew increased with increasing levels of MRP ($P<0.05$). Abundance of volatile compounds associated with MRP in coffee brew fortified with MRP was higher than that of the control (without MRP). Therefore, MRP could be used as antioxidative supplement in instant coffee. Based on sensory evaluation, coffee brew fortified with MRP at all levels showed the similar likeness score for all attributes tested, compared with the control.

Therefore, gelatin hydrolysate with enhanced bioactivities could be prepared by autolysis-assisted process, followed by using glycyl endopeptidase. Subsequently, Maillard reaction was carried out under appropriate condition. The resulting products were shown to be the promising additives for food application or supplementation.

ACKNOWLEDGEMENT

I would like to express my special appreciation and sincere gratitude to my advisor Professor Dr. Soottawat Benjakul of the Department of Food Technology, and also all lecturers in Faculty of Agro-Industry, Prince of Songkla University who have shown the attitude and the substance of a genius in regard to researching and teaching. I would like to thank for their kindness, comments and helpful suggestion. Without his supervision and constant help, this dissertation would not have been possible.

I would like to extend the appreciation and gratitude to the examining committee members, Asst. Prof. Dr. Manee Vittayanont of the Department of Food Technology and Assoc. Prof. Dr. Aran H-Kittikun of the Department of Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Assoc. Prof. Dr. Jirawat Yongsawatdigul of School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology and Prof. Dr. Nora O'Brien of the School of Food and Nutritional Sciences, University College Cork, Ireland, for the useful comments, remarks and engagement through the learning process of this Ph. D. thesis for their significant guidance and sacrifice time to look into this manuscript.

This study could not be succeeded without the financial support from Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program, the Grant-in-Aid for dissertation from Graduate School, Prince of Songkla University, Thailand and The TRF Distinguished Research Professor grant through predoctoral scholarship.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Utilization of seafood processing waste by recovering the high-value constituents, especially protein fraction, has been of increasing interest. The protein recovered in the form of hydrolysates can be used as flavorant or can be incorporated into fish based foods, feed for aquaculture or a nitrogen source in growth media for microorganisms (Cancre *et al.,* 1999; Cavalheiro *et al.,* 2007; Gagńe and Simpson, 1993). In addition, the hydrolysates are sources of biologically active peptides with considerable potential in pharmacology (Udenigwe and Aluko, 2012; Suarez-Jimenez *et al*., 2012). Recovery of the protein fraction from fish processing byproducts has been achieved by enzymatic hydrolysis (Di Bernardini *et al.,* 2011). Certain proteolytic enzymes such as Alcalase (Alemán, Gimenez, Montero *et al.,* 2011; Dong *et al.,* 2008; Ren *et al.,* 2008), trypsin (Kim *et al.,* 2007; Mendis, Rajapakse *et al.,* 2005) and Flavourzyme (Jia *et al.,* 2010; Klompong *et al.,* 2007; Dong *et al.,* 2008) have been used to hydrolyse proteins from these wastes. Although thermal hydrolysis has also been reported to produce hydrolysate with similar specification including yield, protein content and antioxidative activity (Wang, Li *et al.,* 2013; Yang *et al.,* 2008; Yang, Liang *et al.,* 2009), compared with the enzymatic proteolysis process, it needs the longer time with high temperature and pressure (Wang, Li *et al.,* 2013).

Generally, those processes are costly, mainly due to the high expenses of commercial enzymes and energy. Some fish skin such as unicorn leatherjacket has been reported to possess endogenous protease, which caused the degradation of α-chain during gelatin extraction at 50-55 °C (Kaewrueng *et al.,* 2013; Ahmad *et al.,* 2011). Thus, those indigenous proteases should be maximally exploited, in which the cost of commercial enzyme and energy could be reduced. Furthermore, the improvement of functional properties of protein hydrolysate without using the chemicals such as thermal process has been considered. Additionally, the use of

protease with specificity toward gelatin is the most potent method to end up with the intensive cleavage of peptides in gelatin, which may lead to the enhanced bioactivity.

Protein hydrolysates have been known to possess the bioactivities, such as antioxidative activity (Chalamaiah *et al.,* 2012; Nalinanon *et al.,* 2011; Alemán, Giménez, Pérez-Santín *et al.,* 2011; Samaranayaka and Li-Chan, 2008). Peptides from gelatin hydrolysate produced from the skin of *Priacanthus macracanthus* using Alcalase, Neutrase or pyloric caeca extract from *Priacanthus macracanthus* showed the radical scavenging activity (Phanturat *et al.,* 2010). *In vitro* formation of antioxidant peptides from marine sources has been reported. After ingestion, gastrointestinal proteases can produce antioxidant peptide and some peptides are resistant to physiological digestion after oral intake (Samaranayaka *et al.,* 2010; Qian, Jung, Byun *et al.,* 2008).

Most peptides generally serve as functional foods, which can be of health benefit. Furthermore, immunomodulatory and antiprolifertive effects of fish hydrolysate are related to the several preventative effects against different diseases such as cancer, coronary diseases and inflammatory disorders (Vignesh *et al.,* 2012; Khora, 2013). Oyster hydrolysates inhibited tumor growth by improving the immune function in S108-bearing mice, suggesting a potential use for tumor therapy (Wang *et al.,* 2010). An enzymatic hydrolysate from jumbo squid skin gelatin showed cytotoxic effect against MCF-7 and U87 cell lines, with IC_{50} values of 0.13 and 0.10 mg/mL, respectively (Alemán, Pérez-Santín *et al.,* 2011).

Apart of bioactivities of protein hydrolysates, some fish protein hydrolysates have shown antifreezing propertiy. Gelatin hydrolysate was reported to be able control the growth of ice crystal. Damodaran (2007) found that peptide from gelatin hydrolysate with MW of 2,000-5,000 Da had the antifreezing property. The peptide Pro-Ala-Gly-Tyr isolated from Amur surgeon skin gelatin possessed the cryoprotective effect in freeze/thaw-induced Japanese sea bass mince (Nikoo *et al.* 2014). Therefore, peptides in fish protein hydrolysate can be used as natural antifreezing agent with less sweetness, which is different from sugar or sugar algohol having undesirable sweetness, when incorporated in foods.

To maximize the use of peptides, several methods have been implemented for modification of peptides, in which the enhanced bioactivities and functions can be achieved. Protein-saccharide graft reactions which are based on Maillard reactions between the amino group of peptides or protein and the reducing-end carbonyl group of saccharides have gained the interest as the potential tool for improvement of functional property (Guan *et al.,* 2006). The antioxidative effect of Maillard reaction products had been extensively studied (Wang, Bao *et al.,* 2013; Yousef *et al.,* 2013). Moreover, anti-inflammatory and anticancer activities of Maillard reaction products have been also demonstrated (Kitts *et al.,* 2012; Yamabe *et al.,* 2013). Thus, peptides with appropriate modification can serve as the promising additives with multi-functions, e.g. antifreezing agent and antioxidants, which can be used as food additives to maintain the quality or as functional foods.

Therefore, the use of combined methods could be a promising approach to bring about the potential production of gelatin hydrolysate with the maximized bioactivity and functionality. The new natural additive based on gelatin hydrolysate can be produced. Simultaneously, the hydrolysate with bioactivity can be used as functional foods, which can be used as the supplement in drink or foods.

1.2 Review of Literature

1.2.1 Unicorn leatherjacket (*Aluterus monoceros*)

The unicorn leatherjacket, *Aluterus monoceros* (L.), is a widespread species, known from the western Indian Ocean, the eastern Pacific, and the both sides of the Atlantic (De La Cruz-Agüero *et al*., 2007). The unicorn leatherjacket is pale brown or grey mottled with small brown spots on the upper parts of the head and body (Figure 1). The dorsal and anal fins are pale yellow to light brown, while the tail is dark brown. The common name, leatherjacket, comes from their thick, tough, leathery skin, which lacks normal scales and can be peeled off like a jacket (Ben Souissi *et al*., 2011).

Unicorn leatherjacket has been used for fillet production in Thailand, especially for export as frozen fillets. Moreover, in Vietnam, the annual production is

about 14% (200,000 tonnes) of the total catch (1.4 million tonnes). As a consequence, a large amount of skin is produced as a by-product.

Figure 1. Unicorn leatherjacket (*Aluterus monoceros*) (Linnaeus, 1758)
Source: Ben Souissi *et al.* (2011)

1.2.2 Plant proteases

Several plants have been known as the promising source of protease, which have been widely used for foods. However, the types and activity can be varied, depending on plant, maturation, season, etc. (Hasanuzzaman *et al*., 2013)**.**

1.2.2.1 Bromelain

Bromelain (EC 3.4.22.32) is a protease that is derived primarily from the stems of pineapple (*Ananas comosus* or *Ananas bracteatus*) (International Union of Biochemistry, 1984). Bromelain hydrolyses proteins, peptides, amides and esters of amino acids and peptides; preferential cleavage site is the carbonyl end of lysine, alanine, tyrosine and glycine (International Union of Biochemistry, 1984; Godfrey and Reichelt, 1983). Although its stability is lower than papain, bromelain still has a fairly high temperature of inactivation and broad pH activity range. Bromelain has been found to be more active against collagen protein than papain or ficin (Fogle *et al*., 1982). Bromelain was also used for preparing the antioxidative gelatin hydrolysate from cobia (*Rachycentron canadum*) skin (Yang *et al*., 2008).

1.2.2.2 Ficin

Ficin (EC 3.4.22.3) is a protease that is isolated from the latex of species of fig (*Ficus glabrata*) and is composed of several different endopeptidases (Jones and Glazer, 1970). The active enzymes preferentially cleave proteins at tyrosine and phenylalanine residues. Because of this preferential cleavage, ficin tends to produce hydrolsates with low bitterness (Jones and Glazer, 1970). Ficin was used for preparing protein hydrolysate containing peptide with a MW below 1,000 Da which possessed the inhibitory activity toward angiotensin l-converting enzyme (Udenigwe *et al*., 2009; Udenigwe *et al*., 2012).

1.2.2.3 Papain

Papain (EC 3.4.22.2) is a protease derived from the latex of papaya (*Carica papaya*). Papain has fairy broad specificity; it has endopeptidase, amidase and esterase activities. The active site consists of seven subsites (S_1-S_4 and S_1'-S_3') that can each accommodate one amino acid residue of a substrate (P_1-P_4 and P_1'-P_3') (Schechter and Berger, 1967). Specificity is controlled by the S_2 subsite, a hydrophobic pocket that accommodates the P_2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite (Kimmel and Smith, 1954). Antioxidative peptides prepared from Pacific cod skin gelatin using Alcalase, Neutrase, papain, trypsin, pepsin and α-chymotrypsin were comparatively studied (Ngo *et al*., 2011). Among all hydrolysates, papain hydrolysate exhibited the highest antioxidative activity measured by hydroxyl and DPPH redical scavenging activities.

1.2.2.4. Glycyl endopeptidase

Glycyl endopeptidase (EC 3.4.22.25) is one of the four papaya cysteine proteinases. It is a major component which constitutes almost 30% of total protein in the latex of *Carica papaya* (Buttle, 1994; Buttle *et al*., 1989), whereas the most extensively studied papain appears as a minor component at 8% (Azarkan *et al*., 2003). Glycyl endopeptidase has high specificity to cleave only peptide bonds with Gly at P_1 (Buttle, Ritonja, Pearl *et al*., 1990). Unlike other papaya cysteine proteases,

glycyl endopeptidase fails to hydrolyse the usual synthetic substrate, DL-Bz-Arg-pNA (BAPNA), and is not inhibited by cysteine (Buttle, Ritonja, Dando *et al*., 1990; O'Hara *et al*., 1995; Thomas *et al*., 1995).

Gelatin hydrolysate generally has a low degree of hydrolysis (DH), possibly due to the molecular constraint associated with its amino acid sequence/composition. Gelatin with a glycine content of around 33% might not be a preferable substrate for proteases. Glycyl endopeptidase is a major component in papaya latex and has high specificity to cleave gelatin substrate. Kittiphattanabawon *et al*. (2012) used papaya latex enzyme for preparaing gelatin hydrolysate from skin of blacktip shark. Gelatin hydrolysate showed antioxidative activity including radical scavenging activity as well as metal chelating activity.

1.2.3 Gelatin hydrolysate from aquatic sources

Fish skin, a byproduct from fish processing, can be used as a potential source for collagen and gelatin extraction. Collagen and gelatin are currently used in diverse fields including food, cosmetic, and biomedical industries (Kim and Mendis, 2006; Gómez-Guillén *et al*., 2011). Collagen is structurally formed as a triple helix by three extended protein chains that wrap around one another (Kim and Mendis, 2006). Collagen and gelatin are different forms of the same macromolecule. Gelatin is the partially hydrolysed or thermally denatured form of collagen. Heat denaturation easily converts collagen into gelatin. In addition to fish skin, collagen and gelatin could be isolated from bone and fins of fish processing byproducts (Kim and Mendis, 2006; Gómez-Guillén *et al*., 2011). Collagen and gelatin are unique proteins, compared to fish muscle proteins and this uniqueness of fish lies in the amino acid content and they are rich in non-polar amino acids (above 80%) such as Gly, Ala, Val and Pro (Kim and Mendis, 2006). Even though main industrial sources of collagen and gelatin are bovine and porcine skin, many studies have been conducted to extract collagen and gelatin from fish skin and to screen their bioactivities and the potential in pharmacology (Gómez-Guillén *et al*., 2011; Suarez-Jimenez *et al*., 2012). Fish skin has recently attracted the interest of the industry as an alternative source of commercial gelatin. This may be due to comparative unpopularity of porcine skin

collagen and gelatin in relation to some religious reasons. Furthermore, bovine derived collagen and gelatin can be associated with cow disease, bovine spongiform encephalopathy (BSE), leading to the risk for human consumption. In contrast, fish collagen and gelatin have relatively a low risk of unknown pathogens such as BSE (Kim and Mendis, 2006).

Collagen is generally extracted with acid treatment and solubilized without altering its triple helix. However, thermal treatment cleaves hydrogen and covalent bonds that stabilizes the triple helix configuration of collagen and converts its helical conformation into coiled conformation, a gelatin state (Djabourov *et al*., 1993). As a general procedure, hot water treatment is used to solubilize collagen in skin to gelatin. The properties of extracted gelatin may vary with the extraction conditions such as temperature and pH (Kaewrueng *et al*., 2013; Ahmad *et al*., 2011). Optimum conditions for extraction are dependent on fish species and type of collagen (Kim *et al*., 1994). Gelatin can be further hydrolysed to obtain hydrolysate with bioactivity (Figure 2).

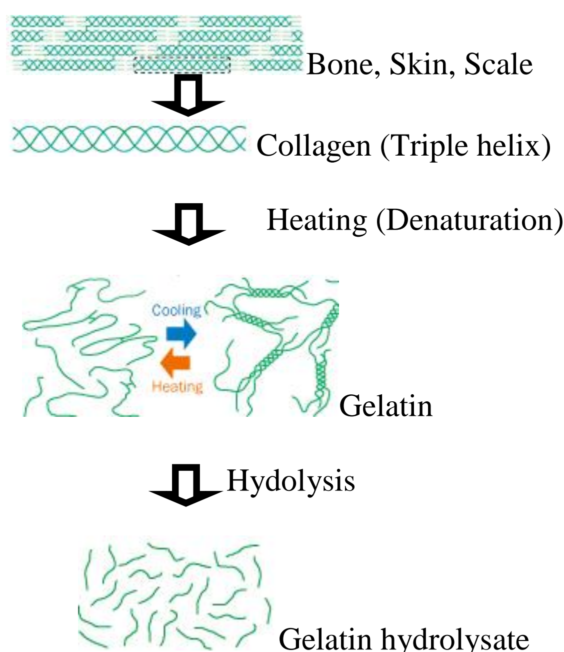

Figure 2 Collagen, gelatin and gelatin hydrolysate

Source: Nitta Gelatin Inc. (2013)

1.2.3.1 Production of gelatin hydrolysate

1.2.3.1.1 Chemical hydrolysis

Chemical hydrolysis of proteins is achieved by cleaving peptide bond with either acid or alkaline (Hale, 1972). It is relatively inexpensive and quite simple to conduct. However, this method is generally difficult to control and almost invariably leads to products with variable chemical composition and functional properties (Blenford, 1994). Protein hydrolysis with strong chemicals and solvents is commonly performed at extreme temperatures and pH. As a consequence, it may yield products with the reduced nutritional qualities, poor functionality and restricted to use as flavor enhancers (Loffler, 1986; Webster *et al*., 1982).

1.2.3.1.2 Thermal hydrolysis

Thermal hydrolysis is conducted using a combination of high temperature and high pressure to break the tissue down to their original small molecular building blocks, resulting in a dramatic reduction in viscosity and the increased biodegradability. Thermal hydrolysis has been reported to produce gelatin hydrolysate with similar specification such as the yield, protein content and DPPH radical scavenging ability (Wang, Li *et al*., 2013; Yang, Liang *et al*. 2009), compared with the enzymatic proteolysis process. However, this thermal hydrolysis needed the long time (170-180 min) and strong condition (121 °C, 100 kPa). Yang *et al*. (2008) prepared the retorted skin gelatin hydrolysate (RSGH) from cobia (*Rachycentron canadum*) skin and enzyme-treated RSGHs (ET-RSGHs) derived from bromelain, papain, pancreatin and trypsin digestion. The obtained gelatin hydrolysates showed DPPH radical scavenging activity and the inhibition effect on lipid peroxidation in linoleic acid model system (Yang *et al*., 2008). ET-RSGHs derived from the RSGH with further enzymatic digestion did not display much more activity than the RSGH. The retorting treatment (121 °C for 30 min) provided a sufficient hydrolysis effect to degrade cobia skin gelatin and produced most antioxidant peptides with smaller molecular sizes, particularly the fractions smaller than 6,511 Da (Yang *et al*., 2008).

1.2.3.1.3 Enzymatic hydrolysis

Biologically active peptides can be produced from precursor proteins using endogenous proteolytic enzymes already present in muscle or viscera of fish and/or by adding exogenous enzymes from other sources (Kristinsson and Rasco, 2000; Kristinsson, 2007). pH and/or temperature of raw material such as fish processing byproducts are generally adjusted to optimal ones to maximize the hydrolysis process using autolysis or by adding an exogenous enzyme (Samaranayaka *et al.*, 2010). In some cases, the raw material is firstly heated to inactivate endogenous proteases before adding the exogenous enzyme (Samaranayaka *et al.*, 2010). Depending on the activity of enzyme, temperature, and other factors such as the target molecular weight range of resultant peptides, the reaction is allowed to proceed for a duration ranging from less than one hour up to several hours. The hydrolysis reaction is then terminated either by using a heat treatment or by adjusting pH, depending on the type of enzyme used. The supernatant after removing solids is often adjusted to neutral pH and then dehydrated to yield the powdered protein hydrolysates (Samaranayaka *et al.*, 2010).

Enzymatic hydrolysis has been used as the most common method of producing bioactive peptides from fish and its by-products (Kim *et al.,* 2013; Guérard, 2007). Industrial food-grade proteinases such as Alcalase, Neutrase, Flavourzyme and Protamex™ derived from microorganisms have been used widely to produce protein hydrolysates with various bioactivities, whereas enzymes from plant (e.g. papain, bromelain, ficin) and animal sources (e.g. pepsin, trypsin) have also been used but to a lesser extent (Guérard, 2007). Depending on the raw material used, endogenous enzymes such as trypsin, chymotrypsin, pepsin, other enzymes of viscera and digestive tract, as well as lysosomal proteases or catheptic enzymes in fish contribute to the breakdown of proteins during autolysis (Kristinsson and Rasco, 2000). Fish protein hydrolysates with antioxidative properties were prepared using Pacific hake (*Merluccius productus*) fish with high endogenous proteolytic activity from *Kudoa paniformis* parasitic infection (Samaranayaka and Li-Chan, 2008). The increased level of cathepsin L-like proteases present in parasitized Pacific hake muscle was successfully used to produce fish protein hydrolysates with antioxidative properties

(Samaranayaka and Li-Chan, 2008). Gender and age dependent changes may lead to variability in endogenous enzymes present in fish and other marine sources (Kristinsson and Rasco, 2000; Guérard, 2007). Despite the technical problems arising from this variability, endogenous enzymes have been used for production of various protein hydrolysates with bioactivities (Samaranayaka and Li-Chan, 2008; Kristinsson and Rasco, 2000; Phanturat *et al*., 2010; Jun *et al*., 2004; Je, Park *et al*., 2005).

Use of exogenous enzymes is preferred in most cases to the autolytic process due to the reduction in time required to achieve similar degree of hydrolysis as well as the better control of hydrolysis to obtain more consistent molecular weight profiles and peptide composition (Chalamaiah *et al*., 2012). Fish protein hydrolysates with ACE-inhibitory activity (IC_{50} 165 μg/mL) could be consistently prepared using different batches of Pacific hake (*Merluccius productus*) fish fillet by using exogenous enzyme, Protamex^{TM} (Cinq-Mars and Li-Chan, 2007; Cinq-Mars *et al.*, 2008). Visceral proteases can be an alternative to produce the hydrolysate with bioactivity. Peptides with antioxidative properties could be prepared from Alaska pollack (*Theragra chalcogramma*) and yellowfin sole (*Limanda aspera*) frame proteins using a crude enzyme mixture from mackerel intestine (Jun *et al*., 2004; Je, Park *et al*., 2005). The pyloric caeca extract (PCE) from bigeye snapper (*P. macracanthus*) was used for the production of gelatin hydrolysate with antioxidative activity (Phanturat *et al*., 2010). Gelatin hydrolysate from bigeye snapper skin prepared using PCE exhibited the increases in DPPH, ABTS radical scavenging activity and ferric reducing antioxidative power (FRAP) as the degree of hydrolysis (DHs) increased (Phanturat *et al*., 2010). Moreover, hydrolysates derived from gelatin using Alcalase in combination with PCE showed the highest ABTS radical scavenging activity (Phanturat *et al*., 2010).

A wide varity of commercial enzyme has been used successfully to hydrolyse fish and other food protein. Proteolystic enzyme from microorganisms such as Alcalase, Neutrase, Pronase E, Flavozyme, and Protamex^{TM} are most suitable to prepare fish protein hydrolysates because of their high productivity (Sae-leaw *et al.,* 2015; Ahn *et al.,* 2010; Ngo *et al.,* 2010; Alemán, Giménez, Pérez-Santín *et al*., 2011). Antioxidative peptides from the skins of tuna and halibut were prepared using

Alcalase, collagenase, trypsin or pepsin (Alemán, Giménez, Pérez-Santín *et al*., 2011). Both squid and tuna gelatin hydrolysates obtained from Alcalase showed the highest ABTS radical scavenging activity (Alemán, Giménez, Pérez-Santín *et al*., 2011). Jia *et al*. (2010) prepared gelatin hydrolysates from Alaska pollack (*Theragra chalcogramma*) skin using Alcalase, Neutrase, trypsin, papain, pepsin, Flavourzyme and Protamex™. Among all proteases used, Protamex™ was the most efficient enzyme for preparing antioxidative peptides (Jia *et al*., 2010). Gelatin hydrolysate of Alaska pollack skin was mainly composed of oligopeptides with two to eight amino acid residues and possessed antioxidative activity (Jia *et al*., 2010). Antioxidative hydrolysates were also prepared from walleye pollock (*Thera chalcogramma*) skin (Zhuang, Li *et al*., 2009). The use of enzyme mix between trypsin and flavourzyme could provide the hydrolysate containing low MW peptides (< 1,000 Da), which were able to scavenge free radical and oxygen species significantly.

Proteolysis induced by heat-activated and heat-stable endogenous enzymes associated with skin matrix can contribute to the destabilization as well as disintegration of collagen structure by disrupting the intra and inter molecular cross-links (Wu *et al*., 2008). Endogenous proteases which are tightly bound with collagenous skin matrix have been known to play an essential role in degrading the native triple helix of collagen under physiological conditions as well as hydrolysing gelatin molecules during extraction at high temperature (Intarasirisawat *et al.*, 2007; Keawruang *et al*., 2013). Intarasirisawat *et al.* (2007) found the autolysis in bigeye snapper (*Priacanthus macracanthus*) skin mediated by a heat-activated serine proteinase with optimal condition at 60 °C and pH 7.5. Indigenous proteases in the skin of unicorn leatherjacket (*Alutherus monoceros*) showed the maximized autolysis at pH 7 and 50-55 °C. Autolysis was markedly inhibited by soybean trypsin inhibitor (SBTI), suggesting that heat activated serine protease was predominant in the skin of unicorn leatherjacket (Keawruang *et al*., 2013; Ahmad *et al*., 2011).

1.2.3.2 Bioactivities of protein hydrolysates

Dietary proteins are a source of biologically active peptides, which are inactive in the parent protein but can be liberated during gastrointestinal digestion,

food processing or fermentation. Once they are released, bioactive peptides can affect numerous physiological functions of the organism (Gómez-Guillén *et al*., 2010). Collagen and gelatin have been focused as a source of biologically active peptides with promising health benefits for nutritional or pharmaceutical applications. Usually, collagen and gelatin hydrolysates and peptides have been produced from pig skin or bovine hide (Jia *et al*., 2010). However, outbreaks of mad cow disease and prohibition of collagen or gelatin from pig skin and bone in some regions have made it necessary to find new marine or poultry sources as alternatives. Discarded collagenous materials from the poultry and fish processing industries have been found to be valuable sources of hydrolysates and peptides with bioactive properties (Cheng *et al*., 2008; Cheng *et al*., 2009; Nam *et al*., 2008; Saiga *et al*., 2008). These collagenous materials include skins, tunics, bones, fins and scales. Furthermore, the isolation of peptides with important biological activities has been reported for collagen and gelatin obtained from other sources such as Alaska pollack skin, jumbo squid skin, Seabass skin, cobia skin or tilapia skin (Kim *et al*., 2001; Byun. *et al*., 2001; Mendis, Rajapakse, Byun *et al*., 2005; Senphan and Benjakul, 2014; Wang, Li *et al*., 2013; Yang *et al*., 2008).

The peptides exhibit different physicochemical properties and biological activities depending on their molecular weight and amino acid sequence, mainly determined by proteases used and source of collagenoues material. The molecular weight of the bioactive peptide is one of the most important factors in producing bioactive peptides with the desired biological activities (Kim and Mendis, 2006; Kim and Wijeselara, 2010). Ultrafiltration, nanofiltration, ionexchange membranes, and column chromatography can be used to separate the peptides that have the desired functional properties from fish protein hydrolysates (Udenigwe and Aluko, 2012; Je, Kim *et al*., 2005; Pihlanto and Korhonen, 2003).

Collagen and gelatin-derived peptides in the area of food science and technology have dealt with their antioxidant and antihypertensive/ACE inhibitory activity (Gómez-Guillén *et al*., 2010). These peptides have repeated unique Gly-Pro-Hyp sequences in their structure, and the observed antioxidative and antihypertensive properties have presumably been associated with this unique amino acid composition

(Kim and Mendis, 2006). Moreover, collagen and gelatin-derived peptides exhibit the numerous bioactivities, namely antimicrobial activity, mineral binding capacity, lipid-lowering effect, anticancer activity, immunomodulatory activity and beneficial effects on skin, bone or joint health (Gómez-Guillén *et al*., 2010; Hou *et al*., 2009; Jung *et al*., 2005; Jung *et al*., 2006; Moskowitz, 2000; Zhang *et al*., 2012; Alemán, Pérez-Santín *et al.,* 2011).

Some studies have been performed to confirm the *in vivo* biological activity of collagen and gelatin peptides, and some convincing data have been obtained for animal models. Fish skin hydrolysates have been reported to affect lipid absorption and metabolism in rats (Saito, 2009). Peptides from marine sources were reported to act protectively against ultraviolet radiation-induced damage on mice skin (Hou *et al*., 2009; Zhuang, Hou *et al*., 2009). Gelatin hydrolysates from Pacific cod (*Gadus macrocephalus*) skin prepared using sequential hydrolysis by pepsin and alkaline protease were fractionated using ultrafiltration membranes. The two polypeptides obtained (2,000 Da<PEP1<6,000 Da; PEP2<2,000 Da) exhibited the protective effects against ultraviolet radiation-induced skin photoaging (Hou *et al*., 2009). Zhuang, Hou *et al*. (2009) reported the protective effects of collagen hydrolysates from jellyfish on mice skin photoaging induced by UV irradiation. The peptide with the lower molecular weight showed much higher effects (Zhuang, Hou *et al*., 2009). However, the *in vivo* bioactivities of marine collagen and gelatin peptide on anticancer and immunomodulatory activities have been barely studied. The oligopeptide peptides of Chum salmon collagen prepared using complex proteases with molecular weight range of 300-860 Da have been reported to stimulate the immune system of the Imprinting Control Region (ICR) mice. Peptide treatment enhanced mitogen-induced lymphocyte proliferation, nature killer (NK) cell activity, spleen $CD4^+$ T helper cells, and secretion of cytokines (IL-2, IL-5, IL-6, IFN-ɣ) (Yang, Zhang *et al.,* 2009). Moreover, these oligopeptide peptides protected against gamma radiation induced immune suppression in the ICR mice (Yang, Zhang *et al.,* 2009).

1.2.3.2.1 Antioxidative activities of gelatin hydrolysates

1.2.3.2.1.1 Radical scavenging activities

The beneficial effects of antioxidative peptides are well known in scavenging free radical and reactive oxygen species or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Rajapkese *et al*., 2005). Kittiphattanabawon *et al*. (2012) produced gelatin hydrolysate from blacktip shark skin possessing antioxidative activity using papaya latex enzyme with different degree of hydrolysis (DH). All gelatin hydrolysates had the increased ABTS and DPPH radical scavenging activities as DH increased. At 40% DH, gelatin hydrolysate showed the highest activity. Gelatin hydrolysate from blacktip shark skin possibly contained substances that were hydrogen donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction (Kittiphattanabawon *et al*., 2012). The ability of gelatin hydrolysates to scavenge DPPH and ABTS radical scavenging gradually increased as % DH increased. Sae-leaw *et al.* (2015) reported the effect of production processes on characteristics and antioxidant activities of seabass skin hydrolysates prepared using Alcalase. Samples hydrolysed during gelatin extraction showed a higher degree of hydrolysis (DH) and yield, compared with those prepared from the hydrolysis of gelatin solution ($p<0.05$). Moreover, the former had the greater DPPH radical scavenging activity than the latter (Sae-leaw *et al.,* 2015). For gelatin hydrolysates from unicorn leatherjacket skin produced using extracellular protease from *Bacillus amyloliquefaciens* H11 (GH-11) or Alcalase (GH-Al), all samples had increased ABTS radical scavenging activity as DH increased (Sai-ut *et al*., 2015). At 20-40% DH, GH-H11 showed higher activity than those of GH-Al. GH-H11 and GH-Al had molecular weights of approximately 750 and 3600 Da, respectively (Sai-ut *et al.,* 2015). Gelatin hydrolysates of farmed Giant catfish skin prepared using visceral alkaline-proteases from Giant catfish, commercial trypsin, and Izyme AL possessed the radical scavenging activity (Ketwana *et al.,* 2016), but Izyme AL-derived sample showed the significantly ($P<0.05$) highest ABTS scavenging activity. In general, an increment in the hydrolysis of gelatin led to higher production of antioxidative hydrolysates/peptides. However, the enzyme used was extremely important to

determine antioxidative potency of the hydrolysates (Ketwana *et al.,* 2016). The ability of hydrolysate to scavenge radical varied with the enzyme used, which possibly resulted from existing differences in the enzyme specificity toward protein substrates. A wide variety of peptides with different modes of actions for inhibiting lipid oxidation were generated during hydrolysis. Changes in size, amount, the exposure of the terminal amino groups of the products obtained and the composition of free amino acids or small peptides affect the antioxidative activity of peptides (Thiansilakul *et al*., 2007; Qian, Jung *et al*., 2008; Wu *et al*., 2003).

1.2.3.2.1.2 Ferrous chelating acticity

Transition metals, such as Fe, Cu and Co, have been known to react with peroxides by acting as one-electron donors to form alkoxyl radicals. Therefore, chelation of transition metal ions would retard the oxidations process (Gordon, 2001). In comparison with other ions, ferrous ion is a key active species responsible for ROS formation in cells, leading to the increased levels of lipid peroxidation (Huang *et al*., 2002). The formation of a violet complex by ferrozine and Fe^{2+} is interrupted in the presence of a chelating agent (Decker and Welch, 1990). Peptides are well known metal chelators and this is one of the antioxidative mechanisms for many active peptides. The activity is affected by size and amino acid sequence (Peng *et al*., 2009). The different molecular weight fractions (3, 5, 10 kDa) of gelatin hydrolysate of cobia skin prepared using Alcalase possessed chelating ability on the ferrous ion (Razali *et al.,* 2015). The hydrolysate with the size of 5 kDa exhibited the highest percentage of ferrous chelating activity (90.69%). Furthermore, the IC_{50} values for hydrolysate with MW of 3, 5 10 kDa were recorded at 0.86, 1.73 and 2.01 mg/mL, respectively. These results suggested that lower molecular weight fraction had greater ability to chelate the ferrous ions (Razali *et al.,* 2015). Taheri *et al.* (2013) found that lower molecular weight fraction of salted herring brine hydrolysate showed higher chelating activity than those with higher molecular weight. Gelatin hydrolysate from unicorn leatherjacket skin prepared using *B. amyloliquefaciens* H11 and Alcalase possessed the increased Fe^{2+} chelating activity as DH increased (Sai-ut *et al*., 2015). Liu *et al.* (2010) reported that the cleavage of peptides led to an enhanced metal ion binding due to the increased concentration of carboxylic groups and amino groups of the peptides.

Carboxyl and amino groups in the side chains of acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids are thought to play an important role in chelating metal ions (Saiga *et al*., 2003). The amino acid composition of gelatin hydrolysates obtained from sole and squid skin prepared using Alcalase showed the abundance of these amino acids, in which both hydrolysates showed high chelating ability. His residues were also reported to contribute to the chelating effect of protein hydrolysates (Chen *et al*., 1995). However, this amino acid is present at low concentration in both hydrolysates.

1.2.3.2.1.3 Ferric reducing antioxidative power

The ferric reducing antioxidant power (FRAP) is generally used to measure the capacity of a substance in reducing TPTZ–Fe(III) complex to TPTZ–Fe(II) complex (Benzie and Strain, 1996; Binsan *et al*., 2008). Since the reducing power of bioactive compounds is associated with their antioxidative activity, the reducing power assay provides a reliable method to study the antioxidative activity of various compounds (Binsan *et al*., 2008).

FRAP of gelatin hydrolysates from skin of seabass (*Lates calcarifer*) prepared using an ammonium sulphate precipitated fraction (ASPF) from Pacific white shrimp hepatopancreas and Alcalase was compared. The increase in FRAP was found with increasing DH. At the same DH, hydrolysates prepared using Alcalase showed a higher activity than those produced using ASPF ($P < 0.05$), except at 40% DH, in which both hydrolysates showed similar FRAP ($P > 0.05$). Increases in reducing power of hydrolysate with increasing DH have been reported in blacktips shark skin hydrolysate prepared using crude extract from papaya latex (Senphan and Benjakul, 2014). Gelatin hydrolysates from farmed giant catfish skin prepared using different enzymes, visceral alkaline-proteases from giant catfish (CTH), commercial trypsin (GHT), and Izyme AL (IZH) showed the difference in FRAP (Ketnawa *et al.,* 2016). CTH showed the highest ($P<0.05$) FRAP followed by GTH and IZH, respectively (Ketnawa *et al.,* 2016). Alemán, Pérez-Santín *et al.* (2011) also found that different peptides generated from the hydrolysis of the giant catfish skin gelatin, by different proteases, had different ability to provide electrons to the radicals.

1.2.3.2.1.4 Prevention of lipid peroxidation

Lipid peroxidation inhibition activity of peptides is dertermined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system and lecithin liposome system, in which corbon-centered, peroxyl radicals and hydroperoxides are involved in the oxidation process (Burton and Ingold, 1986). Peptides can act as antioxidant by retarding the formation of primary oxidation products in the oxidative sequence (Chen *et al*., 1995).

Gelatin hydrolysates from seabass and bigeye snapper skins displayed antioxidative activity in lecithin liposome systems (Senphan and Benjakul 2014; Phanturat *et al.,* 2010). The hydrolysate from seabass skin gelatin prepared using ammonium sulphate precipitated fraction (ASPF) from Pacific white shrimp hepatopancreas with 40% DH retarded the oxidation in a lecithin liposome system as indicated by lowered conjugated dienes (CD) and TBARS values. No pronounced differences in CD were noticeable amongst systems added with hydrolysates at different levels (500-2000 mg/L) ($P > 0.05$) throughout the storage period of 60 h at 37°C. Moreover, TBARS value was lower as the concentration of hydrolysate used increased. Systems containing hydrolysate at 2000 mg/L had the lower TBARS than those added with hydrolysate at levels of 500 and 1000 mg/L ($P < 0.05$).

The antioxidative activity against oxidation β-carotene-linoleate model system of gelatin hydrolysate from blacktip shark skin and North Atlantic lean fish skin had been reported (Kittiphattanabawon *et al.,* 2012; Picot *et al.,* 2010). Kittiphattanabawon *et al.* (2012) found the antioxidative activity of gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme with 40% DH in β-carotene-linoleate model system. The decrease in A_{450} indicates the oxidation of β-carotene in the system caused by free radicals from oxidation of linoleic acid (Chandrasekara and Shahidi, 2010). System containing gelatin hydrolysate at the levels of 500 and 1,000 mg/L effectively retarded the decrease in A_{450}. However, their antioxidative activity was lower than 100 mg/L BHA. The difference in antioxidative activity between gelatin hydrolysate and BHA might be caused by the difference in their polarity. The hydrolphobic antioxidants have higher efficiency than hydrophilic

antioxidants in preventing oxidation in oil-in-water emulsion systems by preferably orienting at the oil-water interface (Wijeratne *et al.*, 2006). Additionally, peptides from Alaska Pollack skin (Kim *et al.*, 2001) and jumbo squid skin gelatin (Mendis, Rajapakse, Byun *et al.*, 2005) also showed inhibitory activity in the linoleic acid model system. In the free radical-mediated lipid peroxidation system, antioxidative activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence (Qian, Jung *et al.*, 2008).

1.2.3.2.2 Bioactivities of protein hydrolysates in cell model system

1.2.3.2.2.1 Cellular antioxidant

Cell culture model systems allow for rapid, inexpensive screening of antioxidative compounds for their bioavailability, metabolism, as well as bioactivity, compared to expensive and time-consuming animal studies and human clinical trials (Liu, 2009). Use of cell culture models for antioxidant research is particularly important since the studies to date have demonstrated that the mechanism or the action of antioxidants in human health promotion go beyond the antioxidant activity of scavenging free radicals (Liu and Finley, 2005). Various cell culture models are therefore an invaluable tool to assess these potential health benefits of food antioxidants, *in vitro*. Cell culture models can also be used to evaluate cytotoxicity of antioxidative compounds at concentrations to be used to exert the desired bioactivity in the body, as well as to study the potential to inhibit intracellular oxidation and to reduce inflammatory responses (Mosmann, 1983; Elisia and Kitts, 2008; Wang *et al.*, 2009). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the metabolic activity of cells through oxidation–reduction activities of mitochondria, is often used to measure the viability of cells during cytotoxicity assays (Mosmann, 1983). During experiments, intracellular oxidation of cells can be induced by using a peroxy radical generator or by using hydrogen peroxide (Elisia and Kitts, 2008).

Yarnpakdee *et al.* (2015) reported that an antioxidant protein hydrolysate prepared from Nile tilapia protein isolate effectively inhibited H_2O_2 and peroxyl radical induced plasmid DNA (pUC18) damage. The protective effect of tuna liver hydrolysates was related to their scavenging of H_2O_2, hydroxyl radical and chelating activity toward Fe^{2+}. Such activities led to the inhibition of the Fenton reaction, and therefore, protected the supercoiled pBR322 plasmid DNA from oxidant-induced strand breaks (Je *et al.*, 2009). Kittiphattanabawon *et al.* (2013) was also reported the inhibition of DNA oxidation by gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme. Gelatin hydrolysate with DH of 40% effectively inhibited hydroxyl and peroxyl radical-induced DNA scission (Kittiphattanabawon *et al.*, 2013).

Gelatin hydrolysates from Nile tilapia (*Oreochromis niloticus*) scale prepared using Alcalase, Pronase E, trypsin and pepsin were studied for their cellular antioxidant activity by monitoring the protective effect on hydroxyl radicals-induced DNA damage and the level of ROS by 20,70-dichlorofluorescein diacetate (DCFH-DA) (Ngo *et al*., 2010). Among hydrolysates, Alcalase-derived hydrolysate exhibited the highest antioxidant activity, compared to other enzymatic hydrolysates and its active peptide was identified as Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe (1,382.57 Da) (Ngo *et al*., 2010). Moreover, this peptide showed no cytotoxic effect on mouse macrophages (RAW 264.7) and human lung fibroblasts (MRC-5). The similar result was reported for protein hydrolysate derived from a salmon by-product and prepared by peptic hydrolysis, which significantly inhibited intracellular reactive oxygen species generation, lipid peroxidation, and enhanced the level of glutathione in Chang liver cells (Ahn *et al.,* 2012).

Hydrolysate derived from Hoki skin gelatin was found to increase the activity of antioxidant enzyme activities, superoxide dismutase (SOD) by 92.8% and catalase (CAT) by 35% in unchallenged human hepatoma cells (Hep3B) following a 24 hr incubation (Mendis *et al*., 2005b). Hoki skin gelatin hydrolysate maintained the cellular redox balance through its radical scavenging activity (Mendis *et al*., 2005b).

1.2.3.2.2.2 Immunomodulatory activity

Immunomodulation involes suppression or stimulation of immune fuctions. Immunomodulaotory food peptides act by enhancing the functions of immune system including regulation of cytokine expression, antibody production and ROS-induced immune functions (Yang, Zhang *et al.,* 2009). Sung *et al*. (2012) studied the anti-inflammatory effect of a sweetfish protein hydrolysate in LPS-induced RAW264.7 cells. The hydrolysate prepared using trypsin and α-chymotrypsin significantly decreased the production of the pro-inflammatory cytokines, IL-1, IL-6 and TNF-α (Sung *et al.,* 2012). Nitric oxide (NO) production has a wide and pervasive regulatory role in the inflammatory response in macrophage cells (Chang *et al.,* 2006). Thus, inhibitors of LPS-induced NO secretion may be effective therapeutically in preventing inflammatory reactions and diseases (Chang *et al.,* 2006). A protein hydrolysate from tilapia muscle protein and salmon byproduct prepared using enzymatic hydrolysis have shown anti-inflammatory activity by inhibition of NO production in LPS-induced cells (Kangsanant *et al.,* 2014; Ahn *et al.,* 2012). The decrease in NO production caused by a protein hydrolysate derived from sweetfish was found to be related to the inhibition of mRNA expression of inducible nitric oxide synthase (Sung *et al.,* 2012).

An enzymatice protein hydrolysate of *Crassostrea gigas* prepared using the protease from *Bacillus* sp. SM98011 showed a strong immunostimulating effects in a dose-dependent maner in sarcoma-S180-baring BALB/c mice (Wang *et al.,* 2010). A fermented fish protein hydrolysate derived from the controlled proteolytic yeast fermentation of Pacific whiting (*Merluccius productus*) induced proliferation and migration in intestinal epithelial cells, which might contribute to anti-inflammatory and healing properties (Fitgerald *et al.,* 2005). Fish protein hydrolysates showed the protective effects on different murine models of colitis, including those induced by dextran sulphate as well as by chronic nonsteroidal anti-inflammatory drugs usage, suggesting their potential applications for curing human disease (Marchbank *et al.,* 2009; Marchbank *et al.,* 2008; Hwang *et al.,* 2012; Bjorndal *et al.,* 2013).

1.2.3.2.2.3 Antiproliferative effects against cancer cells

Peptides with anticancer properties have also been reported from foods. However, the anticancer or antiproliferative activities of marine proteins have been barely studies. A solitary tunicate hydrolysate exhibited both antioxidant activity and anticancer activity in AGS (human breast cancer), DLD-1 (human colon cancer), and HeLa (human cervical cancer) cells (Jumeri and Kim, 2011). The similar result was reported on the antiproliferative activity of an enzymatic hydrolysate from jumbo squid gelatin which demonstrated cytotoxic effects against MCF-7 (human breast carcinoma) and U87 (glioma) cells with IC_{50} values of 130 and 100 μg/mL, respectively (Aleman, Perez-Santin *et al.,* 2011). It was reported that loach protein hydrolysates with the highest antioxidant activity caused the greatest antiproliferative activity against cancer cells (You *et al.,* 2011). Wang *et al*. (2010) found that low molecular size peptides from oyster hydrolysates induced a dose dependent inhibition of growth in transplanted murine sarcoma in BALB/c mice, which they might be attributed to a possible immunostimulatory effect.

Peptide fraction of *Nemipterus japonicas* and *Exocoetus volitans* hydrolysate exerted significant antiproliferative effect on human hepatocellular liver carcinoma cell lines (Hep G2) with IC_{50} values 48.5 and 21.6 mg/mL, respectively. Moreover, these fractions did not show any cytotoxicity effect for Vero (kidney epithelial cells of the African Geen Mondey) cell lines (Naqash and Nazeer, 2010). Hsu *et al*. (2011) isolated 2 peptides from tuna dark muscle by-product hydrolysed with papain and protease XXII. These peptides (Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr) exhibited dose-dependet antiproliferative activities against cultured breast cancer (MCF-7) cells with IC_{50} of 8.1 and 8.8 μM, respectively. Thus, enzymatic hydrolysis of food proteins can release bioactive peptides with anticancer properties.

1.2.3.2.3 Antioxidative peptides from gelatin hydrolysates

Under normal physiological conditions, human health is maintained by the balance of antioxidative and oxidative agents in the body. Reactive oxygen

species (ROS) such as hydroxyl radicals ($^{\bullet}OH$), peroxyl radicals ($^{\bullet}OOR$), superoxide anion ($O_2^{-\bullet}$), and peroxynitrite ($ONOO^-$) are generated during normal biological processes (Decker and Xu, 1998). Endogenous antioxidants help to protect tissues and organs from oxidative damage caused by ROS. These endogenous antioxidative systems in the body include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, α-tocopherol, and vitamin C (Decker and Xu, 1998). Apart from these, amino acids and peptides also contribute to overall antioxidative capacity of cells and help in maintaining the health of biological tissues. Glutathione (γ-Glu-Cys-Gly), carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine), and ophidine (β-alanyl-L-3-methylhistidine) are antioxidative peptides naturally present in muscle tissues (Chan and Decker, 1994; Babizhayev *et al*., 1994).

Antioxidant-prooxidant balance in human body can be altered with the progression of age and due to other factors such as environmental pollutants, fatigue, excessive caloric intake, and high fat diets. With advancing age, the plasma and cellular antioxidant potential as well as the absorption of nutrients, including antioxidants, gradually diminish (Rizvi *et al*., 2006; Elmadfa and Meyer, 2008), leading to an increased vulnerability of proteins to free radical attack. Environmental factors could also weaken the body's immune system and make the body vulnerable to oxidative attack. Use of dietary antioxidants to promote human health by increasing the body's antioxidant load has been recognized as feasible and potentially effective (Gómez-Guillén *et al*., 2011).

Peptide conformation has also been claimed to influence antioxidant capacity, showing both synergistic and antagonistic effects, as far as the antioxidant activity of free amino acids is concerned (Hernández-Ledesma *et al*., 2005). Protease used for hydrolysis may determine the size and the sequence of the peptides, which are assosiacted with their antioxidant activity. Alcalase gelatin-derived hydrolysate showed the higher antioxidant activity than that of other hydrolysates such as those obtained by collagenase, pepsin, trypsin, chymotrypsin, papain or neutrase (Alemán, Gimenez, Montero *et al*., 2011; Qian, Jung *et al*., 2008). Moreover, antioxidant activity is strongly related to peptide molecular weight as demonstrated by Gómez-

Guillén *et al*. (2010) who found antioxidant activity in all the peptide fractions from squid skin hydrolysate, but the activity was higher in the fractions with lower-molecular weight. Similarly, the peptide fraction from Pacific cod skin hydrolysate, with the molecular mass 1300 Da showed the highest hudroxyl radical scavenging activity, being approximately 24% higher than that of the non-fractionated hydrolysate (Himaya, Ngo *et al*., 2012).

Generally, antioxidative properties of peptides are related to their amino acid composition, structure and hydrophobicity. The amino acid composition of gelatin hydrolysates is very similar to that of the parent proteins, being rich in residues of Gly, Ala, Pro, Hyp, Glx and Asx, but poor in Met, Cys, His and Tyr (Alemán, Gimenez, Montero *et al*., 2011; Gómez-Guillén *et al*., 2010; Kim *et al*., 2001; Mendis *et al*., 2005a). Dávalos *et al*. (2004) studied antioxidative activity of individual amino acid and reported that Trp, Tyr and Met showed the highest antioxidant activity, followed by Cys, His and Phe. The rest of the amino acids did not show any antioxidant activity. Mendis *et al*. (2005b) isolated a peptides composed of 7 amino acid residues, from Hoki skin gelain, which contained a His residue at the C-terminus and the repeating motif Gly-Pro-Leu. Furthermore, the antioxidant activity of collagen and gelatin peptides has been linked to the high content of hydrophobic amino acids, which could increase their solubility in lipids and therefore enhance their antioxidative activity (Kim *et al*., 2001). Rajapakse *et al*. (2005) found that fish skin gelatin peptides showed higher antioxidant activity than peptides from meat protein, probably because of the higher percentage of Gly and Pro.

Numerous peptides derived from fish collagenous materials such as skins of Japanese flounder (*Palatichtys olivaceus*) (Himaya, Ryu *et al*., 2012), Hoki (*Johnius belengerii*) (Mendis *et al.,* 2005b), Pacific cod (*Gadus macrocephalus*) (Ngo *et al*., 2011) and sole (Giménez *et al*., 2009), as well as skins of several squid species, such as giant squid (*Dosidicus gigas*) (Alemán, Giménez, Pérez-Santín *et al*., 2011; Giménez *et al*., 2009; Mendis *et al*., 2005a), Jumbo flying squid (*Dosidicus eschrichitii Streenstrup*) (Lin and Li, 2006) or squid (*Todarodes pacificus*) (Nam *et al*., 2008) have been reported. Some antioxidant peptides isolated from fish skin gelatin and other collagenous sources are shown in Table 1.

Table 1 Antioxidative activity of peptides derived from collagenous sources

Sources	Enzymes	Active peptide sequences	Activities	References
Jumbo squid (*Dosidicus gigas*) skin gelatin	Trypsin	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu and Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	hydroxyl and carbon-centered radical scavenging, Inhibition of lipid peroxidation	Mendis, Rajapakse, Byun *et al.* (2005)
Alaska Pollack (*Theragra chalcogramma*) skin	Serial digestion (Alcalase, Pronase E, collagenase)	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly and Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	Inhibition of lipid peroxidation	Kim *et al*. (2001)
Squid tunic (*Dosidicus gigas*) gelatin	Alcalase	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser	Radical scavenging, Ferric reducing power	Alemán, Giménez, Pérez-Santín *et al*. (2011)
Hoki (*Johnius belengerii*) skin gelatin	Trypsin	His-Gly-Pro-Leu-Gly-Pro-Leu	Superoxide, carbon centered radical scavenging	Mendis,Rajapakse *et al*. (2005)
Nile tilapia (*Oreochromis niloticus*) scale	Alcalase	Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe	DPPH, Hydroxyl readical, Superoxide radical scavenging	Ngo *et al.* (2010)
Pacific cod (*Gadus macrocephalus*) skin	Papain	Thr-Cys-Ser-Pro and Thr-Gly-Gly-Gly-Asn-Val	Intracellular free radical scavenging, oxidation-iduced DNA damage	Ngo *et al.* (2011)
Japanese flounder (*Palatichtys olivaceus*) skin	Pepsin	Gly-Gly-Phe-Asp-Met-Gly	ROS-mediated intracellular macromolecule and antioxidative enzyme activity	Himaya, Ryu *et al.* (2012)
Horse mackerel (*Magalaspis cordyla*)	Trypsin	Asn-His-Arg-Tyr-Asp-Arg	Electron spin resonance (ESR) spectrometry using DPPH and hydroxyl (OH$^{\bullet}$) radical scavenging	Sampath Kumar *et al.* (2012)
croaker (*Otolithes ruber*)	Trypsin	Gly-Asn-Arg-Gly-PheAla-Cys-Arg-His-Ala		
Pacific cod (*Gadus macrocephalus*) skin	Gastrointestinal endopeptidases	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	ROS-mediated intracellular macromolecule and antioxidative enzyme activity damage	Himaya, Ngo *et al.* (2012)
Nile tilapia (*Oreochromis niloticus*) skin	Properase E and multifect neutral	Glu-Gly-Leu and Tyr-Gly-Asp-Glu-Tyr	DPPH radical, superoxide anion ($^{\bullet}$OO) and hydroxyl (OH$^{\bullet}$) radical scavenging	Zhang *et al.* (2012)
Nile tilapia (*Oreochromis niloticus*) skin	Properase E	Leu-Ser-Gly-Tyr-Gly-Pro	UV-induced damage skin	Sun *et al.* (2013)
Amur sturgeon (*Acipenser schrenckii)* skin	Alcalase	Pro-Ala-Gly-Tyr	DPPH, ABTS and hydroxyl radical scavenging	Nikoo *et al.* (2014)

1.2.4 Antifreezing agents

Water is one of the major components of food products. Consequently, changes in the physical state of water in food systems and/or the extent of its interactions with other food components during storage cause structural and textural changes in foods, which, in some cases, are detrimental to their quality. This is particularly a problem in frozen foods, such as meat, fish, desserts, and frozen fruits and vegetables (Zhu *et al*., 2005).

During freezing, which is a process of ice crystallization from supercooled water, nucleation of ice occurs first, followed by recrystallization of ice (Mutaftschiev, 1993). The size distribution of ice crystals formed during this recrystallization stage has a strong influence on the texture of frozen foods (Regand and Goff, 2002; Hartel, 1992) and the structural integrity of cell membranes (De Vries, 1974; Beall, 1983). Temperature fluctuations during storage and handling of frozen foods promote ice crystal growth. The crystal growth rate is very slow at lower storage temperatures, especially when the product is stored below its glass transition temperature (Levine and Slade; 1989, Simatos *et al*., 1989; Slade and Levine, 1991). Above the glass transition temperature, the greater molecular mobility of water leads to faster growth of ice crystals. Because the typical average storage temperature in household freezers is well above -20 °C and fluctuates because of automatic defrost cycles (Miller-Livney and Hartel, 1997), formation of large ice crystals and deterioration of textural qualities of frozen foods is a common occurrence under household conditions (Fennema, 1993).

An antifreezinng agent or cryoprotectant is a substance, which protects biological tissue from freezing damage by increasing the surface tension of water as well as the amount of bound water. It can prevent ice crystal growth and migration of water molecules from the protein, thus stabilizing the protein in its native form during frozen storage (Carpenter and Crowe, 1988; Alvarez *et al*., 2010).

1.2.4.1 Classification

1.2.4.1.1 Carbohydrate-based antifreezing agent

Addition of hydrocolloids (such as carboxymethyl cellulose (CMC), locust bean gums and carrageenan) (Regand and Goff, 2003), polydextros, lactitol, glucose syrup (Herrera and Mackie, 2004), as well as trehalose and sodium lactate (Zhou *et al*., 2006) to frozen foods retards the rate of ice crystal growth. This has been attributed to increased viscosity of the serum phase, which slows down molecular mobility of water (Blond, 1988; Budiaman *et al*, 1987; Regand and Goff, 2003), and to a possible increase of the glass transition temperature (Hagiwara and Hartel, 1996).

Hydrocolloids generally have no or only a marginal effect on heterogeneous nucleation temperature of supercooled water (Flores and Goff, 1999). Moreover, products containing carbohydrate-based cryoprotextants would not be acceptable by consumers suffering from diabetes; furthermore, their tendency to impart a sweet taste to the final product might also not be desirable in some cases (Sych *et al*., 1990; Yoon and Lee, 1990). However, there is no consensus on the mechanism because results from various studies have been contradictory (Flores and Goff, 1999; Buyong and Fennema, 1988; Goff *et al*., 1993; Muhr and Blanshard, 1986).

1.2.4.1.2 Antifreeze proteins (AFPs)

AFPs are class of protein and serve as antifreezing agents, by specifically adsorbing to the surface of ice crystals and prevent further growth and recrystallization of ice. Fish, insects, bacteria and plants that live in sub-zero environments all rely on AFPs to survive (Fuller, 2004). AFPs are also important to many industries, including ice cream and frozen yogurt production which relies on AFPs to control ice-crystal growth (Garnham *et al*., 2011).

AFPs were divided into four distinct classes according to molecular mass, molecular structure, protein components, gene copies and natural source. Although AFPs show diverse structural features among certain closely related species,

all have similar effects on ice-crystal growth (Fuller, 2004). Garnham *et al*. (2011) elucidated the ice-binding mechanism through solving the first crystal structure of an Antarctic bacterial AFP. It was found that individual AFP molecules appear to adsorb directly on to ice crystal surfaces which was related to hydrogen bonding and hydrophobic effect of AFP (Garnham *et al*., 2011).

1.2.4.2 The application of protein hydrolysate/peptides as antifreezing agent

The application of protein hydrolysates has attracted much attention in the past decade, mainly due to their high nutritive value and improved functionalities arising from the production of short peptides and free amino acids during hydrolysis (Clemente, 2000). The potential cryopreventive property of protein hydrolysates has recently drawn interest. Optimization of gelatin hydrolysis conditions and molecular weight characterization of the cryoprotective peptide indicated that the most active fraction consisted of cationic peptides in the range of 700 to 1,400 Da (Wang *et al*., 2009). Protein hydrolysates produced from Pacific hake (Cheung *et al*., 2009), blacktip shark skin (Kittiphattanabawon *et al*., 2012), and shrimp waste (Dey *et al.,* (2013; Ruttanapornvareesakul *et al*., 2006) have all been shown to display cryoprotective ability. Fish protein hydrolysates produced by proteolysis of Pacific hake (*Merluccius productus*) with Alcalase R® or Flavourzyme R® showed cryoprotective effect in frozen cod mince (Cheung *et al*., 2009). Both hydrolysates improved the water retention properties with high proportion of unfrozen water in freeze–thawed cod mince. Dey *et al.* (2013) found that protein hydrolysate from shrimp waste could reduce the effect of freeze denaturation on Ca^{2+}-ATPase activity of Croaker fish surimi and the denaturation of myofibrillar Ca^{2+}-ATPase was correlated with the amount of unfrozen water (Dey *et al.* 2013). Kittiphattanabawon *et al*. (2012) examined the cryoprotective effects of gelatin hydrolysate produced by hydrolysis with crude enzyme from papaya (*Carica papaya*) latex from blacktip shark skin with different degrees of hydrolysis (DHs: 5, 10 and 30%). It was found that gelatin hydrolysate with 10% DH was able to prevent the denaturation of surimi protein monitored by Ca^{2+}-ATPase activity and surface hydrophobicity comparable to commercial cryoprotectant (sucrose/sorbitol blend, 3:1).

Dey *et al.* (2013) reported that cryoprotective effect of shrimp waste protein hydrolysate on croaker surimi during frozen storage was caused by the active short chain peptide with molecular weight less than 1600 kDa. Furthermore, hydrophilic amino acids in peptides inhibited denaturaion of myofibrillar protein and had water constraining effect. Damodaran (2007) demonstrated the ability of gelatin hydrolysate to inhibit ice crystal growth in ice cream mix. Peptides produced by hydrolysis with papain were lyophilized and separated into 3 different sized fractions. It was found that peptides with MW greater than 7,000 Da had no inhibitory effect on ice crystal growth in the ice cream mix, while the anti-freeze properties of gelatin were noticeable for peptides with MW smaller than 3,000 Da (Damoraran, 2007).

Cheung *et al*. (2009) also reported that the presence of oligopeptides in both hydrolysates and the high levels of free amino acids including Asp, Glu, Arg, and Lys in FPH-F might be responsible for their cryoprotective action. Chen *et al*. (2005) reported the potential of amino acids on inhibition of protein denaturation by inhibiting eutectic crystallization. Thr, γ-amino-butyric acid, Pro, Arg, Lys, Hyp, Ser, Gly and His were found to strongly inhibit the formation of eutectic crystallization in a NaCl-H_2O system (Chen *et al*., 2005). Nikoo et al. (2014) reported that the tetrapeptide (Pro-Ala-Gly-Tyr) isolated from Amur sturgeon skin hydrolysate, prepared using Alcalase, retared the denaturation of myosin and actin in Japanese seabass mince subjected to 6 freeze/thaw cycles.

1.2.5 Maillard reaction and its products

Several attempts have been made to improve the functional properties of proteins through protein–saccharide graft reactions, based on Maillard reaction between the amino groups of proteins and the reducing-end carbonyl groups of saccharides (Guan *et al*., 2006; Kim and Lee, 2009). It has been reported that protein–saccharide grafts are useful as a new functional biopolymer having the excellent emulsifying, antioxidant and antimicrobial effects for food applications (Guan *et al*., 2006).

Maillard reaction is non-enzymatic browning reaction between amino acids and reducing sugars, which takes place in thermally processed food (Carabasa-Giribet and Ibarz-Ribas, 2000), leading to the formation of a complex series of compounds called Maillard reaction products (MRPs) (Mastrocola and Munari, 2000). Maillard reaction may produce coloured or colourless reaction products, depending on the stage of the reaction, as well as other factors, such as pH, type of reactants, temperature, water activity and concentration of reactants (Billaud *et al*., 2004).

1.2.5.1 Maillard reaction

The Maillard reaction is subdivided into three stages: the early Maillard reaction, the advanced Maillard reaction and the final Maillard reaction (Van Bockel, 1998).

1.2.5.1.1 The early Maillard reaction

A reducing sugar, like glucose (as an aldehyde in open chain form), condenses with a compound possessing a free amino group (of an amino acid or in proteins mainly the ε-amino group of lysine, also the α-amino group of terminal amino acids) to give a condensation product, *N*-substituted glycosylamine via formation of Schiff's base and the Amadori rearrangement, to the so-called Amadori product (Martins *et al*., 2003; Van Bockel, 1998). The degradation and dehydration of Amadori products into amine or carbonyl intermediates, and the reaction of carbonyl intermediates with other amino groups as well as subsequent rearrangement to form advanced glycosylation end-products (AGE-products) also take place (Murthy and Sun, 2000). The early stage of the Maillard reaction can be evaluated by determination of the furosine (ε-*N*-(furoyl-methyl)-*L*-lysine) amino acid formed during acid hydrolysis of the Amadori compounds (Murthy and Sun, 2000).

1.2.5.1.2 The advanced Maillard reaction

The subsequent degradation of amadori product is dependent on the pH of the system (Figure 3). At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural

(HMD), the intermediate product in the Maillard reaction is formed and the degradation of sugars takes place at high temperatures (Ramírez-Jiménez *et al*., 2000). At pH>7, the degradation of Amadori compound is thought to involve mainly 2,3-enolisation, where reductones, including acetol, pyruvaldehyde and diacetyl, are formed. All these compounds are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products. Dicarbonyl compounds will react with amino acids with the formation of aldehydes and α-aminoketones. This reaction is known as the Strecker degradation (Martins *et al.,* 2003).

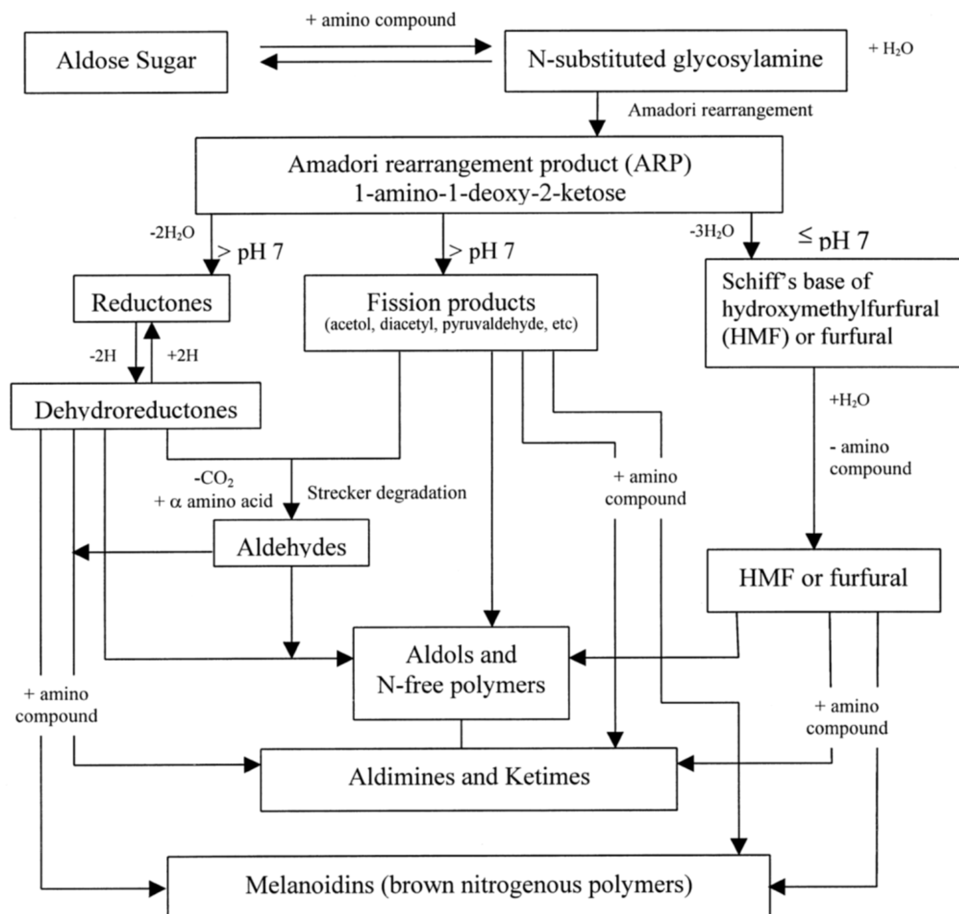

Figure 3 Scheme of Maillard reaction

Source: Hodge (1953)

The degradation of Amadori compound N-(1-deoxy-D-fructose-1-yl)-glycine (DFG) by 1, 2-enolisation and 2, 3-enolisation leads to the formation of 3-deoxy-2-hexosulose and 3-deoxy-2, 3-hexodiulose, respectively. The formation of these intermediates is accompanied by amino acid release (Martins *et al.*, 2003). The degradation of Amadori compound take place through enolisation such as the formation of glucosones by transition-metal catalyzed oxidation of 1, 2-enaminol and 1-amino-1,4-dideoxy-2,3-diulose by elimination of C-4 OH group of 2, 3-enaminal (Huyghues-Despointes and Yaylayan, 1996). Another possible mechanism of degradation of Amadori compound involves a retro-aldol reaction, including that generates 1-glycine-1-deoxy-D-glyceraldehyde and glyceraldehydes through a retro-aldo cleavage at C-3 to C-4. The compound can react with free glycine and produce more compound that subsequently undergoes a β-elimination to form methylglyoxal and releases glycine (Martins *et al.*, 2003).

1.2.5.1.3 The final Maillard reaction

In an advanced stage, a range of reactions including cyclization, dehydrations, retroaldolization, rearrangements, isomerization and further condensations take place, leading to the formation of brown nitrogenous polymers and co-polymers, known as melanoidin (Martins *et al.*, 2003). Melanoidins are of a high molecular weight (up to about 100,000), and they contain nitrogen by definition. However, brown pigments are also formed without nitrogen mainly, from sugar degradation or caramelization (Martins *et al.*, 2003; Phongkanpai *et al.*, 2006).

1.2.5.2 Factors affecting Maillard reaction

Many factors influence the velocity of Maillard reaction such as temperature, time, water activity (a_w) (Buera *et al.*, 1987), reactant source and concentration (Jing and Kitts, 2002), the type and ratio of reducing sugar (Naranjo *et al.*, 1998), amino compounds (Morales and Jemenez-Perez, 2001), pH (Ajandonz *et al.*, 2001) and food composition (Saltmarch and Labuza, 1982).

1.2.5.2.1 Types of sugar and amino acid

Browning rate is significantly influenced by the type of reducing sugar involved in the reaction. The order of reactivity is follows: aldopentoses > aldohexoses > ketohexoses > disaccharides (Naranjo *et al*., 1998). Generally, aldoses are intrinsically more reactive than ketoses (Yoboah *et al*., 1999). Yoboah *et al*. (1999) reported that glucose is more reactive than fructose but Benjakul *et al*. (2005), Giroux *et al*. (2010) and Hwang *et al*. (2011) reported that fructose is more reactive than glucose.

In the Maillard reaction, the concentration of open chain form might be a crucial factor in determining the rate of glycation if the interconversion rate is slower than the reaction rate (Naranjo *et al*., 1998). The reaction rate of each sugar depends on the percentage of acyclic form, but also on the electrophilicity of carbonyl group (Naranjo *et al*., 1998). Among the four sugar studied including fructose, maltose, lactose and glucose, it was observed that fructose has the highest proportion of open chain form, but aldoses would react faster than ketoses because they are more electrophilic (Naranjo *et al*., 1998).

1.2.5.2.2 Temperature and heating time

Temperature and duration of heating determined the rate of the reaction. An increase in temperature leads to an increase of the reactivity between the sugar and the amino agroup (Martins *et al*., 2003). MRPs formation is highly dependent on heating time and temperature. The rate of the Maillard reaction increased exponentially as the heating temperature increases (Billaud *et al*., 2004).

1.2.5.2.3 Concentration of reactant

The rate of the Maillard reaction is governed by concentration of reactant. Matmaroh *et al*. (2006) reported that the increase in fructose concentration led to the increase in A_{294}, fluorescence and A_{420} intensity in the aqueous model system. Benjakul *et al*. (2005) observed that browning and intermediate products increased as increasing sugar concentration. Moreover, the increases in browning and

formation of intermediate products were observed with a concomitant decrease in free amino group. The silver carp protein hydrolysate and glucose with the ratio of 2:1 heated at 60 °C showed high browning intensity and good antioxidant properties, compared with those with 1:1, 1:2 and 1:4 hydrolysate and glucose ratios (You *et al*., 2011).

1.2.5.2.4 pH

In general, the reaction is inhibited by low pH values and is favored by high pH values (Lertittikul *et al*., 2007). The maximum browning was obtained at a pH 12 in porcine plasma protein-glucose model system. Ajandouz *et al*. (2001) revealed that in Maillard reaction, highly UV-absorbing and colorless compounds and brown polymer are formed at higher pH in the fructose-lysine aqueous model system. On the other hand, MRPs could also prepared at pH 7.0-7.5. You *et al*. (2011) prepared the antioxidative MRP using silver carp protein hydrolysate and glucose with the ratio of 2:1 at 60 °C, pH 7.5. The resulting MRP showed high browning intensity and good antioxidative activity. The Maillard reaction of glucose, fructose and ribose-casein was also generated at pH 7.0, 55 °C and the resulting MRPs exhibited antioxidative activity (Jing and Kitts, 2002)

1.2.5.2.5 Water activity (a_w)

Water activity of a given food is dependent on both relative humidity of its surroundings and the presence of various substances. Additionally, water activity or relative humidity is related with the moisture content of the food through the sorption isotherms. The measure of water activity (a_w) is useful to describe a thermodynamic equilibrium state and is often useful for predicting reaction rate (Sherwin and Labuza, 2003). Lievonen and Roos (2002) found that the non-enzymatic browning rate increased as the initial water activity increased in model system of lysine and xylose containing maltodextrin or polyvinylpyrrolidone. Shen and Wu (2004) propose that the reduction of a_w is not the sole major mechanism for ethanol to accelerate Maillard browning in glucose/glycine solution.

1.2.5.3 Bioactivities of Maillard reaction products (MRPs)

1.2.5.3.1 Antioxidative activity

MRPs have been known to prevent oxidation reaction in both model lipid and food system via free radical scavenging action (Morales and Jamenez-Perez, 2001), metal ion chelating property (Wijewickreme and Kitts, 1997) and reducing activity (Tan and Harris, 1995). They also inhibit peroxidase, superoxide dismutase and xenobiotic enzymes (Billaud *et al*., 2004). Morales and Jimenez-Perez (2004) stated that the melanoidins behave as anionic hydrophilic polymers which can form stable complexes with metal ion.

The antioxidant activity of MRPs is summarized in Table 2. Some intermediate Miallard products, such as reductones, have high antioxidative activity in aqueous solutions or emulsion (Serevini and Lerici, 1995). Pyrole-like structures, resulting from the interaction of the amino compounds and furfural, have strong oxygen scavenging effect (Namiki, 1988). The reductone moiety present in the melanoidin structure was reported to exhibit both reducing and chelating properties and oxygen scavenging properties (Namiki, 1988). You *et al.* (2011) examined the effect of substrate ratios, temperature on development of Maillard reaction and antioxidative activity of protein hydrolysate-glucose system. The hydrolysate and glucose heated with the ratio of 2:1 at 60 °C showed high browning intensity and good antioxidative properties. Wang, Bao *et al.* (2013) reported the antioxidant activities of MRPs from the reactions between whey protein isolate and xylose, glucose, fructose, lactose, maltose and sucrose at different initial pH values. MRPs derived from the whey protein isolate-xylose system with increasing pH rendered the highest browning, reducing power and DPPH radical-scavenging activity. Additionally, the antioxidative MRPs were also prepared at neutral condition. The Maillard reaction of Glc–, Fru– and Rib–casein was generated at 55 °C, pH 7.0 (Jing and Kitts, 2002). Glc–, Fru– and Rib–casein all exhibited antioxidant activity against Fenton reactant-induced hydroxyl free radicals. Jiang *et al.* (2013) prepared the MRPs from bovine casein peptide and reducing sugars by heating at 95 °C up to 5 h without pH control. The MRPs derived from ribose showed the highest DPPH radical

scavenging activity and ferrous reducing power, compared with those prepared using other sugars (galactose and lactose) at the same incubation time (Jiang *et al.,* 2013).

Antioxidative xylan–chitosan Maillard reaction products (MRP) were prepared by coheating xylan and chitosan at different time periods (Li *et al*., 2013). MRPs heated for 120 min and 180 min showed much higher antioxidative activity on lecithin liposome peroxidation than chitosan or MRP heated for 60 min. Lipid oxidation of refrigerated pork meat was lower in the samples treated by the MRPs heated for 60 and 120 min (Li *et al*., 2013). Joubran *et al.* (2015) reported that thermally-induced Maillard reaction between bovine alpha-lactalbumin and fructose or fructo-oligosaccharides could potentially modulate its *in vitro* gastro-duodenal digestibility. Particularly, it pertained to ability to interfere with oxidative raction in the lumen, possibly through the generation of bioactive peptides (Joubran et *al.,* 2015).

The antioxidative activities of MRP were also studied in intracellular model systems. Goya *et al.* (2007) found that *in vitro* gastrointestinal digested melanoidin protected against a decrease in antioxidant enzyme activity induced by tert-butylhydroperoxide (*t*-BOOH) in Hep G2 cells. Moreover, antioxidant activities of the MRP were due to their ROS/radical scavenging activity (Goya *et al.,* 2007). The low molecular weight components of MRP prepared using glucose-lysine mixture heated for 60 min, had the marked inhibition toward intracellular oxidation in Caco-2 cells induced with rective oxygen and nitrogen (Kitt *et al.,* 2012). *In vivo*, a MRPs rich diet was able to suppress lipid peroxidation and to increase antioxidative activity of plasma, although it had not modified the antioxidant enzymes activity (superoxide dismutase, glutathione peroxidase and catalase) (Seiquer *et al.,* 2008).

Table 2 Antioxidative activities of Maillard reaction products

Type of compounds	Precursors	Effect on food stability
Imines (Schiff bases)	Sugar, amino acids	Hydroperoxide reduction
Amino deoxy sugars	Schiff bases	Hydroperoxide reduction
Amadori, Heyns products	Amino deoxy sugars	Hydroperoxide reduction
Melanoidins	Premelanoidins	Metal chelation
Dihydrocyclic derivatives	Strecker compounds	Hydroperoxide reduction
Reductones	Dideoxytriulose	Free radical scavenging

Source: Gordon (2001)

1.2.5.3.2 Immunomodulatory and antiproliferation effects

MRPs may have immunomodulatory effect and antiproliferation activity against cancer or tumor cells. Some studies suggest the MRPs exert positive influence on the gut microbiota (Tuohy *et al.,* 2006). Kitts *et al.* (2012) previously reported the anti-inflammatory activity of MRPs prepared using different monosaccharide and amino acid model systems. The MRP derived from glucose-lysine systems showed a marked ability to inhibit intracellular oxidation, the production of inflammatory cytokines and NO in inflamed Caco-2 cells, compared with MRPs from other systems (Kitts *et al.,* 2012). Two components, namely, 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid (HMFA), were identified as bioactive components in this MRP (Kitts *et al.,* 2012).

Yamabe *et al.* (2013) produced an antioxidant MRP with anticancer activity from a ginsenoside-lysine mixture. The increased anticancer effect of MRP upon heat processing was mainly derived from the generation of less-polar compounds (Yamabe *et al.,* 2013). HMFA is a metabolite of HMF and it possesses antitumor activity against SV40-transformed (*in vitro*) and Sacroma 180 implanted intraperitoneally in ICR mice (*in vivo*) (Munekata and Tamura, 1981). An MRP prepared by heating protein hydrolysate and glucose at 145 °C for 20 min demonstrated the potential to decrease Caco-2 cells proliferation (Teodorowicz *et al.,* 2013). Moreover, the anticancer activity of MRP could relate with its immunomodulatory activity (Wang *et al.,* 2010). Sung *et al*. (2011) found that the

thermal sterilization of pepsin hydrolysate from a marine fish half-fin anchovy resulted in enhancing the antiprolifertive activities of resulted product against human prostate cancer, lung cancer andesophagus cancer cell lines with increasing fraction with molecular weight of 3-5 kDa and below 500 Da. These results might be attributed to the Maillard reaction (Sung *et al.,* 2011).

1.2.5.4 The application of MRPs

Maillard reaction products (MRPs) are responsible for essential sensory attributes of thermally processed food products, contributing to their appearance, flavor, aroma and texture. MRPs have been suggested to decrease the lipid oxidation rate, thus highlighting an interesting relationship by which the browning of the food, and thus the process conditions, could affect not only the organoleptic properties but also the oxidative resistance of the food (Lingnert and Hall, 1986).

MRPs have the wide applications in several foods and they showed antioxidative activity in biscuit (Goya, 2009), cereals (Rufiãn-Henares and Delgado-Andrade, 2009), coffee (Goya *et al.*, 2007) and nut (Açar *et al.*, 2009). Li *et al.* (2013) reported the application of MRP in lipid food storage. Antioxidative xylan-chitosan MRPs were able to retard lipid oxidation in the refrigerated pork meat (Li *et al.*, 2013). Tarr and Cooke (1949) reported that enediols, intermediate products of the Maillard reaction, strongly retarded fat oxidation in frozen minced salmon or herring meats stored at -10 or -20 °C. Although investigations of MRP as antioxidants have shown exceptionally potent antioxidative activity to a variety of foods, the practical use of MRP as an antioxidant in food industry has not been so far carried out. This is mainly due to facts that MRPs have dark color, bitter taste and peculiar flavor and solubility which could be problem for the application of MRPs as antioxidants in foods.

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1.4 Objectives

1.4.1 To study the impact of autolysis assisted process on production of antioxidative gelatin hydrolysates from unicorn leatherjacket skin in combination with thermal hydrolysis or hydrolysis using papain.

1.4.2 To isolate and investigate the role of glycyl endopeptidase enzyme from papaya latex in production of antioxidative gelatin hydrolysate.

1.4.3 To enhance the antioxidative activity of gelatin hydrolysate from autolysed unicorn leatherjacket skin using partially purified glycyl endopeptidase enzyme from papaya latex.

1.4.4 To investigate the cryoprotective and antioxidative properties of gelatin hydrolysate in washed mince model system subjected to multi freeze-thaw cycle.

1.4.5 To modify the antioxidative activity of gelatin hydrolysate via Maillard reaction.

1.4.6 To study the antioxidative, immonomodulatory and antiproliferative activities of gelatin hydrolysate and its Maillard reaction using *in vitro* cell culture assay.

1.4.7 To investigate the supplimentation of antioxidative Maillard reaction base on gelatin hydrolysate in instant coffee.

CHAPTER 2

Characteristics and antioxidative activity of gelatin hydrolysates from unicorn leatherjacket skin as affected by autolysis-assisted process

2.1 Abstract

Autolysis-assisted process mediated by indigenous protease in combination with thermal hydrolysis and hydrolysis using papain was used for production of gelatin hydrolysate from skin of unicorn leatherjacket. Prior autolysis could enhance the yield and antioxidative activity of gelatin hydrolysates, especially when subsequent hydrolysis by 2% papain was implemented (AU+HP2.0). Hydrolysates possessed ABTS radical and H_2O_2 scavenging activities as well as metal chelating activity. AU+HP2.0 and gelatin hydrolysate prepared using papain without prior autolysis (HP2.0) could retard oxidation in lecithin liposome system and antioxidative activity of hydrolysates at 10.0 g/L was comparable to that of 0.1 g/L trolox, in which the oxidation was almost completely inhibited. Base on size exclusion chromatography, antioxidative peptide in AU+HP2.0 had molecular weight of 2,200 Da. Therefore, autolysis assisted process showed the potential for the production of gelatin hydrolysates with enhanced antioxidative activity.

2.2 Introduction

Protein hydrolysates have gained increasing attention due to their bioactivities and the potential in pharmacology (Gómez-Guillén *et al.,* 2011; Suarez-Jimenez *et al.,* 2012). Protein hydrolysates especially from marine resources are of interest since there is less constraint for consumption by some religions. Gelatin and derivative including hydrolysate or peptide from bovine and porcine originals are prohibited by Hindu and Muslim, respectively (Gómez-Guillén *et al.,* 2011).

Enzymatic hydrolysis has been intensively employed and the enzymes used have been reported to determine the bioactivities of resulting hydrolysates (Gómez-Guillén *et al.,* 2011). Certain proteolytic enzymes such as Alcalase (Kim *et al.* 2001), trypsin (Mendis *et al.,* 2005a, 2005b) and collagenase (Kim *et al.,* 2001; Byun and Kim, 2001) have been used to produce hydrolysate from fish gelatin. Papain is a cheap cysteine protease obtained from the latex of papaya. It has broad specificity and has been widely used for improving the bioactivity and functionality of protein hydrolysate (Li *et al.,* 2012; Ngo *et al.,* 2011). Antioxidative peptides prepared from Pacific cod skin gelatin using Alcalase, Neutrase, papain, trypsin, pepsin and α-chymotrypsin were comparatively studied (Ngo *et al.,* 2011). Among all hydrolysates, that produced using papain exhibited the highest antioxidative activity. In addition, thermal hydrolysis was reported to produce gelatin hydrolysate with similar yield and antioxidative activity, compared with enzymatic process (Yang *et al,.* 2009, 2008). However, thermal hydrolysis needed the longer time (170-180 min) and harsh condition (121 °C, 10^5 kg/m.s^2) (Yang *et al.,* 2009; Wang *et al.,* 2013). Enzymes, energy consumed and process used directly contribute to the cost of production. To reduce such a cost, the indigenous proteases should be maximally exploited, in which the cost and time of hydrolysis processes could be lowered. Samaranayaka and Li-Chan (2008) showed the feasibility of preparing fish protein hydrolysate with antioxidative activity from Pacific hake via autolysis mediated by indigenous protease. Hydrolysate from capelin was also prepared by autolysis (Shahidi *et al*., 1995).

Skin of unicorn leatherjacket *(Aluterus monoceros)* has been reported to possess indigenous protease which caused the degradation of α-chain during gelatin extraction at 50-55 °C (Kaewruang *et al.,* 2013; Ahmad *et al.,* 2011). The autolysis mediated by indigenous protease can be used as the aid for production of gelatin hydrolysate along with other hydrolysis methods, in which the amount of commercial proteases and energy used can be reduced. Moreover, gelatin hydrolysate can be produced directly from fish skin without prior gelatin extraction. Nevertheless, there is no information regarding the production of fish gelatin hydrolysate from unicorn leatherjacket skin as assisted by indigenous proteases.

2.3 Objective

To investigate the potential of autolysis-assisted process for production of gelatin hydrolysate with antioxidative activity from the skin of unicorn leatherjacket in combination with thermal hydrolysis and enzymatic hydrolysis using papain.

2.4 Materials and methods

2.4.1 Chemicals

Papain from papaya (*Carica papaya*) latex (E.C. 3.4.22.2) with the activity of ≥3 unit/g, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bathophenanthroline disulphonic acid, 2,2′-azinobis (3- thylbenzothiazoline-6-sulfonic acid) (ABTS), sodium dodecyl sulphate (SDS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4´´-disulfonic acid sodium salt (ferrozine) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol, trichloroacetic acid (TCA), ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Ammonium thiocyanate was obtained from Lab-Scan (Bangkok, Thailand). Coomassie Blue R-250, and *N,N,N´,N´*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Bis-acrylamide was purchased from Fluka (Buchs,

Switzerland). Low molecular weight marker was obtained from GE healthcare UK Limited (Buckinghamshire, UK). All chemicals were of analytical grade.

2.4.2 Preparation of fish skins

The skins of unicorn leatherjacket (*A. monoceros*) with the size of 1.0-1.5 kg/fish were obtained from a dock, Songkhla, Thailand, during June-August, 2013. Fish were killed by ice-shock after capture. Three different lots of skins were collected. For each lot, skins were pooled and used as the composite sample. Skins were 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, Hat Yai within 1 h. Upon arrival, the skins were washed with iced tap water (0–2°C) and cut into small pieces (0.5×0.5 cm^2), placed in polyethylene bags and stored at −20°C. The storage time was less than 2 weeks.

2.4.3 Pretreatment of fish skin

Removal of non-collagenous proteins was carried out according to the method of Ahmad *et al*. (2011) with a slight modification. Fish skins (0.5×0.5 cm^2) were soaked in 0.05 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Germany) at a speed of 150 rpm. The alkaline solution was changed after 2 h and total pretreatment time was 4 h. Pretreated skins were washed with tap water until neutral or faintly basic pH of wash water was obtained.

2.4.4 Preparation of gelatin hydrolysate by autolysis assisted processes

2.4.4.1 Preparation of gelatin hydrolysate using autolysis

The pretreated skins were mixed with deionised (DI) water at a ratio of 1:5 (w/v). The autolysis was conducted by incubating the mixture in a water bath (Model W350, Memmert, Schwabach, Germany) at 55°C for 12 h and terminated by heating at 90°C for 15 min. The mixture was centrifuged at $5000 \times g$ at 4°C using a

refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) for 10 min to remove the debris. Gelatin hydrolysate obtained by autolysis process was collected and referred to as AU.

2.4.4.2 Preparation of gelatin hydrolysate by thermal process in combination with prior autolysis

The autolysis was conducted as described above. After inactivation of indigenous protease, the mixture was subjected to thermal hydrolysis by autoclave (121°C, 10^5 kg/m/s^2) for different times (15, 30, 60 min). The mixture was then centrifuged at 5000 × g at 4°C using a refrigerated centrifuge for 10 min to remove debris. The gelatin hydrolysates obtained were referred to as AU+TH15, AU+TH30 and AU+TH60. Gelatin hydrolysates prepared by thermal hydrolysis for 15, 30 and 60 min (without prior autolysis) were also prepared and referred to as TH15, TH30 and TH60, respectively.

2.4.4.3 Preparation of gelatin hydrolysate by hydrolysis using papain with prior autolysis

After autolysis and inactivation of indigenous protease, the mixture was added with papain at different concentrations (0.5, 1.0 and 2.0% of protein substrate). The mixture was incubated at 50°C for 1 h and the hydrolysis was terminated by heating at 90°C for 15 min. The mixture was centrifuged at 5000 × g at 4°C for 10 min to remove debris. The gelatin hydrolysates were collected and referred to as AU+HP0.5, AU+HP1.0 and AU+HP2.0. The gelatin hydrolysates prepared by hydrolysis using papain at levels of 0.5, 1.0 and 2.0% (without prior autolysis) were also prepared and referred to as HP0.5, HP1.0 and HP2.0, respectively.

2.4.5 Analyses

All gelatin hydrolysates were subjected to analyses.

2.4.5.1 Determination of yield

Yield of gelatin was calculated as follows:

Yield% = [hydroxyproline content of supernatant (g/mL)×volume of supernatant(mL)]/ [hydroxyproline content of initial skin (g/g)×weight of initial skin (g)] ×100

Hydroxyproline content in both supernatant (gelatin hydrolysate) and the initial skins was determined according to the method of Bergman and Loxley (1963).

2.4.5.2 Determination of α–amino group content

The α–amino group content was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µl), 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixture was cooled at room temperature for 15 min. L-leucine standard solutions with concentrations ranging from 0.5 to 5.0 mM were used. The absorbance was read at 470 nm and α–amino group content was expressed in terms of L-leucine.

2.4.5.3 Determination of antioxidative activity

2.4.5.3.1 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as described by Binsan *et al*. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The solution obtained (1 mL) was then diluted with 50 mL DI water, in order to obtain an absorbance of 1.1±0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a

spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600μM was prepared. The activity was expressed as μmol Trolox equivalent (TE)/g solid.

2.4.5.3.2 Ferrous chelating activity

Chelating activity of samples towards ferrous ion (Fe^{2+}) was measured by the method of Thiansilakul *et al*. (2007) with a slight modification. Sample (200 μl) was mixed with 800 μl of distilled water. Thereafter, 0.1 mL of 2.0 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as μmol EDTA equivalents (EE)/g solid.

2.4.5.3.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000) with a slight modification. Briefly, the sample (1 mL) was mixed with 83 μl of 100 mM hydrogen peroxide (prepared in 0.1 M phosphate buffer, pH 7.4). The mixture was allowed to react for 40 min at room temperature. The absorbance at 230 nm of the reaction mixture was read and the sample blank (devoid of hydrogen peroxide) was used for background subtraction. Trolox (0-10 mM) was used as the standard. The hydrogen peroxide scavenging activity was expressed as μmol TE/g solid.

2.4.6 Characterisation of gelatin hydrolysates

Gelatin hydrolysates with high ABTS radical scavenging activity including AU+TH30 and AU+HP2.0 were determined for protein patterns in comparison with AU, TH30 and HP2.0.

Protein patterns of gelatin hydrolysates were determined by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Samples solution (15 mL) was mixed with 5 mL of 5% SDS solution and then heated at 85°C for 1 h, followed by centrifugation at 8500 × g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS and 20% glycerol). Samples (10 µg proteins) were loaded onto polyacrylamide gel made of 15% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA gel^{-1}. After electrophoresis, gels were stained for 1 h with a mixture of 0.05% (w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained for 1 h with a mixture of 30% (v/v) methanol and 10% (v/v) acetic acid and destained again with the same mixture for 30 min. Low molecular weight markers including phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used to estimate the molecular weight of proteins or peptides.

2.4.7 Antioxidative activity and molecular weight distribution of the selected gelatin hydrolysates

HP2.0 and AU+HP2.0 were determined for antioxidative activity and size distribution.

2.4.7.1 Lecithin liposome model system

Antioxidative activity of the selected gelatin hydrolysates in a lecithin liposome model system was determined according to the method of Thiansilakul *et al*. (2007). Lecithin liposome system was prepared by suspending lecithin in deionised water at a concentration of 8 mg/mL. The mixture was stirred with a glass rod, followed by sonification for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Gelatin hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain a final concentration of 1.0, 2.0 and 10.0 g/L. The mixture was sonicated for 2 min. To initiate the reaction, 40 mL of 0.15 M cupric acetate were

added. The mixture was shaken in the dark at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37°C. The system containing 0.10 g/L Trolox was also prepared. The control was prepared in the same manner, except that distilled water was used instead of gelatin hydrolysates or Trolox. Oxidation in lecithin liposome systems was monitored during 24 h of incubation by determining of PV and TBARS values.

2.4.7.1.1 Determination of peroxide value (PV)

PV was measured according to the method of Wu *et al*. (2003). Liposome sample (0.1 mL) was mixed with 4.7 mL of ethanol/distilled water (3:1), 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride solution in 3.5% HCl. After incubation at 40°C for 3 min, the absorbance was read at 500 nm using a spectrophotometer. PV was calculated from a standard curve of cumene hydroperoxide (0-100 mg/L) and expressed as mg cumene hydroperoxide/L lecithin liposome.

2.4.7.1.2 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978) with a slight modification. Liposome sample (0.5 mL) was mixed using a vortex mixture (Vortex-Genie2 mixer, Scientific Industries, Bohemia, NY, USA) with 2.5 mL of TBARS solution (0.375% thiobarbituric acid, 15% TCA and 0.25 M HCl). The mixture was heated in boiling water for 10 min to develop the pink colour. The mixture was then cooled with running water and centrifuged at 5000 x *g* for 10 min at room temperature using Hettich centrifuge (Hettich Model MIKRO-20, Tuttlingen, Germany). The supernatant was collected and the absorbance at 532 nm was measured using a spectrophotometer. TBARS value was calculated from a standard curve of malondialdehyde (MDA) (0–10 mg/L) and expressed as mg MDA/L lecithin liposome.

2.4.7.2 Molecular weight distribution

Gelatin hydrolysates (HP2.0 and AU+HP2.0) were separated by size exclusion chromatography using a Sephadex G-25 gel filtration column (2.5 × 50 cm) (17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). The experiment was conducted in walked in cold room (4 °C) and 2 mL of sample solution (50 mg/mL) were loaded. After being loaded, the elution was performed using a low pressure chromatography system (BioLogic LP system, Bio-RAD Laboratories Ltd., Hercules, CA, USA) coupled with a fraction collector (Model 2128, Bio-RAD Laboratories Ltd., Hercules, CA, USA). Fifty mM sodium phosphate buffer (pH 7.0) was use as the elution buffer at a flow rate of 0.5 mL/min. The fractions of 3 mL were collected. The absorbance was recorded at 220 and 280 nm. ABTS radical scavenging activity of each fraction was determined. Blue dextran (2,000,000 Da) was use for void volume measurement. The molecular weight markers included insulin chain B (3495.89 Da), vitamin B_{12} (1355.4 Da), glycine-tyrosine (238.25 Da) and tyrosine (181.2 Da). MW of fraction with the highest ABTS radical scavenging activity was estimated from the plot between available partition coefficient (K_{av}) and the logarithm of the molecular weight of the protein standards.

2.4.8 Statistical analysis

All experiments were run in triplicate using different three lots of skin. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The data with $P<0.05$ were considered to be statistically significant.

2.5 Results and discussion

2.5.1 Effect of different processes on yield, α-amino group content and antioxidative activity of gelatin hydrolysate

2.5.1.1 Yield and α-amino group content

Yields of gelatin hydrolysates prepared by various processes are shown in Figure 4 (a). With autolysis, the resulting gelatin hydrolysate (AU) had the yield of 57.6%. During incubation at 55°C for 12 h, the heat applied was able to destroy hydrogen bonds stabilising triple helix of collagen in the pretreated skin. As a result, the conversion of collagen to gelatin took place. Simultaneously, indigenous protease with the optimum temperature of 55°C was activated (Kaewruang *et al*., 2013), thereby inducing hydrolysis of released gelatin. Based on inhibitor study, indigenous proteases in the skin of unicorn leatherjacket were classified as serine protease (Ahmad *et al*., 2011). When thermal hydrolysis was implemented, the yield of gelatin hydrolysate with heating time of 60 min (TH60) increased, compared with those of TH15 and TH30 ($P<0.05$). It was noted that yield of AU was not different from TH15 and TH30 ($P>0.05$). Autoclave used in the process more likely provided high energy for destroying the hydrogen bonds stabilising the collagen localised in the skin matrix (Yang *et al*., 2008; Wang *et al*., 2013). Peptide bonds of α- or β-chain were also cleaved to some extent with the sufficient autoclave time. When autolysis was performed prior to thermal hydrolysis using autoclave, the higher yield was obtained, compared with process without prior autolysis. Autolysis might loosen the skin matrix to some degrees. This might facilitate the cleavage of peptide by subsequent autoclave process. The highest yield (72.02%) was found for AU+TH60 sample ($P<0.05$) and was higher than that of TH60 sample (65.44%). When papain was used for preparing gelatin hydrolysate without and with prior autolysis, it was found that the yield increased with increasing papain levels. Prior autolysis generally resulted in the increased yield when the same level of papain was used. AU+HP2.0 sample had the yield of 69.12%, whereas HP2.0 sample showed the yield of 61.62%. Papain has been used for hydrolysis of gelatin (Yang *et al*., 2008; Wang *et al*., 2013). It was reported that papain preferably cleaved α-chain with the abundance of glycine residues

(Kamphuis *et al*., 1985). Yang *et al*. (2008) reported that papain could hydrolyse the retorted gelatin from cobia skin within 0.5 h using 1:100 enzyme substrate ratio. With prior autolysis, peptides released or loosen skin matrix could serve as the preferable substrate, rather than compact structure. This was evidenced by the higher yield when autolysis was conducted before the hydrolysis using papain.

The α-amino group content of hydrolysates prepared using different processes with and without prior autolysis is depicted in Figure 7 (b). Different α-amino group content was found amongst gelatin hydrolysates prepared by different processes. It was noted that α-amino group content of AU was similar to TH15 and TH30 ($P>0.05$). Prior autolysis did not increase α-amino group content of gelatin hydrolysate prepared by thermal hydrolysis process for all autoclave times used ($P>0.05$). When papain was used, α-amino group content in gelatin hydrolysate increased with increasing papain levels ($P<0.05$). This result was in accordance with Abdulazeez *et al*. (2012) who reported that the increase of papain/substrate (1.0, 2.0 and 4.0%) resulted in the increase of yield and degree of hydrolysis of protein hydrolysate from king fish. It was obvious that the marked increase in α-amino group content was observed in AU+HP samples, compared with HP samples. The result indicated that prior autolysis more likely provided the preferable substrate for papain. The partially hydrolysed peptides or loosen skin matrix could favour the migration of papain to substrate. Also, the exposed substrates were readily available for hydrolysis by both indigenous protease and papain. The increases in α-amino group content were in accordance with yield. The differences in α-amino group content amongst gelatin hydrolysate were observed, whilst the similar yield was noticeable. This reflected the difference in degree of hydrolysis in gelatin hydrolysates prepared with different processes. In other word, gelatin extracted was cleaved to varying degrees. It was noted that AU+HP showed the highest cleavage of peptides, especially when papain at the higher level was used.

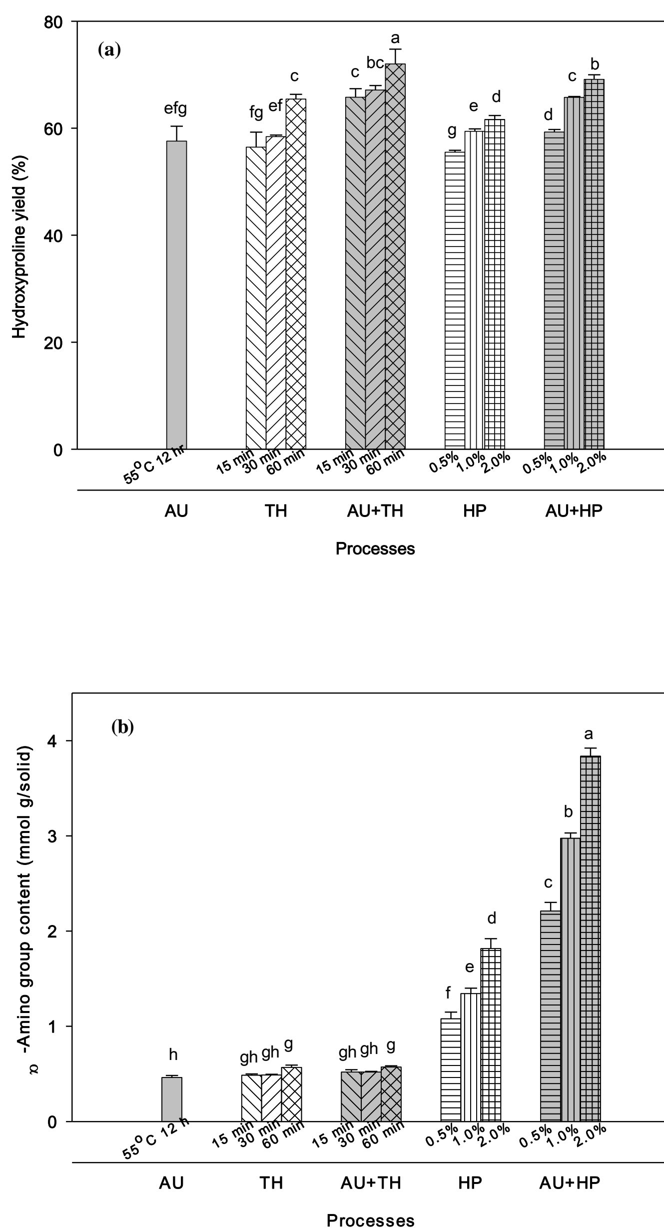

Figure 4 Yield (a) and α-amino group content (b) of gelatin hydrolysates from unicorn leatherjacket skin obtained from different processes. AU; Autolysis (55°C, 12 h), TH; Thermal hydrolysis (autoclave at 121°C), AU+TH; Prior autolysis followed by thermal hydrolysis, HP; Hydrolysis using papain, AU+PH; Prior autolysis followed by hydrolysis using papain. Bars represent standard deviation (n=3). Different letters on the bars indicate significant differences ($P<0.05$).

2.5.1.2 Antioxidative activity

Table 3 shows the antioxidative activity of gelatin hydrolysates obtained from different processes as examined by different assays. ABTS radical scavenging activity of AU sample was 120.28 μmol TE/g solid. For TH sample, a slight increase in activity was found with increasing autoclave time ($P<0.05$). Prior autolysis could enhance activity of hydrolysate in some TH samples. This might reflect the change in antioxidative peptides caused by autolysis in conjunction with autoclave. ABTS radical scavenging assay is based on the measurements using $ABTS^{+}$/ABTS redox couple. The ability of antioxidants to scavenge the preformed $ABTS^{+}$ was estimated by the decolourisation (Re *et al*., 1999). Prior autolysis, followed by autoclave generally resulted in the enhanced ABTS radical scavenging activity of resulting hydrolysate. For HP samples, the increase in ABTS radical scavenging activity was obtained when papain above 1% was used ($P<0.05$). HP samples showed the activity of 126.64-146.44 μmol TE/g solid. AU+HP possessed higher ABTS radical scavenging activity (135.62-159.71 μmol TE/g solid) ($P<0.05$), compared with HP. The results were in accordance with increasing α-amino group content in hydrolysate, especially AU+HP sample (Figure 7 (b)). Smaller peptides in AU+HP samples more likely had the higher ability in scavenging ABTS radicals than those present in hydrolysates produced by other processes.

Ferrous chelating activity of gelatin hydrolysates is shown in Table 3. AU sample had chelating activity of 8.94 μmol EE/g solid. For TH samples, TH30 and TH60 showed higher activity than TH15 ($P<0.05$). Nevertheless, the decrease in chelating activity ($P<0.05$) was observed in AU+TH60, compared with AU+TH15 and AU+TH30 ($P<0.05$). The result indicated that different peptides in hydrolysates might have varying ability in complexing with Fe^{2+} (Balti *et al*., 2011). Similar trend was observed for AU+HP samples, in which papain at higher levels caused the decreases in Fe^{2+} chelating activity of AU+HP sample. However, the increase of chelating activity was observed in HP samples with papain at higher level. Liu *et al*. (2010) reported that the cleavage of peptides led to an enhanced metal ion binding due to the increased concentration of carboxylic groups and amino groups of the peptides.

In addition, the chelating activity of gelatin hydrolysate was much higher than that of gelatin, approximately by 7.23-25.08 folds (Liu *et al*., 2010). It is well-known that transition metal ions such as iron or copper may catalyse the formation of reactive oxygen species that accelerate lipid oxidation. Thus, gelatin hydrolysates obtained in the present study were able to prevent lipid oxidation via metal chelating ability.

H_2O_2 scavenging activity of different gelatin hydrolysates is shown in Table 3. AU sample had H_2O_2 scavenging activity of 739.73 μmol TE/g solid. TH and AU+TH samples possessed the lower H_2O_2 scavenging activity than AU sample ($P<0.05$), regardless of thermal conditions or prior autolysis. On the other hand, HP sample had the decrease in activity as papain at levels of 1.0 and 2.0% was used ($P<0.05$). However, the increase in H_2O_2 scavenging activity was obtained in AU+HP2.0 sample (766.84 μmol TE/g solid) and its activity was higher than AU+HP0.5 and AU+HP1.0 samples ($P<0.05$). This was coincidental with the increase in α-amino group content in AU+HP2.0 sample (Figure 7 (b)). The small peptides might exhibit the higher ability in scavenging H_2O_2. H_2O_2 has been known to be generated *in vivo* by several oxidising enzymes, such as superoxide dismutase. Together with reactive oxygen species (ROS), it can be broken down to hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($O_2^{\bullet -}$) via Fenton reaction in the presence of Fe^{2+}. $^{\bullet}OH$ and $O_2^{\bullet -}$ can initiate lipid peroxidation and are toxic to cells. Therefore, it is crucial for cells to remove H_2O_2 via an antioxidant defense (Wang *et al*., 2007).

In the present study, it was found that prior autolysis could enhance the yield and antioxidative activity of gelatin hydrolysates. Therefore, AU+TH30 and AU+HP2.0 were determined for protein pattern in comparison with AU, TH30 and HP2.0samples.

Table 3 Antioxidative activity of gelatin hydrolysates from unicorn leatherjacket skin obtained from different processes

Processes	ABTS radical scavenging activity (µmol TE/g solid)	Ferrous chelating activity (µmol EE/g solid)	H_2O_2 scavenging activity (µmol TE/g solid)
AU	120.28±2.16*fgh**	8.94±0.13d	739.73±7.74b
TH15	115.84±1.55h	6.95±0.08e	582.70±6.06d
TH30	124.11±2.23efg	10.84±0.28b	548.33±5.72e
TH60	127.44±6.05ef	10.10±0.01bc	548.09±0.09e
AU+TH15	122.30±4.67cd	9.58±0.04cd	602.79±3.62c
AU+TH30	132.33±3.25de	10.67±0.57b	580.93±6.47d
AU+TH60	117.05±3.41gh	4.83±0.78f	507.59±7.47f
HP0.5	126.64±1.25de	9.16±0.02d	764.33±5.16a
HP1.0	127.40±1.25de	10.81±0.04b	746.61±4.91b
HP2.0	146.44±5.74b	12.35±0.51a	744.60±2.41b
AU+HP0.5	135.62±3.24c	10.28±0.24bc	742.84±4.90b
AU+HP1.0	143.09±3.87b	6.33±0.19e	745.35±5.71b
AU+HP2.0	159.71±3.43a	4.92±0.01f	766.84±0.04a

AU; Autolysis lysis, 55°C, 12 hr. TH15, TH30, TH60; Thermal hydrolysis, autoclave at 121°C for 15, 30, 60 min, respectively. HP0.5, HP1.0, HP2.0; Hydrolysis using papain at 0.5, 1.0, 2.0%, respectively. * mean ± standard deviation (n=3). ** Different letters in the same column indicate significant different ($P<0.05$).

2.5.2 Protein patterns of the selected gelatin hydrolysates

Protein patterns of gelatin hydrolysates as analysed by SDS-PAGE are shown in Figure 5. Pretreated skin, a starting material, contained α_1-chain and α_2-chain with the ratio of approximately 2:1, suggesting the presence of type I collagen in the skin. This result was in agreement with Kaewruang *et al*. (2013) who reported that gelatin extracted from unicorn leatherjacket contained α-chains as the major components. The gelatin hydrolysate obtained from autolysis process (AU) showed some degradation, with the appearance of peptides with MW below β- and α-chains. This suggested the role of the indigenous proteases in hydrolysis of skin components. A similar electrophoretic profile was reported for unicorn leatherjacket gelatin obtained by extracting at 55°C (Kaewruang *et al*., 2013). Degradation due to the action of indigenous proteases was found at 55°C (Kaewruang *et al*., 2013). When thermal process (autoclave) was implemented, TH30 sample showed some degradation of both α- and β-chains. For AU+TH30, the higher degradation was observed and no α- and β-chains were retained. In addition, AU+TH30 contained peptides with MW of 66 kDa. This result indicated that prior autolysis could provide more proteolysis, compared with AU and TH30 samples. HP2.0 had no high molecular weight molecules (β-, α- and γ-chain) retained with coincidental formation of peptides, ranging from 20 to 25 kDa. However, AU+HP2.0 sample had no peptides detected by SDS-PAGE with 15% separating gel. This result indicated the drastic degradation occurring in AU+HP2.0. This result was in accordance with the highest α-amino group content in AU+HP2.0 sample. Papain is a one of the four cysteine proteinases from papaya latex. It has high specificity to cleave peptide bonds with Gly at P_1 (Kamphuis *et al*., 1985). Moreover, fish skin gelatin with a glycine content of around 1/3 of amino acid composition could be a preferable substrate for papain.

The results revealed that enzymatic processes were appropriate for production of gelatin from pretreated skin with antioxidative activity, especially when prior autolysis was implemented. Therefore, HP2.0 and AU+HP2.0 samples were prepared and subjected to characterisation and determination for antioxidative activity in lecithin liposome model system.

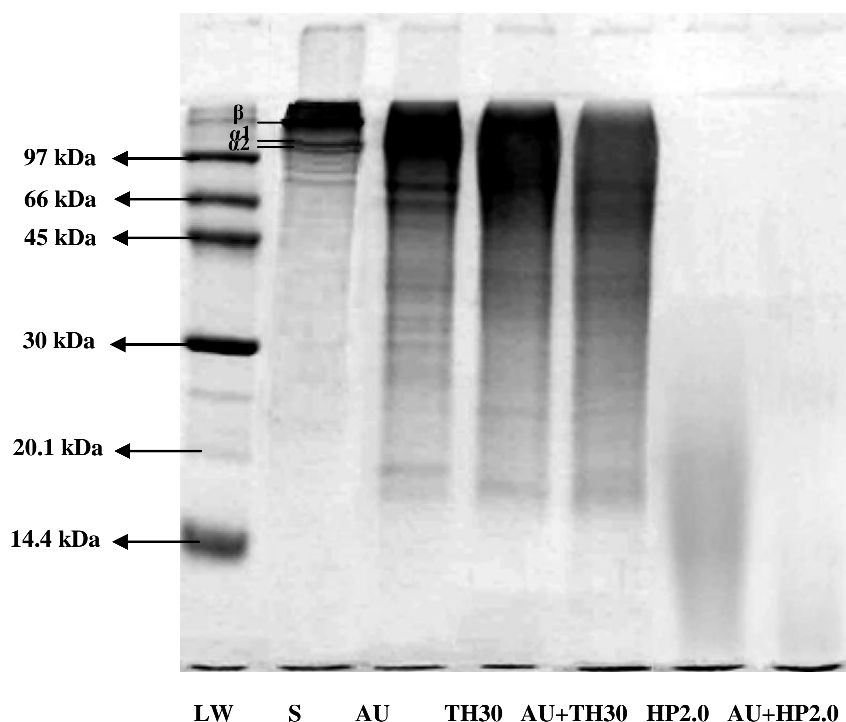

Figure 5 SDS-PAGE patterns of pretreated skin (PS) and gelatin hydrolysates from unicorn leatherjacket skin obtained from different processes. AU; Autolysis (55°C, 12 h), TH30; Thermal hydrolysis (autoclave at 121°C, 30 min), AU+TH30; Prior autolysis followed by thermal hydrolysis (autoclave at 121°C, 30 min), HP2.0; Hydrolysis using 2.0% papain and AU+HP2.0; Prior autolysis followed by hydrolysis using 2.0% papain.

2.5.3 Antioxidative activity and molecular weight distribution of the selected gelatin hydrolysates

2.5.3.1 Lecithin liposome system

Oxidation of lecithin liposome system containing HP2.0 and AU+HP2.0 at different concentrations (1.0-10.0 g/L) was monitored by determining peroxide value (PV) (Figure 6 (a)) and TBARS values (Figure 6 (b)) during incubation for 24 h at 37°C. For the control and systems added with 1.0-2.0 g/L gelatin hydrolysates, the sharp increase in PV was found within the first 6 h of incubation. PV remained unchanged during 6-12 h, followed by a sharp decrease up to 24 h. The systems added with AU+HP2.0 showed the lower PV than those containing HP2.0 ($P<0.05$). In addition, the control system showed the higher PV than

those added with hydrolysates and trolox (P<0.05). Notably, very low increases in PV of liposome system added with 10.0 g/L HP2.0 and AU+HP2.0 samples were observed throughout 24 h of incubation. This preventive effect on lipid oxidation was comparable to that of 0.1 g/L trolox. The results indicated that AU+HP2.0, especially at high concentration, could retard the formation of hydroperoxide in liposome system. The sharp decreases in PV after 12 h of incubation were more likely caused by the decomposition of hydroperoxides to the secondary products (Frankel *et al*., 1997). Several peptides from gelatin hydrolysate were reported to possess antioxidative activity. Phanturat *et al*. (2010) also reported that gelatin hydrolysate from bigeye snapper skin could retard the oxidation of lecithin liposome system.

Changes in TBARS of liposome system during incubation for 24 h are depicted in Figure 6 (b). The increase in TBARS indicated the formation of the secondary lipid oxidation products. The marked increases in TBARS were observed in the first 18 h of incubation (P<0.05) in the control and all systems containing gelatin hydrolysates at levels of 1.0 and 2.0 g/L. Thereafter, the gradual decreases in TBARS were observed during 18-24 h. However, the lower value of TBARS was observed for all systems containing gelatin hydrolysates than that of the control system (P<0.05). It was noted that the slight change in TBARS of liposome system added with 10.0 g/L gelatin hydrolysates, both HP2.0 and AU+HP2.0, was observed throughout 24 h of incubation and was comparable to that of 0.1 g/L trolox. The result suggested that HP2.0 and AU+HP2.0 could retard the formation of the secondary oxidation products effectively, especially at the high concentration (10.0 g/L). The formation of TBARS indicated that the primary products were decomposed to the secondary products, especially aldehydes. The decrease in TBARS when the incubation time increased was probably due to the loss in those volatile secondary products (Phanturat *et al*., 2010). Thus, HP2.0 and AU+HP2.0 samples could retard the early stages as well as the advanced stage of oxidation, mainly via their radical scavenging activity.

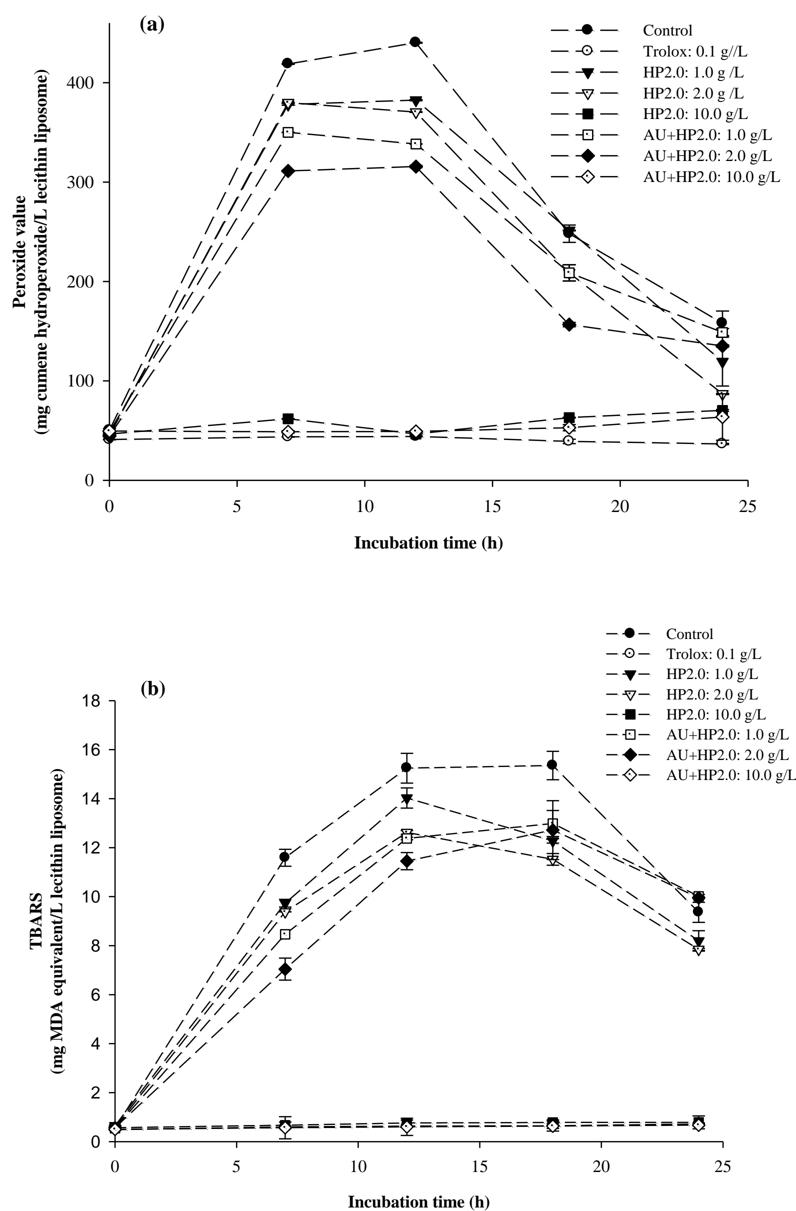

Figure 6 The formation of peroxide value (a) and TBARS (b) in lecithin liposome system containing gelatin hydrolysates from unicorn leatherjacket skin. HP2.0; Hydrolysis using 2.0% papain and AU+HP2.0; Prior autolysis followed by hydrolysis using 2.0% papain. Bars represent standard deviation (n=3).

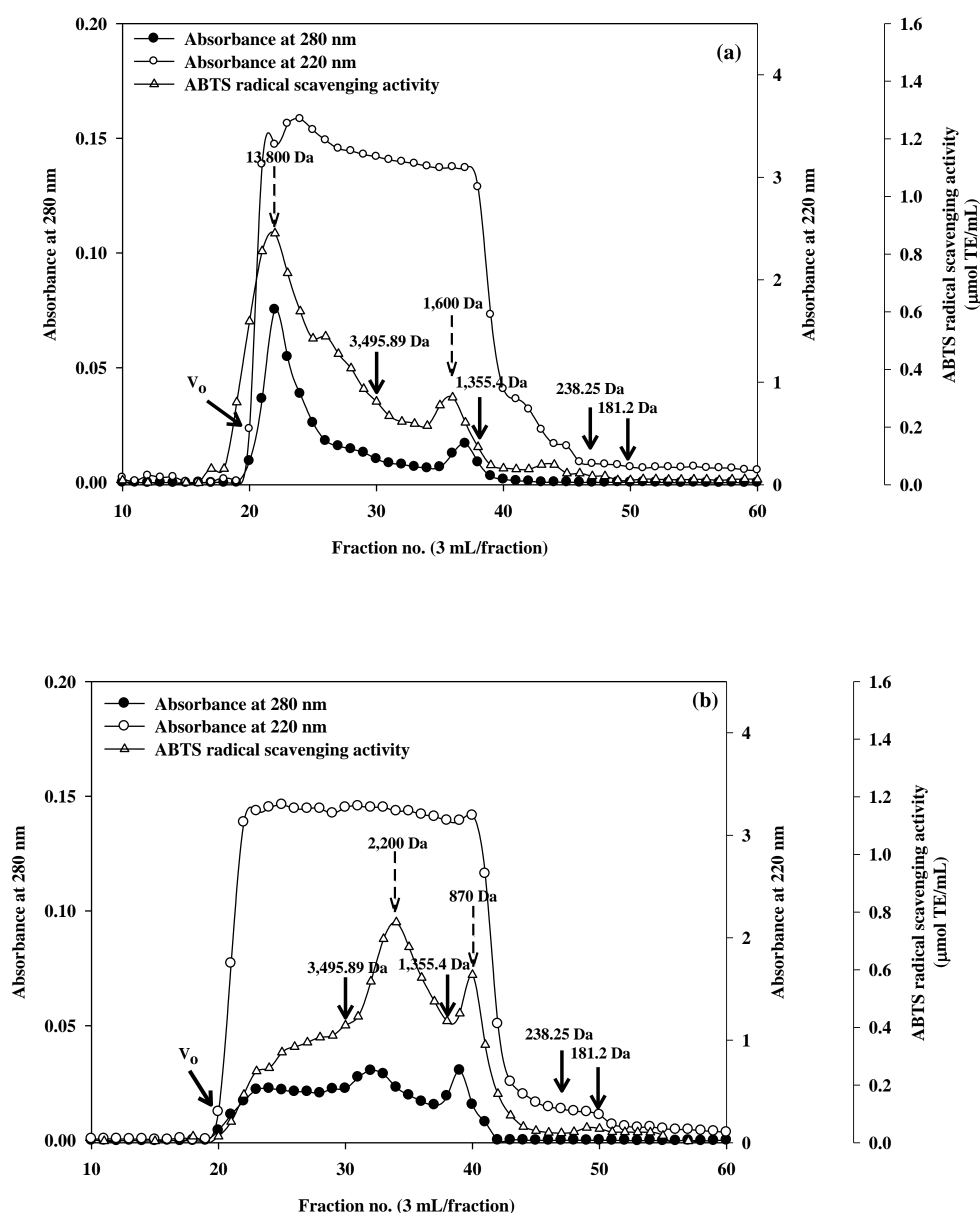

Figure 7 Elution profile by Sephadex G-25 size exclusion chromatography and antioxidative activity of gelatin hydrolysates from unicorn leatherjacket skin. (a) HP2.0; Hydrolysis using 2.0% papain and (b) AU+HP2.0; Prior autolysis followed by hydrolysis using 2.0% papain. Absorbance at 280 nm (●), 220 nm (○), ABTS radical scavenging activity (Δ), molecular weight markers (→) molecular weight of fraction (-->).

2.5.3.2 Molecular weight distribution

Elution profiles of HP2.0 and AU+HP2.0 samples on the Sephadex™-G25 gel filtration chromatography are shown in Figure 7. Each hydrolysate had two major peaks of A_{280}, indicating the presence of peptides containing aromatic amino acids with varying MW (Figure 7). Peaks of A_{220} represent the peptides in the fractions collected. As shown in Figure 7 (a), HP2.0 sample contained peptide with MW of 13,800 Da, which showed the highest ABTS radical scavenging activity. Additionally, HP2.0 also contained antioxidative peptides with MW of 1,600 Da. For AU+HP2.0 sample, it showed the different elution profile from that of HP2.0. Peptide with MW of 2,200 Da was the dominant peptide with ABTS radical scavenging activity in AU+HP2.0 sample. In addition, peptides with MW of 870 Da also had ABTS radical scavenging activity. In general, small peptides possess high antioxidative activity (Liu *et al*., 2010; Phanturat *et al*., 2010). Kim *et al*. (2001) found that peptides in gelatin hydrolysate peptides with MW ranging from 1,500 to 4,500 Da showed antioxidative activity. In addition, the gelatin hydrolysates from cobia (*Rachycentro canadum*) skin containing peptides having a MW below 700 Da exhibited antioxidative activity (Yang *et al*., 2008). The result suggested that hydrolysate most likely contained certain peptides with radical scavenging activity, which could terminate radical chain reaction.

2.6 Conclusion

Autolysis assisted process had the potential for the production of gelatin hydrolysates with antioxidative activity from unicorn leatherjacket skin. The hydrolysis using papain with prior autolysis, especially AU+HP2.0, could enhance the yield and antioxidative activity of gelatin hydrolysates. Peptide with MW of 2,200 Da was the potential antioxidative peptide in AU+HP2.0. Thus, autolysis mediated by indigenous protease in the skin could be exploited for production of peptides with antioxidative activity.

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CHAPTER 3

Glycyl endopeptidase from papaya latex: Partial purification and the use for production of fish gelatin hydrolysate

3.1 Abstract

An aqueous two-phase system (ATPS) in combination with ammonium sulphate ($(NH_4)_2SO_4$) precipitation was applied to fractionate glycyl endopeptidase (GE) from the papaya latex of Red Lady and Khack Dum cultivars. ATPS containing polyethylene glycol (PEG 2000 and 6000) and salts ($(NH_4)_2SO_4$ and $MgSO_4$) at different concentrations were used. GE with high purity fold (PF) and yield was found in the salt-rich bottom phase of ATPS with 10% PEG 6000-10% $(NH_4)_2SO_4$. When ATPS fraction from Red Lady cultivar was further precipitated with 40-60% saturation of $(NH_4)_2SO_4$, PF of 2.1-fold with 80.23% yield was obtained. Almost offensive odourous compounds, particularly benzyl isothiocyanate, were removed from partially purified GE. The fish gelatin hydrolysates prepared using GE showed higher ABTS radical scavenging activity with the lower enzyme odourous compounds, compared with those of crude extract (CE). Thus antioxidative gelatin hydrolysate with negligible undesirable odour could be prepared with the aid of GE.

3.2 Introduction

Carica papaya is widely cultivated in tropical and subtropical regions all around the world. Apart from the edible fruits, enzymes stored in its lactiferous cells can be produced and have found several applications (de Oliveira and Vitória, 2011). When these cells rupture, the coagulation of latex occurs. This represents an important defense mechanism of the plant against pathogens and other harmful attacks. In addition, the latex of *C. papaya* is a rich source of the cysteine endopeptidases, including papain, glycyl endopeptidase, chymopapain and caricain, constituting more than 80% of whole enzyme (Azarkan *et al*., 2003). Papaya latex was used for preparing protein hydrolysates with bioactivities (Kittiphattanabawon *et al.*, 2012; Ngo *et al.*, 2011). Due to the abundance of glycine in gelatin molecules, glycyl endopeptidase, a major component which constitutes almost 30% of total protein in the latex of *C. papaya*, can serve as potential protease, which preferably cleaves the peptide bonds in gelatin. However, undesirable off-odour of crude papaya latex leads to the offensive odour or flavour in the resulting gelatin hydrolysates, thereby causing the consumer rejection.

The volatile compounds of various papaya cultivars have been extensively investigated (Pino *et al*., 2003). Twenty-five odorants were considered as odour-active compounds and contribute to the typical papaya aroma. The pungent-sour and green-note odours found in the green fruit are due to benzyl isothiocyanate and some C6 compounds (e.g., 1-hexen-3-one), respectively (Pino, 2014). Ulrich and Wijiya (2010) found that stinky and smokey odours were caused by butanoic acid and benzyl isothiocyanate. Therefore, the crude enzyme obtained from green fruit latex might contain those odourous compounds. When papaya latex proteases were employed, those compounds mostly contribute to offensive offodour in resulting gelatin hydrolysate, thus obstructing the extensive utilisation and consumption of hydrolysate.

Among the recent studies, there are many techniques were applied in protein concentration and purification, such as membrane-aided filtration. However, the adverse effects of operating condition have been concerned on enzyme

denaturation (Nakkeeran and Subramanain, 2010; Krstiic *et al*., 2007,). Aqueous-two-phase system (ATPS) is a good choice which offers mild and non-disruptive purification conditions for biomolecules, especially enzyme (Prinz *et al*., 2012). ATPS has been widely employed as an effective and economical process for the separation, purification and concentration of enzymes (Subathra *et al*., 2012; Rawdkuen *et al*., 2011; Ketnawa and Rawdkuen, 2011). ATPS can remove the undesirable compounds present in the system including unidentified polysaccharides, interfering protein and contaminants (Dubey and Jagannadham, 2003). Chaiwut *et al.* (2007) used ATPS followed by salt precipitation for isolation of glycyl endopeptidase from papaya latex. Therefore, ATPS can be used to fractionate glycyl endopeptidase in papaya latex and remove the offensive odourous compounds under the appropriate condition. As a consequence, the more active fraction without undesirable odour could be prepared from papaya latex and further used as a processing aid in production of gelatin hydrolysate.

3.3 Objectives

To fractionate glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars grown in Thailand, using ATPS and ammonium sulphate precipitation, and to determine odorous compounds in the obtained fraction.

To determine the antioxidative activities and odourous compound of fish gelatin hydrolysate prepared using glycyl endopeptidase rich fraction.

3.4 Materials and methods

3.4.1 Chemicals

Fish skin gelatin from tilapia was purchased from Lapi Gelatine S.p.A (Empoli, Italy). Polyethylene glycol (PEG) was obtained from Fluka (Buchs, Switzerland). 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2′-azinobis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), sodium dodecyl sulphate (SDS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4´´-disulfonic acid sodium salt (ferrozine) were

purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol and trichloroacetic acid (TCA), ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Ammonium thiocyanate was purchased from Lab-Scan (Bangkok, Thailand). Coomassie Blue R-250, and *N,N,N´,N´*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Boc-Ala-Ala-Gly-pNA was obtained from Bachem Inc. (Torrance, CA, USA). Low molecular weight marker was purchased from GE healthcare UK, Limited (Buckinghamshire, UK). All chemicals were of analytical grade.

3.4.2 Preparation of crude extract from papaya (*C. papaya*) latex

Fresh papaya latex was collected from two cultivars (Red Lady and Khack Dum) in Hat Yai, Thailand. Four to six longitudinal incisions were made on the green papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

To prepare crude extract, the latex was mixed with cold distilled water (≤4 °C) with a latex to water ratio of 1:1 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000×g at 4 °C for 20 min using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant was filtered using a Whatmam No.1 filter paper, followed by freeze-drying (Kittiphattanabawon *et al*., 2012). These crude extract powders from papaya latex of Red Lady and Khack Dum cultivars referred to as CE-RL and CE-KD, respectively, were kept at -40 °C until use.

3.4.3 Fractionation of glycyl endopeptidase using aqueous two-phase system (ATPS)

ATPS was prepared in 10-mL centrifuge tube according to the method of Nitsawang *et al*. (2006) and Rawdkuen *et al*. (2011). Crude extract powder (1 g) was dissolved in 8 mL of distilled water. The pH of solution was adjusted to 6.0 using

6 M HCl and the volume was made up to 10 mL by distilled water to obtain a concentration of 100 mg/mL prior to fractionation using ATPS.

3.4.3.1. Effect of salts on fractionation of glycyl endopeptidase from papaya latex

To study the effect of salts on the partitioning of glycyl endopeptidase from papaya latex using ATPS, $(NH_4)_2SO_4$ or $MgSO_4$ at different concentrations (10, 15, 20 and 25% w/w) was added in conjunction with 10% PEG (2000 and 6000 Da) in an aqueous system. Crude extract solution (100 mg/mL) was added into the system to obtain 20% (w/w). Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixture was mixed continuously for 15 min using a Vortex mixer (Vortex-genie2, G-560E, USA). Phase separation was achieved by centrifuging the mixture at 9,000×g for 20 min at 4 °C. The salt-rich bottom phase was carefully separated using a pipette. Volumes of both top and bottom phases were measured and recorded. The enzyme activity was evaluated and protein content was determined using the Bradford method (Bradford, 1976) in both phases. The phase composition giving the highest yield and purification was chosen for further study.

3.4.3.2 Effect of PEG on fractionation of glycyl endopeptidase from papaya latex

$(NH_4)_2SO_4$ at 10% was used in the system. PEG (2000 and 6000 Da) at different concentrations (10, 15 and 20%, w/w) were used. The biphasic systems were generated after addition of crude extract and distilled water as described previously.

3.4.3.3 Calculation of ATPS parameters

Top and bottom phases from all tested ATPS were subjected to calculation of ATPS parameters. Yield, specific activity (SA), purification fold (PF), partition coefficient of protein concentration (KP) and volume ratio (VR) were calculated as follows:

$$\text{Yield}(\%)=\frac{A_T}{A_i}\times 100$$

where A_T is total glycyl endopeptidase activity in the protease rich phase and A_i is the initial glycyl endopeptidase activity of the crude extract before being partitioned.

$$SA(\text{unit/mg protein})=\frac{\text{Glycyl endopeptidase activity}}{\text{protein concentration}}$$

$$PF=\frac{SA_e}{SA_i}$$

where SA_e is the SA of each phase and SA_i is the initial SA of the crude extract before being partitioned.

$$KP=\frac{C_T}{C_B}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively.

$$VR=\frac{V_T}{V_B}$$

where V_T and V_B are top and bottom phase volume, respectively.

Based on purity and recovery yield, the ATPS containing PEG at the concentration rendering the most effective partitioning was chosen for further study.

3.4.4 Ammonium sulphate precipitation

Glycyl endopeptidase was further precipitated from the selected ATPS fraction by ammonium sulphate at different saturations (28-80% saturation). After centrifugation at 9,000×g at 4 °C for 20 min, the pellet was re-dissolved in distilled water and dialysed against 20 volumes of distilled water for 6 times. After lyophilisation, enzyme powder was stored at -40 °C until use. The partially purified glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars referred to as “GE-RL” and “GE-KD”, respectively, were subjected to characterisation.

3.4.5 Assay for glycyl endopeptidase activity

Activity of glycyl endopeptidase was determined following the method of Buttle (1994). The enzyme solution (200 μL) was mixed with 500 μL of activating agent (40 mM cysteine/20 mM Na_2·EDTA in 0.5 M phosphate buffer, pH 7.5). The phosphate buffer (275 μL, pH 7.5) was added and the mixture was incubated in a water bath (Model W350, Memmert, Schwabach, Germany) at 40 °C for 5 min. The reaction was then started by adding 25 μL of substrate solution (50 mM Boc-Ala-Ala-Gly-pNA in dimethylsulfoxide). After 8 min, 1 mL of stopping reagent (50% TCA, w/v) was added. The reaction mixture was centrifuged at 5,000 ×g for 10 min, and the absorbance of the supernatant containing the released *p*-nitroaniline was measured at 410 nm. Blank was prepared in the same manner, except the substrate was added after addition of stopping reagent. One unit of enzyme activity was defined as the amount of enzyme causing an increase of 0.1 in absorbance per min under the assayed condition.

3.4.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

3.4.6.1 Protein staining

SDS-PAGE of crude extract and partially purified glycyl endopeptidase from both cultivars were performed according to the Laemmli method (Laemmli, 1970). Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The mixture was boiled for 3 min. The samples (15 μg protein) were loaded onto the gel made of 4% stacking and 15% separating gels. They were subjected to an electrophoresis set at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was stained overnight with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid. Protein patterns were then visualised after destaining with 30% methanol and 10% acetic acid until a clear background was obtained.

3.4.6.2 Protease activity staining

The protease separated using SDS-PAGE was subjected to activity staining as per the method of Garcia-Carreno *et al*. (1993). Sample was mixed with sample buffer as mentioned previously. However, the mixture was not boiled prior to loading onto the gel (4% stacking and 15% separating gel). After electrophoresis, the gel (3 µg protein each lane) was washed in 2.5% Triton X-100 at 4 °C for 15 min to remove SDS and renature the proteins. The gels were then washed again with distilled water and incubated with 2% casein in 50 mM phosphate buffer pH 7.5, containing 40 mM cysteine in a water bath for 1 h at 40 °C. The gels were washed again with distilled water, fixed, stained and destained as described above. The appearance of a clear zone on the dark background indicated protease activity. The bands with protease activity were calculated for their molecular weights.

3.4.7 Preparation of gelatin hydrolysate using crude extract and partially purified glycyl endopeptidase from papaya latex

3.4.7.1 Determination of protease activity

Both crude extract and partially purified glycyl endopeptidase (1.0 mg/mL) were determined for protease activity as per the method of Vallés *et al.* (2007) with a slight modification. The enzyme solution (0.1 mL) was mixed with 1.1 mL of 1% (w/v) casein in 0.1M Tris–HCl, pH 7.0 containing 12 mM cysteine. The mixture was incubated at 37 °C for 20 min. The reaction was stopped by adding 1.8 mL of 5% TCA. After centrifugation at 3,000×g for 15 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit was defined as the amount of enzymes causing an increase of 1.0 absorbance unit per min under the assayed condition (Vallés *et al*., 2007).

3.4.7.2 Comparative study on gelatin hydrolysis

Crude extract or partially purified glycyl endopeptidase from both cultivars were added to the commercial fish skin gelatin solution (3%, w/v) at a level of 40 units/g protein. During hydrolysis at 40 °C, the sample was taken every 10 min

for 2 h, followed by enzyme inactivation by heating at 90 °C for 15 min in a temperature controlled water bath. The mixture was then centrifuged at 5,000×g for 10 min. The supernatant was determined for α-amino group content and ABTS radical scavenging activity. Crude extract and partially purified glycyl endopeptidase from the cultivar yielding the highest hydrolysis were selected. The hydrolysis time providing the highest α-amino group content within the range of initial velocity was chosen for further study.

3.4.7.3 Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

The crude extract and partially purified glycyl endopeptidase from Red Lady cultivar papaya latex were used to produce gelatin hydrolysate with different DHs (10, 15, 20 and 25% DH) as per the method of Benjakul and Morrissey (1997). Fish gelatin solution (3%, w/v) was added with enzyme at different concentrations (40, 80, 160, 320 and 640 Unit/g protein). The mixture was incubated at 40 °C for 1 h, the enzyme was then inactivated by heating at 90 °C for 15 min in a temperature controlled water bath. DH of the gelatin hydrolysates was measured. Log_{10} (enzyme concentration) vs. DH was plotted and enzyme concentrations required to hydrolyse fish gelatin solution to obtain the desired DHs were calculated from the regression equation

After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and freeze-dried. The gelatin hydrolysate powder was placed in polyethylene bag and stored at -40 °C. Hydrolysate powder was also determined for antioxidative activities.

3.4.7.4 Determination of α–amino group content

The α–amino group content was determined according to the method of Benjakul *et al*. (1997). To diluted samples (125 μL), 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50°C for 30

min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixture was cooled at room temperature for 15 min. L-leucine standard solutions with concentrations ranging from 0.5 to 5.0 mM were used. The absorbance was read at 420 nm and α–amino group content was expressed in terms of L-leucine.

3.4.7.5 Estimation of degree of hydrolysis (DH)

The obtained hydrolysates were subjected to the determination of DH according to method of Benjakul *et al*. (1997). DH was calculated using the following equation:

$$\mathrm{DH} = \frac{(L_t - L_0)}{(L_{max} - L_0)} \times 100$$

where L_t corresponds to the amount of α-amino acid released at time t. Lo is the amount of α–amino acid in original sample. L_{max} is the maximum amount of α–amino acid in sample obtained after acid hydrolysis (6 N HCl for 12 h at 100 °C).

3.4.8 Determination of antioxidative activities

3.4.8.1 ABTS radical scavenging activity

ABTS radical scavenging activity of gelatin hydrolysates was determined as described by Binsan, Benjakul, Visessanguan, Roytrakul, Tanaka and Kishimura (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The solution obtained (1 mL) was then diluted with 50 mL distilled water, in order to obtain an absorbance of 1.1±0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 μL) was mixed with 2850 μL of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard

curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalent (TE)/g protein.

3.4.8.2 Ferrous chelating activity

Chelating activity of gelatin hydrolysates towards ferrous ion (Fe^{2+}) was measured by the method of Thiansilakul, Benjagul and Shahidi (2007) with a slight modification. Sample (200 μL) was mixed with 800 μL of distilled water. Thereafter, 0.1 mL of 2.0 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as μmol EDTA equivalents (EE)/g protein.

3.4.8.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000) with a slight modification. Briefly, the sample (1 mL) was mixed with 83 μl of 100 mM hydrogen peroxide (prepared in 0.1 M phosphate buffer, pH 7.4). The mixture was allowed to react for 40 min at room temperature. The absorbance at 230 nm of the reaction mixture was read and the blank (devoid of hydrogen peroxide) was used for background subtraction. Trolox (0-10 mM) was used as the standard. The hydrogen peroxide scavenging activity was expressed as μmol TE/g protein.

3.4.9 Measurement of volatile compounds

The volatile compounds in crude extract and partially purified glycyl endopeptidase as well as their corresponding gelatin hydrolysates with 25% DH were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Pino (2014) with a slight modification.

3.4.9.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 80 mg of sample were mixed with 4 mL of deionised water and stirred continuously to dissolve the sample. The mixture was heated at 60 ºC in 20 headspace vial with equilibrium time of 10 h. The SPME fibre (50/30 μm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC for 15 min before use and then exposed to the headspace. The 20 mL-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

3.4.9.2 GC–MS analysis

GC–MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m ± 0.25 mm ID, with film thickness of 0.25 μm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas with a constant flow of 1 mL/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150μA, and the electron multiplier voltage at 500 V.

3.4.9.3 Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds including aldehydes, ester, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

3.4.10 Statistical analysis

All experiments were run in triplicate using different three lots of latex. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The data with $P<0.05$ were considered to be statistically significant.

3.5 Results and discussion

3.5.1 Effect of ATPS and ammonium sulphate precipitation on fractionation of glycyl endopeptidase from papaya latex

3.5.1.1 Effect of ATPS

To fractionate glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars, several ATPS comprising PEG (2000 and 6000) at 10% in the presence of $(NH_4)_2SO_4$ and $MgSO_4$ at various concentrations (10, 15, 20 and 25% (w/w)) were used (Table 4 and 5). It was found that ATPS containing 10% PEG 2000 and 10% $(NH_4)_2SO_4$ or 10-15% $MgSO_4$ had no phase separation. Both salts in the range used could not generate the two-phase formation. The mechanism of biphasic generation in PEG-salt system is dependent on balancing of enthalpic and entropic

Table 4 Effect of phase composition in PEG-salts ATPS on partitioning of glycyl endopeptidase from papaya latex of Red Lady cultivar

Phase composition (%, w/w)	VR	KP	SA	PF	Yield (%)
10%PEG 2000–10% $(NH_4)_2SO_4$	ns	ns	ns	ns	ns
10%PEG 2000–15% $(NH_4)_2SO_4$	0.32±0.04e	0.67±0.02e	1965.30±26.41d	1.58±0.02d	96.60±1.30bc
10%PEG 2000–20% $(NH_4)_2SO_4$	0.25±0.04f	1.36±0.13c	1534.36±7.46f	1.24±0.01f	40.72±0.20f
10%PEG 2000–25% $(NH_4)_2SO_4$	0.21±0.06f	4.94±0.46a	260.65±1.58i	0.21±0.00g	1.61±0.01hi
10%PEG 6000–10% $(NH_4)_2SO_4$	0.67±0.06c	0.76±0.01d	2442.71±38.91b	1.97±0.03b	98.97±1.58a
10%PEG 6000–15% $(NH_4)_2SO_4$	0.56±0.00d	1.01±0.14c	2524.89±48.39a	2.04±0.04a	90.29±1.73e
10%PEG 6000–20% $(NH_4)_2SO_4$	0.32±0.03e	2.93±0.06b	908.58±10.68g	0.73±0.01h	10.87±0.13g
10%PEG 6000–25% $(NH_4)_2SO_4$	0.32±0.00e	5.00±0.28a	494.60±10.33h	0.40±0.01i	2.93±0.06h
10%PEG 2000–10% $MgSO_4$	ns	ns	ns	ns	ns
10%PEG 2000–15% $MgSO_4$	ns	ns	ns	ns	ns
10%PEG 2000–20% $MgSO_4$	0.88±0.07a	0.55±0.01f	1836.33±7.87e	1.48±0.01e	92.29±0.39d
10%PEG 2000–25% $MgSO_4$	0.78±0.00b	0.52±0.01f	1820.33±0.88e	1.47±0.00e	95.17±0.04c
10%PEG 6000–10% $MgSO_4$	ns	Ns	ns	ns	ns
10%PEG 6000–15% $MgSO_4$	0.88±0.07a	0.56±0.00f	2029.50±0.99c	1.64±0.00c	99.32±0.05a
10%PEG 6000–20% $MgSO_4$	0.78±0.00b	0.49±0.01f	1801.44±4.18e	1.45±0.00e	98.79±0.23a
10%PEG 6000–25% $MgSO_4$	0.67±0.00c	0.50±0.02f	1816.62±41.19e	1.46±0.03e	98.73±2.23ab

VR: volume ratio (top/bottom); KP: partition coefficient of protein in the top phase; SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery; ns: no phase separation. Different lowercase letters in the same column indicate significant.

Table 5 Effect of phase composition in PEG-salts ATPS on partitioning of glycyl endopeptidase from papaya latex of Khack Dum cultivar

Phase composition (%, w/w)	VR	KP	SA	PF	Yield (%)
10%PEG 2000–10% $(NH_4)_2SO_4$	ns	ns	ns	ns	ns
10%PEG 2000–15% $(NH_4)_2SO_4$	0.98±0.17c	0.43±0.01e	1193.70±0.52b	1.17±0.00b	99.97±0.04a
10%PEG 2000–20% $(NH_4)_2SO_4$	0.88±0.00c	0.60±0.01d	694.11±13.05i	0.68±0.01i	48.94±0.92f
10%PEG 2000–25% $(NH_4)_2SO_4$	0.88±0.08c	3.09±0.01a	282.88±16.02j	0.28±0.01j	3.81±0.21h
10%PEG 6000–10% $(NH_4)_2SO_4$	1.50±0.00ab	0.48±0.03e	1325.18±0.96a	1.30±0.00a	99.90±0.07a
10%PEG 6000–15% $(NH_4)_2SO_4$	0.92±0.00c	0.49±0.01e	1143.42±0.64c	1.12±0.00c	99.40±0.05a
10%PEG 6000–20% $(NH_4)_2SO_4$	0.92±0.00c	1.87±0.06c	947.73±10.57d	0.93±0.01d	21.06±0.23g
10%PEG 6000–25% $(NH_4)_2SO_4$	0.92±0.00c	3.03±0.02b	299.10±4.73j	0.29±0.00j	4.10±0.06h
10%PEG 2000–10% $MgSO_4$	ns	ns	ns	ns	ns
10%PEG 2000–15% $MgSO_4$	ns	ns	ns	ns	ns
10%PEG 2000–20% $MgSO_4$	1.11±0.18bc	0.35±0.01f	834.64±1.23g	0.83±0.00g	66.82±0.10d
10%PEG 2000–25% $MgSO_4$	1.04±0.00bc	0.32±0.00f	907.20±19.53e	0.89±0.02e	73.62±1.58b
10%PEG 6000–10% $MgSO_4$	ns	ns	ns	ns	ns
10%PEG 6000–15% $MgSO_4$	1.63±0.09a	0.34±0.00f	864.49±10.54f	0.84±0.01f	69.74±0.85c
10%PEG 6000–20% $MgSO_4$	1.38±0.00b	0.34±0.00f	791.62±10.70h	0.78±0.01h	62.69±0.85e
10%PEG 6000–25% $MgSO_4$	1.27±0.00b	0.32±0.00f	846.58±1.35f	0.83±0.00f	70.46±0.11c

VR: volume ratio (top/bottom); KP: partition coefficient of protein in the top phase; SA: specific activity (unit/mg protein) of the top phase; PF: purification factor; yield: activity recovery; ns: no phase separation. Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

forces involved in the aqueous hydration of the solutes (Huddleston *et al*., 1991). The partitioning of proteolytic enzyme is also dependent on “volume exclusion effect” of the polymer and “salting-out effect” of salts (Huddleston *et al*., 1991).

In the present study, the partitioning of glycyl endopeptidase from papaya latex was strongly dependent on the type and concentration of salts. The increase in salt concentration from 10 to 25% resulted in the decreases in most partition parameters, except for KP. Increasing salt concentration led to the higher proportion of salt-rich bottom phase as indicated by the decreased VR. The distribution of the protein in ATPS was indicated by KP. The ATPS containing 10% PEG 6000-25% $(NH_4)_2SO_4$ provided the highest KP of 5.00 and 3.03 for latex from Red Lady and Khack Dum cultivars, respectively. These results suggested that most of proteins from latex were preferably partitioned to the top phase under such a condition. In contrast, the ATPS having 10% PEG 6000-10% $(NH_4)_2SO_4$ showed the lower KP (0.48-0.76) with the higher SA, PF and yield ($P<0.05$), indicating that most of target protease, glycyl endopeptidase, more likely shifted to the bottom phase. The SA, PF and yield were generally decreased, when salt concentration increased. Increase in salt concentration provided the salting-out effect (Rawdkuen *et al*., 2011). The phase system containing $(NH_4)_2SO_4$ generally showed the superior partitioning efficiency to those containing $MgSO_4$. Huddleston *et al*. (1991) concluded that the effectiveness of various salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out ability). For the latex of Red Lady cultivar, the highest SA (2524.89 Unit/mg protein) and PF (2.04-fold) were obtained from ATPS containing 10% PEG 6000-15% $(NH_4)_2SO_4$, whilst the highest recovery was obtained in ATPS having 10% PEG 6000-10% $(NH_4)_2SO_4$, in which SA of 2442.71 Unit/mg protein and PF of 1.97-fold were obtained. For Khack Dum cultivar (Table 5), a phase system containing 10% PEG 6000 and 10% $(NH_4)_2SO_4$ gave the highest SA (1325.18 Unit/mg protein) and PF (1.30-fold) with the highest yield (99.90%). This result was in agreement with Chaiwut *et al*. (2007) who reported that glycyl endopeptidase from papaya latex was successfully fractionated using ATPS comprising PEG 6000 (6%, w/w) and $(NH_4)_2SO_4$ (15%,

w/w). Due to the high SA, PF and yield, $(NH_4)_2SO_4$ was found to be appropriate for ATPS containing PEG.

Influences of PEG with different molecular weights (2000 and 6000) and concentrations (10, 15 and 20% (w/w)) on partitioning of glycyl endopeptidase from papaya latex of Red Lady and Khack Dum cultivars were also studied. The highest SA (1325.18-2524.89 units/mg protein) and yield (98.73-99.90) could be obtained from the ATPS with PEG 6000, regardless of salt and papaya cultivar, compared with those found in ATPS containing PEG 2000. These results were in accordance with Subathra *et al*. (2012) who reported that the best ATPS for separation of protease from Neem leaves was found in the system having PEG with higher MW (8000). However, Rawdkuen *et al*. (2011) found that when protease from the latex of *Calotropic procera* was partitioned by ATPS, PEG 1000 gave a higher yield than PEG 2000 and 3000. PEG concentration (10, 15 and 20%, w/w) had no significant effect on protease partition (data not shown). Moreover, the lower yield was obtained from the ATPS with higher PEG concentration (20%, w/w). Similar trend was observed for the fractionation of papain from papaya latex using ATPS (Nitsawang *et al*., 2006). Due to a high viscosity of mixture containing high PEG concentration, the lower yield was obtained (Nitsawang *et al*., 2006).

Amongst all ATPS tested, the system comprising 10% PEG 6000-10% $(NH_4)_2SO_4$ effectively partitioned glycyl endopeptidase to the salt-rich bottom phase and undesired proteins to the PEG-rich top phase. Under this condition, the resulting glycyl endopeptidase fraction from papaya latex of Red Lady and Khack Dum cultivars had SA of 1325.18-2442.71 Units/mg protein, PF of 1.30 to 1.97-fold and yield of 98.97-99.90%.

3.5.1.2 Effect of ammonium sulphate precipitation

The selected ATPS fractions (10% PEG 6000-10% $(NH_4)_2SO_4$) of Red Lady and Khack Dum latex were subjected to ammonium sulphate precipitation at different %saturations (Table 6). The ATPS fraction from latex of Red Lady cultivar obtained from ammonium sulphate precipitation using 50–60% saturation showed the

highest SA (2806.37 units/mg protein) and PF (2.26-fold) with 55.33% yield. However, the sufficient yield is the one factor considered for enzyme fractionation. Fraction with 40-50% saturation also had the high yield (24.90%) with slightly lower specific activity and purity. Therefore, the glycyl endopeptidase from Red Lady cultivar was precipitated using ammonium sulphate (40-60% saturation) to obtain the higher yield (80.23%) with the compromising SA (2646.64 units/mg protein) and PF (2.14-fold). For Khack Dum cultivar, the increases in SA (1892.34 units/mg protein) and PF (1.86-fold) with 73.08% yield were obtained when ammonium sulphate at 60-70% saturation was used. Ammonium sulphate precipitation is widely used to isolate water-soluble proteins of either plant or animal origin (Brovko and Zagranichnaya, 1998). Ammonium sulphate precipitation is rapid, inexpensive and convenient for protein separation (Eursakun *et al*., 2012). Ammonium sulphate precipitation separated different protein components with diverse properties and characteristics (Achouri and Boye, 2013). Different amounts of ammonium sulphate can precipitate the proteins with different properties, in which the protein with similar properties and characteristics could be concentrated and pooled at the same ammonium sulphate saturation (Achouri *et al*., 2013). The high SA, PF and yield, the ammonium sulphate saturation of 40–60% and 60-70% were shown to be the optimum range for the recovery of the glycyl endopeptidase from ATPS fraction of papaya latex from Red Lady and Khack Dum cultivars, respectively. Therefore, these conditions were selected for preparing the partially purified glycyl endopeptidase.

Table 6 Ammonium sulphate precipitation of glycyl endopeptidase from 10% PEG 6000-10% $(NH_4)_2SO_4$ ATPS fraction

Ammonium sulphate (% saturation)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Red Lady Cultivar			
ATPS: 10%PEG 6000–10% $(NH_4)_2SO_4$	2442.71±38.91b	1.97±0.03b	98.97±1.58a
28-40%	nd	nd	nd
40-50%	2486.90±21.41b	2.01±0.02b	24.90±0.32c
50-60%	2806.37±27.49a	2.26±0.02a	55.33±0.77b
60-70%	1427.15±31.61c	1.15±0.02c	1.82±0.10d
70-80%	1097.12±26.23d	0.88±0.02d	0.36±0.03d
>80%	1138.16±6.47d	0.92±0.00d	0.49±0.01d
Khack Dum Cultivar			
ATPS: 10%PEG 6000–10% $(NH_4)_2SO_4$	1325.18±0.96c	1.30±0.00c	99.90±0.07a
28-40%	nd	nd	nd
40-50%	nd	nd	nd
50-60%	1463.34±44.69b	1.44±0.04b	7.79±0.26c
60-70%	1892.34±25.74a	1.86±0.02a	73.08±1.07b
70-80%	992.16±11.20d	0.97±0.01d	6.37±0.08c
>80%	395.96±2.86e	0.39±0.00e	2.55±0.03d

nd: no detected.
Different lowercase letters in the same column within the same cultivar indicate significant differences ($p < 0.05$).

3.5.2 Protein pattern and activity staining of crude extract and partially purified glycyl endopeptidase from papaya latex

Protein pattern and activity staining of crude extract (CE) and partially purified glycyl endopeptidase (GE) are shown in Figure 8 (a) and (b). The crude extract obtained from papaya latex of Red Lady (CE-RL) and Khack Dum (CE-KD) cultivars showed the major protein bands with MW between 33 and 66 kDa (Figure 8 (a)). Bands with MW below 14 kDa were also observed. This was in agreement with Azarkan *et al*. (2003) who reported the pattern of the whole protein fraction from papaya latex using SDS-PAGE. After partial purification using ATPS-ammonium sulphate precipitation, the proteins with MW lower than 14 kDa were mostly eliminated. For activity staining (Fig 1 (b)), protein bands with proteolytic activities were observed with MW around 23 kDa and higher. There were several proteases in papaya latex (Azarkan *et al*., 2003). It was noted that CE obtained from papaya latex with different cultivars showed the different activity bands, indicating the presence of varying proteases with different MWs. CE-RL had the higher intensity of protease bands with MW >45 kDa, compared with those of CE-KD. On the other hand, GE-RL showed the less protease bands, compared with those of CE-RL. The result suggested that some proteases might be removed during partitioning using ATPS or ammonium sulphate precipitation. The visible four distinctive protease bands with MW of 23, 33, 40 and 50 kDa were obtained for GE-RL. GE-KD possessed the increasing numbers of protease bands, especially with MW >30 kDa, compared with those of CE-KD. The results suggested that other proteases might co-migrate along with GE to the bottom phase. These results were in agreement with their protein pattern as shown in Fig 1. (a). Zerhouni *et al*. (1998) studied the protein pattern of papaya cysteine proteases using SDS-PAGE. The GE band was observed at MW of 23 kDa, whereas papain and chymopapain had MW lower than 14 kDa (Zerhouni *et al*., 1998). In the present study, activity bands with MW less than 14 kDa were observed as smear bands, suggesting that papain might be constituted to some extent in the fraction. In addition, Chaiwut *et al*. (2007) used an ATPS of 6% PEG 6000-15% $(NH_4)_2SO_4$ for removing papain from crude papaya latex to the PEG-rich top phase. Chymopapain was also separated from the salt-rich bottom phase using ammonium sulphate

precipitation. From Figure 8 (a) and (b), high intensity of glycyl endopeptidase band with MW of 23 kDa was observed in GE-RL, compared with GE-KD. This was in agreement with the higher specific activity (Table 6). The GE-RL (2646.64 units/mg protein) showed the higher specific enzyme activity (P<0.05) than those of GE-KD (1892.34 units/mg protein). Thus, the fractionation used in the present study could be used for partitioning the GE, a target enzyme, and also removed the undesired proteins or enzyme contaminants.

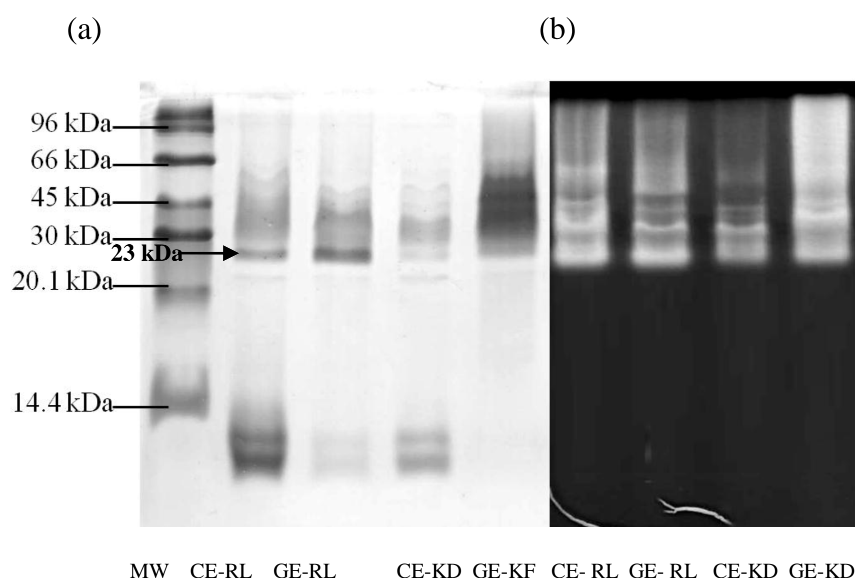

Figure 8 SDS-PAGE patterns (a) and activity staining (b) of crude extract and partially purified glycyl endopeptidase from papaya latex. MW: molecular weight marker; CE-RL: Crude extract-Red Lady; GE-RL: Partially purified glycyl endopeptidase-Red Lady; CE-KD: Crude extract-Khack Dum and GE-KD: Partially purified glycyl endopeptidase-Khack Dum.

3.5.3 Fish skin gelatin hydrolysates prepared using crude extract and partially purified glycyl endopeptidase and their antioxidative activities

The comparative study of crude extract (CE-RL) and partially purified glycyl endopeptidase (GE-RL) obtained from papaya latex of Red Lady cultivar on gelatin hydrolysis was conducted. Based on the α-amino group content of resulting

gelatin hydrolysates, CE-RL and GE-RL showed the highest hydrolysis toward gelatin, in comparison with those of Khack Dum cultivar. Additionally, the hydrolysates prepared by protease from Red Lady cultivar exhibited the higher ABTS radical scavenging activity ($P<0.05$). Therefore, CE-RL and GE-RL were selected and used for preparing gelatin hydrolysate with different DHs. Gelatin hydrolysates were also determined for antioxidative activities.

ABTS radical scavenging activity of gelatin hydrolysates with different DHs obtained from CE-RL and GE-RL is shown in Figure 9 (a). In general, the increases in radical scavenging activity were found in hydrolysate, compared with gelatin ($P<0.05$). The results indicated that antioxidative peptides were produced during the hydrolysis. ABTS radical scavenging activity gradually increased as %DH increased up to 25% ($P<0.05$), especially those prepared using GE-RL. However, ABTS radical scavenging activity of gelatin hydrolysate obtained from CE-RL with 20%DH and 25%DH was not different ($P>0.05$). When comparing the ABTS radical scavenging activity of gelatin hydrolysate prepared using CE-RL and GE-RL at the same %DH, gelatin hydrolysate prepared using the latter showed the higher activity ($P<0.05$). Due to the different proteases between CE-RL and GE-RL as shown in Figure 9, the resulting gelatin hydrolysates containing different antioxidative peptides could be obtained. In general, the peptides exhibit different physicochemical properties and biological activities, depending on their molecular weight and amino acid sequence, mainly determined by proteases used (Kim and Wijeselara, 2010). It was found that GE-RL had higher GE (Table 4). As a result, peptide bonds with Gly at P_1 (Buttle *et al.*, 1990) were more cleaved. Therefore, the resulting gelatin hydrolysates prepared using GE-RL probably contained more Gly residue at the C-terminus, comparing with those prepared using the crude extract. Antioxidative peptides isolated from Alaska pollack skin contained a Gly residue at the C-terminus (Kim *et al.*, 2001).

Ferrous chelating activity of gelatin hydrolysates was also investigated. Ferrous ion (Fe^{2+}) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl ($OH^{\bullet}$). All gelatin

hydrolysates prepared using CE-RL and GE-RL had no ability in complexing with Fe^{2+}, regardless of DHs (data not shown).

H_2O_2 scavenging activity of gelatin hydrolysates with different DHs is shown in Fig 2 (b). Commercial fish gelatin had H_2O_2 scavenging activity of 743.53 μmol TE/g protein. The gelatin hydrolysates with 20-25% DH had the decrease in H_2O_2 scavenging activity ($P<0.05$). It was noted that gelatin hydrolysate prepared using GE-RL had the lower activity, compared with those using CE-RL at the same DH tested ($P<0.05$). Therefore, it was possibly that short peptide chain obtained from hydrolysis had the low ability in scavenging H_2O_2. Wu, Chen and Shiau (2003) found that size, level and composition of free amino acids of peptides affected the antioxidative activity. Peptides generated, when GE-RL was used, might show lower potential in binding H_2O_2, compared with those prepared using CE-RL. Nevertheless, no changes in H_2O_2 scavenging activity was obtained in hydrolysate prepared using CE-RL with DHs of 10% and 15%, in comparison with gelatin.

The results suggested that gelatin hydrolysates contained peptides or proteins, which served as hydrogen or electron donors, which could convert the radicals to more stable forms. Thus, they could reduce and retard the oxidation, mainly via their radical scavenging activity. Moreover, the partially purified glycyl endopeptidase fractionated from papaya latex of Red Lady cultivar had the potential in production of antioxidative gelatin hydrolysates, especially at 25% DH.

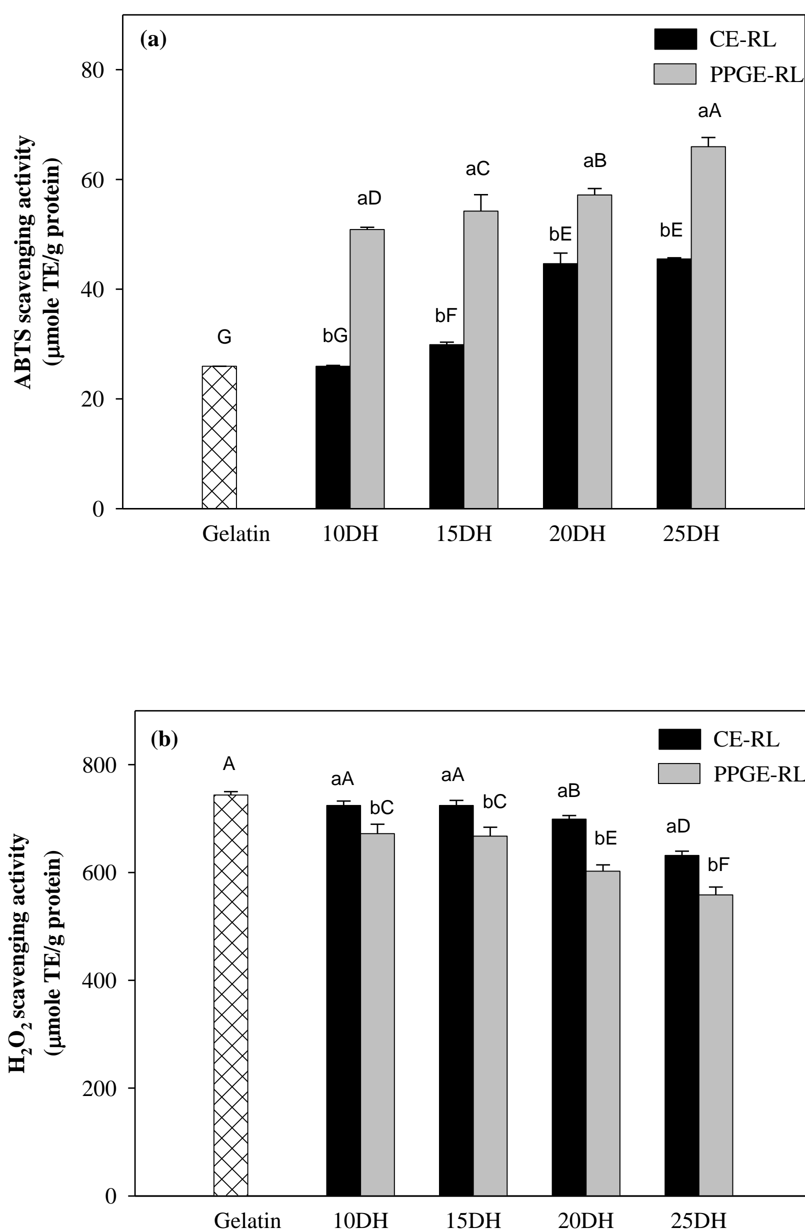

Figure 9 ABTS radical scavenging activity (a) and H_2O_2 scavenging activity (b) of gelatin and gelatin hydrolysates prepared using crude extract (CE-RL) and partially purified glycyl endopeptidase (GE-RL) from papaya latex of Red Lady cultivar with different DHs. Different lowercase letters on the bars within the same DH indicate significant difference ($p<0.05$). Different uppercase letters on the bars indicate significant difference ($p<0.05$).

3.5.4 Effect of partitioning on removal of odourous compounds in papaya latex and gelatin hydrolysate

Crude extract (CE-RL) and partial purified glycyl endopeptidase (GE-RL) from papaya latex of Red Lady cultivar were determined for odourous compound (Table 7). Benzyl isothiocyanate (42% abundance) was the major odourous compound in CE-RL, followed by benzene acetonitrile and ethyl hexadecanoate, respectively. It was reported that benzyl isothiocyanate at high amount was associated with the pungent-sour odour in the green papaya fruit (Fischer, 1996). Benzyl isothiocyanate was defined as an important odourant in papaya odour (Pino, 2014). Jirovetz *et al.* (2003) reported that the green-notes of green papaya were due to some C6 compounds (e.g., (E)-3-hexen-1-ol). In the present study, 1-hexanol, 2-ethyl was found in CE-RL. Benzene acetonitrile, hexadecanoic acid and ethyl hexadecanoate were also isolated and quantified from fresh papaya (Pino, 2014). In general, all compounds detected in GE-RL were markedly lower in abundance than those of CE-RL, especially 1-hexanol, 2-ethyl, which was not found in GE-RL. These results indicated the potential of ATPS and ammonium sulphate precipitation in removal of odourous compounds in papaya latex.

The corresponding gelatin hydrolysates produced using CE-RL showed the high content of odourous compounds, which were in accordance with those found in CE-RL. Benzyl isothiocyanate was found as a major odourous compound (15% abundance) in gelatin hydrolysate with small amount of ethyl hexadecanoate and methyl hexadecanoate. On the other hand, the gelatin hydrolysate prepared using GE-RL had the lowered odourous compounds. These results suggested that use of GE-RL yielded gelatin hydrolysate with negligible off-odour compounds.

Table 7 Odourous compounds in crude extract, partial purified gylcyl endopeptidase and their corresponding gelatin hydrolysates

Compounds	Peak area (Abundance) × 10^7			
	Enzymes		Corresponding gelatin hydrolysates	
	CE-RL	GE-RL	CE-RL	GE-RL
Hexanol, 2-ethyl	3.1	nd	3.7	nd
Benzene acetonitrile	285.8	0.4	4.0	nd
Benzyl isothiocyanate	533.1	1.1	43.1	8.2
Methyl hexadecanoate	39.9	0.9	23.7	7.5
ethyl hexadecanoate	84.2	1.1	38.5	11.6
1,2-Benzene dicarboxylic acid, dibutyl ester	37.2	0.7	35.8	29.6
Hexadecanoic acid	17.6	0.5	28.5	20.3
1,2-Benzene dicarboxylic acid	45.3	7.4	5.7	5.6

nd: no detected.

3.6 Conclusions

The glycyl endopeptidase from papaya latex was partitioned using aqueous two-phase (10% PEG 6000-10% $(NH_4)_2SO_4$) in combination with ammonium sulphate precipitation (40-60% saturation). The partially purified glycyl endopeptidase showed the potential in production of antioxidative gelatin hydrolysates. Enzyme fraction contained lower odourous compounds in papaya latex. The gelatin hydrolysate produced using the selected fraction had negligible odourous compounds. This would increase the exploitation of papaya latex for production of antioxidative gelatin hydrolysate.

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CHAPTER 4

Antioxidative gelatin hydrolysate from unicorn leatherjacket skin as affected by prior autolysis

4.1 Abstract

Gelatin hydrolysates from autolysed non-swollen and swollen unicorn leatherjacket skin prepared using partially purified glycyl endopeptidase (GE) from papaya latex were examined for their antioxidative activities. Autolysed swollen skin was more hydrolysed by GE as indicated by higher α-amino group content, compared with autolysed non-swollen skin. ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of hydrolysates were increased with increasing levels of GE used. Antioxidative gelatin hydrolysate from autolysed skin, both non-swollen and swollen skins, using 8% GE termed 'NS-8GE' and 'SS-8GE' exhibited different modes of action. When both hydrolysates were tested in lecithin liposome system, the efficiency in retardation of lipid oxidation was in a dose dependent manner. Antioxidative activity of hydrolysates at 5.0 g/L was comparable to that of 0.1 g/L Trolox, in which the oxidation was almost completely inhibited. NS-8GE and SS-8GE also showed their antioxidative activities in gastrointestinal tract model system (GIMs). ABTS radical scavenging activity of both hydrolysates increased, but FRAP decreased in a duodenal condition. Based on size exclusion chromatography, major antioxidative peptides in NS-8GE and SS-8GE had molecular weight of 1,170 and 750 Da, respectively. Therefore, GE could enhance antioxidative activity of autolysed skin. Additionally, swelling process directly determined the modes of actions of resulting gelatin hydrolysates. NS-8GE had high ABTS radical scavenging activity, whereas SS-8GE showed high FRAP.

4.2 Introduction

Lipid oxidation has been known as a serious cause of food deterioration as well as various human diseases, such as cardiovascular diseases and cancers (Lobo *et al.*, 2010). The reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^{\bullet -}$), lipid peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), and singlet oxygen ($^1O_2$) are the main initiators of lipid oxidation through free radical chain reaction (Min and Ahn, 2005). Antioxidants are substances that can inhibit the generation of free radicals in a system and prevent or delay the lipid oxidation reaction. Generally, the most common synthetic antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been used to enhance the oxidative stability and inhibit lipid oxidation in lipid containing foods (Min and Ahn, 2005; Xu, 2008).

Nowadays, natural additives involving antioxidants have gained attention from consumers. Protein hydrolysates, especially from marine processing byproducts, have attracted interest due to their safety as well as bioactivities (Kim and Wijesekara, 2010; Gómez-Guillén *et al.*, 2011). Enzymatic hydrolysis has been intensively employed to produce antioxidative gelatin hydrolysates (Gómez-Guillén *et al.*, 2011). However, the high cost of production is mainly associated with enzyme, energy consumed and production process. Recently, autolysis mediated by indigenous protease was used as the aid for production of gelatin hydrolsates with antioxidative activity from unicorn leatherjacket skin that could reduce the amount of commercial proteases and energy used (chapter 2). The selection of proper enzyme to substrate was crucial for production of antioxidative gelatin hydrolysate from Alaska pollack skin (Kim *et al.*, 2001). Kittiphattanabawon *et al.* (2012) found that glycyl endopeptidase, the major cysteine protease in papaya latex, showed the effectiveness in cleaving peptide bonds with Gly at P_1 of fish gelatin from blacktip shark skin, yielding antioxidative gelatin hydrolysate with high degree of hydrolysis. Additionally, the active fraction of glycyl endopeptidase from papaya latex was

prepared using aqueous-two-phase system to remove offensive odour compounds (chapter 3).

To reduce the amount of enzyme and energy used, the autolysis-assisted process mediated by indigenous protease in unicorn leatherjacket skin could be employed prior to further hydrolysis by glycyl endopeptidase from papaya latex. Additionally, pretreatment of skin via swelling process might affect the hydrolysis by protease used. As a consequence, gelatin hydrolysate with different antioxidative activities could be obtained.

4.3 Objective

To investigate the impact of autolysis-assisted process of non-swollen and swollen skin of unicorn leatherjacket and effect of partially purified glycyl endopeptidase at different levels on antioxidative activities of resulting gelatin hydrolysates.

4.4 Materials and methods

4.4.1 Chemicals

Polyethylene glycol (PEG) 6000 was obtained from Fluka (Buchs, Switzerland). 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2′-azinobis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4´,4´´-disulfonic acid sodium salt (ferrozine), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas, trypsin from bovine pancreas (EC 3.4.21.4) and bile extract porcine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Ammonium thiocyanate was purchased from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

4.4.2 Preparation of skin and autolysed skin

4.4.2.1 Preparation of skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock, Songkhla, Thailand. The fish with the size of 1.0-1.5 kg/fish (30-35 cm of body length) were caught from the Gulf of Thailand, stored in ice and off-loaded approximately 2-3 days after capture. Three different lots of skins were collected. For each lot, skins were pooled and used as the composite sample. Skins were 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, Hat Yai within 1 h. Upon arrival, the skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2), placed in polyethylene bags and stored at -20°C until use. The storage time was less than 2 weeks.

4.4.2.2 Pretreatment of skins

The skins were pretreated to remove non-collagenous proteins following the method of Kaewruang *et al.* (2013). Fish skins (0.5×0.5 cm^2) were soaked in 0.05 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Staufen, Germany) at a speed of 150 rpm. The alkaline solution was changed after 2 h and total pretreatment time was 4 h. Pretreated skins were washed with tap water until neutral or faintly basic pH of wash water was obtained.

4.4.2.3 Preparation of autolysed skin

4.4.2.3.1 Use of non-swollen skin

Autolysis was conducted using pretreated skin (non-swollen skin) following the method of chapter 2.4.3. The pretreated skins were mixed with deionised water at a ratio of 1:5 (w/v). The autolysis was conducted by incubating the mixture in a water bath (Model W350, Memmert, Schwabach, Germany) at 55 °C for

12 h and terminated by heating at 90°C for 15 min. The mixture was centrifuged at 5000×g at 4 °C using a refrigerated centrifuge model Avanti J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) for 10 min to remove the debris. The supernatant was collected, freeze-dried and referred to as autolysed non-swollen skin (NS).

4.4.2.3.2 Use of swollen skin

To prepare swollen-skin, the pretreated skin was soaked in 0.1 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) for 6 h with a gentle stirring at room temperature as per the method of Kaewruang *et al.* (2013). The acidic solution was changed every 3 h. Acid-treated skin was washed thoroughly with tap water until wash water became neutral or faintly acidic. To prepare autolysed skin, the swollen skin was mixed with deionised water at a ratio of 1:5 (w/v) and subjected to autolysis as previously described. Autolysed swollen-skin obtained was referred to as 'SS'.

4.4.3 Production of gelatin hydrolysate from autolysed skin using partially purified glycyl endopeptidase (GE)

4.4.3.1 Preparation of crude extract from papaya (*C. papaya*) latex

Fresh papaya latex was collected from Red Lady green papaya fruit cultivated in Songkhla, Thailand. Four to six longitudinal incisions were made on the green papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

The crude extract was prepared using the method of Kittiphattanabawon *et al.* (2012). The latex was mixed with cold distilled water (≤4 °C) with a latex to water ratio of 1:3 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000×g at 4 °C for 20 min using a refrigerated centrifuge. The supernatant was filtered using a Whatman No.1 filter paper (Whatman International, Maidstone, Kent, UK), followed by lyophilisation (Scanvac Model Coolsafe 55 freeze dryer, Coolsafe, Lynge, Denmark). The powder obtained was referred to as 'crude extract'.

4.4.3.2 Fractionation of glycyl endopeptidase using aqueous two-phase system (ATPS) in combination with ammonium sulphate precipitation

Crude extract powder (1 g) was dissolved in 8 mL of distilled water. The pH of solution was adjusted to 6.0 using 6 M HCl and the volume was made up to 10 mL by distilled water to obtain a concentration of 100 mg/mL prior to fractionation using ATPS.

The glycyl endopeptidase was fractionated using the method of chapter 3. ATPS with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of glycyl endopeptidase. The salt-rich bottom phase was collected and further precipitated using ammonium sulphate at 60% saturation. After centrifugation at 9,000×g at 4 °C for 20 min, the pellet was re-dissolved in distilled water and dialysed against 20 volumes of distilled water for 6 times. After lyophilisation, the powder (713 Unit/mg protein) referred to as 'partially purified glycyl endopeptidase, GE' was stored at -40 °C until use.

4.4.3.3 Production of gelatin hydrolysates using GE

Autolysed fish skins, both NS and SS, were used as substrates. The solutions (3%, w/v) were then added with GE at different concentrations (2, 4, 6 and 8%, based on solid matter). The reaction was proceeded at 40 °C for 60 min. After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and lyophilised. The gelatin hydrolysate powders were placed in polyethylene bag and stored at -40 °C. Hydrolysate powders were subjected to analyses.

4.4.4 Analyses

4.4.4.1 Determination of α–amino group content

The α–amino group content was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µL), 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution was added. The

solution was mixed thoroughly and placed in a temperature-controlled water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixture was cooled at room temperature for 15 min. L-leucine standard solutions with concentrations ranging from 0.5 to 5.0 mM were used. The absorbance was read at 420 nm and α–amino group content was expressed in terms of L-leucine.

4.4.4.2 Determination of *in vitro* antioxidative activities

4.4.4.2.1 ABTS radical scavenging activity

ABTS radical scavenging activity of gelatin hydrolysates was determined as described by Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The solution obtained (1 mL) was then diluted with 50 mL distilled water in order to obtain an absorbance of 1.1±0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µL) was mixed with 2850 µL of ABTS solution and the mixture was allowed to stand at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalent (TE)/g sample.

4.4.4.2.2 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000) with a slight modification. Briefly, the sample (1 mL) was mixed with 83 µl of 100 mM hydrogen peroxide (prepared in 0.1 M phosphate buffer, pH 7.4). The mixture was allowed to react for 40 min at room temperature. The absorbance at 230 nm of the reaction mixture was read. The blank, in which 0.1 M phosphate buffer was used instead of hydrogen peroxide, was

prepared. Trolox (0-10 mM) was used as the standard. The hydrogen peroxide scavenging activity was expressed as μmol Trolox equivalent (TE)/g sample.

4.4.4.2.3 Ferric reducing antioxidant power (FRAP)

FRAP was determined according to the method of Benzie and Strain (1996). FRAP reagent was prepared by mixing acetate buffer (30 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v). The sample solution (100 μL) was mixed with 3 mL of working FRAP reagent and incubated in dark condition at room temperature for 30 min. The absorbance of the reaction mixture was read at 593 nm using a spectrophotometer. The standard curve was prepared using Trolox ranging from 0 to 500 μM. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

4.4.4.2.4 Ferrous chelating activity

Chelating activity of gelatin hydrolysates towards ferrous ion (Fe^{2+}) was measured by the method of Thiansilakul *et al.* (2007) with a slight modification. Sample (200 μL) was mixed with 800 μL of distilled water. Thereafter, 0.1 mL of 2.0 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0-1.0 mM) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed as μmol EDTA equivalents (EE)/g sample.

4.4.5 Antioxidative activity of gelatin hydrolysate in lecithin liposome model system

Gelatin hydrolysates prepared using 8% GE rendering the highest *in vitro* antioxidative activity were tested in a lecithin liposome model system according to the method of Thiansilakul *et al.* (2007). Both gelatin hydrolysates were named 'NS-8GE' and 'SS-8GE' where NS and SS were used as substrates, respectively.

Lecithin liposome system was prepared by suspending lecithin in deionised water at a concentration of 8 mg/mL. The mixture was stirred with a glass rod, followed by sonification for 30 min in a sonicating bath (Elma Model S30H, Singen, Germany). Gelatin hydrolysate (3 mL) was added to the lecithin liposome system (15 mL) to obtain the final concentrations of 2.5 and 5.0 g/L. The mixture was sonicated for 2 min. To initiate the reaction, 40 µL of 0.15 M cupric acetate were added. The mixture was shaken in the dark at 120 rpm using a shaker (Unimax 1010, Heidolph Model, Schwabach, Germany) at 37°C. The system containing 0.10 g/L Trolox was also prepared. The control was prepared in the same manner, except that distilled water was used instead of gelatin hydrolysates or Trolox. Oxidation in lecithin liposome systems was monitored during 24 h of incubation by determining PV and TBARS values.

4.4.5.1 Determination of peroxide value (PV)

PV was measured according to the method of Wu *et al.* (2003). Liposome sample (0.1 mL) was mixed with 4.7 mL of ethanol/distilled water (3:1), 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride solution in 3.5% HCl. After incubation at 40°C for 3 min, the absorbance was read at 500 nm using a spectrophotometer. PV was calculated from a standard curve of cumene hydroperoxide (0-100 mg/L) and expressed as mg cumene hydroperoxide/L lecithin liposome.

4.4.5.2 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978) with a slight modification. Liposome sample (0.5 mL) was homogenised with 2.5 mL of TBARS solution (0.375% thiobarbituric acid, 15% TCA and 0.25 M HCl). The mixture was heated in boiling water for 10 min to develop the pink colour. The mixture was then cooled with running water and centrifuged at 5000x*g* for 10 min at room temperature using Hettich centrifuge (Hettich Model MIKRO-20, Tuttlingen, Germany). The supernatant was collected and the absorbance at 532 nm was read using a spectrophotometer. TBARS value was calculated from a standard curve of

malondialdehyde (MDA) (0–10 mg/L) and expressed as mg MDA/L lecithin liposome.

4.4.6 Changes in antioxidative activities of gelatin hydrolysate in gastrointestinal tract model system

Gastrointestinal tract model system mimicing the conditions of the digestive organs was prepared according to the method of Enari *et al.* (2008) with slight modifications. Gelatin hydrolysate solution (10 mg/mL in distilled water; 100 mL) was mixed with 0.5 ml of 1 M HCl-KCl buffer (pH 1.5), followed by adding 32 U/mL of pepsin solution in 1 M HCl-KCl buffer (pH 1.5) (5 mL) and incubating for 60 min at 37 °C (stomach condition) with a continuous shaking using a Unimax 1010 shaker (Heidolph Model, Schwabach, Germany). Thereafter, the pH of the reaction mixture was raised to 6.8 with 1 M $NaHCO_3$ (1 mL), and the enzyme mixture of bile and pancreatic juice (1 mL) that contained pancreatin (10 mg/mL), trypsin (14600 U/mL) and bile extract (13.5 mg/mL) in 10 mM phosphate buffer (pH 8.2), was added to the solution, followed by incubation at 37 °C for 3 h (duodenal condition) with a continuous shaking. The digestion was terminated by placing in a boiling water bath for 10 min. During the simulated gastrointestinal digestion, the mixture was randomly taken at 0, 0.5, 1, 2, 3 and 4 h for determination of ABTS radical scavenging activity and FRAP.

4.4.7 Molecular weight distribution

Gelatin hydrolysates (NS-8GE and SS-8GE) were separated by size exclusion chromatography using a Sephadex G-25 gel filtration column (2.5×50 cm) (17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). The experiment was conducted in walked in cold room (4 °C) and 2 mL of sample solution (60 mg gelatin/mL) were loaded. After being loaded, the elution was performed using a low pressure chromatography system (BioLogic LP system, Bio-RAD Laboratories Ltd., Hercules, CA, USA) coupled with a fraction collector (Model 2128, Bio-RAD Laboratories Ltd., Hercules, CA, USA). Fifty mM sodium phosphate buffer (pH 7.0) was used as the elution buffer at a flow rate of 0.5 mL/min. The fractions of 3 mL

were collected. The absorbance was recorded at 220 and 280 nm. ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of each fraction was determined. Blue dextran (2,000,000 Da) was use for void volume measurement. The molecular weight markers included insulin chain B (3496 Da), vitamin B_{12} (1355 Da), glycine-tyrosine (238 Da) and tyrosine (181 Da). MW of fraction with the highest ABTS radical scavenging activity and FRAP was estimated from the plot between available partition coefficient (K_{av}) and the logarithm of the molecular weight of the protein standards.

4.4.8 Statistical analysis

All experiments were run in triplicate using the three different lots of skin. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The data with $P<0.05$ were considered to be statistically significant.

4.5 Results and discussion

4.5.1 Effect of GE on hydrolysis of autolysed skin

The hydrolysis of autolysed non-swollen and swollen skin by GE at different levels monitored by α-amino group content is shown in Figure 10. Without hydrolysis using GE, NS and SS had the similar α-amino group content ($P>0.05$). The α-amino group content of resulting gelatin hydrolysates increased with increasing GE levels for both substrates (NS and SS) used. This result was in accordance with those from chapter 2 that the increase in enzyme/substrate (0.5, 1.0 and 2.0%) resulted in the increase in degree of hydrolysis of gelatin hydrolysate from unicorn leatherjacket skin prepared using papain. When NS was used as a substrate, α-amino group contents in resulting hydrolysates were lower than those found in hydrolysates from SS at all enzyme levels used ($P<0.05$). The result indicated that peptides in SS were more likely the preferable substrate for GE. Autolysis of gelatin in unicorn leatherjacket skin at 55 °C caused by indigenous protease (Kaewruang *et al.*, 2013)

yielded available peptides for further hydrolysis by GE. For swollen skin (SS), α-chains or their cross-link, e.g. β- or γ-chains, could be more repulsed. As a result, those proteins could be cleaved by indigenous protease easily. The resulting peptides might have free exposed glycine along the chain to a higher extent, in comparison with peptides produced from non-swollen skin. When the skin matrix was still compact, cleavage sites might be limited and the resulting peptides were plausibly present as bundles, in which cleavage site, especially glycine, could be hindered. This might result in the lower hydrolysis as indicated by lower α-amino group content.

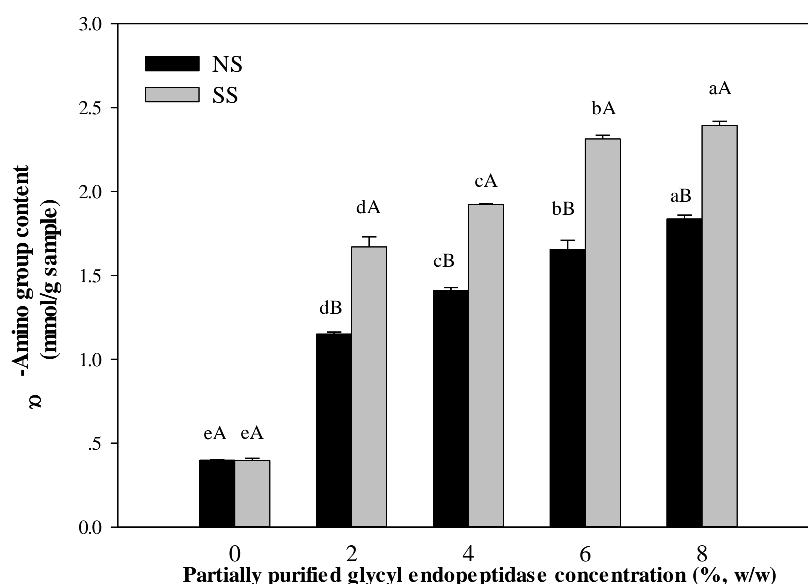

Figure 10 α-amino group content of gelatin hydrolysates prepared using partially purified glycyl endopeptidase at different concentrations (2%, 4%, 6% and 8% of solid matter). NS; Autolysed non-swollen skin, SS; Autolysed swollen skin. Different uppercase and lowercase letters on the bars within the same enzyme concentration and the same substrate, respectively, indicated significant different ($P<0.05$). Bars represent standard deviation (n=3).

4.5.2 *In vitro* antioxidative activities of gelatin hydrolysates

Figure 11 shows the antioxidative activities of gelatin hydrolysates prepared using GE at different levels (2-8% of solid matter) as examined by different assays. ABTS radical scavenging activity of NS (52.72 μmol/g sample) was higher than SS (29.50 μmol/g sample) ($P<0.05$). Notably, NS and SS had similar α-amino group content (Figure 10). Different peptides with various chain length and amino acid sequence might be obtained as influenced by prior swelling. Therefore, the difference in antioxidative activity of autolysed skin (NS and SS) could be observed. The increase in ABTS radical scavenging activity of gelatin hydrolysates was obtained as GE was applied for the second step of hydrolysis. The higher activity was found when GE levels used increased ($P<0.05$), regardless of substrate. At the same level of GE, gelatin hydrolysates obtained from SS possessed the lower ABTS radical scavenging activity, compared with those from NS ($P<0.05$). It was noted that ABTS radical scavenging activity of resulting gelatin hydrolysates was not in accordance with α-amino group content (Figure 10). This might reflect the difference in antioxidative peptides in hydrolysate when different substrates were used. Wu *et al.* (2003) reported that size, level and composition of free amino acids of peptides affected the antioxidative activity. ABTS radical scavenging assay is based on biamperometric measurements using $ABTS^+$/ABTS redox couple. The ability of antioxidants to scavenge the preformed $ABTS^+$ was estimated by the decolourisation (Re *et al.*, 1999). Therefore, hydrolysate prepared from NS using GE more likely showed higher ability of scavenging ABTS radical, compared with those from SS.

H_2O_2 scavenging activity of gelatin hydrolsates prepared using GE at different concentrations (2-8% of solid matter) is depicted in Figure 11 (b). NS (1.17 mmol/g sample) and SS (1.16 mmol/g sample) had a similar H_2O_2 scavenging activity ($P>0.05$). The decrease in H_2O_2 scavenging activity of gelatin hydrolysates was observed after further hydrolysis by GE, regardless of substrates. However, the resulting gelatin hydrolysate prepared with 4% GE had a similar H_2O_2 scavenging activity, compared with the substrates, NS and SS ($P>0.05$). These results suggested that the peptides generated after hydrolysis by GE had low ability in scavenging

H_2O_2. From chapter 2, we found that H_2O_2 scavenging activity of gelatin hydrolysates from unicorn leatherjacket prepared using papain decreased when the level of papain increased. It was reported that degree of hydrolysis had no relationship with antioxidative activity of protein hydrolysate (Chen *et al.*, 1995). The extensive hydrolysis probably caused the lower antioxidative activity, due to the generation of free amino acids which had lower antioxidative activity, compared to peptides (Hernández-Ledesma *et al.*, 2005). H_2O_2, which is a weak oxidising agent, is not directly involved in the initiation of lipid oxidation because its reduction potential is lower than that of unsaturated fatty acid (Choe and Min, 2005). However, H_2O_2 shows indirect contribution to lipid oxidation. H_2O_2 is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Choe and Min, 2005). Thus, further hydrolysis of autolysed skin by GE slightly lowered H_2O_2 scavenging activity of resulting hydrolysates.

Ferric reducing antioxidant power (FRAP) of gelatin hydrolsates prepared using GE at different concentrations is shown in Figure 11 (c). NS had low FRAP (0.04 μmol/g sample). On the other hand, SS had no FRAP. The activity of gelatin hydrolysates increased when GE levels used increased ($P<0.05$), regardless of substrates. Generally, gelatin hydrolysate obtained from SS exhibited the higher FRAP than those from NS, especially when GE at levels higher than 2% was used. The increases in FRAP were in accordance with increasing α-amino group content of gelatin hydrolysate (Figure 10). It was suggested that smaller peptides in hydrolysate obtained from SS more likely had the higher ability in reducing TPTZ-FE(III) complex. FRAP is generally used to measure the capacity of a substance in reducing TPTZ-FE(III) complex to TPTZ-Fe(II) complex (Binsan *et al.*, 2008). This was in agreement with Kittiphattanabawon *et al.* (2012) who reported that FRAP of hydrolysate from blackstip shark skin prepared using papaya latex enzyme increased with increasing degree of hydrolysis. After hydrolysis using GE, the resulting gelatin hydrolysates from both substrates, NS and SS, possibly contained high amounts of peptides which donated electron to free radicals, thereby terminating the chain reaction.

Ferrous chelating activity of gelatin hydrolsates is shown in Figure 11 (d). NS (6.79 μmol/g sample) showed the higher ferrous chelating activity than SS (3.46 μmol/g sample) ($P<0.05$). After further hydrolysis by GE, the resulting gelatin hydrolysates from NS had the decreases in chelating activity, especially when GE levels increased ($P<0.05$). However, the increase in ferrous chelating activity was obtained when 4% GE was used for hydrolysis. When SS was used as substrate, the ferrous chelating activity of resulting hydrolysates increased as GE levels increased up to 4% ($P<0.05$). Nevertheless, the decrease in chelating activity was observed in hydrolysate prepared using GE level higher than 4% ($P<0.05$). From chapter 2, we found that ferrous chelating activity of gelatin hydrolysate from unicorn leatherjacket skin decreased with increasing papain levels. The result indicated that different peptides in hydrolysates might have varying ability in sequestering Fe^{2+}. It was noted that gelatin hydrolysates from NS after further hydrolysis using GE showed the higher chelating activity, compared with those from SS. It is well-known that transition metal ions such as iron or copper may catalyse the formation of reactive oxygen species that accelerate lipid oxidation. Thus, gelatin hydrolysates obtained in the present study were able to prevent lipid oxidation via metal chelating ability. Nevertheless, their ability varied, depending upon the level of GE used for hydrolysis.

From the above measurements, gelatin hydrolysate prepared with 8% GE from both NS (NS-8GE) and SS (SS-8GE) showed the highest radical scavenging activity and electron donating activity, which are crucial for termination of chain reaction. Therefore, NS-8GE and SS-8GE were selected for determination of antioxidative activity in lecithin liposome model system in comparison with NS and SS.

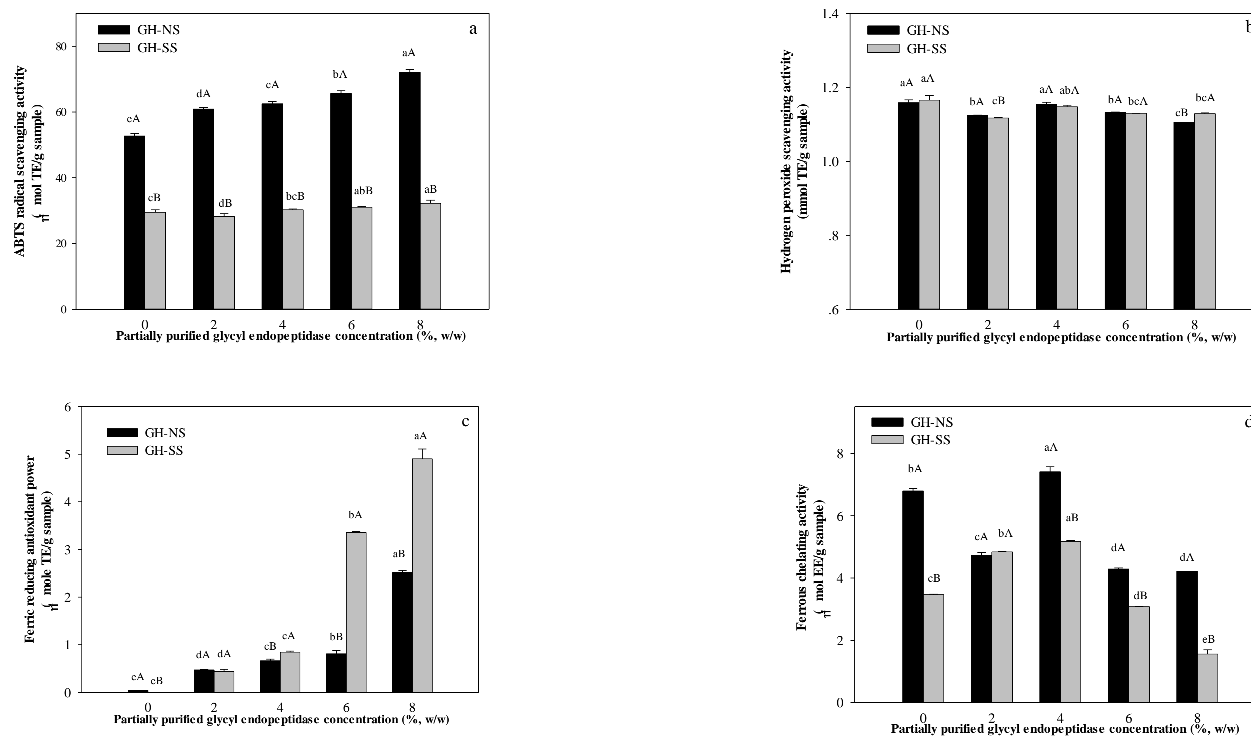

Figure 11 ABTS radical scavenging activity (a), hydrogen peroxide scavenging activity (b), ferric reducing antioxidant power (c) and ferrous chelating activity (d) of gelatin hydrolysates prepared using partially purified glycyl endopeptidase at different concentrations (2%, 4%, 6% and 8% of solid matter). NS; Autolysed non-swollen skin, SS; Autolysed swollen skin. Different uppercase and lowercase letters on the bars within the same enzyme concentration and the same substrate, respectively, indicated significant different (P<0.05). Bars represent standard deviation (n=3).

4.5.3 Antioxidative activity of selected gelatin hydrolysates in lecithin liposome model system

Oxidation of lecithin liposome system added with gelatin hydrolysates at different concentrations (2.5 and 5.0 g/L) was monitored by determining peroxide value (PV) (Figure 12 (a)) and TBARS values (Figure 12 (b)) during incubation of 48 h at 37 °C. For the control (without hydrolysate or trolox), the sharp increase in PV was found within the first 42 h of incubation (P<0.05), in which the highest value (138.74±3.64 mg cumene hydroperoxide/L lecithin liposome) was found at 42 h of incubation. Thereafter, the sharp decrease in PV was observed up to 48 h. The sharp decrease in PV after 42 h of incubation was plausibly caused by the decomposition of hydroperoxide to the secondary products (Frankle *et al.*, 1997). The system added with NS and SS showed the lower PV than the control throughout 48 h of incubation (P<0.05). In general, the rate of increase varied with the concentration of gelatin hydrolysate used. On the other hand, the system added with NS-8GE and SS-8GE showed very low increase in PV throughout the incubation of 48 h. Ability in prevention of hydroperoxide formation was comparable to that of 0.1 g/L trolox, especially for NS-8GE and SS-8GE at high concentration tested (5.0 g/L). NS-8GE and SS-8GE possessed the higher antioxidative activity in lecithin liposome system than their substrates (NS and SS). This was in accordance with higher antioxidative activities of hydrolysates, especially ABTS radical scavenging activity and FRAP, as shown in Figure 11 (a) and (c), respectively. NS-8GE and SS-8GE showed both radical scavenging activity and reducing antioxidant power. Gelatin hydrolysates from seabass and bigeye snapper skins displayed antioxidative activity in lecithin liposome systems (Senphan and Benjakul, 2014; Phanturat *et al.*, 2010).

Changes in TBARS of liposome system during incubation of 48 h are depicted in Figure 12 (b). The sharp increase in TBARS was observed after 18 h of incubation in the control and systems added with NS and SS at all levels (P<0.05), indicating the formation of the secondary lipid oxidation products. The systems containing gelatin hydrolysate at 5.0 g/L had lower TBARS than those added with hydrolysate at a lower level (P<0.05). There were no marked changes in TBARS of

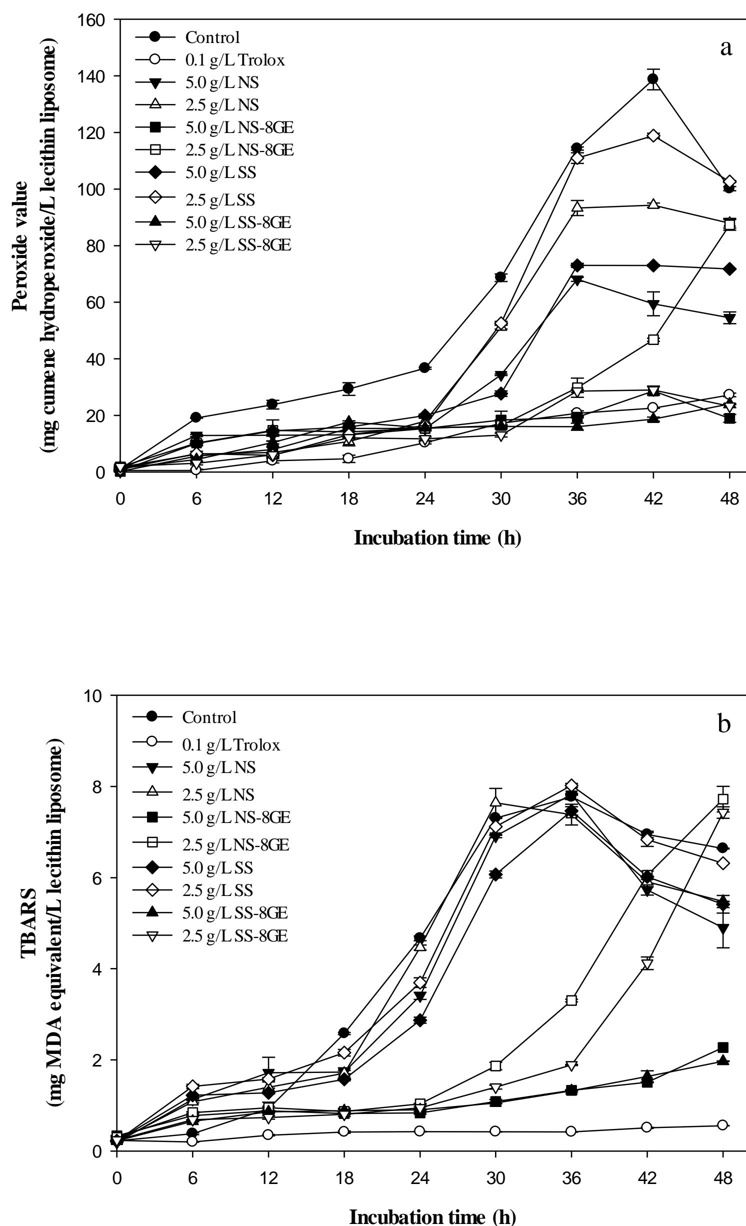

Figure 12 The formation of peroxide value (a) and TBARS (b) in lecithin liposome system containing gelatin hydrolysates from unicorn leatherjacket skin. NS; Autolysed non-swollen skin, NS-8GE; Autolysed non-swollen skin hydrolysed using 8% partially purified glycyl endopeptidase, SS; Autolysed swollen skin. SS-8GE; Autolysed swollen skin hydrolysed using 8% partially purified glycyl endopeptidase. Bars represent standard deviation (n=3).

lecithin liposome system added with 1.0 g/L trolox. Slight increases in TBARS were observed in the system containing 5.0 g/L NS-8GE and SS-8GE throughout 48 h of incubation. The result suggested that NS-8GE and SS-8GE, especially at the high concentration (5.0 g/L) could retard the formation of the secondary oxidation products effectively. After 36 h of incubation time, the decreased TBARS was noticeable, suggesting the loss of oxidation products. The secondary products with low molecular weight could be lost easily (Stahnke, 1995). Thus, NS-8GE and SS-8GE were able to retard the early stages as well as the advanced stage of oxidation in lecithin liposome system.

4.5.4 Changes in antioxidative activities of gelatin hydrolysate in gastrointestinal tract model system

The gastrointestinal tract model system (GIMs) was used to mimic the digestive system containing several proteases. The changes in antioxidative activity of NS-8GE and SS-8GE, as monitored by ABTS radical scavenging activity and FRAP, throughout the system is presented in Figure 13. ABTS radical scavenging activity decreased after pepsin digestion ($P<0.05$) but increased under duodenal condition during 1 and 6 h. After digestion in duodenum, proteases in pancreatin juice might cleave the peptides and release some new peptides with higher antioxidative activities, especially radical scavenging activity as evidenced by the increased ABTS radical scavenging activity. It was found that ABTS radical scavenging activity was higher for SS-8GE than NS-8GE throughout ingestion system. The results suggested that NS-8GE and SS-8GE had different peptides, which could be further hydrolysed at varying degree. As a result, the generated peptides more likely had different chain length as well as amino acid sequence. This directly determined ABTS radical scavenging activity. Kittiphattanabawon *et al*. (2012) also found the increased antioxidative activity of gelatin hydrolysate from blacktip shark skin prepared using papaya latex after being ingested in the simulated model system. The gelatin hydrolysate from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp showed the increase of antioxidative activity with further hydrolysis in

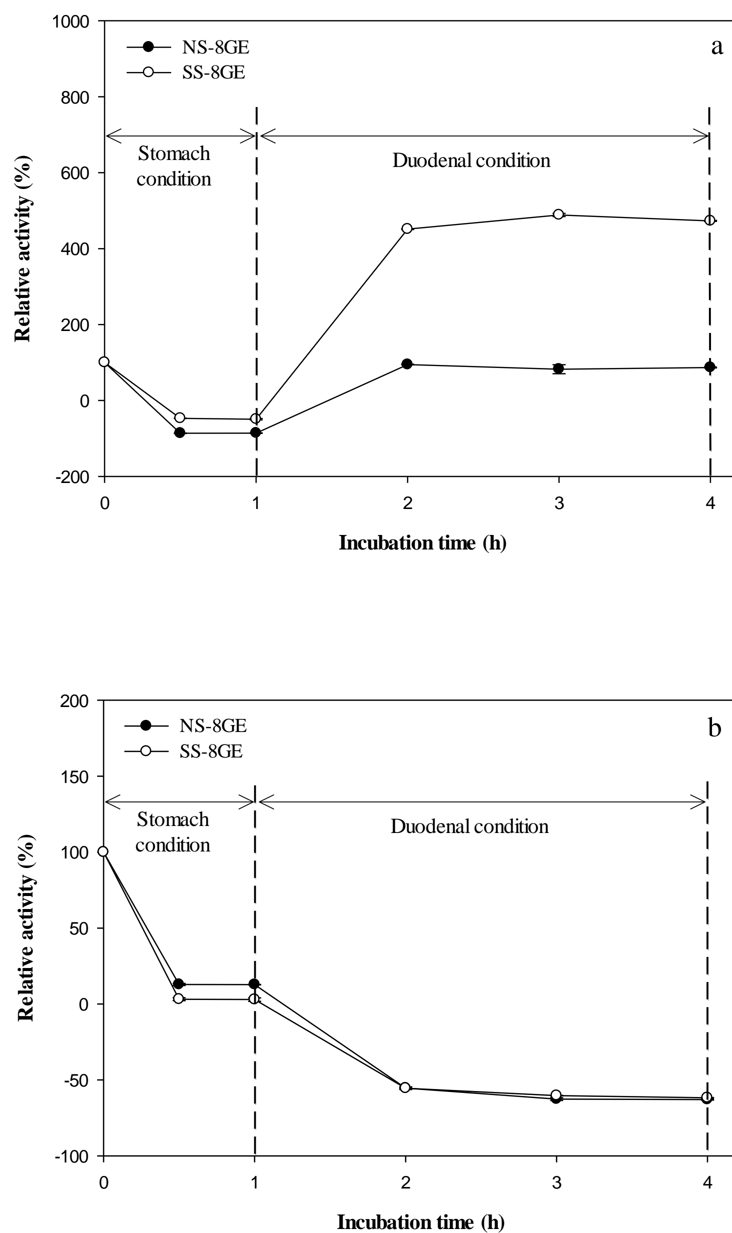

Figure 13 ABTS radical scavenging activity (a) and ferric reducing antioxidant power (b) of gelatin hydrolysates from unicorn leatherjacket skin in gastrointestinal tract model system. NS-8GE; Autolysed non-swollen skin hydrolysed using 8% partially purified glycyl endopeptidase, SS-8GE; Autolysed swollen skin hydrolysed using 8% partially purified glycyl endopeptidase. Bars represent standard deviation (n=3).

intestinal simulated system (Senphan and Benjakul, 2014). On the other hand, FRAP of both NS-8GE and SS-8GE decreased after ingestion in the GIMs over digestion time of 0-4 h. The extensive hydrolysis during digestion might provide small peptides with low ability on donation of electron to free radicals. The result suggested that radical scavenging activity of NS-8GE and SS-8GE could be increased in the gastrointestinal tract.

4.5.5 Molecular weight distribution

Elution profiles of NS-8GE and SS-8GE on the Sephadex^{TM}-G15 gel filtration chromatography are shown in Figures 14 and 15, respectively. Based on A_{280}, gelatin hydrolysates showed three major peaks, indicating the presence of peptides containing aromatic amino acid with varying MW (Figure 14 (a) and 15 (a)). Different elution profiles between NS-8GE and SS-8GE were generally obtained. SS-8GE showed the higher peak of peptides containing aromatic amino acids with low MW than NS-8GE. Based on A_{220} representing peptides, it was noted that NS-8GE showed higher content of larger peptides, in comparison with SS-8GE. The result indicated that prior autolysis and swelling process used for preparing substrate might enhance hydrolysis by GE. Moreover, this result was in accordance with free α-amino group content of SS-8GE which was higher than that of NS-8GE as shown in Figure 10. ABTS radical scavenging activity and FRAP of fractions of NS-8GE and SS-8GE are depicted in Figure 14 (b) and 15 (b), respectively. NS-8GE fraction with MW of 1,170 Da exhibited the highest ABTS radical scavenging activity, followed by the peptide with MW of 3,380 Da (Figure 14 (b)). Additionally, NS-8GE contained peptides (2,150 Da, 1,010 Da and 220 Da) which possessed the ability in donating electron to free radicals. For SS-8GE, the fraction with MW of 750 exhibited the highest FRAP, followed by that with MW of 1,590 Da. ABTS radical scavenging activity was also found in fractions with MW of 1,850 and 1,010 Da. Both gelatin hydrolysates showed ABTS radical scavenging activity and FRAP, in which NS-8GE and SS-8GE possessed the ability to retard the oxidation mainly via their radical

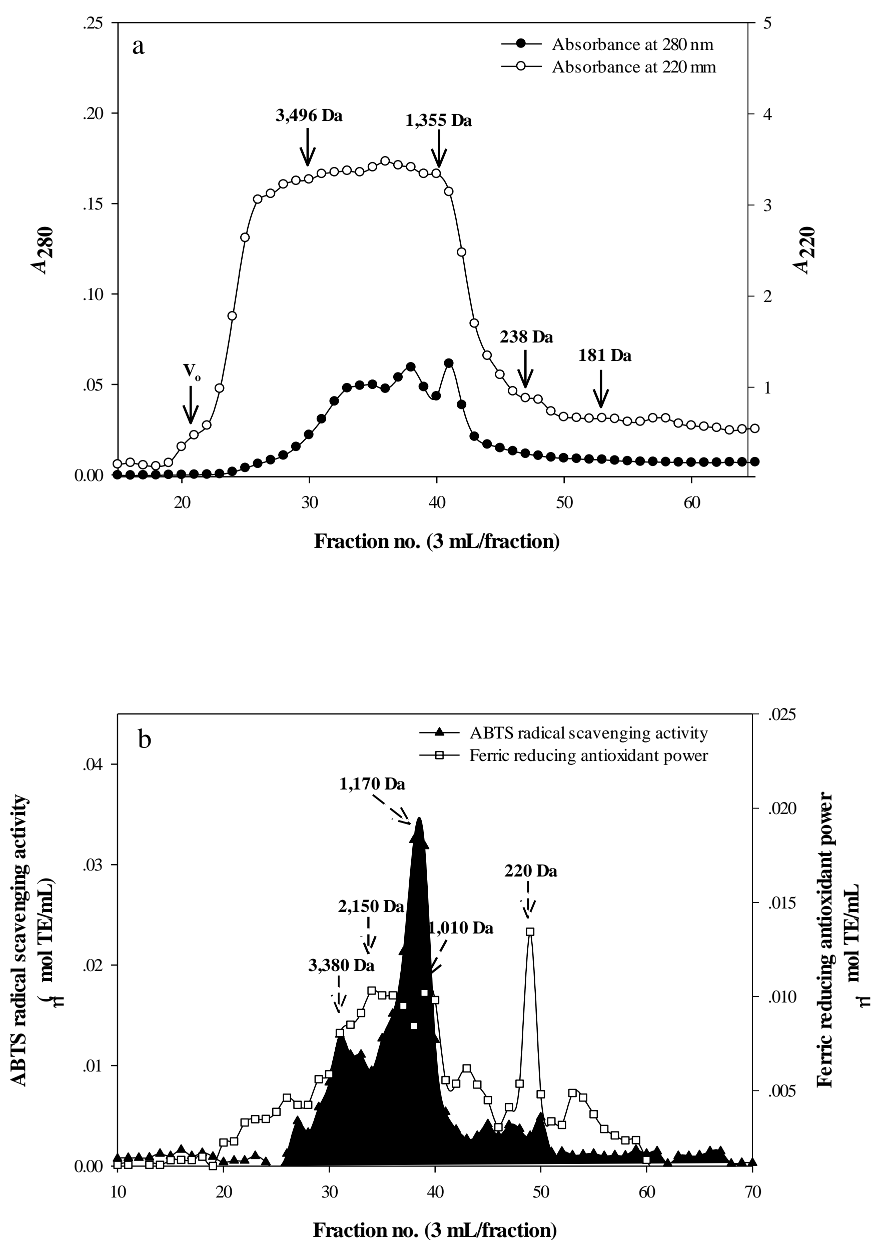

Figure 14 Elution profile of gelatin hydrolysate (NS-8GE) from unicorn leatherjacket skin subjected to Sephadex G-25 size exclusion chromatography. Absorbance at 280 nm (●), 220 nm (○) (a); ABTS radical scavenging activity (Δ) and ferric reducing antioxidant power (□) (b); molecular weight markers (→) molecular weight of fraction (-->).

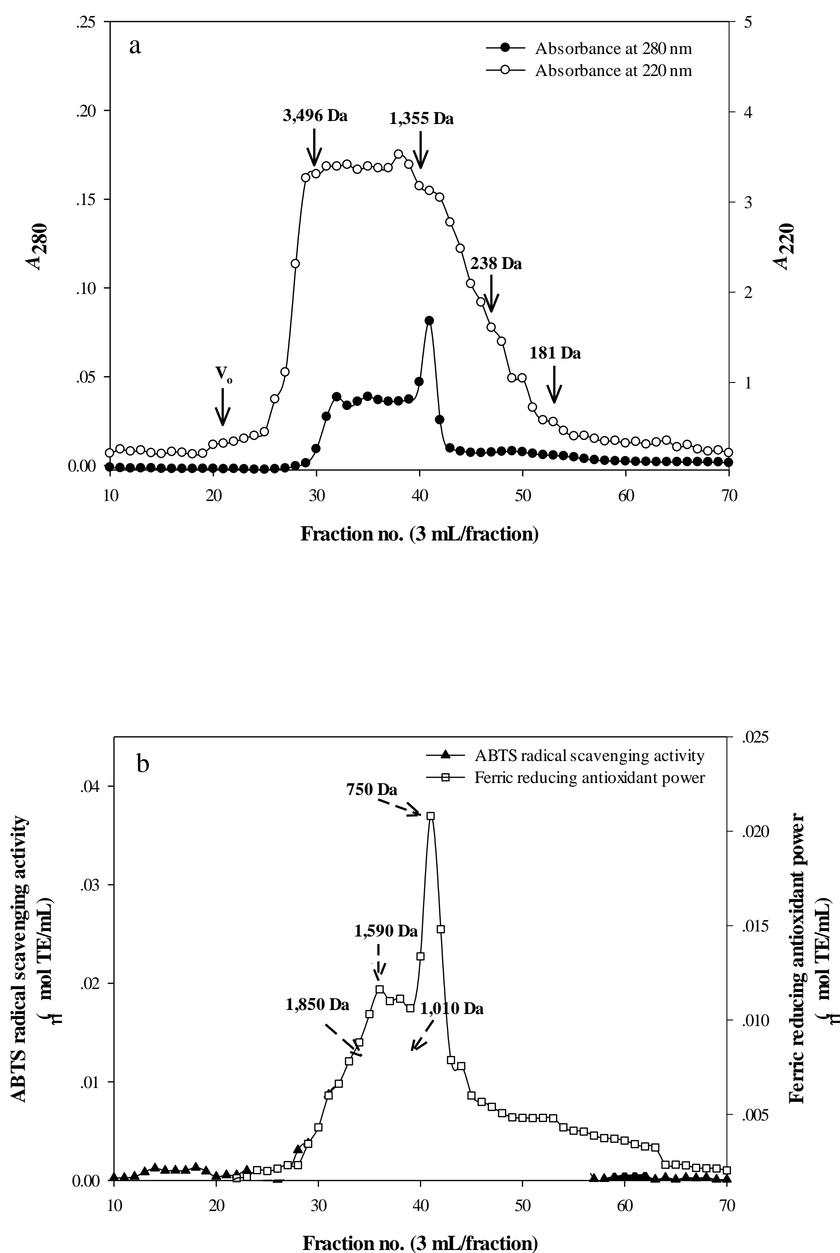

Figure 15 Elution profile of gelatin hydrolysate (SS-8GE) from unicorn leatherjacket skin subjected to Sephadex G-25 size exclusion chromatography. Absorbance at 280 nm (●), 220 nm (○) (a); ABTS radical scavenging activity (Δ) and ferric reducing antioxidant power (□) (b); molecular weight markers (→) molecular weight of fraction (-->).

scavenging and electron donating activities, respectively. The small peptides generally possess high antioxidative activity (Kittiphattanabawon *et al.*, 2012; Phanturat *et al.*, 2010). However, the level and amino acid composition of peptide also affected the antioxidative activity (Wu *et al.*, 2003). Gelatin hydrolysate from skin of unicorn leatherjacket prepared using autolysis-assisted process with MW of 2,200 Da exhibited higher antioxidative activity than those with MW of 870 Da (chapter 2). Yarnpakdee *et al.* (2014) found that Nile tilapia protein hydrolysates contained antioxidative peptides with different modes of action. Fraction containing peptides with MW of 513 Da and 1,484 Da showed the strongest ABTS radical scavenging activity and metal chelating activity, respectively (Yarnpakdee *et al.*, 2014). The result suggested that NS-8GE and SS-8GE contained peptides with different sizes and varying modes of action.

4.6 Conclusion

Antioxidative gelatin hydrolysate could be produced from both autolysed non-swollen and swollen skin from unicorn leatherjacket skin with 8% partially purified glycyl endopeptidase from papaya latex. Gelatin hydrolysates possessed antioxidative activities which showed the effective in retarding lipid oxidation. However, modes of action of both hydrolysates were different, more likely governed by swelling process. Peptide having MW of 1,170 Da with the dominant radical scavenging activity was found in NS-8GE, whereas that with MW of 750 Da having the profound FRAP was observed in SS-8GE. Therefore, autolysis mediated by indigenous protease in the skin could be applied for production of gelatin hydrolysate with antioxidative activity.

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CHAPTER 5

Cryoprotective and antioxidative effects of gelatin hydrolysate from unicorn leatherjacket skin

5.1 Abstract

Cryoprotective properties of gelatin hydrolysates from autolysed non-swollen and swollen unicorn leatherjacket skin using partially purified glycyl endopeptidase (GE) from papaya latex were examined. Gelatin hydrolysates from autolysed non-swollen skin showed higher cryoprotective property in salt solution system than that from autolysed swollen skin as indicated by lower enthalpy for melting of eutectic and ice crystal. The cryoprotective effect of gelatin hydrolysate (0.5 and 1.0%) was in dose-dependent manner. Gelatin hydrolysate retarded physicochemical changes of natural actomyosin from washed mince system, as evidenced by lower changes in Ca^{2+}-ATPase activity, surface hydrophobicity and disulfide bond formation, compared with the control. Based on DSC analysis, the enthalpy of myosin and actin was also higher in the presence of gelatin hydrolysate. Gelatin hydrolysate could prevent lipid oxidation in washed mince system as shown by lower TBARS value and less abundance of hexanal, heptanal and 1-pentene-3-ol.

5.2 Introduction

Freezing and frozen storage have been widely used to retain sensory quality and nutrients of fish. During frozen storage, proteins undergo denaturation associated with quality loss. Furthermore, lipid oxidation in frozen food can be retarded but still occurs at a lower rate (Zarzycki and Swiniarska, 1993). Moreover, temperature fluctuation and abuse during transportation and storage lead to the deterioration of fish muscle quality. This is due to the destabilisation of bondings and interactions between protein molecules (Benjakul and Sutthipan, 2009). To alleviate or retard protein denaturation and lipid oxidation in muscle food caused by the formation of ice crystals as well as the changes in salt concentration in unfrozen phase during the frozen storage, cryoprotectants and antioxidants have been widely used, especially in fish mince or surimi (Benjakul and Visessanguan, 2011; Alvarez *et al*., 2010). As cryoprotectants, sucrose and sorbitol are amongst the common additives to maintain quality of frozen foods, however they may contribute to sweetness in products. Synthetic antioxidants, e.g., butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tertiary butylhydroquinone, etc. have been widely used in foods. Recently, synthetic chemicals and ingredients pose the adverse effects on the product, particularly in term of safety concern (Kim and Wijesekar, 2010). Natural and safe additives without negative effect on quality have therefore been searched.

Gelatin hydrolysate, especially from fish skin, has gained increasing interest as the additives with multi-functions. Protein hydrolysates and peptides have been shown to exhibit cryoprotective effect (Kittiphattanabawon *et al*., 2012a) and antioxidative activity (Qiu *et al*., 2014) in fish products during frozen storage. Nikoo *et al*. (2014) reported that tetrapeptide isolated from Amur sturgeon skin gelatin showed the antioxidative and cryoprotective effects in Japanese sea bass mince subjected to repeated freeze-thawing. Several proteases have been used to produce gelatin hydrolysates (Gómez-Guillén *et al*., 2011). Autolysis-assisted process mediated by indigenous protease has recently shown the potential for production of gelatin hydrolysates from skin of unicorn leatherjacket with antioxidative activity

(chapter 2). Kittiphattanabawon *et al*. (2012b) reported that gelatin hydrolysate from blacktip shark skin prepared using crude enzyme from papaya latex acted as an alternative cryoprotectant with the lower sweetness in fish mince product. Recently, partially purified glycyl endopeptidase (GE) from papaya latex could serve as potential protease and yielded hydrolysates with antioxidative activity (chapter 3). Additionally, gelatin hydrolysate does not have the strong bitter taste and can thus be used in a wide range of products (Phillips and Williams, 2011). Since both protein denaturation and lipid oxidation cause the loss in quality and consumer's acceptance of fish, natural additives having both cryoprotective and antioxidative properties, especially gelatin hydrolysate, could be promising to burden such deteriorative processes.

5.3 Objective

To study the cryoprotective and antioxidative effects of gelatin hydrolysate from unicorn leatherjacket skin prepared using partially purified glycyl endopeptidase from papaya latex.

5.4 Materials and methods

5.4.1 Chemicals

Polyethylene glycol (PEG) 6000 was obtained from Fluka (Buchs, Switzerland). Tris-maleate, adenosine triphosphate (ATP), 1-anilinonaphthalene-8-sulfonic acid (ANS) and 2-nitro-5-thiosulfobenzoate (NTSB) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sucrose, sorbitol and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). 2-thiobarbituric acid was procured from Fluka (Buchs, Switzerland). All chemicals were of analytical grade.

5.4.2 Preparation of gelatin

5.4.2.1 Preparation of fish skins

The skins of unicorn leatherjacket (*A. monoceros*) were obtained from a dock, Songkhla, Thailand. Three different lots of skins were collected. For each lot, skins were pooled and used as the composite sample. Skin were 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, Hat Yai within 1 h. Upon arrival, the skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2), placed in polyethylene bags and stored at -20°C until use. The storage time was less than 2 weeks.

5.4.2.2 Pretreatment of fish skin

The skins were pretreated to remove non-collagenous proteins using the method of Kaewruang *et al*. (2013). Fish skins (0.5×0.5 cm^2) were soaked in 0.05M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Staufen, Germany) at a speed of 150 rpm. The alkaline solution was changed after 2 h and total pretreatment time was 4 h. Pretreated skins were washed with tap water until neutral or faintly basic pH of wash water was obtained.

5.4.2.3 Preparation of autolysed fish skin

5.4.2.3.1 Use of non-swollen skin

Autolysis was conducted using pretreated skin (non-swollen skin) following the method of chapter 2.4.3. The pretreated skins were mixed with deionised water at a ratio of 1:5 (w/v). The autolysis was conducted by incubating the mixture in a water bath (Model W350, Memmert, Schwabach, Germany) at 55°C for 12 h and terminated by heating at 90°C for 15 min. The mixture was centrifuged at 5000×g at 4°C using a refrigerated centrifuge model Avanti J-E (Beckman Coulter,

Inc., Palo Alto, CA, USA) for 10 min to remove the debris. Autolysed skin was collected and referred to as 'NS'.

5.4.2.3.2 Use of swollen skin

To prepare swollen-skin, the pretreated skin was soaked in 0.1 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) for 6 h with a gentle stirring at room temperature. The acidic solution was changed every 3 h. Acid-treated skin was washed thoroughly with tap water until wash water became neutral or faintly acidic. To prepare autolysed skin, the swollen skin was mixed with deionised water at a ratio of 1:5 (w/v) and subjected to autolysis as previously described. Autolysed skin obtained was referred to as 'SS'.

5.4.3 Production of gelatin hydrolysate from autolysed skin using partially purified glycyl endopeptidase (GE)

5.4.3.1 Preparation of crude extract from papaya (*C. papaya*) latex

Fresh papaya latex was collected from green papaya fruit cultivated in Songkhla, Thailand. Four to six longitudinal incisions were made on the green papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

The crude extract was prepared using the method of Kittiphattanabawon *et al*. (2012b). The latex was mixed with cold distilled water (≤4 °C) with a latex to water ratio of 1:3 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000×g at 4 °C for 20 min using a refrigerated centrifuge. The supernatant was filtered using a Whatmam No.1 filter paper, followed by lyophilisation (Scanvac Model Coolsafe 55 freeze dryer, Coolsafe, Lynge, Denmark). The powder obtained was referred to as 'crude extract'.

5.4.3.2 Fractionation of glycyl endopeptidase using aqueous two-phase system (ATPS) in combination with ammonium sulphate precipitation

Crude extract powder (1 g) was dissolved in 8 mL of distilled water. The pH of solution was adjusted to 6.0 using 6 M HCl and the volume was made up to 10 mL by distilled water to obtain a concentration of 100 mg mL^{-1} prior to fractionation using ATPS.

The glycyl endopeptidase was fractionated using the method of chapter 3. ATPS with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of glycyl endopeptidase. The salt-rich bottom phase was collected and further precipitated using ammonium sulphate at 60% saturation. After centrifugation at 9,000×g at 4 °C for 20 min, the pellet was re-dissolved in distilled water and dialysed against 20 volumes of distilled water for 6 times. After lyophilisation, the powder referred to as 'partially purified glycyl endopeptidase, GE' was stored at -40 °C until use.

5.4.3.3 Production of gelatin hydrolysates

Autolysed skins, both NS and SS, were used as the substrates. Both NS and SS solutions (3%, w/v) were added with GE (8%, based on solid matter). The reaction was taken place at 40 °C for 60 min. After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and lyophilised. The gelatin hydrolysate powders prepared using NS and SS were referred to as 'NS-8GE' and 'SS-8GE', respectively. The powders were placed in polyethylene bag and stored at -40 °C until use. The storage time was not longer than 2 months.

5.4.4 Cryoprotective effect of gelatin hydrolysate in salt solution model system

The effects of both gelatin hydrolysates (NS-8GE and SS-8GE) on the eutectic crystallisation of a NaCl/water model system were studied using a differential scanning calorimetry (DSC) (Perkin–Elmer, Model DSCM, Norwalk, CT, USA)

following the method of Chen *et al*. (2005) and Izutsu *et al*. (1995) with a slight modification. Gelatin hydrolysates were dissolved in 0.15 M NaCl solution at different concentrations (0.5 and 1.0%, w/v). The sample (approximately 2 mg) was sealed in an aluminum pan and cooled from room temperature to -80 °C at the rate of 5 °C/min. During rewarming at 2 °C/min, the heating scans were recorded. The cryoprotective effects of gelatin hydrolysate were evaluated from heat absorption by the melting of eutectic and ice crystal (the maximum transition temperature (T_{max}) and enthalpy (ΔH)) in comparison with the control (0.15 M NaCl).

5.4.5 Cryoprotective and antioxidative effects of gelatin hydrolysate in washed mince model system

Gelatin hydrolysate (NS-8GE) yielding the high cryoprotective effects in a salt solution model system was selected. Its cryoprotective and antioxidative effects in washed mince model system were studied.

5.4.5.1 Preparation of washed mince added with gelatin hydrolysate

Short-bodied mackerel (*Rastrelliger brachysoma*) was purchased from a market in Songkhla, Thailand. The fish were placed in ice with a fish to ice ratio of 1:2 (w/w) in a polystyrene box and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, the fish was deheaded and filleted. The flesh was minced using a mincer with the hole diameter of 5 mm. The mince was suspended in 3 volumes of cold water (4 °C). The mixture was stirred gently for 5 min and the washed mince was filtered with two layers of cheese cloth. Washing was repeated three times. Finally, the washed mince was centrifuged at 700×g for 10 min using a basket centrifuge (model CE 21 K, Grandiumpiant, Belluno, Italy).

Gelatin hydrolysate, commercial cryoprotectant (sucrose/sorbitol blend (1:1, w/w)) or gelatin hydrolysate/cryoprotectant blend (1:1, w/w) was dissolved in distilled water (5 mL) and added into mince (100 g) to obtain a final concentration of 6% (based on washed mince). For the control sample, 5 mL of distilled water was added. The samples added with gelatin hydrolysate, commercial cryoprotectant and

gelatin hydrolysate/cryoprotectant blend were referred to as 'NS-8GE', 'S/S' and 'Blend', respectively. The mixtures were mixed well for 1 min, placed in polyethylene bags and heat-sealed.

Sample (30 g) in polyethylene bag was frozen at -20 °C using an air-blast freezer for 24 h. The frozen samples were thawed with running tap water until the temperature reached 0-2 °C (approximately 30 min). The core temperature of sample was measured using a digital thermometer (DE-3004 Type K, *Der Ee* Electrical Instrument Co., Ltd., Taiwan, China). The freeze-thawing was performed for 0, 3 and 6 cycles. Thawed samples were kept in ice and use for analysis within 4 h.

5.4.5.2 Analyses

5.4.5.2.1 Physicochemical properties of muscle proteins

5.4.5.2.1.1 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul *et al.* (1997) with a slight modification. To avoid any interfering effect from gelatin hydrolysate or cryoprotectant on the measurement of physicochemical properties of NAM, gelatin hydrolysate or cryoprotectant was removed before NAM extraction (Kittipattanabawon *et al.*, 2012a). Washed mince (4 g) was homogenised in 40 mL of chilled distilled water (2 °C) using a homogeniser (model T25 basic, IKA Labotrecnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was centrifuged at 10,000×g for 10 min at 2 °C.

To extract NAM, washed sample was further homogenised in chilled 0.6 M KCl, pH 7.0, at a ratio of 1:10 (w/v), using a homogeniser. To avoid overheating , the sample was placed in ice and homogenised for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The extract was centrifuged at 5000×g for 30 min at 2 °C. Three volumes of chilled deionised water were added to the supernatant to precipitate NAM. NAM was then collected by centrifuging at 5000× g for 20 min at 2 °C, and the pellet was dissolved in an appropriate volume of

chilled 0.6 M KCl, pH 7.0 by stirring for 30 min at 2 °C. The extracted NAM was kept at 4 °C and used within 12 h.

5.4.5.2.1.2 Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of NAM was determined using the method of Benjakul *et al*. (1997). The prepared NAM with the protein concentration of 3 mg/mL (250 μL) was mixed with 150 μL of 0.5 M Tris-maleate (pH 7), 250 μL of 0.1 M $CaCl_2$ and 1725 μL of distilled water. The mixture was vigorously mixed using a vortex mixer (model G-560E, Scientific Industries, Inc., Bohemia, NY, USA). To initiate the reaction, 20 mM ATP (125 μL) was added into the mixture and mixed thoroughly. The mixture was incubated at room temperature for 10 min and the reaction was terminated by adding 1250 μL of chilled 15% (w/v) trichloroacetic acid (TCA, 4 °C). The reaction mixture was centrifuged at 5000× g for 5 min at room temperature using a centrifuge (model Allegra® 25R, Beckman Coulter, Inc., Kerfeld, Germany). The inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as mmol inorganic phosphate released/mg protein·min. A blank solution was prepared by adding chilled TCA prior to addition of ATP.

5.4.5.2.1.3 Surface hydrophobicity

Surface hydrophobicity of NAM was determined by the method of Benjakul *et al*. (1997). NAM solution was diluted to 0.125, 0.25, 0.5 and 1 mg mL^{-1} using 10 mM phosphate buffer, pH 7.0 containing 0.6 M NaCl. The diluted NAM solutions (4 mL) were mixed well with 20 μL of 8 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer, pH 7.0. The relative fluorescence intensity of ANS-protein conjugates was measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at the excitation wavelength of 374 nm and the emission wavelength of 485 nm. Protein surface hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity versus protein concentration (mg/mL) using a linear regression analysis. The initial slope was referred to as "SoANS".

5.4.5.2.1.4 Disulphide bond content

Disulphide bond content in NAM was determined using 2-nitro-5-thiosulfobenzoate (NTSB) assay, according to the method of Thannhauser *et al.* (1987). To 0.5 mL of NAM sample (1 mg/mL), 3 mL of freshly prepared NTSB assay solution were added. The mixture was mixed thoroughly and incubated in the dark at room temperature for 25 min. Absorbance at 412 nm was measured using a spectrophotometer. A blank was conducted by replacing the sample with 0.6 M KCl. Disulphide bond content was calculated using the extinction coefficient of 13,600 L/mol·cm and was expressed as mol/g protein.

5.4.5.2.1.5 Thermal transitions of muscle protein

Thermal transition of protein in all samples was determined using the differential scanning calorimetry (DSC) (Perkin–Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg wet weight) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. The samples were scanned at 5 °C min^{-1} over the range of 20–85 °C. The maximum transition temperature (T_{max}) was measured. Enthalpy (ΔH) was estimated by measuring the area under the DSC transition curve. The residual enthalpy was expressed as the residual (%), relative to that of fresh washed mince (without additives). The system was calibrated using indium.

5.4.5.2.2 Lipid oxidation

5.4.5.2.2.1 Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined using a distillation method as described by Tarladgis *et al.* (1960). Ten g sample, 97.5 mL of distillated water and 2.5 mL of 6 N HCl were transferred to a Kjeldahl flask. The mixture was heated until 200 mL of distillate were obtained. To determine TBARS, the distillate (0.2 mL) was added with 1 mL of TBAR solution (0.375% thiobarbituric acid, 15% TCA and 0.25M HCl) and heated in boiling water for 10 min. After cooling with running water and centrifugation at 5000xg for 10 min at

room temperature, the absorbance of the pink solution was read at 532 nm. TBARS value was calculated from a standard curve of malondialdehyde (MDA) (0-10 mg/L) and expressed as mg MDA/kg sample.

5.4.5.2.2.2 Volatile compounds

Volatile lipid oxidation compounds in samples were determined by solid-phase micro-extraction/gas chromatography–mass spectrometry (SPME/GC–MS) (Iglesias and Medina 2008).

- Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of sample were homogenised at a speed of 13,500 rpm for 2 min with 8 mL of ultra-pure water. The mixture was centrifuged at 2000×g for 10 min at 4 °C. The supernatant (6 mL) was heated at 60 °C with equilibrium time of 10 h in a 20-mL headspace vial (Agilent Technologies, Palo Alto, CA, USA). The SPME fibre (75 µm Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC for 15 min before use and then exposed to the headspace. The 20 mL-vial containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60 ºC for 30 min. The volatile compounds were then desorbed in the GC injector port for 10 min at 260 ºC.

-GC-MS analysis

GC–MS analysis was performed in a Trace Ultra gas chromatograph coupled with a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific Inc., San Jose, CA, USA) and equipped with a splitless injector. Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m ± 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC min^{-1} to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 10 ºC/min to a final temperature of 260 ºC and holding for 5 min. Helium was employed as a carrier gas with a constant flow of 1.5 mL/min. The injector was operated in the

splitless mode and its temperature was set at 260 ºC. Transfer line temperature was maintained at 265 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 200 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 10-200 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 μA, and the electron multiplier voltage at 500 V.

-Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. The identified volatile compounds including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

5.4.6 Statistical analysis

All experiments were run in triplicate using the three different lots of skin. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The data with $P<0.05$ were considered to be statistically significant.

5.5 Results and discussion

5.5.1 Cryoprotective effect of gelatin hydrolysates in salt solution model system

Figure 16 shows DSC thermogram of NaCl solution containing NS-8GE and SS-8GE at different concentrations (0.5 and 1.0%, w/v). For the control

system (0.15 M NaCl solution), there were two endothermic melting transition (T_{max}) occurring at around -22 and 0 °C, corresponding to the melting of the eutectic and ice crystal, respectively (Izutsu *et al*., 1995). Thermal analysis of frozen salt solution model system has been performed to determine the eutectic and ice melting behavior (Chen *et al*., 2005; Izutsu *et al*., 1995). When the gelatin hydrolysates (NS-8GE and SS-8GE) were added, the melting temperatures of ice crystal occurred in the same region (around 0 °C), regardless of levels used. Nevertheless, the endothermic peak corresponding to the eutectic melting was found to differ. The eutectic melting temperature was slightly shifted to a lower temperature, especially for the system containing SS-8GE, compared with the control counterpart. Notably, the peak almost disappeared with the addition of 1.0% gelatin hydrolysate, indicating the inhibition of eutectic crystallisation. Therefore, both gelatin hydrolysates affected the eutectic formation. The result was in accordance with Chen *et al*. (2005) who reported the effect of amino acids on the eutectic behaviour of NaCl solution. Their impact on eutectic formation was governed by chemical structure (Chen *et al*., 2005).

The control system showed the highest ΔH for eutectic (8.71 J/g) and ice (304.17 J/g) endothermic peaks, compared with those incorporated with gelatin hydrolysates (Figure 16). The system added with gelatin hydrolysate possessed the lower ΔH for both endothermic peaks, especially at the higher concentration used. Salt solution incorporated with 1.0% NS-8GE required enthalpy for disruption of eutectic (2.14 J/g) and ice (163.86 J/g) crystal structures, which was lower than those from 1.0% SS-8GE (2.20 J/g and 232.09 J/g, respectively). ΔH or the area under endothermic melting peak also correlated with the amount of order-phase fraction in the eutectic and ice crystals. The result suggested that the control system contained higher fraction of order-phase structure, compared to those added with gelatin hydrolysate. Moreover, gelatin hydrolysate might obstruct the eutectic and ice crystal formation, in which the ordered phase was decreased as indicated by lower ΔH. Izutsu *et al*. (1995) reported that the addition of cryoprotectants (sugar and polymers) inhibited the eutectic crystallisation of the NaCl solution and also decreased the area under the endothermic peak. Therefore, gelatin hydrolysate, especially NS-8GE,

showed the cryoprotective effect, in which the eutectic and ice crystallisation of NaCl solution model system was prevented.

Based on the above results, NS-8GE with the higher cryoprotective effect in salt solution model systems was selected for study in washed mince model system.

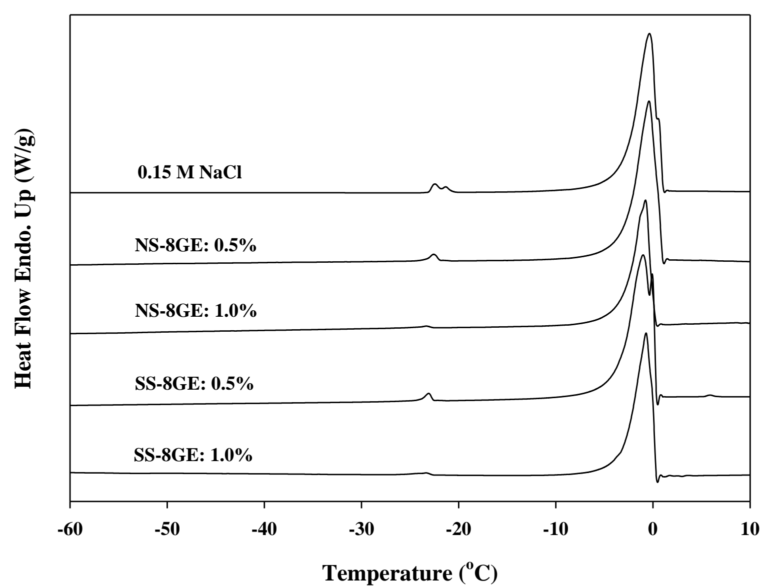

Figure 16 DSC heating curve for NaCl solution composed of gelatin hydrolysates with different concentrations (0.5 and 1.0% w/v). NS-8GE; Gelatin hydrolysate from non-swollen skin, SS-8GE; Gelatin hydrolysate from swollen skin.

5.5.2 Cryoprotective and antioxidative effects of NS-8GE in washed mince model system

Washed mince system containing 6% (w/w) NS-8GE was subjected to freeze-thawing for 0, 3 and 6 cycles. Physiochemical properties of muscle protein and lipid oxidation in the system were monitored in comparison with the control system (without additive) and those added with 6% (w/w) commercial cryoprotectant (sucrose:sorbitol, 1:1) (S/S) or 6% (w/w) NS-8GE/cryoprotectant blend (blend).

5.5.2.1 Ca^{2}+-ATPase activity

Ca^{2+}-ATPase activity of NAM from washed mince added without and with NS-8GE, S/S or blend subjected to multiple freeze-thaw cycles is shown in Figure 17 (a). The similar Ca^{2+}-ATPase activity was found in all samples at 0 freeze-thaw cycle (P>0.05), indicating that the cryoprotectants tested had no effect on Ca^{2+}-ATPase activity of fresh mince. A continuous decrease in Ca^{2+}-ATPase activity in all samples was found with increasing freeze-thaw cycles (P<0.05). Ca^{2+}-ATPase activity of NAM from the control (without additive) showed a sharp decrease after freeze-thawing process, especially with 6 freeze-thaw cycles, in which less than 10% activity was retained. Kittiphattanabawon *et al*. (2012a) reported that surimi from threadfin bream without cryoprotectant was particularly susceptible to denaturation as induced by the repeated freeze-thaw cycles. The decrease in Ca^{2+}-ATPase activity was possibly due to the conformational change of myosin globular head responsible for ATPase activity as well as the aggregation of this portion (Ochaiai and Chow, 2000). Generation of ice crystals and the increase in ionic strength of the system induced myosin denaturation and disruption of the actin-myosin couples during frozen or freeze-thaw process (Benjakul and Sutthipan, 2009). The system containing NS-8GE showed more than 60% residual activity after 6 freeze-thaw cycles. The system added with S/S or blend showed higher residue activity (P<0.05), indicating the higher preventive effect on myosin heavy chain. Dey *et al*. (2013) found that protein hydrolysate from shrimp waste could reduce the effect of freeze denaturation on Ca^{2+}-ATPase activity of croaker surimi and the denaturation of myofibrillar Ca^{2+}-ATPase was correlated with the amount of unfrozen water (Dey *et al*., 2013). Cryoprotective

effect of NS-8GE could be attributed to the high proportion of hydrophilic amino acids, which had water constraining effect, thereby lowering the migration of water to form ice crystal. The results suggested that NS-8GE exhibited cryoprotective effect on myosin in washed mince system. However, the effectiveness was markedly increased when used in combination with commercial cryoprotectant (blend).

5.5.2.2 Surface hydrophobicity

Surface hydrophobicity (SoANS) of washed mince system added without and with NS-8GE, S/S or blend after freeze-thawing with different cycles is depicted in Figure 17 (b). Different SoANS of NAM added with various additives was noticeable. After mixing the washed mince with S/S and NS-8GE, the lower SoANS was observed in NAM. This was plausibly due to the interaction between NAM and those cryoprotectants via hydrophilic-hydrophilic interaction. The increase in SoANS of all samples was observed after freeze-thawing, especially for the control. With repeated freeze-thawing, unfolding of proteins and exposure of hydrophobic residues took place. The increase in SoANS of all samples was consistent with the decrease in Ca^{2+}ATPase (Figure 17 (a)). The increase in SoANS value of system containing NS-8GE or blend was distinctively lower than the control ($P<0.05$), especially at 6 freeze-thaw cycles. Similar SoANS was found amongst all samples containing NS-8GE, S/S or blend after 3 and 6 freeze-thaw cycles ($P>0.05$). Thus, the addition of NS-8GE could retard the conformational change of NAM as indicated by lowered change in SoANS. As a result, the denaturation of fish protein could be suppressed by addition of NS-8GE. Dey *et al*. (2013) reported that cryoprotective effect of shrimp waste protein hydrolysate on croaker surimi during frozen storage was caused by the active short chain peptide with molecular weight less than 1600 kDa. Furthermore, hydrophilic amino acids in peptides inhibited denaturaion of myofibrillar proteins and had water constraining effect. Thus, NS-8GE and blend could retard the change in protein conformation as induced by repeated freeze-thawing.

5.5.2.3 Disulphide bond content

Disulphide bond content in NAM extracted from washed mince system without and with NS-8GE, S/S or blend after multiple freeze-thaw process is depicted in Figure 17 (c). Disulphide bond content of NAM from the control increased sharply with increasing freeze-thaw cycles. Nevertheless, disulphide bond content in systems containing S/S, NS-8GE or blend increased to a lower extent. The increase in disulphide bond content was generally coincidental with the decrease in Ca^{2+}ATPase (Figure 17 (a)). When myosin molecules underwent conformational change, the reactive sulfhydryl groups could be more exposed. This could favour the formation of disulphide bond through oxidation. It was reported that the oxidation of sulfhydryl groups, especially in the head region, caused the decrease in Ca^{2+}ATPase activity (Benjakul *et al*., 1997). Cryoprotective effects of trehalose and sodium lactate on tilapia surimi were reported during frozen storage (Zhou *et al*., 2006). Kittiphattanabawon *et al*. (2012a) found that the decrease in sulfhydryl groups of NAM extracted from threadfin bream with a concomitant disulphide bond formation was coincidental with the decrease in Ca^{2+}ATPase activity during freeze-thawing process. The lower disulphide bond content ($P<0.05$) was observed for NS-8GE containing system at 0 freeze-thaw cycle, compared with others. NS-8GE with the reducing power (chapter 4) could reduce the disulphide bond to free sulfhydryl groups after mixing. After freeze-thawing process, NS-8GE and blend containing systems showed the lower disulphide bond content, especially with 6 freeze-thaw cycles, compared with those containing S/S and the control sample ($P>0.05$). In addition, the lowest disulphide bond content was observed for NS-8GE containing sample ($P<0.05$). The result suggested that freeze-thawing process possibly induced the oxidation of exposed sulfhydryl groups to disulphide bonds and the incorporation of NS-8GE exhibited the most effectiveness on retardation of disulphide bonds formation in washed mince system.

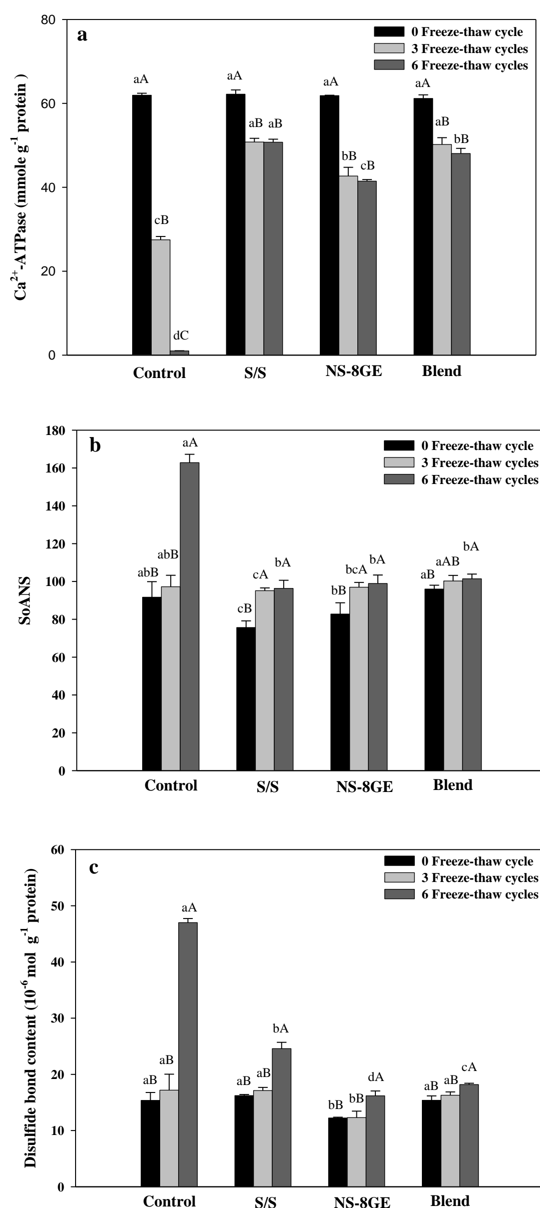

Figure 17 Ca^{2+}-ATPase activity (a), surface hydrophobicity (b) and disulphide bond content (c) of natural actomyosin from various washed mince systems subjected to different freeze-thaw cycles. Control; no additives, S/S; added with commercial cryoprotectant, NS-8GE; added with NS-8GE, Blend; added with NS-8GE/commercial cryoprotectant (1:1, w/w). Different uppercase and lowercase letters on the bars within the same additive and the same freeze-thaw cycle, respectively, indicate significant differences ($P< 0.05$). Bars represent standard deviation (n=3).

5.5.2.4 Thermal transitions of muscle protein

From DSC thermograms, the control system (without additive), had two major endothermic transition temperatures (T_{max}), corresponding to myosin and actin with T_{max} of 50.12 and 66.75 °C and ΔH of 0.46 and 2.02 J/g, respectively (Figure 18). This result was in accordance with the previous study on DSC thermograms of Japanese sea bass and black tiger shrimp muscle proteins, which showed two major endothermic transitions, corresponding to myosin and actin, respectively (Nikoo *et al*., 2014; Jantakoson *et al*., 2012). After 3-6 cycles of freeze-thawing, there was no marked change in T_{max} of myosin (49.80-50.82 °C) and actin (65.84-66.74 °C) in all systems tested. Relative ΔH of myosin and actin in the system added without (control) and with NS-8GE, S/S or blend after multiple freeze-thawing is shown in Figure 19 (a) and (b), respectively. Decrease in ΔH of myosin and actin was found with increasing freeze-thaw cycles. The control sample had the lowest ΔH for myosin and actin, compared with others. The highest residual ΔH of myosin and actin was found in the system added with S/S, followed by those containing blend and NS-8GE, respectively, at the same freeze-thaw cycle ($P<0.05$). Nevertheless, NS-8GE could retard the denaturation of myosin and actin more effectively, as indicated by higher residual ΔH, compared with the control. The decrease in ΔH of myosin in all samples was in accordance with the decrease in Ca^{2+}ATPase activity (Figure 17 (a)). For actin (Figure 19 (b)), ΔH of system containing NS-8GE was higher than that found for myosin (Figure 19 (a)). The results suggested that actin was more stable to denaturation than myosin as influenced by repeated freeze-thawing. Similar ΔH of actin was also observed between the system added with NS-8GE and blend. ΔH is a measure of remaining native protein, which reflects the status of ordered conformation of protein (Koshiyama *et al*., 1981). Thus, NS-8GE could decrease the denaturation of myosin and actin in washed mince induced by multiple freeze-thaw cycles.

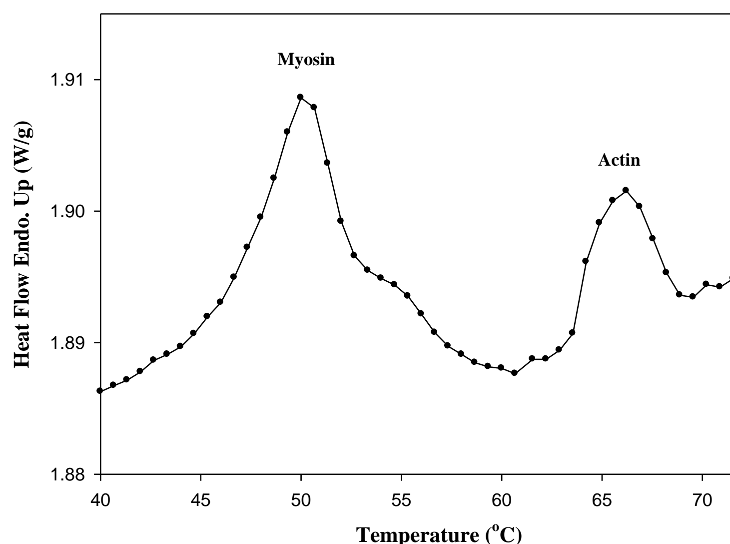

Figure 18 DSC thermogram of mackerel washed mince.

5.5.2.5 TBARS

As shown in Figure 20, TBARS value of all systems without freeze-thawing ranged from 9.83 to 11.24 mg MAD/kg sample, indicating that lipid oxidation occurred during handling or processing of mackerel to some extent. The increase in TBARS value of all samples was observed with increasing freeze-thaw cycle, except the sample added with blend, which showed a decrease after 6 freeze-thaw cycles (P<0.05). The decrease in TBARS was probably due to their reaction with free amino acids, proteins and peptides present in washed mince to form Schiff's bass (Dillard and Tapple, 1973). In addition, volatile oxidation products with low molecular weight could be lost during repeated freeze-thawing. Control sample (without additives) showed the higher formation of TBARS at all freeze-thaw cycles tested, compared with other samples (P<0.05). However, the sample containing blend possessed the highest TBARS value at 3 freeze-thaw cycles (P<0.05), compared with other samples. When S/S or NS-8GE was incorporated in the washed mince system, the formation of TBARS was retarded, especially those with NS-8GE. NS-8GE could retard the oxidation mainly via its radical scavenging activity (chapter 4). Apart from radical scavenging activity, NS-8GE also had metal chelating activity and electron donatin g ability, which were able to prevent lipid oxidation effectively (chapter 4).

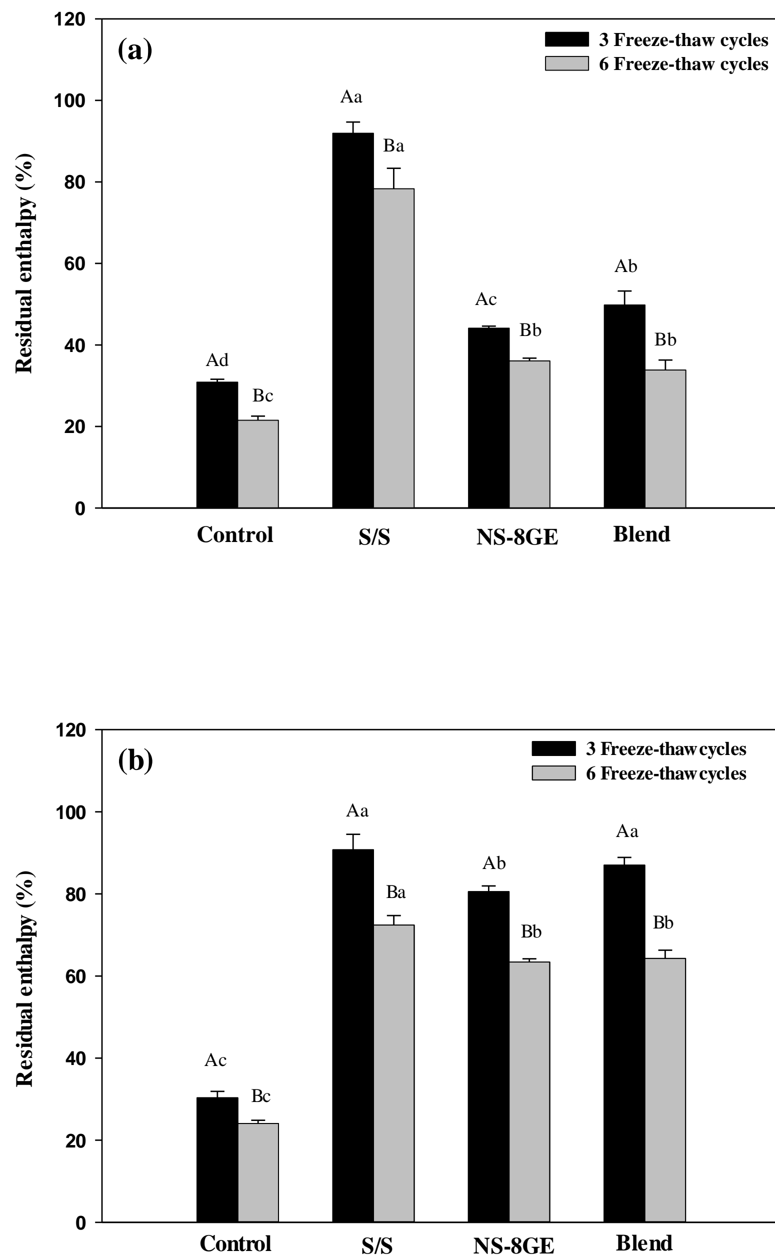

Figure 19 Residual enthalpy (ΔH) of myosin (a) and actin (b) of various washed mince systems subjected to multiple freeze-thaw cycles. Control; no additives, S/S; added with commercial cryoprotectant, NS-8GE; added with NS-8GE, Blend; added with NS-8GE/commercial cryoprotectant (1:1, w/w). Different lowercase and uppercase letters on the bars within the same freeze-thaw cycle and the same additive,

respectively, indicate significant differences (P<0.05). Bars represent standard deviation (n=3).

Qiu *et al*. (2014) found that silver carp antioxidant peptide could prevent lipid oxidation of fish fillets during frozen storage, in which conjugated diene value, TBARS value and fluorescence compounds were reduced during the storage. Antioxidative tetrapeptide isolated from Amur sturgeon skin gelatin prevented lipid oxidation in fish mince subjected to repeated freeze-thawing, as indicated by lower TBARS formation (Nikoo *et al*., 2014). Thus, NS-8GE exhibited the preventive effect against the lipid oxidation in washed mince system induced by freeze-thawing process.

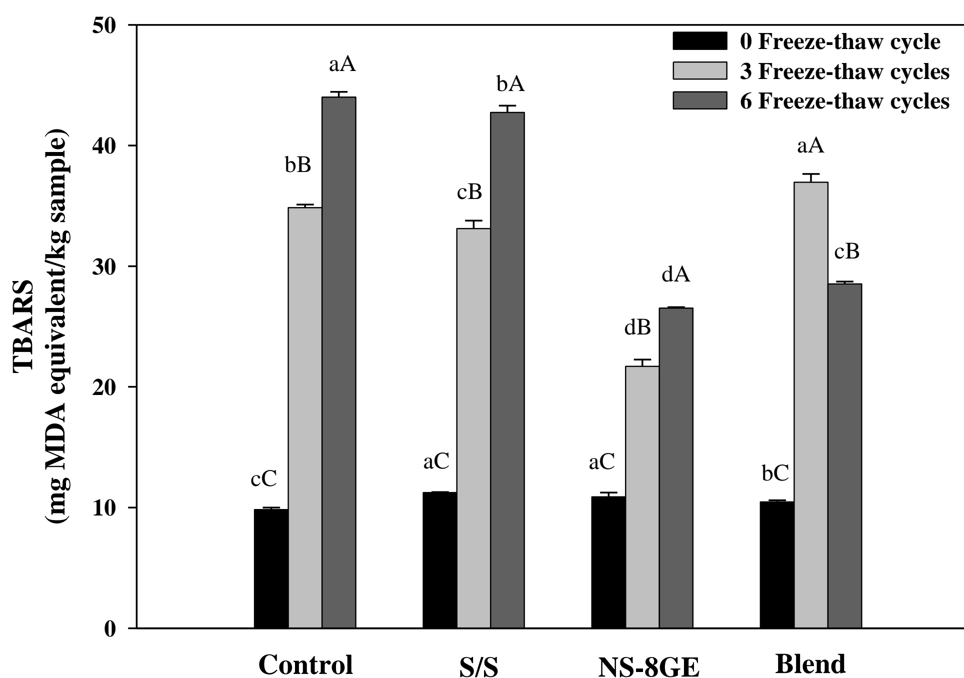

Figure 20 TBARS (a) of various washed mince systems subjected to different freeze-thaw cycles. Control; no additives, S/S; added with commercial cryoprotectant, NS-8GE; added with NS-8GE, Blend; added with NS-8GE/commercial cryoprotectant (1:1, w/w). Different uppercase and lowercase letters on the bars within the same additive and the same freeze-thaw cycle, respectively, indicate significant differences (P<0.05). Bars represent standard deviation (n=3).

5.5.2.6 Volatile compounds

The washed mince system added with NS-8GE yielding the lowest lipid oxidation as indicated by the lowest TBARS value (Figure 20) was selected for determination of volatile compound in comparison with the control (without additives) after 0 and 6 freeze-thaw cycles (Table 8). The volatiles identified in washed mince model system were mostly aldehydes, alcohols, and ketone. At 0 freeze-thaw cycle, hexanal was the major volatile compound. Heptanal, 1-pentene-3-ol, 1-octen-3-ol, 2-octenal, pentanal, butanal and 2-nonenal were also found at low level. Hexanal, heptanal and 2-nonenal were reported as the secondary oxidation products of linoleic acid (Grosch, 1987). Aliphatic alcohol contributed to off-flavour produced by oxidative deterioration of food lipid, such as 1-pentene-3-ol and 1-octen-3-ol which were described as oxidised and musty flavour, respectively (Badings, 1970). The formation of (E)-2-alkenals ranging from butenal to undecenal (such as 2-octenal and 2-decenal) indicated the oxidation of n-3, n-6 and n-9 fatty acids in fish oil (Grosch, 1987). Additionally, 1-alkanols and 1-alkanals (such as pentanol, pentanal and hexanal) can occur by the decomposition of the primary hydroperoxides of fatty acids (Kellard *et al*., 1985). Alkadienals (2, 4-decadienal and 2, 4-heptadienal), secondary lipid oxidation products, were found as a potent odourant in fish oil (Venkateshwarku *et al*., 2004). Unsaturated ketones (1-octen-3-one and 1-penten-3-one) were reported as off-flavours in oxidised lipid (Venkateshwarlu *et. al*., 2004). In the present study, 3-undecen-2-one was also detected as low content. The formation of volatile compounds was in agreement with TBARS detected in the sample without freeze-thawing (Figure 20). The increase in abundance of volatile compounds after 6 freeze-thaw cycles was observed. In general, all compounds detected in NS-8GE containing system were markedly lower than those of control. The control showed the marked increase in 2, 4-heptadienal, approximately 10-fold after 6 freeze-thaw cycles. It was reported that the increase in 2, 4-heptadienal yielded an extremely rancid odour in fish oil (Hu and Pan, 2000). Moreover, pentanol was detected after freeze-thawing process in both control and sample added with NS-8GE. The result suggested that freeze-thawing process could induce lipid oxidation in the system as indicated by the increase in abundance of lipid oxidation products and

formation of new volatile compounds. Nevertheless, those volatile compounds were generated to a low extent in the system containing NS-8GE. The result reconfirmed the antioxidative acitivity of NS-8GE. Therefore, NS-8GE could retard oxidative deterioration and prevent the formation of offensive odorous compounds in the washed mince system. Thus, NS-8GE could act as an alternative additive having both cryoprotective and antioxidative properties.

Table 8 Volatile compounds in washed mince before and after freeze-thawing

Compounds	Peak area (Abundance)×10^7			
	Control (Freeze-thaw cycles)		NS-8GE (Freeze-thaw cycles)	
	0	6	0	6
Butanal	11.42	31.98	2.73	3.83
Pentanal	20.97	46.61	4.50	5.79
Hexanal	326.79	455.30	178.10	217.63
1-Pentene-3-ol	26.02	42.01	17.60	21.14
Heptanal	30.52	64.50	10.63	15.9
Pentanol	nd	14.62	nd	7.84
Hexanol	4.32	13.17	4.36	18.56
Nonanal	7.81	9.85	3.16	4.61
2-Octenal	22.92	26.62	3.54	5.12
1-Octen-3-ol	27.31	45.88	15.82	17.07
2,4-Heptadienal	10.71	118.99	6.62	7.01
2-Nonenal	11.97	16.90	2.90	9.43
2-Decenal	4.56	6.31	1.90	1.41
3-Undecen-2-one	10.61	22.20	3.11	11.43
2,4-Decadienal	15.30	19.92	1.81	4.10

nd: not detected.
Control: without additive.
NS-8GE: added with NS-8GE.

5.6 Conclusion

The cryoprotective gelatin hydrolysate with antioxidative property could be produced from unicorn leatherjacket skin using autolysis of non-swollen skin in combination with partially purified glycyl endopeptidase from papaya latex. Gelatin hydrolysate was effective in protecting protein denaturation of mackerel

washed mince induced by freeze-thawing process and in retarding lipid oxidation. Therefore, resulting gelatin hydrolysate could be used as an alternative cryoprotectant with antioxidative potential in surimi or related products.

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CHAPTER 6

Production of antioxidative Maillard reaction product from gelatin hydrolysate of unicorn leatherjacket skin

6.1 Abstract

Antioxidative activities of Maillard reaction products (MRPs) derived from gelatin hydrolysate (NS-8GE) of unicorn leatherjacket skin as affected by types of saccharides, NS-8GE to saccharide ratio, incubation temperatures, relative humidity (RH) and times were investigated. MRPs prepared using the mixture of NS-8GE and galactose showed the highest antioxidative activity, compared with those derived from other saccharides tested (glucose or fructose). NS-8GE to galactose ratio of 2:1 (w/w) yielded the MRPs with the highest antioxidative activity. When various incubation temperatures (50, 60, 70 °C) and RHs (55, 65, 75%) were used for MRPs preparation, the highest browning index and the absorbance at 280 nm were found at 70 °C ($P<0.05$), regardless of RH. Antioxidative activity of MRPs generally increased with increasing temperatures. The highest antioxidative activity was found when RH of 55% was used. Based on electrophoresis, the formation of high molecular weight polymers were observed in MRPs. FTIR spectra revealed that the advanced glycation took place in MRPs as evidenced by the changes in wavenumbers and amplitude of amide bands. The optimal condition for preparing antioxidative MRPs included heating the mixture of NS-8GE and galactose (2:1) at 70 °C and 55% RH for 36 h.

6.2 Introduction

Gelatin hydrolysate, especially from marine resources, has been known to possess antioxidative activity and has gained increasing interest as the food additives with multi-functions (Nikoo *et al.*, 2014; Kittiphattanabawon *et al.*, 2012). Several proteases have been used to produce gelatin hydrolysates (Gómez-Guillén *et al.*, 2011). Protease from papaya latex is one of the common enzymes used for preparing protein hydrolysates with bioactivities (Kittiphattanabawon *et al.*, 2012). Recently, the used of partially purified glycyl endopeptidase (GE) from papaya latex successfully yielded antioxidative hydrolysates without undesirable odor (chapter 3).

Skin of unicorn leatherjacket (*Aluterus monoceros*) has been reported to possess indigenous protease, causing the degradation of α-chain during gelatin extraction at 50-55 °C (Kaewruang *et al.*, 2013; Ahmad *et al.*, 2011). Autolysis-assisted process mediated by indigenous protease has recently shown the potential for production of gelatin hydrolysates from skin of unicorn leatherjacket with enhanced antioxidative activity (chapter 2). Moreover, the use of indigenous protease in combination with GE from papaya latex could increase antioxidative activity of gelatin hydrolysate from unicorn leatherjacket skin (chapter 4 and 5). Nevertheless, the activity of gelatin hydrolysate was still lower in comparison with the commercially available antioxidant.

Maillard reaction has gained the interest as the effective tool without the use of conventional chemical reagent for improvement of both sensory characteristics and bioactivity of food proteins or peptides (Eric *et al.*, 2013; Wang *et al.*, 2013; Liu *et al.*, 2014). Maillard reaction is based on the condensation between carbonyls and amines (Jing and Kitts, 2004). Maillard reaction products (MRPs) have been known to have strong antioxidative activity, including radical chain breaking, electron donating and metal chelating ability (Dong *et al.*, 2012; Liu *et al.*, 2014). Several antioxidative MPRs derived from protein hydrolysates have been investigated (Liu *et al.*, 2014; Sumaya-Martinez *et al.*, 2005; You *et al.*, 2011). Generally, the numerous factors, such as heating condition, type and concentration of saccharides or

protein, etc., have been shown to determine antioxidative activity of MRPs. However, a little information on MRPs from fish skin gelatin hydrolysate has been reported.

6.3 Objective

To investigate the characteristics and antioxidative activity of MRPs derived from gelatin hydrolysate from unicorn leatherjacket skin as affected by several processing parameters.

6.4 Materials and methods

6.4.1 Chemicals

D-glucose, D-fructose, D-galactose and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were procured from Fluka (Buchs, Switzerland). 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2′-azinobis (3- thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Coomassie Blue R-250, and *N,N,N´,N´*-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Low molecular weight marker was purchased from GE healthcare UK, Limited (Buckinghamshire, UK). All chemicals were of analytical grade.

6.4.2 Preparation of autolysed skins from unicorn leatherjacket skin

Skins of unicorn leatherjacket (*A. monoceros*) were obtained from a dock, Songkhla, Thailand. Three different lots of skins were collected. For each lot, skins were pooled and used as the composite sample. Skins were 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, Hat Yai within 1 h. Upon arrival, the skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm2), placed in polyethylene bags and stored at -20°C until use. The storage time was less than 2 weeks.

6.4.2.1 Pretreatment of fish skins

Fish skins (0.5×0.5 cm^2) were soaked in 0.05M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins (Kaewruang *et al.*, 2013). The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Staufen, Germany) at a speed of 150 rpm. The alkaline solution was changed after 2 h and total pretreatment time was 4 h. Pretreated skins were washed with tap water until neutral or faintly basic pH of wash water was obtained.

6.4.2.2 Preparation of autolysed fish skin

The pretreated skins were mixed with deionised water at a ratio of 1:5 (w/v). The autolysis was performed by incubating the mixture in a water bath (Model W350, Memmert, Schwabach, Germany) at 55°C for 12 h and terminated by heating at 90°C for 15 min (chapter 5.4.2.3). The mixture was centrifuged at 5000×g at 4°C using a refrigerated centrifuge, model Avanti J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) for 10 min to remove the debris. The suppernatant was collected, freeze-dried and referred to as autolysed non-swollen skin (NS).

6.4.3 Production of gelatin hydrolysate from autolysed skin using partially purified glycyl endopeptidase (GE)

6.4.3.1 Preparation of crude extract from papaya (*C. papaya*) latex

The crude extract was prepared from papaya latex (Kittiphattanabawon *et al.*, 2012) and used for preparation of glycyl endopeptidase. The glycyl endopeptidase was fractionated using ATPS in combination with ammonium sulphate precipitation (chapter 3).

6.4.3.2 Production of gelatin hydrolysates

Solution of autolysed skin (3%, w/v) was added with GE (8%, based on solid matter). After the reaction proceeded at 40 °C for 60 min (chapter 4), enzyme inactivation was carried out by heating the mixture at 90 °C for 15 min. Thereafter,

the mixture was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and freeze-dried using Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The gelatin hydrolysate powder was referred to as 'NS-8GE'.

6.4.4 Preparation of Maillard reaction products (MRPs) with antioxidative activities

6.4.4.1 Study on the effects of type of saccharides and NS-8GE to saccharide ratios

NS-8GE powder was mixed with different saccharides (glucose, fructose and galactose) at various ratios (1:2, 1:1 and 2:1; w/w). Those mixtures were dissolved in distilled water to obtain the final solid concentration of 60 mg/mL. Thereafter, the pH of solutions was adjusted to 7.0 with 1 M NaOH or 1 M HCl, followed by freeze-drying. Resulting NS-8GE-saccharide powders were placed on the glass petridish and incubated at 65% relative humidity at 60 °C in an environmental chamber (WTB Binder, Tuttlingen, Germany). At 12, 24, 36 and 48 h of incubation, the MRP was collected and subjected to analyses.

6.4.4.2 Study on the effects of temperature and relative humidity

The powder of NS-8GE:galactose (2:1) was transferred into the petridish. The powder was incubated at different temperatures (50, 60 and 70 °C) and relative humidity (55, 65 and 75%) for 0-48 h. The MRP was collected and analysed.

6.4.5 Analyses

6.4.5.1 Determination of free amino group content

MRP solution with appropriate dilution (125 µL) was mixed with 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution was added. The mixtures were mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Bavaria, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was measured at 420 nm

(Benjakul and Morrissey, 1997). Free amino acid content was expressed in term of L-leucine.

6.4.5.2 Measurement of UV-absorbance and browning index

Appropriate dilution (50-fold) was made from MRP sample (60 mg/mL) using distilled water and the absorbance was measured at 294 and 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) for determining UV-absorbance and browning intensity, respectively (Ajandouz *et al.*, 2011).

6.4.5.3 Measurement of fluorescence

Fluorescence intensity of MRP samples (60 mg/mL) with an appropriate dilution (50-fold) was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 Fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) (Morales and Jimenez-Perez, 2001).

6.4.5.4 Measurement of pH

Prior to determination, MRP was dissolved in distilled water to obtain the concentration of 60 mg/mL. The pH value of solution was measured using a pH meter (Sartorius PB-10, Göttingen, Germany).

6.4.5.5 Antioxidative activity

6.4.5.5.1 ABTS radical scavenging activity

The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution (Binsan *et al.*, 2008). The working solution was prepared by mixing two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The solution obtained (1 mL) was then diluted with 50 mL of distilled water, in order to obtain an absorbance of 1.10±0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 μL) was mixed with 2850 μL of ABTS solution and the mixture was left at room temperature

for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer (Binsan *et al.*, 2008). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalent (TE)/g solid.

6.4.5.5.2 Ferric reducing antioxidant power (FRAP)

FRAP reagent was prepared by mixing acetate buffer (30 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v/v) (Benzie and Strain, 1996). The sample solution (100 μL) was mixed with 3 mL of working FRAP reagent and incubated in the dark at room temperature for 30 min. The absorbance of the reaction mixture was read at 593 nm using a spectrophotometer (Benzie and Strain, 1996). The standard curve was prepared using Trolox ranging from 0 to 500 μM. The activity was expressed as μmol Trolox equivalents (TE)/g solid.

6.4.6 Characterization of the selected MRP

MRP with the highest ABTS radical scavenging activity and FRAP obtained from NS-8GE:Gal incubated at 70 °C, 55% RH for 36 h were characterized in comparison with NS-8GE and NS-8GE:Gal powder (without heating).

6.4.6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein solutions were mixed at a 1:1 (v/v) ratio with the sample buffer (0.125M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (20 μg protein) were loaded onto the gel made of 4% stacking and 17.5% separating gels. They were subjected to an electrophoresis at a constant current of 15 mA per gel using a Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the gel was stained overnight with staining solution (0.02% (w/v) Coomassie Brilliant Blue R-250) in 50% (v/v) methanol, and 7.5% (v/v) acetic acid.

Protein patterns were then visualised after destaining with 30% methanol and 10% acetic acid until a clear background was obtained (Laemmli, 1970).

6.4.6.2 FT-IR measurements

FT-IR spectra were obtained using an FT-IR spectrometer (EQUINOX 55, Bruker, Germany). Diffusive reflectance of the IR was measured using the powder specimens diluted with potassium bromide (KBr) powder of spectroscopic grade by one-tenth and recorded with an average of 32 scans at a resolution of 4 cm^{-1}. Background noise was corrected with pure KBr data (Wang *et al.*, 2013).

6.4.7 Statistical analysis

A completely randomized design (CRD) was used throughout the study. All data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using the Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The result with $P<0.05$ was considered to be statistically significant.

6.5 Results and discussion

6.5.1 Effects of types of saccharides and NS-8GE to saccharide ratios on antioxidative activities of MRPs

The effects of three different saccharides and NS-8GE to saccharide ratios on antioxidative activities of MRPs as determined by ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) are shown in Figure 21. The mixture of NS-8GE and saccharides (glucose, fructose or galactose) at a ratio of 1:1 (w/w) was used to prepare the MRPs by heating the mixture at 60 °C and 65% RH. Both ABTS radical scavenging activity (Figure 21 (b)) and FRAP (Figure 21 (d)) of all MRPs increased with increasing heating time ($P<0.05$). Amongst all mixtures, NS-8GE:galactose yielded MRPs with the highest antioxidative activity ($P<0.05$), followed by NS-8GE:glucose and NS-8GE:fructose, respectively. It was reported that

Maillard reaction rate of the protein and galactose system was faster than that of the systems containing other sugars (Kato *et al.*, 1986). The configuration of the reaction intermediate (Amadori compound) played an important role in the reaction rate. With the proper configuration of protein-galactose system, the reaction intermediate compound could form the energetically stable chair form, which smoothly and rapidly converted into glyceraldehyde compound and brown polymer, respectively (Kato *et al.*, 1986). Antioxidative activity of the heated protein-sugar systems was correlated with the formation of MRPs, in which galactose system showed the greater activity than those from other saccharides (Liu *et al.*, 2014a; Benjakul *et al.*, 2005). Therefore, MRPs derived from NS-8GE:galactose system with the highest antioxidative activity was further studied.

Antioxidative activities of resulting MRPs from NS-8GE:galactose system at different ratios (1:2, 1:1, 2:1, w/w), subjected to heating at 60 °C and 65% RH, are depicted in Figure 21 (a) and Figure 21 (b). Both ABTS radical scavenging activity and FRAP of MRPs increased with increasing heating time. As the ratios between NS-8GE and galactose decreased, the antioxidative activity decreased ($P<0.05$). Amongst all ratios tested, NS-8GE to galactose ratio of 2:1 rendered MRPs with the highest antioxidative activity ($P<0.05$). Antioxidative activity of MRPs derived from silver carp protein hydrolysate-glucose system decreased as protein concentration decreased (You *et al.*, 2011). The difference in antioxidative activity of MRPs observed in different studies was possibly due to the different compositions of amino acid and conformation of protein and saccharides as well as the conditions used for MRPs preparation. Therefore, MRPs from NS-8GE:galactose with a ratio of 2:1 (NS-8GE:Gal) had high free radical scavenging activity and were capable of donating electron.

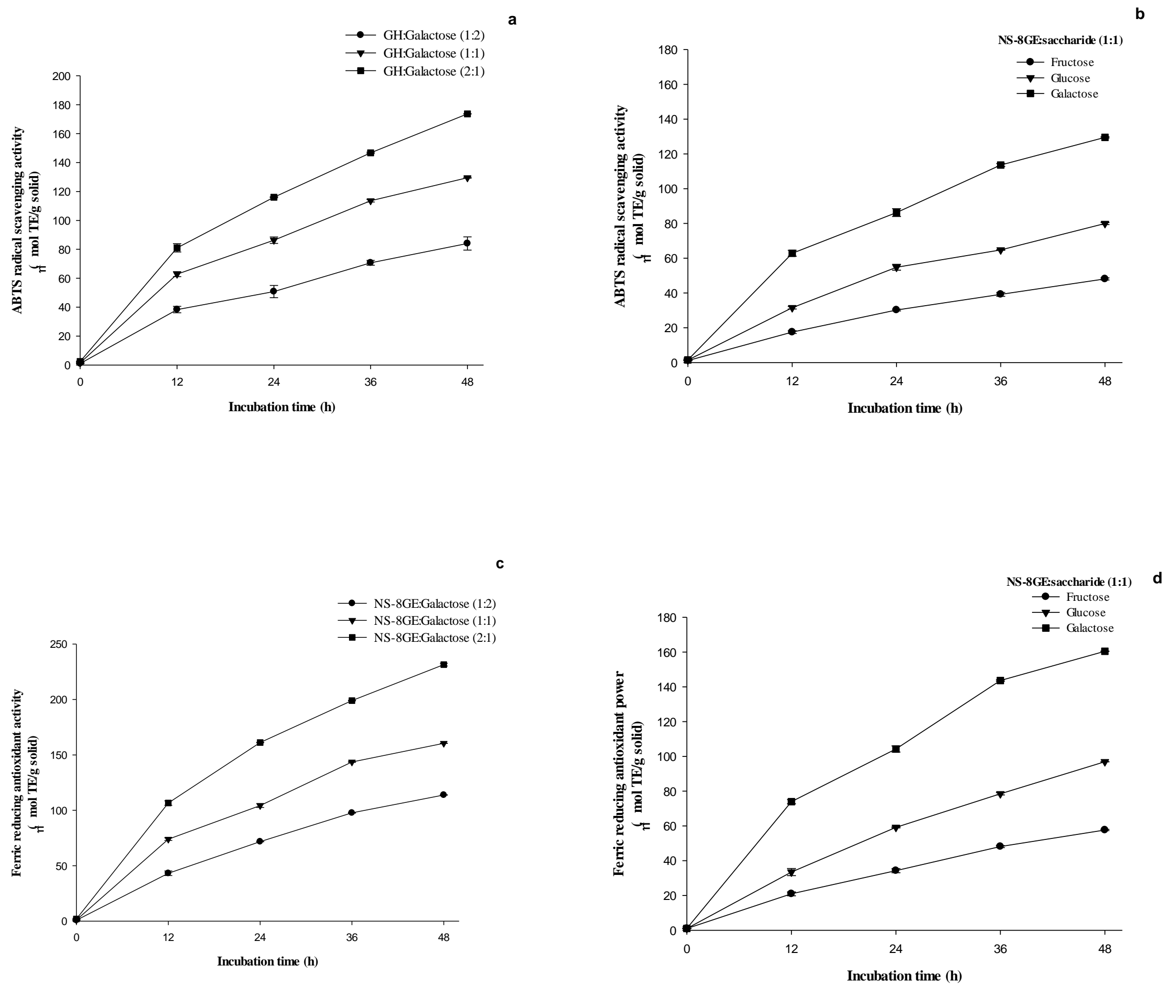

Figure 21 The effects of different saccharides (b and d) and protein saccharide ratio (a and c) on antioxidative activity of MRPs during heat treatment (0-48 h) at 60 °C, 65% RH. a and b; ABTS radical scavenging activity, c and d; Ferric reducing antioxidant power. Bars represent standard deviation (n=3).

6.5.2 Effects of incubation temperatures and relative humidity (RH) on characteristics and antioxidative activity of MRPs

6.5.2.1 Browning index and absorbance at 294 nm

The effects of incubation temperatures and RH on Maillard reaction were monitored as a function of incubation time as shown in Figure 22. The absorbance at 420 was used as a measure of browning index due to the formation of final product and the absorbance at 294 nm represented the generation of intermediate product of Maillard reaction. Generally, the absorbance at 420 and 294 nm of NS-8GE:Gal system increased as the incubation time increased (P<0.05), regardless of temperature and RH used. It was found that the highest formation rate of MRPs (P<0.05) was obtained when the mixture was incubated at the highest temperature (70 °C), followed by 60 °C and 50 °C, respectively. The similar result was reported for silver carp protein hydrolysate-glucose system (You *et al.*, 2011). At the same incubation temperature, the final products of Maillard reaction were generated at the lowest rate when RH of 55% was used (P<0.05). On the other hand, the highest browning index with coincidentally lowest intermediate product was obtained in MRP prepared at 75% RH (P<0.05), especially when heated at 70 °C, compared with those from other RH used. It was suggested that the conversion of intermediate to final products was more likely affected by moisture content and water activity of NS-8GE:Gal system as governed by different RHs used. Non-enzymatic browning rate increased as the initial water activity increased in lysine and xylose containing maltodextrin or polyvinylpyrrolidone model system (Lievonen and Roos, 2002). Therefore, both incubation temperature and RH were the crucial factors affecting the production of final and intermediate products in MRPs.

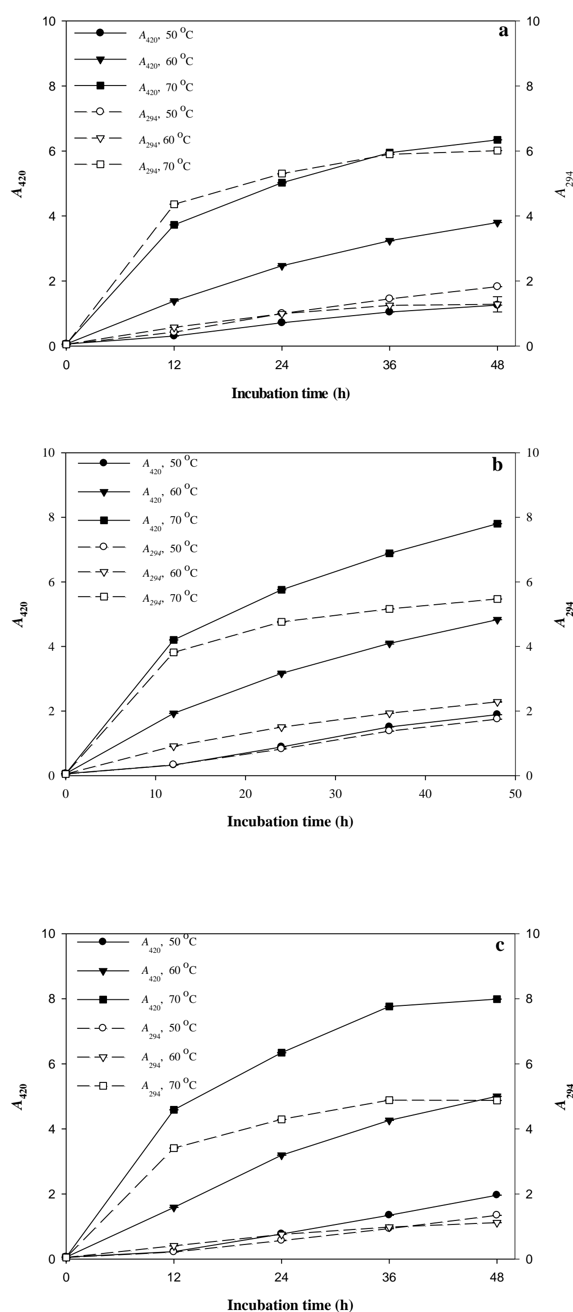

Figure 22 Changes in absorbance at 420 nm (—) and 294 nm (- - -) of MRPs at different temperatures and RH. a; 55% RH, b; 65% RH, c; 75% RH. Bars represent standard deviation (n=3). within the same additive and the same freeze-thaw cycle, respectively, indicate significant differences (P<0.05). Bars represent standard deviation (n=3).

6.5.2.2 Fluorescence intensity

Fluorescence intensity of MRPs derived from NS-8GE:Gal system is shown in Table 9. NS-8GE:Gal system had the sharp increases in fluorescence intensity within the first 12 h. Thereafter, the rate of increase was decrease. The decrease in fluorescence intensity was also found in some samples, depending on the condition used. The lowest fluorescence intensity (P<0.05) was found when the highest temperature was used (70 °C) under the same RH used. The similar result was also found in porcine plasma protein-sugar model system, in which a sharp increase in fluorescence intensity was observed within the first hour of heating, followed by a continuous decrease up to 5 h of heating (Lertittikul *et al.*, 2007). On the other hand, MRPs prepared at 50 °C showed a continuous increase during incubation (0-48 h). Nevertheless, a slight change in fluorescence intensity was observed after 24 h of heating time (P<0.05). The lower intensity of fluorescence intensity was related well with lower browning index of MRPs when incubated at 50 °C (P<0.05), compared with higher temperature. The fluorescence development in Maillard reaction is generally used as an indicator of reaction rate and MRPs formation (Yeboah et al, 1999). The development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Jing and Kitts, 2002). It was noted that the different RH used had varying impacts on reaction rate, in which MRPs prepared at 75% RH showed the higher reaction rate as evidenced by the high fluorescence intensity, followed by the subsequent decrease. The change was generally more pronounced at 70 °C. The results indicated the different degree of conversion of intermediates to polymer compounds when various incubation temperatures and RH were used for MRP preparation.

6.5.2.3 pH

pHs of MRPs derived from NS-8GE:Gal system under different incubation temperatures and RHs are shown in Table 9. The initial pH of NS-8GE:Gal system was 6.81 and it decreased gradually as incubation times increased (P<0.05), at all RH and temperature used. Nevertheless, different pHs of MRPs were found when various incubation temperatures and RH were used. Amongst MRPs prepared at

Table 9 Characteristics of MRPs derived from NS-8GE:Gal systems as affected by heating temperature and RH at various incubation times

Relative humidity (%)	Temperature (°C)	Time (h)	Fluorescent intensity	pH	Free amino group content (mmol/g solid)
NS-8GE:Gal (2:1, w/w)		0	1.82±0.02	6.81±0.01	2.11±0.05
55	50	12	73.98±0.21^{dCy}	5.41±0.01^{aAz}	0.64±0.00^{aAz}
		24	113.45±0.50^{cAy}	5.02±0.01^{bAy}	0.41±0.01^{bAz}
		36	124.92±0.10^{bAz}	4.82±0.01^{cAy}	0.30±0.00^{cAz}
		48	128.69±0.44^{aAz}	4.69±0.01^{dAy}	0.26±0.00^{cAz}
	60	12	117.07±0.44^{aAz}	4.61±0.01^{aBz}	0.32±0.02^{aBz}
		24	114.19±0.62^{bAz}	4.40±0.01^{bBy}	0.27±0.01^{bCz}
		36	95.70±0.80^{cBx}	4.31±0.01^{cBy}	0.26±0.00^{bAy}
		48	85.91±0.82^{dBx}	4.24±0.01^{dBy}	0.26±0.00^{bAx}
	70	12	86.36±0.10^{aCx}	4.26±0.01^{aCz}	0.45±0.00^{aCz}
		24	62.18±0.95^{bBx}	4.14±0.01^{bCy}	0.34±0.00^{bBz}
		36	48.23±0.06^{cCx}	4.11±0.01^{cCx}	0.31±0.00^{bAz}
		48	42.96±0.68^{dCx}	4.06±0.01^{dCy}	0.30±0.01^{bAz}
65	50	12	90.20±0.05^{dBx}	5.56±0.02^{aAy}	0.82±0.01^{aAy}
		24	144.11±0.22^{cAx}	5.02±0.03^{bAy}	0.51±0.01^{bAy}
		36	160.52±0.41^{aAy}	4.82±0.01^{cAy}	0.40±0.01^{cAy}
		48	156.56±1.78^{bAy}	4.69±0.01^{dAy}	0.36±0.02^{cAy}
	60	12	141.77±0.07^{aAy}	4.67±0.01^{aBy}	0.64±0.01^{aBx}
		24	116.02±0.50^{bBy}	4.44±0.02^{bBy}	0.37±0.01^{bBy}
		36	81.110.57^{cBz}	4.28±0.01^{cBy}	0.19±0.01^{cCz}
		48	63.97±0.64^{dBz}	4.22±0.01^{dBxy}	0.18±0.01^{cBy}
	70	12	83.27±0.78^{aCy}	4.33±0.01^{aCy}	0.55±0.01^{aCy}
		24	52.90±1.11^{bCy}	4.17±0.01^{bCxy}	0.38±0.02^{bBy}
		36	38.43±0.19^{cCy}	4.12±0.01^{cCx}	0.37±0.01^{bBy}
		48	30.26±0.12^{dCy}	4.10±0.01^{cCx}	0.38±0.01^{bAy}
75	50	12	70.10±0.05^{cCz}	5.76±0.02^{dAx}	1.10±0.02^{aAx}
		24	143.42±0.59^{bAx}	5.31±0.01^{cAx}	0.89±0.01^{bAx}
		36	169.58±1.78^{aAx}	5.02±0.02^{bAx}	0.66±0.01^{cAx}
		48	168.66±2.19^{aAx}	4.81±0.03^{aAx}	0.54±0.00^{dAx}
	60	12	148.01±0.40^{aAx}	4.84±0.01^{dBx}	0.54±0.00^{aBy}
		24	125.11±0.32^{bBx}	4.53±0.01^{cBx}	0.45±0.01^{bBx}
		36	93.35±0.43^{cBy}	4.35±0.01^{bBx}	0.30±0.01^{cBx}
		48	75.61±0.55^{dBy}	4.25±0.01^{aBx}	0.25±0.01^{cAx}
	70	12	82.44±0.35^{aBy}	4.36±0.01^{aCx}	0.78±0.01^{aCx}
		24	50.46±2.52^{bCy}	4.19±0.01^{bCx}	0.56±0.00^{bCx}
		36	32.51±0.64^{cCz}	4.11±0.01^{cCx}	0.55±0.01^{bCx}
		48	29.43±1.86^{cCy}	4.06±0.01^{dCy}	0.52±0.01^{bBx}

Data are mean ± standard deviation (n=3). $^{a, b, c, d}$ indicate significant differences ($p < 0.05$) between values in the same column within the same temperature and relative humidity. $^{A, B, C}$ indicate significant differences ($p < 0.05$) between values in the same column within the same incubation time and relative humidity. $^{x, y, z}$ indicate significant differences ($p < 0.05$) between values in the same column within the same incubation time and temperature.

different temperatures, that incubated at 70 °C showed the highest rate of pH decrease ($P<0.05$), followed by those prepared at 60 °C and 50 °C, respectively. In addition, MRPs obtained from different RHs had the slight differences in pH, when the same temperature was used. The decrease in pH of MRPs might be due to the production of formic and acetic acids from the reducing sugar, which is partially degraded into these compounds via Maillard reaction (Rufian-Henares *et al.*, 2006). Moreover, the reaction of amines to form less basic reaction products or acids could be the mechanism underlying the pH decrease in Maillard reaction system (Liu *et al.*, 2008). The decrease in pH was in accordance with the formation of MRPs (Figure 22). It has been known that reaction rate between sugar and amino group in dry system was much faster than that found in an aqueous solution (Kato *et al.*, 1986). Lower water activity in sugar-protein in dry system more likely favored Maillard reaction, in which the reactants were aligned closely for glycation. Therefore, the incubation temperature and RH affected the formation of MRPs in NS-8GE:Gal system during heat treatment as monitored by the varying decreases in pH of system.

6.5.2.4 Free amino group content

The effect of incubation temperature and RH on the change of free amino group content in MRPs as a function of time is shown in Table 9. Free amino group content of NS-8GE:Gal without heating was 2.11 mmol/g solid and it decreased as heating time increased. The marked decrease in free amino group content was observed within the first 24 h of heating ($P<0.05$). Thereafter, the rate of decrease was lower up to 48 h. The decrease in free amino group content was lower in MRPs prepared at 50 °C, compared with those with higher temperatures ($P<0.05$). In general, MRPs prepared at 55% RH exhibited the highest decreasing rate in free amino group content ($P<0.05$), especially those heated at 70 °C (0.30-0.45 mmol/g solid). This was in accordance with the higher increase in browning index and absorbance at 294 nm of MRPs prepared at 70 °C (Figure 22). The results indicated that α- or ε-NH_2 group in the NS-8GE covalently attached to the galactose to form advanced glycation end products to a greater extent with increasing reaction time, especially at low RH and high temperature. Free amino groups of porcine plasma

protein hydrolysate in the presence of reducing sugar decreased gradually via Maillard reaction during the extended heating (Liu *et al.*, 2014a). However, the browning reaction and the protein polymerization depended on the formation of degradation compounds rather than upon the sugar binding reaction to protein amino groups (Kato *et al.*, 1986). Therefore, the decrease in free amino group content in resulting MRPs could be used as an index for glycation of NS-8GE:Gal system, which was affected by temperature, RH and time.

6.5.2.5 Antioxidative activity

ABTS radical scavenging activity and FRAP of MRPs derived from NS-8GE:Gal system prepared by heating at different temperatures and RH are shown in Table 10. ABTS radical scavenging activity of NS-8GE:Gal system without heating (0 h) was 14.13 μmol TE/g solid. After heating, the activity of resulting MRPs generally increased as the incubation time increased ($P<0.05$). The role of Maillard reaction in enhancement of free radical scavenging activity of β-lactoglobulin and α-lactalbumin was reported (Jiang and Brodkorb, 2012). The sharp increase in ABTS radical scavenging activity was found in MRPs prepared by heating at 70 °C, especially those with 55% RH which showed the highest activity (173.16-230.12 μmol TE/g solid) ($P<0.05$). However, no difference in activity was observed between MRPs prepared at 70 °C and 55% RH for 36 h and 48 h ($P>0.05$). Maillard reaction could be a good method to improve radical scavenging activity of silver carp hydrolysate (You *et al.*, 2011). Furthermore, the antioxidative activity was increased when Maillard reaction was performed at higher temperature (You *et al.*, 2011). When the different RH was used, the resulting MRPs prepared at 75% RH showed the lower ABTS radical scavenging activity ($P<0.05$). The radical scavenging activity correlated well with absorbance at 294 nm (Table 9). Free radical scavenging activity of glycated proteins was not directly related to the glycation degree (Chevalier *et al.*, 2001). However, the correlation between radical scavenging activity and fluorescence intensity or browning intensity were reported for some systems (Morales and Jimenes-Perez, 2001; Benjakul *et al.*, 2005). The results confirmed that radical

activity of NS-8GE could be improved via Maillard reaction under the optimal condition.

The similar results were obtained for FRAP (Table 10). FRAP of NS-8GE:Gal system without heat treatment (1.96 μmol TE/g solid) increased when Maillard reaction was introduced. MRPs prepared at 70 °C showed the higher increase in FRAP ($P<0.05$), followed by those heated at 60 °C and 50 °C, respectively. Nevertheless, no change in FRAP was observed after 36 h of heating (314.61-315.36 μmol TE/g solid) ($P>0.05$). When Maillard reaction was performed at 75% RH, FRAP was lower, whereas the greatest increase in FRAP was found when 55% RH was employed ($P<0.05$). Hydroxyl and pyrrole groups of advanced MRPs might act as reducing agents (Yanagimoto *et al.*, 2002). Reducing capacity of MRPs could be produced during the formation of heterocyclic products in the Maillard reaction (Kim and Lee, 2009). Amadori products in the primary phase of Maillard reaction were reported as the reducing agent in MRPs derived from β-lactogluculin-glucose system (Dong *et al.*, 2012). The discrepancies in the literature may be related to the diversity of conditions used for MRP preparation as well as the variation of reactants in the system. Due to high ABTS radical scavenging activity and reducing power, MRP derived from the NS-8GE:Gal system heated at 70 °C with 55% RH for 36 h could be used as an alternative antioxidant to prevent lipid oxidation in food products.

Table 10 Antioxidative activity of MRPs derived from NS-8GE:Gal systems as affected by heating temperature and RH at various incubation times

Relative humidity (%)	Temperature (°C)	Incubation time (h)	ABTS radical scavenging activity (µmol TE/g solid)	FRAP (µmol TE/g solid)
NS-8GE:Gal (2:1, w/w)		0	14.13±0.84	1.96±0.03
55	50	12	22.93±6.47^{dCx}	33.89±0.47^{dCx}
		24	43.56±7.04^{cCx}	66.46±0.49^{cCx}
		36	64.56±2.24^{bCx}	87.99±0.63^{bCy}
		48	76.00±0.92^{aCy}	104.42±0.60^{aCy}
	60	12	77.41±0.58^{dBx}	102.65±4.85^{dBxy}
		24	107.74±6.60^{cBx}	165.10±8.75^{cBx}
		36	136.06±5.65^{bBy}	204.46±6.51^{bBx}
		48	154.00±8.66^{aBy}	236.52±5.94^{aBx}
	70	12	173.16±2.02^{cAx}	238.84±1.76^{cAx}
		24	197.84±2.60^{bAx}	282.46±5.98^{bAx}
		36	226.36±5.82^{aAx}	314.61±6.01^{aAx}
		48	230.12±2.80^{aAx}	315.36±2.09^{aAx}
65	50	12	23.52±0.53^{dCx}	33.11±0.74^{dCx}
		24	48.07±1.36^{cCx}	65.94±0.85^{cCx}
		36	66.17±4.71^{bCx}	92.01±0.99^{bCx}
		48	81.92±1.89^{aCx}	110.00±0.25^{aCx}
	60	12	81.00±2.85^{dBx}	106.57±2.02^{dBx}
		24	116.02±0.33^{cBx}	161.18±0.20^{cBx}
		36	146.77±0.41^{bBx}	199.02±0.30^{bBx}
		48	173.69±0.04^{aBx}	231.46±1.24^{aBx}
	70	12	172.75±3.75^{dAx}	224.37±2.02^{dAy}
		24	199.62±1.73^{cAx}	260.85±3.19^{cAy}
		36	218.72±5.82^{bAxy}	282.64±1.36^{bAy}
		48	237.21±3.01^{aAx}	300.25±0.21^{aAy}
75	50	12	15.37±0.18^{dCx}	23.49±0.06^{dCy}
		24	42.37±1.58^{cCx}	55.05±0.36^{cCy}
		36	58.96±0.22^{bCx}	78.32±0.82^{bCz}
		48	75.94±0.04^{aCy}	100.37±0.06^{aCz}
	60	12	70.29±2.23^{cBy}	93.35±3.36^{dBy}
		24	100.36±9.78^{bBx}	153.61±2.32^{cBx}
		36	121.77±1.11^{aBz}	176.91±1.35^{bBy}
		48	127.98±5.69^{aBz}	188.98±0.17^{aBy}
	70	12	170.04±4.45^{cAx}	217.87±4.71^{cAy}
		24	194.28±4.33^{bAx}	257.48±0.13^{bAy}
		36	203.88±0.58^{aAy}	265.91±6.96^{abAy}
		48	205.28±0.33^{aAy}	269.95±6.12^{aAz}

Data are mean ± standard deviation (n=3).

$^{a, b, c, d}$ indicate significant differences ($p < 0.05$) between values in the same column within the same temperature and relative humidity.

$^{A, B, C}$ indicate significant differences ($p < 0.05$) between values in the same column within the same incubation time and relative humidity.

$^{x, y, z}$ indicate significant differences ($p < 0.05$) between values in the same column within the same incubation time and temperature.

6.5.3 Characteristics of the selected MRP

6.5.3.1 Electrophoretic patterns

Electrophoretic patterns of MRP from NS-8GE:Gal system heated at 70 °C with 55% RH for 36 h (MRP) in comparison with NS-8GE and NS-8GE:Gal (without heating) are shown in Figure 23. For NS-8GE, no β- and α-chain were retained, suggesting the compelete degradation of those components during hydrolysis. Peptides with MW lower than 14.4 kDa were predominant in NS-8GE. A similar electrophoretic profile was found with NS-8GE:Gal without heating (NS-8GE:Gal). Moreover, the band with MW of 25 kDa was found in NS-8GE:Gal with coincidental decrease in band intensity of protein or peptides with MW lower than 10 kDa. This result suggestted that Maillard reaction at the early stage might occured with the formation of the condensation product, *N*-substituted glycosylamine or Amadori product, between galactose and NS-8GE during preparation of the NS-8GE:Gal powder. When heat treatment was implemented, the smear bands with much higher MW as well as bands with MW of 10-20 kDa were observed with a coincidental disappearance of band with MW less than 10 kDa. Thus, heat treatment used induced the formation of some new products with the various MW. It was presumed that proteins or peptides with MW of 10-20 kDa might be the degradation products of previously formed glycated peptides (MW of 40-66 kDa). Additionally, the degradation and dehydration of Amadori products into amine or carbonyl intermediates, and the reaction of carbonyl intermediates with other amino groups as well as subsequent rearrangement to form advanced glycosylation end-products were reported by Murthy and Sun (2000). This change was in agreement with the decrease in free amino group content, indicating that Maillard reaction proceeded (Table 9). Kato *et al.* (1986) reported that MRPs from ovalbumin-saccharides systems showed the formation of both higher and lower MW compounds, compared with native ovalbumin, via rearrangement and polymerization of Maillard reaction. Maillard conjugation of protein and saccharide resulted in the appearance of high MW constituents (Wang *et al.*, 2013; Liu *et al.*, 2014b). It was found that the formation of these high MW polymers was related with the increased antioxidative activity (Wang

et al., 2013). The formation of MRP more likely contributed to the improvement of ABTS radical scavenging activity and FRAP of resulting MRPs.

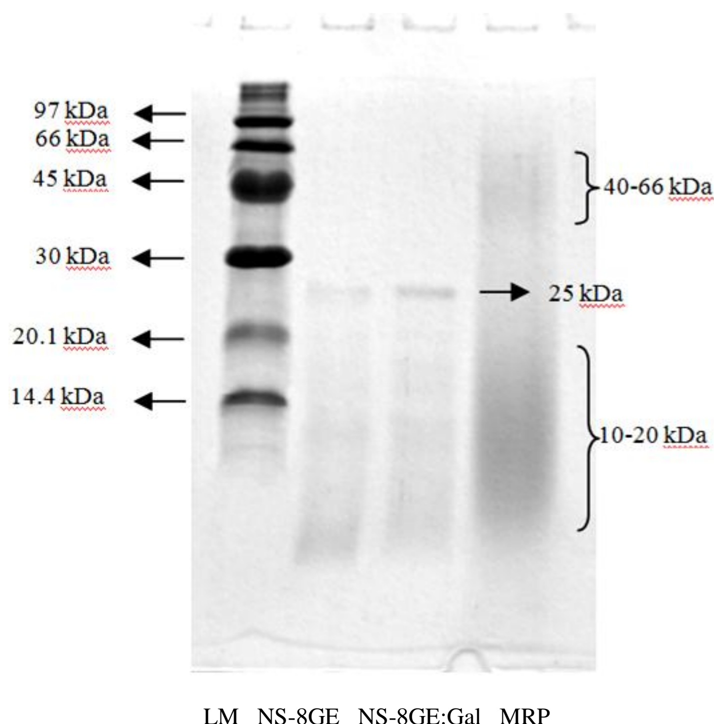

Figure 23 SDS-PAGE of MRP derived from gelatin hydrolysate of unicorn leatherjacket skin. LM; Low molecular weight markers, NS-8GE; Gelatin hydrolysate, NS-8GE:Gal; the mixture of gelatin hydrolysate and galactose (2:1) without heat treatment, MRP; MRP of NS-8GE:Gal prepared at 70 °C with 55% RH for 36 h.

6.5.3.2 FT-IR

FT-IR spectra in the region of 4000-500 cm^{-1} for MRP derived from NS-8GE:Gal system in comparison with NS-8GE and NS-8GE:Gal without heating are presented in Figure 24. NS-8GE showed the typical character of amide bands, including amide I (1652 cm^{-1}), amide II (1545 cm^{-1}), amide III (1245 cm^{-1}), amide A (3310 cm^{-1}) and amide B (2940 cm^{-1}). The spectrum was similar to that reported from gelatin of unicorn leatherjacket skins (Kaewruang *et al.*, 2014). Amide-I vibration mode is primarily C=O stretching vibration (Mohd Nasir *et al.*, 2006). Amide-II

vibration is attributed to combination of the N-H in plane bend and the C-N stretching vibration (Muyonga *et al.* 2004). The amide III band could be described as a combination peaks mainly arisen from C-N stretching and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Mohd Nasir *et al.*, 2006). Amide-A represents the stretching vibrations of the N-H group coupled with hydrogen bonding (Muyonga *et al.*, 2004). Amide-B is attributed to the asymmetric stretching vibration of =C–H, as well as $–NH_3^+$ (Kaewruang *et al.*, 2014).

For NS-8GE:Gal, the similar spectrum was observed to that of NS-8GE. However, peaks associated with galactose at the region of 955-1154 cm^{-1}were noticeable. These bands were mainly due to the C-C and C-O stretchings as well as to the deformations of atomic groups OCH, COH, CCH of galactose (Wang *et al.*, 2013; Buslov *et al.*, 1999). Those bands associated to saccharide appeared with the less intensity of absorbance in MRP, compared with NS-8GE:Gal. The remarkable shift of amide A band to higher wavenumber (3390 cm^{-1}) was observed from MRP spectrum with the higher amplitude than NS-8GE:Gal. This was probably denoted not only the formation of new amino group ($-NH_2$) caused by degradation or fragmentation during Maillard reaction but also the present of –OH group of carboxylic acid in MRPs. The similar result was reported by Fernández *et al.* (2014) who found that the absorption bands ranged from 2000 to 3500 cm^{-1} represented a characteristic of the OH^- carboxylic acid constituents in MRP of bovine plasma- oxidized soybean oil. In general, similar spectra for amide I, II and III were observed for all three samples. However, some differences in amplitude were noticeable. Higher amplitude of MRP was observed in comparison with NS-8GE:Gal. This was plausibly governed by the formation of MRPs, which consisted of C=O, C=N, C-N and C=C group of Amadori compound and Schiff base generated via Maillard reaction with the absorbance bands ranged from 1750 to 1200 cm^{-1}. The similar result was found in MRPs from whey protein isolate-sugar system (Wang *et al.*, 2013). The result from present study indicated the chemical structure and functional group of NS-8GE and galatose could be modified by Maillard reaction. Amino group of NS-8GE and aldehyde group of galactose were consumed with the appearance of new compounds in MRPs. This was

in accordance with the formation of peptides or proteins with MW around 40-66 and 10-20 kDa (Figure 23). Thus, heat treatment under dry condition applied to NS-8GE:Gal in the present study could foster the progress of Maillard reaction. This might be associated with the improvement of antioxidative activity of resulting MRPs.

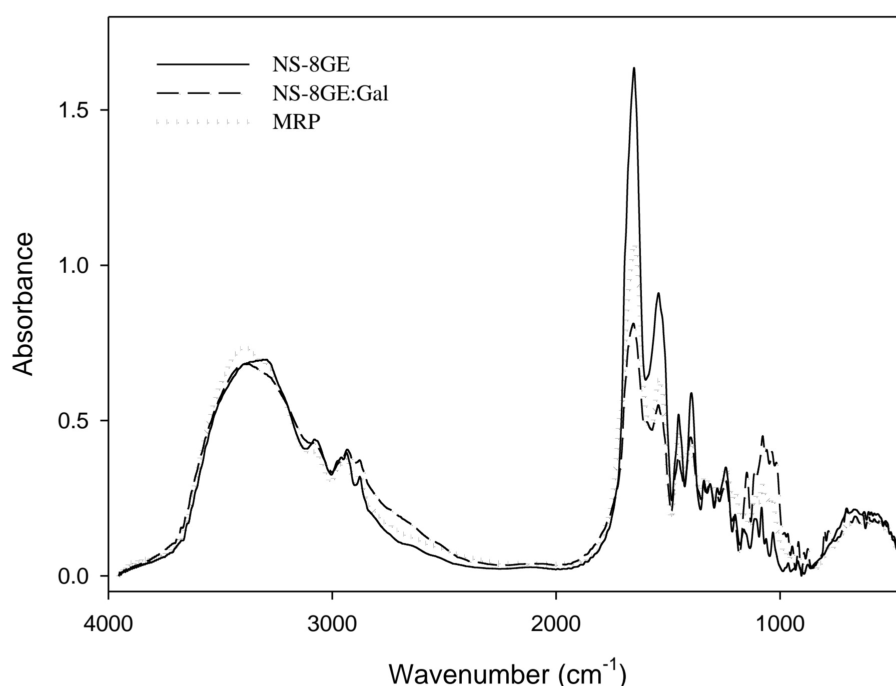

Figure 24 Fourier transform infrared spectra of MRP derived from gelatin hydrolysate of unicorn leatherjacket skin. NS-8GE; Gelatin hydrolysate, NS-8GE:Gal; the mixture of gelatin hydrolysate and galactose (2:1) without heat treatment, MRP; MRP of NS-8GE:Gal prepared at 70 °C with 55% RH for 36 h.

6.6 Conclusion

Maillard reaction between NS-8GE and galactose (2:1) under dry condition prepared by heating at 70°C with 55% RH for 36 h could effectively improve the antioxidative activity of NS-8GE. The formation of MRPs was evidenced by the decreases in pH and free amino group content during heat treatment. The degradation, rearrangement and polymerization occurred via Maillard reaction might

be associated with the enhancement of radical scavenging activity and reducing power of MRPs. MRPs could therefore be used as alternative antioxidants in food products.

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CHAPTER 7

Antioxidant, immunomodulatory and antiproliferative effects of gelatin hydrolysate from unicorn leatherjacket skin

7.1 Abstract

The antioxidant, immunomodulatory and antiproliferative effects of a gelatin hydrolysate (NS-8GE) prepared from unicorn leatherjacket skin using partially purified glycyl endopeptidase were investigated in cell culture model systems. NS-8GE under the tested concentrations (750-1500 μg/mL) protected against H_2O_2-induced DNA damage in U937 cells. NS-8GE also protected against the H_2O_2-induced reduction in cellular antioxidant enzyme activities, superoxide dismutase (SOD) and catalase (CAT), in HepG2 cells. NS-8GE demonstrated immunomodulatory potential by reducing pro-inflammatory cytokine (interleukin-6 (IL-6) and IL-1β) production and nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Cell proliferation in human colon cancer (Caco-2) cells was significantly reduced in dose-dependent manner following the incubation with NS-8GE. These results indicated that NS-8GE had several bioactivities which supported its potential as a promising functional food ingredient.

7.2 Introduction

The enzymatic hydrolysis of proteins has become an established method for producing peptides with potentially enhanced bioactivity (Karamać *et al.*, 2014). Numerous studies have shown that marine hydrolyses, prepared using various enzymes, are novel sources of bioactive peptides. Several enzymes such as digestive, microbial and plant proteases, including trypsin, pepsin, collagenase, protamex, alcalase, papain, etc., have been used to prepare hydrolysates (Aleman *et al.*, 2011b; Aleman, Perez-Santin *et al.*, 2011). Hydrolysates of several skin gelatins derived from tuna (*Thunnus* spp.), hoki (*Johnius belengerii*), unicorn leatherjacket (*Aluterus monoceros*) and jumbo flying squid (*Rhopilema esculentum*) have been shown to possess antioxidant activity (Aleman *et al.*, 2011a; Mendis *et al.*, 2005). Bioactive peptides with antioxidant activity have also exhibited immunomodulatory and anticancer potential (Chakrabarti *et al.*, 2014; Suarez-Jimenez *et al.*, 2012). A protein hydrolysate derived from a salmon by-product and prepared by peptic hydrolysis significantly inhibited intracellular reactive oxygen species generation, lipid peroxidation, and enhanced the level of glutathione in Chang liver cells. Additionally, this hydrolyste possessed anti-inflammatory activity by inhibiting nitric oxide production and pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-6 (IL-6) and interleukin-1β (IL-1β) in RAW264.7 macrophage cells (Ahn *et al.*, 2012). Flying squid skin gelatin hydrolysates prepared using different commercial proteases demonstrated anti-proliferative effects in human breast carcinoma (MCF-7) and glioma (U87) cell lines, along with ABTS radical scavenging activity and ferric ion reducing power (Aleman *et al.*, 2011b).

Gelatin hydrolysate has been known to have the antioxidative activities. Partially purified glycyl endopeptidase (GE) from papaya latex was used in combination with autolysis-assited process to enhance antioxidative activity of gelatin hydrolysate from unicorn leatherjacket skin (chapter 3 and 4). Moreover, the gelatin hydrolysate prepared using GE in combination with the autolysis-assisted process acted as a multifunctional additive which retarded the deterioration of frozen fish mince by acting as both an antifreeze and antioxidant agent (chapter 5).

7.3 Objective

To elucidate the potential of gelatin hydrolysate for use as functional food ingredients, the present study aimed to investigate the antioxidant activity, immunomodulatory and anticancer effects of a gelatin hydrolysate from unicorn leatherjacket skin in various cell culture model systems.

7.4 Materials and methods

7.4.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), Hanks balanced salt solution (HBSS) and non-essential amino acids were purchased from Sigma-Aldrich Chemical Co. (Dublin, Ireland). Human histiocytic lymphoma cells (U937 cells), mouse leukaemic macrophages (RAW264.7 cells), human hepatoma cells (HepG2 cells) and human carcinoma cells (Caco-2 cells) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Fetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Costar cell culture plastics were supplied by Fisher Scientific (Dublin, Ireland). All other cell culture chemicals and reagents were from Sigma Chemical Co. (Dublin, Ireland). All solvents used were of HPLC grade.

7.4.2 Preparation of gelatin hydrolysate from unicorn leatherjacket skin

7.4.2.1 Prepation of fish skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock, Songkhla, Thailand. Three different lots of skins were collected. For each lot, skins were pooled and used as a composite sample. The skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm2).

The prepared skins were then prepared by removing non-collagenous proteins using the method of Kaewruang *et al.* (2013). Fish skins (0.5×0.5 cm2) were soaked in 0.05M NaOH with a skin/alkaline solution ratio of 1:10 (w/v). Following pretreatment, skins were washed with tap water until neutral or faintly basic pH of wash water was obtained. The autolysis of pretreated skins was then conducted

following the method of chapter 5.4.2.3. The resulting autolysed skin was used as substrate for preparation of gelatin hydrolysate.

7.4.2.2 Preparation of partially purified glycyl endopeptidase (GE) from papaya (*Carica papaya*) latex

Fresh papaya latex was collected from green papaya fruit cultivated in Songkhla, Thailand. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h. The crude extract was prepared using the method of Kittiphattanabawon *et al.* (2012).

The glycyl endopeptidase was fractionated from crude extract using the method of chapter 3. Aqueous two phase system (ATPS) with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of glycyl endopeptidase. The obtained GE was stored at -40 °C until use.

7.4.2.3 Production of gelatin hydrolysates

Autolysed skins solutions (3%, w/v) were mixed with GE (8%, based on solid matter) and incubated at 40 °C for 60 min (chapter 4). After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and lyophilised. The gelatin hydrolysate powders were referred to as 'NS-8GE'. The powder was placed in polyethylene bag and stored at -40 °C until use. The storage time was not longer than 2 months.

7.4.3 Cell culture

U937, HepG2 and RAW264.7 cells were grown in RPMI-1640 supplemented with 10% FBS. Cells were cultured in the absence of antibiotics. The cells were grown at 37 °C in a 5% (v/v) CO_2 atmosphere in a humidified incubator. Reduced serum media (25 mL/L FBS) was used for all experiments.

7.4.3.1 Cell proliferation

U937, HepG2 and RAW264.7 cells (2×10^4 cells/mL) was supplemented with increasing concentrations of gelatine hydrolysate (NS-8GE) in 96-well flat-bottom plates with a final volume of 200 µL at 37 ºC for 24 h. Following incubation, cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) according to the manufactures instructions. Absorbance was read at 570 nm using a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The IC_{50} value (the concentration of sample that induced 50% decrease in viable cells) for each sample was calculated using the data obtained from the MTT assay and Prism software (version 4.0, GraphPad Inc., San Diego, CA, USA).

7.4.4 Determination of DNA damage (Comet assay)

U937 cells (1×10^5 cells/mL) were treated with NS-8GE (2.5, 5.0 and 7.5 mg/mL) for 24 h in a 24-well plate with a final volume of 1 mL media, containing reduced FBS (25 mL/L) at 37 °C. Following incubation, cells was treated with 40 and 60 µmol/L H_2O_2 for 30 min. Oxidative DNA damage in the U937 cells was assessed using the Comet assay as described by McCarthy *et al*. (2012). Briefly, slides were prepared by coating with 10 g/L normal gelling agarose (NGA). Cells (30 µL) were then mixed with 10 g/L low melting point (LMP) agarose, placed on a microscope slides, covered with a coverslip and the mini-gels were allowed to solidify on ice. Slides were then placed in cold lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L tri(hydroxymethyl)-aminomethane, fresh 10 mL/L Triton® X-100 and 100 mL/L dimethyl sulfoxide) for 1.5 h at 4 ºC. Slides were aligned in a horizontal gel electrophoresis tank (Horizon® 20·25, GIBCO BRL Life Technologies, Gaithersburg, MD, USA) which was filled with fresh electrophoresis solution (1 mmol/L EDTA, 300 mmol/L NaOH; pH 13). Slides were allowed to sit in this buffer for 30 min. Electrophoresis was conducted at 20V, 300 mA for 25 min at 4 ºC. After electrophoresis, the slides were neutralized using 0.4 mol/L Tris for 5 min (x3) and rinsed with distilled water. Slides were stained with ethidium bromide (20 mg/L) for 5

min and rinsed with distilled water. The Komet 5.5 image analysis software (Kinetic Imaging, Liverpool, UK) was used to score 50 cells for each slide using a fluorescence microscope (Optiphot-2, Nikon). DNA damage was expressed as percentage tail DNA.

7.4.5 Antioxidant enzyme activity assays: Superoxide dismutase (SOD) and catalase (CAT) activities

HepG2 cells (2×10^5 cells/mL, 5 mL) were incubated with gelatin hydrolysate (125 and 250 µg/mL) for 24 h at 37 °C. Following incubation, cells were exposed to 2 mM H_2O_2 for 2 h. Cells were harvested, sonicated and centrifuged (15000 rpm, 30 min) at 4 °C and the supernatant was collected for the determination of antioxidant enzyme activity.

The activity of total cellular SOD was determined using the method of Misra and Fridovich (1977). The supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase, and cytochrome c were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome c, and this reaction is inhibited by SOD. The reduction in cytochrome c was used to determine the activity of SOD present in the samples from a standard curve. Samples were read at 550 nm at 20 min intervals for at least five readings.

Catalase (CAT) activity was determined using a modification of the method of Baudhuin *et al.*, (1964), where any remaining H_2O_2 was determined as a yellow 'peroxy titanium sulfate'. One unit of catalase activity was defined as the amount of catalase required to decompose 1µmol H_2O_2 per min at pH 7.5 and 25 °C. The absorbance was measured at 465 nm.

SOD and CAT activities were determined relative to the protein content as SOD and CAT units/mg protein in cell homogenate, respectively. The protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay as described by Smith *et al.* (1985). Enzyme activity was expressed as a percentage of untreated, control cells.

7.4.6 Cytokine production (Immunomodulatory activity)

RAW264.7 cells, at a density of 2×10^5 cells/mL, were seeded in 96-well plates in the presence of lipopolysaccharide (LPS, 0.5 mg/L) and treated with test samples (750, 1000 and 1500 mg/mL) for 24 h at 37 ºC. Production of the cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) was determined using ELISA kits (eBioscience mouse IL-6 and IL-1β ELISA Ready-SET-Go kits, Insight Biotechnology, Wembley, U.K.). Absorbance was read at 450 nm using a microplate reader. Data were expressed as a percentage of the LPS-stimulated RAW264.7 cell control.

7.4.7 Nitric oxide (NO) secretion

NO secretion was determined using the Griess reagent. RAW264.7 cells were plated at a density of 1×10^5 cells/mL in a 96-well plate and incubated for 48 h. NO production was induced with LPS (2 µg/mL) and cells were co-treated with different concentrations of gelatin hydrolysate for 24 h. The cultured supernatant (50 µL) was plated into a 96 well plate and 50 µL of Griess reagent (1:1 of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-naphtyl-ethylenediamine dichloride in water) was added. Sodium nitrite was used to generate a standard curve. The plate was incubated at room temperature for 10 min and the absorbance was measured at 540 nm using a Thermo Scientific Varioskan Flash microplate reader.

7.4.8 Antiproliferative effect

Caco-2 cells (2×10^4 cells/mL) were cultured in DMEM supplemented with 100 mL/L FBS and 10 mL/L non-essential amino acids in 96-well flat-bottom plates. Gelatin hydrolysate at different concentrations (0-1.0 mg/mL) was then added with a final volume of 200 µL. Following the incubation at 37 ºC for 24 h. Cell viability was assessed using the MTT assay and the IC_{50} value was calculated as mentioned above.

7.4.9 Statistical analysis

A completely randomized design (CRD) was used for the statistical analysis of physical and chemical analysis. All data was subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The results with $P<0.05$ were considered to be statistically significant.

7.5 Results and discussion

7.5.1 Effect of gelatin hydrolysate (NS-8GE) on cell viability in U937, HepG2 and RAW264.7 cells

The effect of NS-8GE from unicorn leatherjacket skin, at different concentrations, on cell proliferation in U937, HepG2 and RAW264.7 cells viability is depicted in Figure 25. Cell proliferation initially increased in the presence of NS-8GE at 5 µg/mL and 10 µg/mL NS-8GE in U937 cells but decreased significantly at concentrations above 10 µg/mL (Figure 25 (a)). The cell proliferation was above 50% at the highest concentration investigated (40 µg/mL) and therefore, an IC_{50} value for NS-8GE could not be determined in U937 cells. In the human hepatoma, HepG2 cells, cell proliferation decreased in a dose dependant manner to a concentration of 400 µg/mL, after which point a plateau in cell proliferation was observed, an IC_{50} value of 343.5 µg/mL was quantified for this cell line. In the murine macrophage, RAW 264.7 cells, there was a dose-dependent decrease in cell proliferation up to a concentration of 600 µg/mL, cell proliferation then plateaued between 600 and 1200 µg/mL before decreasing to approximately 50% at the highest concentration (1500 µg/mL).Similar to the U937 cells, the decrease in cell proliferation was not sufficient to allow the calculation of an IC_{50} value. Differences in the effect of NS-8GE on cell proliferation were observed in the different cell lines. Cell proliferation was inhibited at the lowest concentrations in U937 cells followed by the HepG2 cells. RAW 264.7 cells were the most resistant to the anti-proliferative effects of NS-8GE. This is in

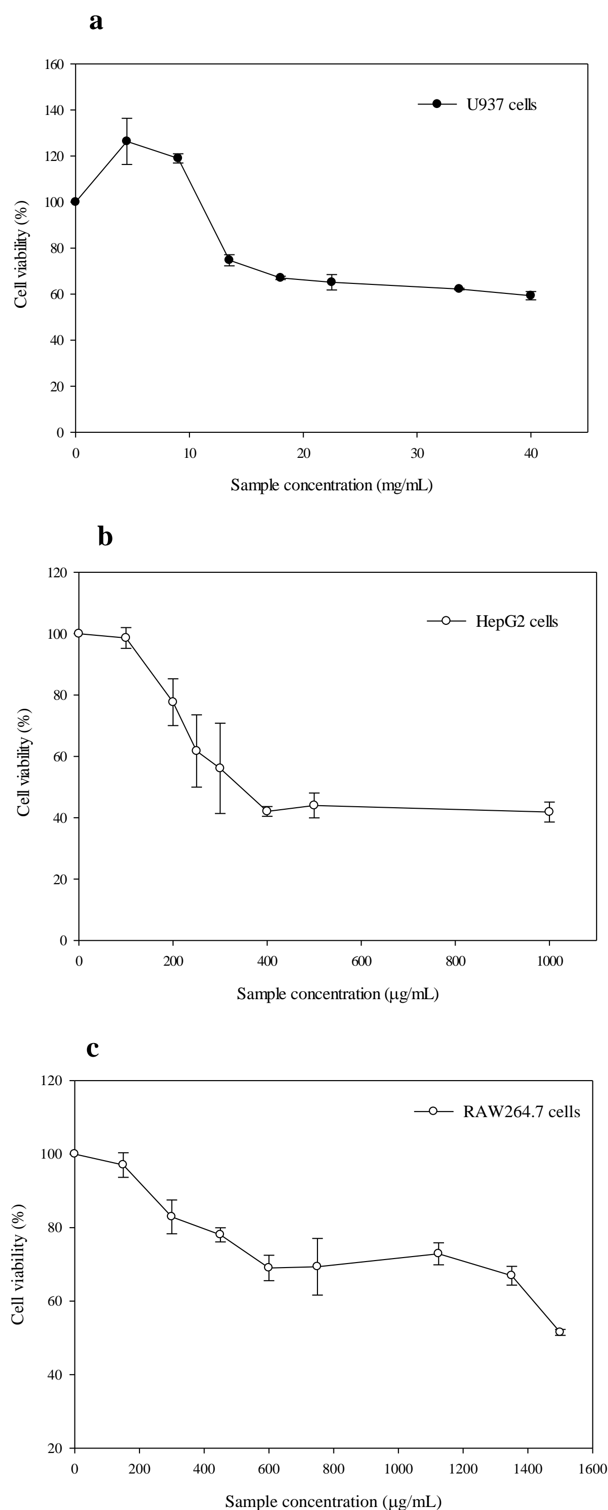

Figure 25 Cell viability (%) of U937 (a), HepG2 (b) and RAW264.7 (c) cells at different concentration of gelatin hydrolysate (NS-8GE). Cell viability was measured using the MTT assay. Values represent the mean ± SD for three independent experiments.

accordance with a previous report which found that the effects of fish protein hydrolysates (1 mg/mL) on human breath cancer (MCF-7/6 and MDA-MB-231 cells) cell proliferation were dependent on the cell line used (Picot *et al.*, 2006). Nazeer *et al.* (2012) tested the cytotoxic effects of an antioxidant peptide derived from croaker muscle hydrolysates in cell lines using MTT assay. The peptide was toxic to Vero cells at a concentration of 125 µg/mL, with a cell viability of less than 50% of control. The cell proliferation data obtained for U937, HepG2 and RAW264.7 was used to select non-cytotoxic concentrations for the determination of the bioactive effects of NS-8GE in these cell lines.

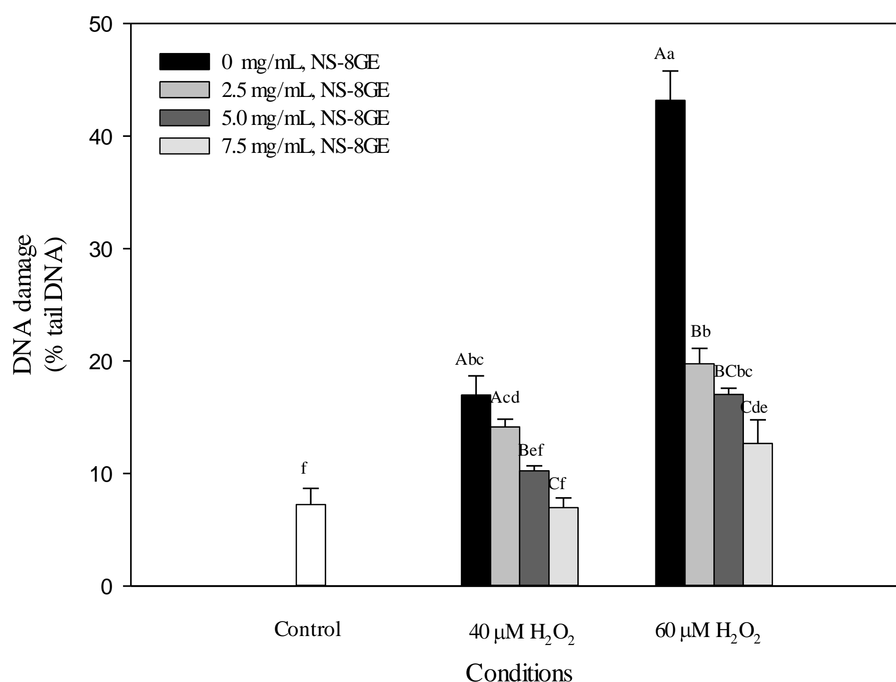

Figure 26 The ability of gelatin hydrolysate (NS-8GE) to protect against DNA damage induced by different concentration of H_2O_2. DNA damage was measured using the Comet assay. Values are mean ± SD of 3 independent experiments. [A, B, C] indicate significant differences ($p < 0.05$) between values in the same H_2O_2 concentration. [a, b, c, d, e, f] indicate significant differences ($p < 0.05$) between values.

7.5.2 Ability of gelatin hydrolysate (NS-8GE) to protect against oxidant-induced DNA damage in U937 cells

The protective effect of NS-8GE against oxidant induced DNA damage was assessed using the comet assay in the U937 cell model system (Figure 26). U937 cells were treated with NS-8GE at different concentrations (2.5-7.5 mg/mL) for 24 h and DNA damage was induced using H_2O_2 at different concentrations (40 and 60 µM) for 30 min. The oxidant (H_2O_2) at 40 and 60 µM significantly increased the percentage of DNA damage, expressed as % tail DNA, to 17 and 43%, respectively. DNA damage was not induced by incubation with NS-8GE at tested concentration (2.5-7.5 mg/mL) in U937 cells (data not shown). NS-8GE protected against H_2O_2-induced increases in tail DNA in a dose dependent manner in U937 cells (Figure 26). At 40 µM H_2O_2, the % tail DNA was significantly ($P<0.05$) reduced to control levels at the highest concentration of NS-8GE used (7.5 mg/mL). A similar trend was observed in cells exposed to 60 µM H_2O_2. Few studies have previously investigated the DNA protective effects of fish protein hydrolysates. Yarnpakdee *et al.* (2015) reported that an antioxidant protein hydrolysate prepared from Nile tilapia protein isolate effectively inhibited H_2O_2 and peroxyl radical induced plasmid DNA (pUC18) damage. The protective effect of tuna liver hydrolysates was related to their scavenging of H_2O_2, hydroxyl radical and chelating activity toward Fe^{2+}. Such activities led to the inhibition of the Fenton reaction, and therefore, protected the supercoiled pBR322 plasmid DNA from oxidant-induced strand breaks (Je *et al.*, 2009). In our previous study, the antioxidant gelatin hydrolysate (NS-8GE) prepared using glycyl endopeptidase from unicorn leatherjacket skin possessed an ability to donate electrons and scavenge free radicals (chapter 4). This was likely the mechanism by which NS-8GE protects against oxidant-induced DNA damage in U937 cells as found in the present study.

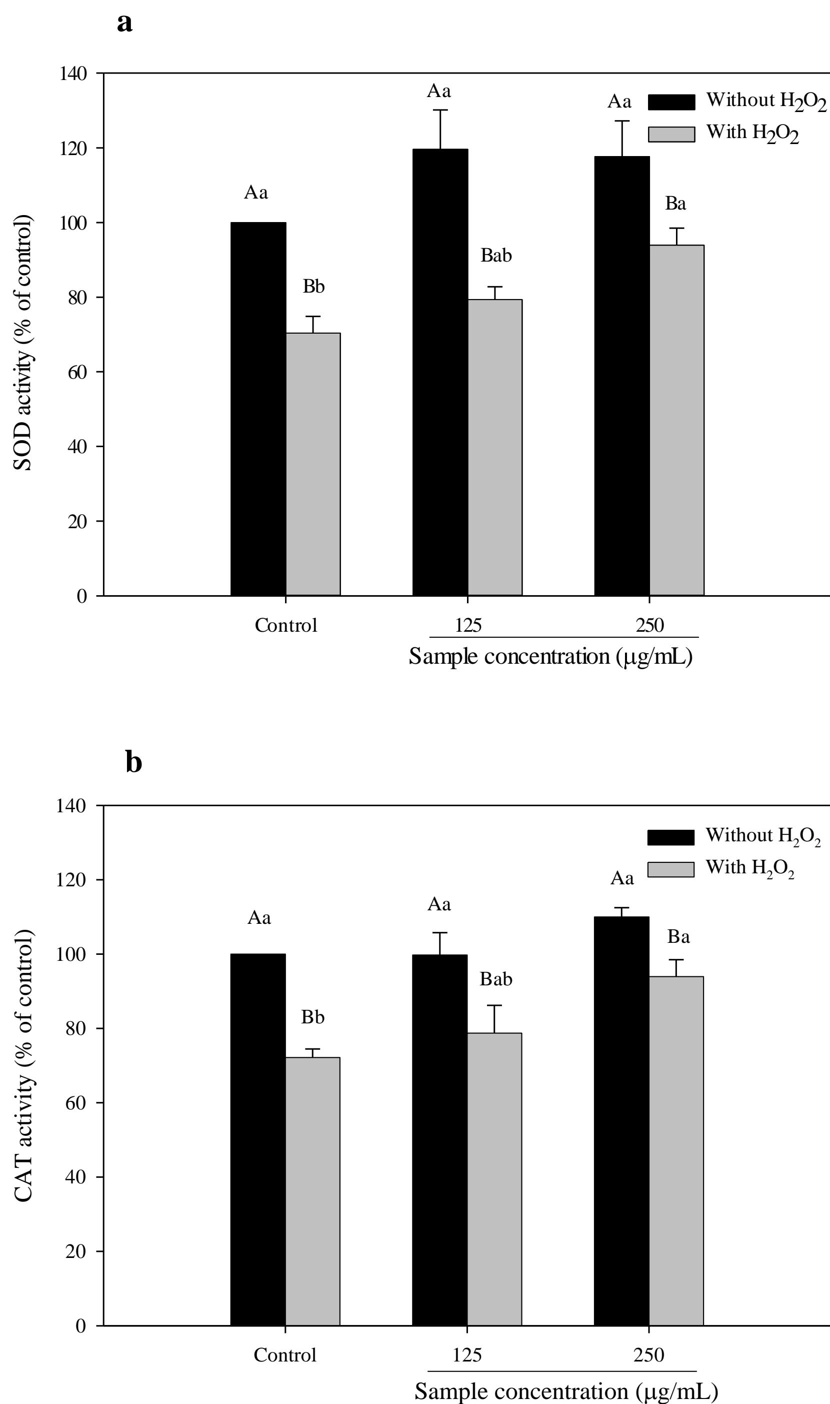

Figure 27 Effects of gelatin hydrolysate (NS-8GE) on antioxidant enzyme activity, superoxide dismutase (SOD) (a) and catalase (CAT) (b) activities in HepG2 cells without and with H_2O_2 treated. Values represent the mean ± SD for three independent experiments. $^{A, B}$ indicate significant differences ($p < 0.05$) between values in the same sample concentration. $^{a, b, c}$ indicate significant differences ($p < 0.05$) between values in the same H_2O_2 concentration.

7.5.3 Effect of gelatin hydrolysate (NS-8GE) on induction of antioxidant enzyme activities in HepG2 cells

The ability of NS-8GE to protect against a H_2O_2-induced depletion of the cellular antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) was investigated in HepG2 cells (Figure 27). The levels of SOD and CAT activity were significantly decreased ($P<0.05$) by approximately 30% in HepG2 cells, following exposure to H_2O_2. In the absence of H_2O_2, NS-8GE did not affect the activity of SOD or CAT. However, NS-8GE demonstrated an ability to prevent the decreases in SOD and CAT activity induced by H_2O_2, especially at the highest concentration used (250 μg/mL) with approximately 17% and 20% increase, respectively ($P<0.05$), compared with that of cells incubated with H_2O_2 alone. The radical scavenging activity of NS-8GE, as previously described (chapter 4), may be the mechanism involved in its protective effect against the H_2O_2 mediated decrease in antioxidant enzyme activity. An antioxidant hydrolysate derived from Hoki skin gelatin was found to increase the activity of SOD by 92.8% and CAT by 35% in unchallenged human hepatoma cells (Hep3B) following a 24 hr incubation (Mendis *et al.*, 2005). Hoki skin gelatin hydrolysate maintained the cellular redox balance through its radical scavenging activity. The chain reaction of superoxide to H_2O_2 and followed by water in the presence of SOD and CAT was not taken place. This might be the reason behind the induction of enzymes in H_2O_2-induced cell treated with gelatin hydrolysate (Mendis *et al.*, 2005).

7.5.4 Pro-inflammatory cytokines production

The immunomodulatory potential of gelatin hydrolysate (NS-8GE) from unicorn leatherjacket skin was investigated in LPS-stimulated RAW264.7 macrophage cells. In LPS-induced RAW264.7 cells, IL-1β and IL-6 levels were increased more than 30 and 80 folds, respectively, compared with that of control (without LPS) cells (data not shown). The effects of NS-8GE on the production of pro-inflammatory cytokines, IL-1β (Figure 28 (a)) and IL-6 (Figure 28 (b)), was examined. It was noted that the levels of both pro-inflammatory cytokines were

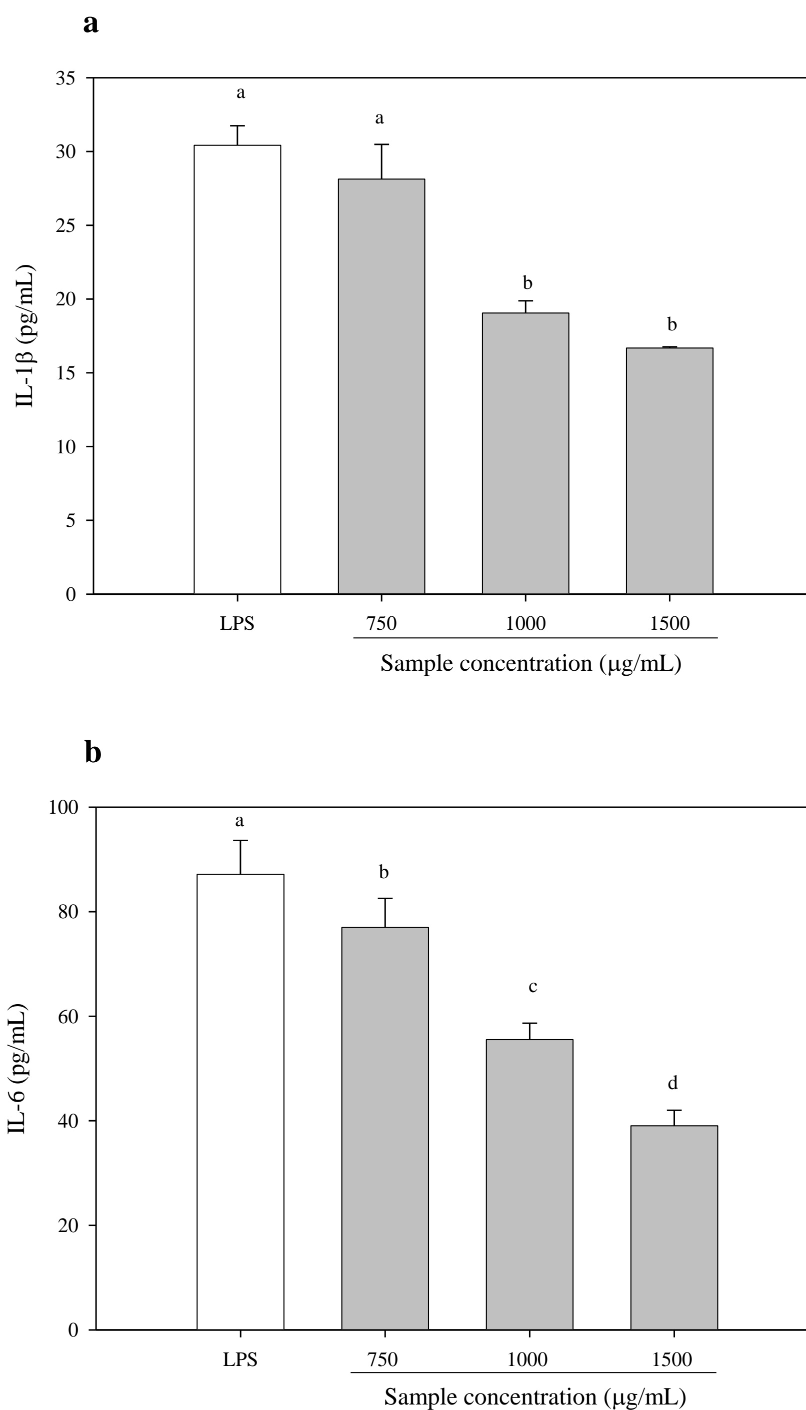

Figure 28 Effect of gelatin hydrolysate on production of IL-1β (a) and IL-6 (b) in LPS-induced RAW264.7 cells. Values represent the mean for three independent experiments ± SD. [a, b, c, d] indicate significant differences ($p < 0.05$) between values.

decreased in dose dependent manner following NS-8GE treatment. IL-6 was significantly reduced (P<0.05) by greater than 50% at the highest NS-8GE concentration used (1500 µg/mL). A similar result was reported by Sung *et al.* (2012) who studied the anti-inflammatory effect of a sweetfish protein hydrolysate in LPS-induced RAW264.7 cells. The hydrolysate prepared using trypsin and α-chymotrypsin significantly decreased the production of the pro-inflammatory cytokines, IL-1, IL-6 and TNF-α (Sung *et al.*, 2012). It is well known that these pro-inflammatory cytokines play a key role in the process of inflammatory diseases (Lanan *et al.*, 2012). Excessive production of these cytokines can result in systemic inflammatory response syndrome, such as septic shock (Lanan *et al.*, 2012) and prolonged supra-optimal levels of pro-inflammatory cytokines are associated with chronic inflammatory disorders such as allergic, autoimmune, rheumatologic and cardiovascular disorders. It has been reported that the intake of dietary antioxidants could prevent chronic progressive autoimmune diseases by inhibiting oxidative damage and thereby reducing inflammation (Ford *et al.*, 2003).

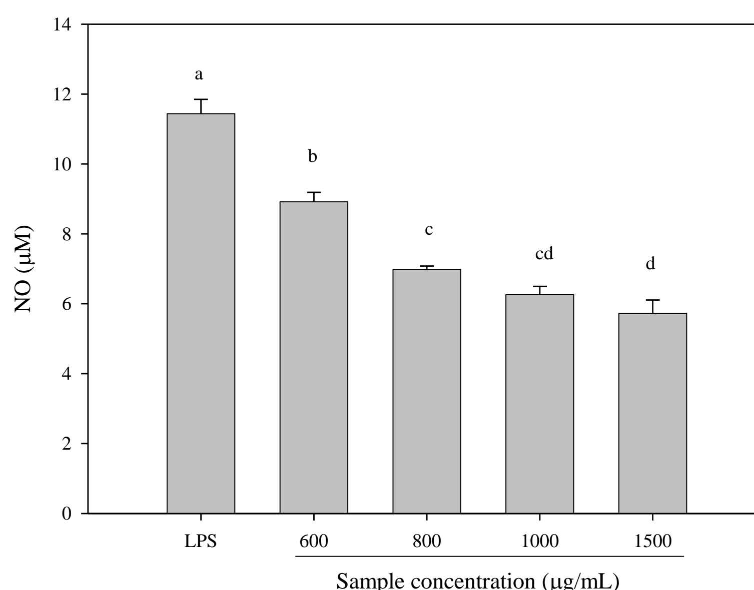

Figure 29 Effect of gelatin hydrolysate at different concentrations on inhibition of nitric oxide (NO) secretion in LPS-induced RAW264.7 cells. Values represent the mean ± SD for three independent experiments. [a, b, c, d] indicate significant differences ($p < 0.05$) between values.

7.5.5 Nitric oxide (NO) secretion

The effect of gelatin hydrolysate from unicorn leatherjacket skin on nitric oxide secretion was assessed in LPS-treated RAW264.7 macrophage cells (Figure 29). NO production in RAW264.7 cells was significantly enhanced to 11.73 μM (P<0.05) upon stimulation with LPS, compared with that of control (0.45 μM, without LPS-induced). The cells treated with NS-8GE showed a considerable dose dependent decrease in NO production. NS-8GE at the highest concentration used (1500 μg/mL) inhibited NO secretion more than 60%. NO production has a wide and pervasive regulatory role in the inflammatory response in macrophage cells (Chang *et al*., 2006). Thus, inhibitors of LPS-induced NO secretion may be effective therapeutically in preventing inflammatory reactions and diseases (Chang *et al*., 2006). A protein hydrolysate from tilapia muscle protein and salmon byproduct prepared using enzymatic hydrolysis have shown anti-inflammatory activity by inhibition of NO production in LPS-induced cells (Kangsanant *et al*., 2014; Ahn *et al*., 2012). The decrease in NO production caused by a protein hydrolysate derived from sweetfish was found to be related to the inhibition of mRNA expression of inducible nitric oxide synthase (Sung *et al*., 2012).

7.5.6 Antiprolifertive effect of gelatin hydrolysate (NS-8GE)

Human colon cancer (Caco-2) cells were used as the model cell culture system for studying the effect of gelatin hydrolysate (NS-8GE) from unicorn leatherjacket skin on antiproliferative activity (Figure 30). NS-8GE showed a notable antiproliferative activity by decreasing cancer cell viability in a dose dependent manner. At 300 μg/mL NS-8GE, a significant decrease in cell viability was observed with 20% inhibition (P<0.05) in colon carcinoma (Caco-2) cell growth. In addition, the growth inhibition was more than 50% at concentrations of 400-1000 μg/mL NS-8GE. This observation is similar to the previous data reported on the antiproliferative activity of an enzymatic hydrolysate from jumbo squid gelatin which demonstrated cytotoxic effects against MCF-7 (human breast carcinoma) and U87 (glioma) cells with IC_{50} values of 130 and 100 μg g/mL, respectively (Aleman, Perez-Santin *et al*., 2011). A solitary tunicate hydrolysate exhibited both antioxidant activity and

anticancer activity in AGS (human breast cancer), DLD-1 (human colon cancer), and HeLa (human cervical cancer) cells (Jumeri and Kim, 2011). It was reported that loach protein hydrolysates with the highest antioxidant activity caused the greatest antiproliferative activity against cancer cells (You *et al.*, 2011). Wang *et al.* (2010) found that low molecular size peptides from oyster hydrolysates induced a dose dependent inhibition of growth in transplanted murine sarcoma in BALB/c mice, which they attributed to a possible immunostimulatory effect.

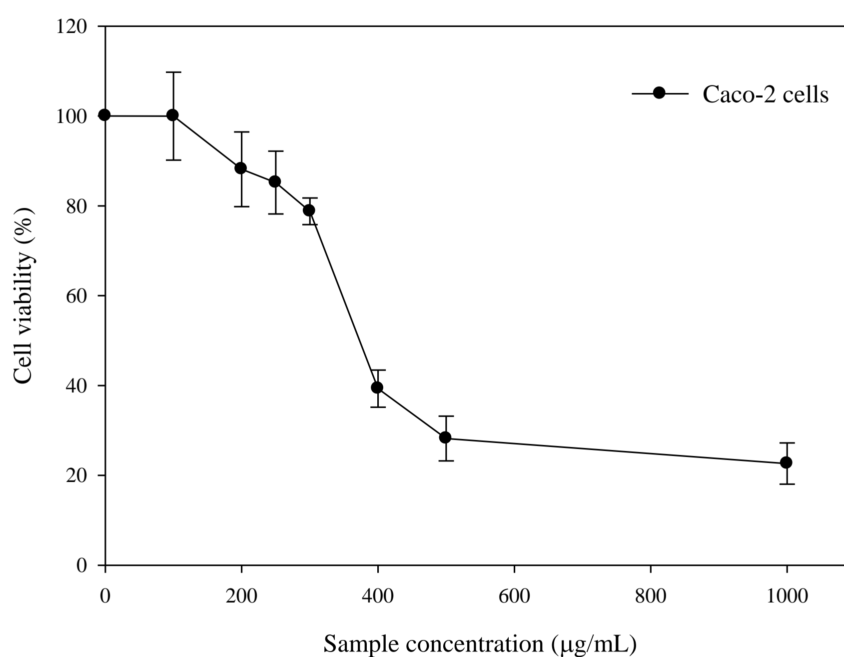

Figure 30 Antiproliferative effect of gelatin hydrolysate at different concentration on Caco-2 cell viability. Values represent the mean ± SD for three independent experiments.

7.6 Conclusion

A gelatine hydrolysate (NS-8GE) from unicorn leatherjacket skin prepared using partially purified glycyl endopeptidase possessed cellular antioxidant activity by protecting DNA damage and enhancing intracellular antioxidant enzyme activity in H_2O_2-challenged cells. NS-8GE also showed immunomodulatory and antiproliferative activity in cell culture model systems. These findings indicate the potential of NS-8GE for use as a functional food ingredient with various health benefits.

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CHAPTER 8

Antioxidant, immunomodulatory and anticancer effects of Maillard reaction products from gelatin hydrolysate of unicorn leatherjacket skin

8.1 Abstract

The effects of a Maillard reaction product (MRP), produced from a gelatin hydrolysate (NS-8GE) which was derived from unicorn leatherjacket skin, on *in vitro* cellular antioxidant activity, immunomodulatory properties and anticancer activity were investigated. MRP (750-1500 μg/mL) protected against H_2O_2-induced DNA damage in U937 cells. DNA damage was reduced by approximately 50% at the highest MRP concentration (1500 μg/mL). The activities of cellular antioxidant enzymes, superoxide dismutase and catalase , were decreased in HepG2 cells exposed to H_2O_2 but were induced in the presence of MRP. MRP reduced the production of pro-inflammatory cytokines (IL-1β and IL-6) and nitric oxide in LPS-induced RAW264.7 cells. The proliferation of colon carcinoma, Caco-2 cells was inhibited in the presence of MRP with an IC_{50} of 1.7 mg/mL. Therefore, MRP demonstrated bioactive potential through the suppression of oxidative stress and inflammation *in vitro* in addition to inhibiting the proliferation of human colon cancer cells.

8.2 Introduction

Maillard reaction products (MRPs) have been proven to be potential candidates as the effective antioxidants (Amarowicz, 2009). Maillard derived antioxidants can be prepared using various model systems, such as tuna stomach hydrolysate-sugar, lysine-honey, chitosan-sugar, porcine plasma protein–glucose and casein hydrolysate-glucose (Sumaya-Martinez *et al.,* 2005; Antony *et al.,* 2000; Phisut and Jiraporn, 2013; Lertittikul *et al.,* 2007; Guerard and Sumaya-Martinez, 2003). Glycosylation of fish protein hydrolysates with monosaccharides or oligosaccharides has been performed under various conditions. The antioxidant activities of a protein hydrolysate from silver carp were effectively improved using Maillard reaction under the powdered state (You *et al.,* 2011). In addition to their antioxidant potential, MRPs have also demonstrated to exhibit the promising immunomodulatory and antitumor effects. Teodorowicz *et al.* (2013) reported that the Maillard reaction between glucose and peanut 7S globulin resulted in a product which reduced pro-inflammation in intestinal cells (Caco-2 cells) as evidenced by a reduction in IL-8 secretion. Song *et al.* (2011) found that heating hydrolysate from a marine fish half-fin anchovy prepared using pepsin resulted in the formation of a Maillard reaction product with enhanced antiproliferative activity in DU-145 human prostate cancer cells, 1299 human lung cancer cells and 109 human esophagus cancer cells. However, Maillard reaction products have also caused the production of some undesirable compounds including cytotoxic substances. The cytotoxic effects of MRP have been reported in different model systems and are associated with the use of high temperatures in the production of the MRP (Jing and Kitts, 2002).

Recently, gelatin hydrolysate from skin of unicorn leatherjacket with antioxidative activity has been successfully produced using autolysis assisted process in combination with glycyl endopeptidase (chapter 4). To enhance the antioxidative activity as well as other bioactivites, the use of Maillard reaction to prepare MRPs based on gelatin hydrolysate can be a promising and effective means.

8.3 Objective

To investigate the antioxidant activity, immunomodulatory potential and anticancer effects of a Maillard reaction product (MRP) derived from gelatin hydrolysates of unicorn leatherjacket skin in cell culture model systems in order to determine bioactivity and potential of this MRP as a functional food ingredient. The antioxidant potential of the MRP following *in vitro* gastrointestinal digestion was also investigated to determine its stability during gastro-intestinal digestion.

8.4 Materials and methods

8.4.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), Royal Park Memorial Institute (RPMI)-1640 medium, Hanks balanced salt solution (HBSS) and non-essential amino acids were purchased from Sigma-Aldrich Chemical Co. (Dublin, Ireland). Mouse leukaemic macrophages (RAW264.7 cells), human histiocytic lymphoma cells (U937 cells), human hepatoma cells (HepG2 cells) and human colon cancer cells (Caco-2 cells) were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Fetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Costar cell culture plastics were supplied by Fisher Scientific (Dublin, Ireland). 2,2′-Azinobis (3- thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), cell culture chemicals and reagents were from Sigma Chemical Co. (Dublin, Ireland). All solvents used were of HPLC grade.

8.4.2 Preparation of gelatin hydrolysate from unicorn leatherjacket skin

8.4.2.1 Prepation of fish skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock, Songkhla, Thailand. Three different lots of skins were collected. For each lot, skins were pooled and used as a composite sample. The skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2).

The pretreated skins were then prepared by removing non-collagenous proteins using the method of Kaewruang *et al.* (2013). Fish skins ($0.5 \times 0.5\ cm^2$) were soaked in 0.05M NaOH with a skin/alkaline solution ratio of 1:10 (w/v). Following pretreatment, skins were washed with tap water until neutral or faintly basic pH of wash water was obtained. The autolysis was then conducted using pretreated skin following the method of chapter 5.4.2.3. The resulting autolysed skin was used as substrate for preparation of gelatin hydrolysate.

8.4.2.2 Preparation of partially purified glycyl endopeptidase (GE) from papaya (Carica papaya) latex

Fresh papaya latex was collected from green papaya fruit cultivated in Songkhla, Thailand. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h. The crude extract was prepared using the method of Kittiphattanabawon *et al.* (2012b).The glycyl endopeptidase was fractionated from crude extract using the method of chapter 3. Aqueous two phase system (ATPS) with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of glycyl endopeptidase. The obtained GE was stored at -40 °C until use.

8.4.2.3 Production of gelatin hydrolysates

Autolysed skin solution (3%, w/v) was mixed with GE (8%, based on solid matter) and incubated at 40 °C for 60 min (chapter 4). After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min. The supernatant was collected and lyophilised. The gelatin hydrolysate powder was referred to as ‘NS-8GE’. The powder was placed in polyethylene bag and stored at -40 °C until use. The storage time was not longer than 2 months.

8.4.3 Preparation of Maillard reaction product (MRP)

The powder of NS-8GE was mixed with galactose (2:1, w/w) and the mixture was heated at 70 °C with 55% relative humidity for 36 h in an environmental

chamber (WTB Binder, Tuttlingen, Germany). The resulting MRP was collected and subjected to analyses.

8.4.4 Cell culture

U937 cells were grown in RPMI-1640 medium supplemented with 100 mL/L FBS. RAW264.7 and HepG2 cells were maintained in DMEM supplemented with 100 mL/L FBS. Caco-2 cells were cultured in DMEM supplemented with 100 mL/L FBS and 10 mL/L non-essential amino acids. Cells were cultured in the absence of antibiotics. The cells were grown at 37 °C in a 5% (v/v) CO2 atmosphere in a humidified incubator. Reduced serum media (25 mL/L FBS) was used for all experiments.

8.4.4.1 Cell proliferation

U937, RAW264.7 and HepG2 cells (5×10^4 cells/mL) was supplemented with increasing concentrations of MRP in 96-well flat-bottom plates with a final volume of 200 µL at 37 ºC for 24 h. Following incubation, cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) according to the manufactures instructions. Absorbance was read at 570 nm using a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The IC_{50} value (the concentration of sample that induced 50% decrease in viable cells) for each sample was calculated using the data obtained from the MTT assay and Prism software (version 4.0, GraphPad Inc., San Diego, CA, USA).

8.4.5 Determination of antioxidant activity in cell model systems

8.4.5.1 Determination of DNA damage (Comet assay)

U937 cells (1×10^5 cells/mL) were treated with MRP (750, 1000 and 1500 µg/mL) for 24 h in a 24-well plate with a final volume of 1 mL media, containing reduced FBS (25 mL/L) at 37 °C. Following incubation, cells were treated

with 40 or 60 µmol/L H_2O_2 for 30 min. Oxidative DNA damage in the U937 cells was assessed using the Comet assay as described by McCarthy *et al*. (2012). Briefly, slides were prepared by coating with 10 g/L normal gelling agarose (NGA). Cells (30 µL) were then mixed with 10 g/L low melting point (LMP) agarose, placed on a microscope slides, covered with a coverslip and the mini-gels were allowed to solidify on ice. Slides were then placed in cold lysis solution (2.5 moL/L NaCl, 100 mmoL/L EDTA, 10 mmoL/L tri(hydroxymethyl)-aminomethane, 10 mL/L Triton® X-100 and 100 mL/L dimethyl sulfoxide) for 1.5 h at 4 ºC. Slides were aligned in a horizontal gel electrophoresis tank (Horizon® 20·25, GIBCO BRL Life Technologies, Gaithersburg, MD, USA) which was filled with fresh electrophoresis solution (1 mmoL/L EDTA, 300 mmoL/L NaOH; pH 13). Slides were allowed to equilibrate in this buffer for 30 min. Electrophoresis was conducted at 20V, 300 mA for 25 min at 4 ºC. After electrophoresis, the slides were neutralised using 0.4 moL/L Tris for 5 min (x3) and rinsed with distilled water. Slides were stained with ethidium bromide (20 mg/L) for 5 min and rinsed with distilled water. The Komet 5.5 image analysis software (Kinetic Imaging, Liverpool, UK) was used to score 50 cells for each sample using a fluorescence microscope (Optiphot-2, Nikon). DNA damage was expressed as percentage tail DNA.

8.4.5.2 Antioxidant enzyme activity assays: Superoxide dismutase (SOD) and catalase (CAT) activities

HepG2 cells (2×10^5 cells/mL, 5 mL) were incubated with different concentrations of MRP (250 and 500 µg/mL) for 24 h at 37 °C. Following incubation, cells were exposed to 2 mM H_2O_2 for 2 h. Cells were harvested, sonicated and centrifuged (15000 rpm, 30 min) at 4 °C and the supernatant was collected for the determination of antioxidant enzyme activity.

The activity of total cellular SOD was determined using the method of Misra and Fridovich (1977). The supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase, and cytochrome c were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome c, and this reaction is inhibited by SOD. The reduction in cytochrome c

was used to determine the activity of SOD present in the samples from a standard curve. Samples were read at 550 nm at 20 min intervals for at least five readings.

Catalase activity was determined using a modified method of Baudhuin *et al.* (1964), where any remaining H_2O_2 was determined as a yellow 'peroxy titanium sulfate'. One unit of catalase activity was defined as the amount of catalase required to decompose 1µmol H_2O_2 per min at pH 7.5 and 25 °C. The absorbance was measured at 465 nm.

SOD and CAT activities were determined relative to the protein content and expressed as SOD and CAT units/mg protein in cell homogenate, respectively. The protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay as previously described (Smith *et al.,* 1985). Data were expressed as a percentage of untreated, control cells.

8.4.6 Immunomodulatory effect of Maillard reaction product

8.4.6.1 Cytokine production: Interleukin-6 (IL-6) and interleukin-1β (IL-1β)

RAW264.7 cells, at a density of 2×10^5 cells/mL, were seeded in 96-well plates in the presence of lipopolysaccharide (LPS, 0.02 µg/mL for IL-6 and 0.1 µg/mL for IL-1β) and treated with MRP (0.75, 1.00 and 1.50 mg/mL) for 24 h at 37 ºC. Production of the cytokines IL-6 and IL-1β was determined using ELISA kits (eBioscience mouse IL-6 and IL-1β ELISA Ready-SET-Go kits, Insight Biotechnology, Wembley, U.K.). Absorbance was read at 450 nm using a microplate reader (Thermo Scientific Varioskan® Flash).

8.4.6.2 Nitric oxide (NO) secretion

NO secretion was determined using the Griess reagent. RAW264.7 cells were plated at a density of 1×10^5 cells/mL in a 96-well plate and incubated for 48 h. NO secretion was induced with LPS (2 µg/mL) and cells were co-treated with different concentration (600-1500 µg/mL) of MRP for 24 h. The cultured supernatant

(50 µL) was plated into a 96 well plate and 50 µL of Griess reagent (1:1 of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-naphtyl-ethylenediamine dichloride in water) were added. Sodium nitrite (0-100 µM) was used to generate a standard curve. The plate was incubated at room temperature for 10 min and the absorbance was measured at 540 nm using a Thermo Scientific Varioskan Flash microplate reader.

8.4.7 Anticancer activity

Caco-2 cells (2×10^4 cells/mL) were supplemented with increasing concentrations (0.0–5.0 mg/mL) of MRP in 96-well flat-bottom plates with a final volume of 200 µL at 37 ºC for 24 h. Following incubation, cell viability was assessed using the MTT assay and the IC_{50} value was calculated as mentioned above.

8.4.8 *In vitro* gastrointestinal digestion

The samples were subjected to *in vitro* digestion, using a procedure previously described (O'Connell *et al.,* 2007). Briefly, sample (20 mg) was added to 10 mL Hank's balanced salt solution (HBSS) and digested with pepsin (0.04 g/mL) at pH 2 and 37 °C for 1 h in a shaking water bath. The pH of the mixture was adjusted to 5.3 using 0.9 M $NaHCO_3$. Bile salts, consisting of glycodeoxycholate (0.8 mmoL/L), taurodeoxycholate (0.45 mmoL/L), taurocholate (0.75 mmoL/L); and pancreatin (0.08 g/mL) were then added. The pH was adjusted to 7.4 using 1.0 M NaOH. The final volume was adjusted to 20 mL by adding HBSS followed by a digestion period of 2 h at 37 °C, in a shaking water bath. Samples were then centrifuged at 15,000 rpm at 4 °C for 30 min and the aqueous supernatant was divided into two groups, with and without filtration through a 0.22 µm filter. ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) of samples before and after *in vitro* digestion were measured.

8.4.8.1 ABTS radical scavenging activity

Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulphate were prepared (Binsan *et al.,* 2008). The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was allowed to react for 12 h at room temperature in the dark. The working solution (1 mL) was then diluted with 50 mL of distilled water, in order to obtain an absorbance of 1.10±0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Fresh ABTS solution was prepared for each assay. Sample (150 µL) was mixed with 2850 µL of ABTS solution and the mixture was incubated at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer (Binsan *et al.,* 2008). The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox at concentrations ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalent (TE)/g solid.

8.4.8.2 Ferric reducing antioxidant power (FRAP)

FRAP reagent was prepared by mixing acetate buffer (30 mM, pH 3.6), 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v/v) (Benzie and Strain, 1996). The sample solution (100 µL) was mixed with 3 mL of working FRAP reagent and incubated in the dark at room temperature for 30 min. The absorbance of the reaction mixture was measured at 593 nm using a spectrophotometer (WPA Lightwave S2000, Scinteck Instruments, Centreville, Virginia USA). The standard curve was prepared using $FeSO_4$ at concentrations ranging from 0 to 100 µM. The activity was expressed as µmol Fe(II) equivalents/g solid.

8.4.9 Statistical analysis

A completely randomized design (CRD) was used throughout the study. All data was subjected to analysis of variance (ANOVA) and mean comparisons was carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social

Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA). The results with $P<0.05$ were considered to be statistically significant.

8.5 Results and discussion

8.5.1 Effect of Maillard reaction product (MRP) on cell viability in U937, HepG2 and RAW264.7 cells

The cytotoxicity of MRP derived from gelatin hydrolysate of unicorn leatherjacket skin was tested in the U937, HepG2 and RAW264.7 cell lines to determine the non-cytotoxic concentration for the use in the cell-based assays. Cells were treated without or with increasing concentrations of MRP at 37 °C for 24 h and the cell viability was measured by the MTT assay. A reduction in cell viability was found in all cell models, in a dose-dependent manner. Cell viability of U937 cells was stimulated with significant increase in cell viability at 150-400 µg/mL MRP ($P<0.05$), compared with untreated cells. The markedly decreased ($P<0.05$) cell viability was found when using MRP at the concentration higher than 1300 µg/mL and IC_{50} value of 1250 µg/mL was determined. A similar trend was observed in HepG2 cells, in which the decrease of cell viability was observed in the presence of MRP and an IC_{50} value of 1.0 mg/mL MRP was determined. It was noted that MRP had a low cytotoxicity in RAW264.7 cells (Figure 31) and cell viability was greater than 80%, compared with untreated cells, at the highest MRP concentration tested (1500 µg/mL). Moreover, the cell viability of RAW264.7 cells was higher than 60% when the MRP concentration was 10 mg/mL (data not shown). Therefore an IC_{50} value for MRP could not be determined in RAW264.7 cells. For the further experiments, a non-toxic concentration of MRP was selected.

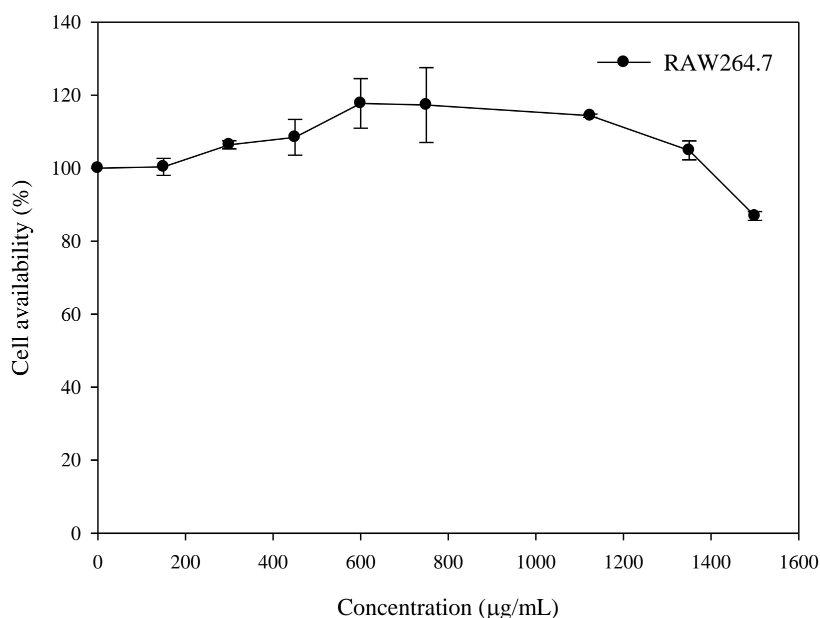

Figure 31 Cell viability (%) of RAW 264.7 cells at different concentrations of Maillard reaction product (MRP). Values are mean ± SD of 3 independent experiments.

8.5.2 Ability of Maillard reaction product (MRP) to protect against oxidant-induced DNA damage in U937 cells

The ability of MRP to protect against oxidant induced DNA damage was assessed using the comet assay in the U937 cell model system (Figure 32). The U937 cells were pretreated with different concentrations of MRP (500-1500 µg/mL) and DNA damage was then induced by exposing the cells to different concentrations (40 and 60 µM) of H_2O_2 for 30 min. The viability of the cells under these experimental conditions was determined to be greater than 80% by means of the fluorescein diacteate-ethidium bromide staining method (data not shown). The oxidant (H_2O_2) at 40 and 60 µM significantly increased the percentage DNA damage (expressed as % tail DNA) to 17 and 43%, respectively, compared with untreated cells (control). MRP at the tested concentrations (500-1500 µg/mL) did not induce DNA damage in U937 cells in the absence of H_2O_2 (data not shown). At 40 µM H_2O_2, a significant decrease in DNA damage was observed in cells treated with the highest

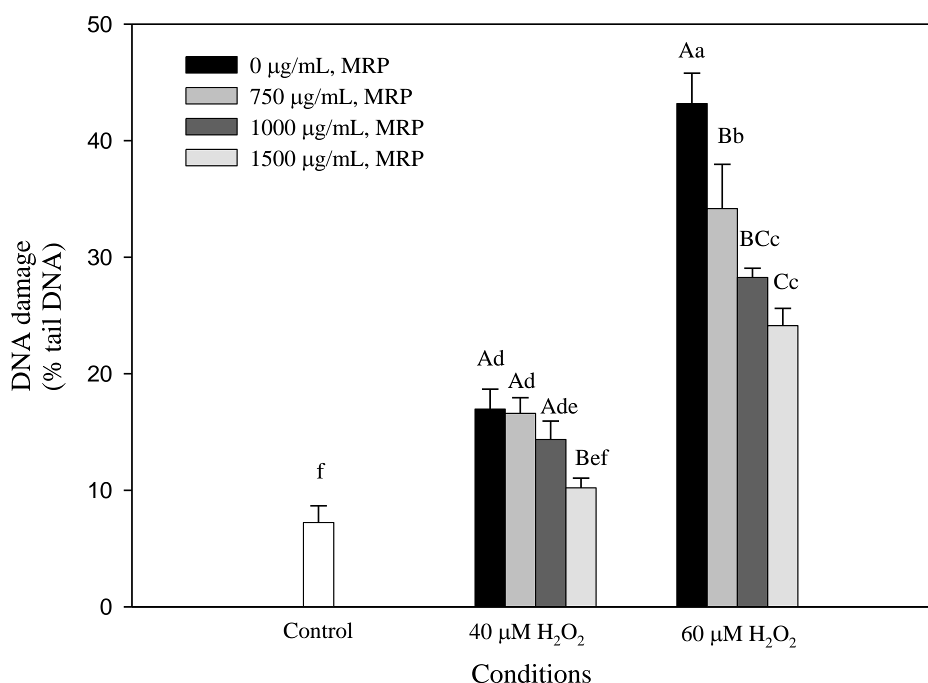

Figure 32 The ability of Maillard reaction product (MRP) to protect against DNA damage induced by different concentrations of H_2O_2. Values are mean ± SD of 3 independent experiments. [A, B, C] indicate significant differences ($p < 0.05$) between values in the same H_2O_2 concentration. [a-f] indicate significant differences ($p < 0.05$) between values.

MRP concentration (1500 µg/mL) and it was also noted that tail DNA was comparable to control levels at MRP concentration (1500 µg/mL) and it was also noted that tail DNA was comparable to control levels at this concentration of MRP. A similar trend was observed in U937 cells exposed to 60 µM H_2O_2. MRP decreased the tail DNA ($P<0.05$) in a dose dependent manner. The DNA damage was reduced to approximately 25% tail DNA at the highest MRP concentration used (1500 µg/mL) compared with a level of 43% tail DNA in cells exposed to H_2O_2 in the absence of MRP. Wijewickreme and Kitts (1998) reported that a coffee MRP lowered the degree of supercoiled PM2 bacteriophage DNA breakage induced by Fe^{2+} at a concentration of 0.001% (w/v) but the protective effects of resulting MRPs were absent at higher MRP concentrations (0.01% (w/v)). Moreover, in many instances, compared with the use of lower coffee MRP concentrations, higher coffee MRP concentrations

significantly enhanced the degree of DNA breakage than the original DNA in the control (Wijewickreme and Kitts, 1998). Yen *et al.* (2002) studied the effect of MRPs prepared using different monosaccharides in combination with lysine on DNA damage in human lymphocytes. The MRPs produced from different sources and preparation methods induced DNA damage at a range of concentrations (Yen *et al.,* 2002). Therefore, MRP derived from gelatin hydrolysate of unicorn leatherjacket skin under the tested concentration had the ability to protect against H_2O_2-induced DNA damage in U937 cells.

8.5.3 Effect of Maillard reaction product (MRP) on antioxidant enzyme activities in HepG2 cells

Cellular antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are regarded as the first line of defence against ROS generated during oxidative stress (Meerson *et al.,* 1982). In order to investigate the antioxidant activity of MRP, oxidative stress, resulting in a reduction in the activity of catalase and SOD, was induced in HepG2 cells using 2 mM H_2O_2. The ability of MRP at different concentrations (250 and 500 µg/mL) to protect against this reduction in antioxidant enzyme activity was assessed. H_2O_2 significantly reduced SOD (Figure 33 (a)) and CAT (Figure 33 (b)) activities ($P<0.05$) to approximately 70 and 72% of control (without H_2O_2 treated), respectively (Figure 33). MRP had no effect on SOD and CAT activities in HepG2 in the absence of H_2O_2 exposure. This result was similar to that reported by Yen *et al.* (2002) and Seiquer *et al.* (2008), in which MRPs had no effect on antioxidant enzyme activity in unchallenged human lymplcytes and erythrocytes. MRPs prepared by heating lysine with different monosaccharides at pH 9.0 and 100 °C for 3 h did not affect the activity of glutathione peroxidise in human lymphocytes at the concentrations of 0.05-0.80 mg/mL (Yen *et al.*, 2002). In the present study, the two antioxidant enzyme activities were increased in H_2O_2-challenged cells following a pre-incubation with MRP, in dose dependent manner (Figure 33). SOD activity was significantly increased ($P<0.05$) to 84% of control,

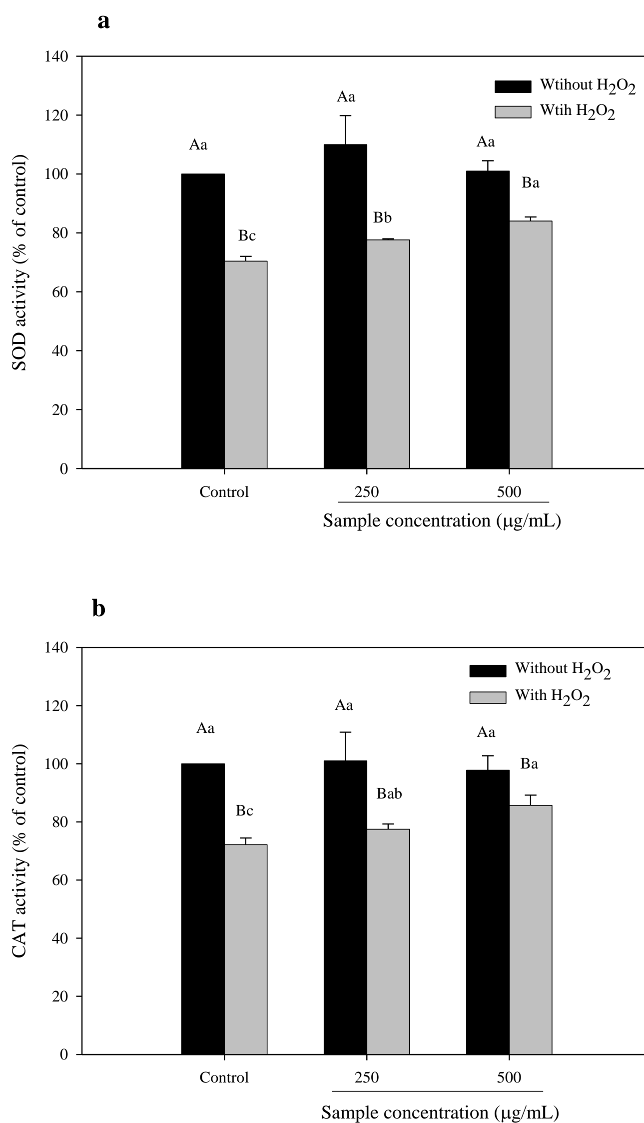

Figure 33 Effects of Maillard reaction product (MRP) on superoxide dismutase (SOD) (a) and catalase (CAT) (b) activities in HepG2 cells without and with H_2O_2-induced. Values are mean ± SD of 3 independent experiments. $^{A, B}$ indicate significant differences ($p < 0.05$) between values in the same sample concentration. $^{a, b, c}$ indicate significant differences ($p < 0.05$) between values in the same H_2O_2 concentration.

while CAT activity was increased to 86% of control at the highest MRP tested (500 μg/mL). The result was in agreement with Goya *et al*. (2007) who found that a *in vitro* gastrointestinal digested melanoidin protected against a decrease in antioxidant enzyme activity induced by tert-butylhydroperoxide (t-BOOH) in HepG2. Antioxidant activities of the MRP were due to their ROS/radical scavenging activity.

8.5.4 Immunomodulatory effects of Maillard reaction product (MRP) in RAW264.7 cells

The immunomodulatory effects of MRP were investigated by assessing their ability to reduce the secretion of pro-inflammatory cytokines in LPS challenged RAW264.7 cells. The production of cytokines (IL-1β and IL-6) and nitric oxide secretion were quantified (Figure 34). LPS, which is derived from the outer membrane of Gram-negative bacteria, stimulated RAW264.7 macrophage cells to produce IL-1β (Figure 34 (a)) and IL-6 (Figure 34 (b)) at concentrations of 31.4 and 93.1 pg/mL ($P<0.05$), respectively (data not shown). There was a dose dependent decrease in IL-1β and IL-6 production in LPS-stimulated cells incubated with MRP, compared with cells incubated with LPS alone. The production of both cytokines was reduced by approximately 50% in the presence of 1500 μg/mL MRP (Figure 34 (a) and (b)). A similar result was found for NO secretion (Figure 34 (c)). The production of this pro-inflammatory marker was significantly increased ($P<0.05$) following the exposure to LPS, to 11-fold of control (without LPS). In the presence of MRP, NO secretion was decreased in a dose dependent manner (Figure 34 (c)). It was observed that NO production from LPS-induced RAW264.7 cells was inhibited almost 70% by MRP at the highest concentration tested (1500 μg/mL). Kitts *et al.* (2012) previously reported the anti-inflammatory activity of MRPs prepared using different monosaccharide and amino acid model systems. The MRP derived from glucose-lysine systems showed a marked ability to inhibit intracellular oxidation, the production of inflammatory cytokines and NO in inflamed Caco-2 cells, compared with MRPs from other systems (Kitts *et al.,* 2012). Two components, namely, 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid (HMFA), were identified as bioactive components in this MRP (Kitts *et al.,* 2012). The results

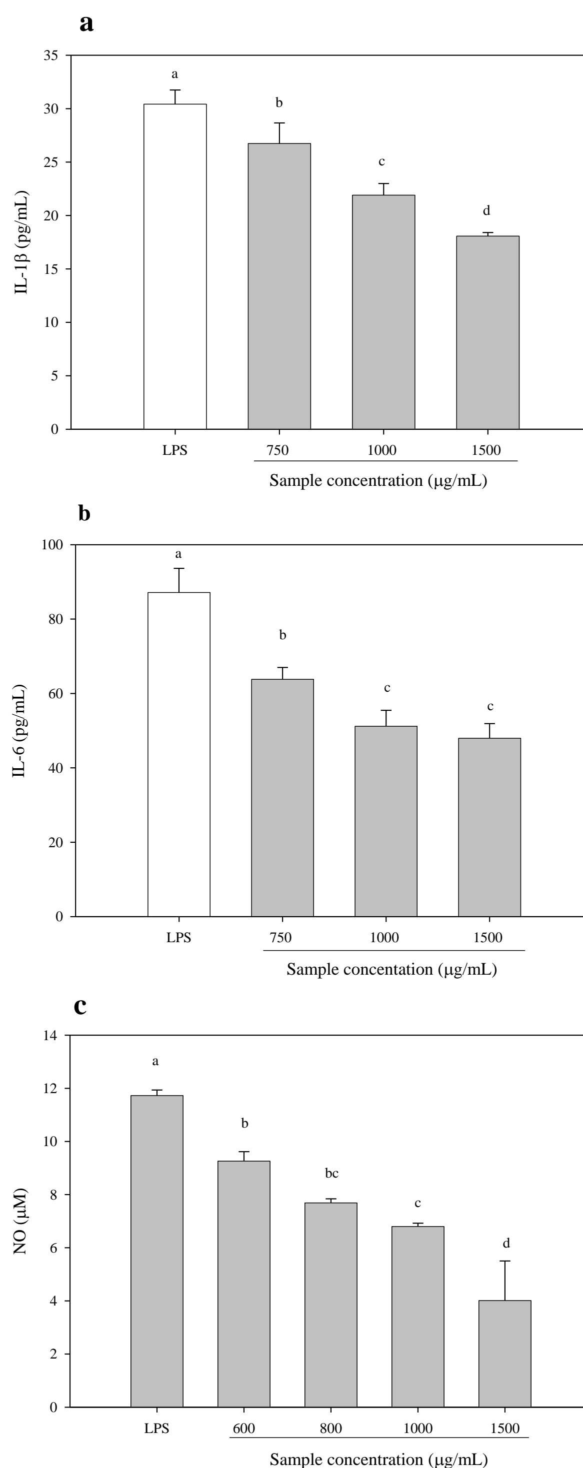

Figure 34 Immunomodulatory effects of Maillard reaction product (MRP) on production of cytokine IL-1β (a) and IL-6 (b) and nitric oxide (NO) secretion (c) in LPS-induced Raw264.7 cells. Values are mean ± SD of 3 independent experiments. $^{a\text{-}d}$ indicate significant differences ($p < 0.05$) between values.

indicated that an antioxidant MRP derived from a gelatin hydrolysate of unicorn leatherjacket skin has bioactive potential, particularly with regard to the suppression of inflammation in LPS-induced RAW264.7 cells.

8.5.5 Antiproliferative effect of Maillard reaction product (MRP) against human colon cancer cells (Caco-2)

The anticarcinogenic potential of MRP was accessed through inhibitory effect on cancer cell growth or cytotoxicity on cancer cell lines. The antiproliferative activity of MRP was studied in human colon cancer (Caco-2) cells using the MTT assay. A dose-dependent inhibition of cancer cell growth was found (Figure 35). It was noted that the cell viability was significantly decreased ($P<0.05$) when MRP was added at concentrations higher than 1.0 mg/mL, compared with control (without treated) and an IC_{50} value of 1.7 mg/mL was obtained. Moreover, cell viability was lower than 40% with 2.5 to 5.0 mg/mL MRP. Yamabe *et al.* (2013) produced an antioxidant MRP with anticancer activity from a ginsenoside-lysine mixture. They found that the increased anticancer effect of MRP upon heat processing was mainly derived from the generation of less-polar compounds (Yamabe *et al.,* 2013). HMFA is a metabolite of HMF and it possesses antitumor activity against SV40-transformed (*in vitro*) and Sacroma 180 implanted intraperitoneally in ICR mice (*in vivo*) (Munekata and Tamura, 1981). An MRP prepared by heating protein hydrolysate and glucose at 145 °C for 20 min demonstrated the potential to decrease Caco-2 cells proliferation (Teodorowicz *et al.,* 2013). Moreover, the anticancer activity of MRP could relate with its immunomodulatory activity (Wang *et al.,* 2010).

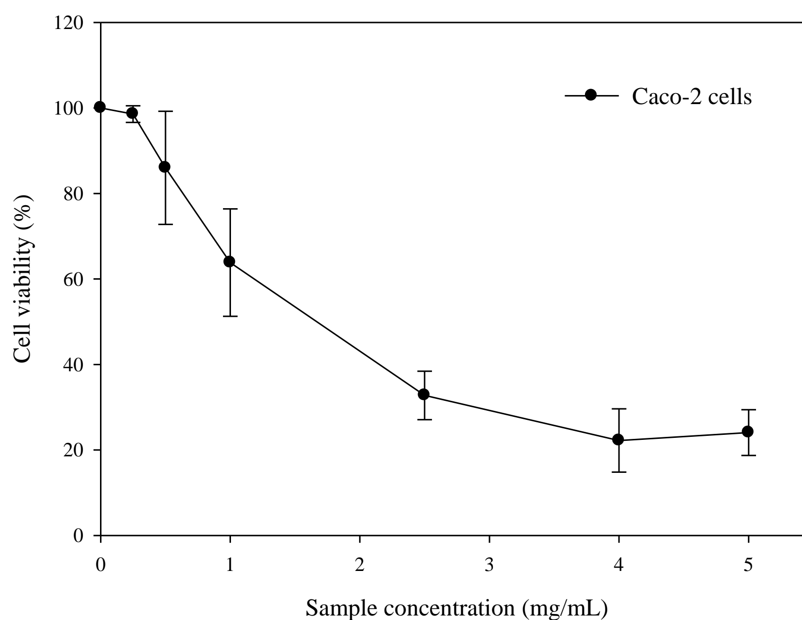

Figure 35 The anticancer activity of Maillard reaction product (MRP) at different concentrations on cell viability (%) of Caco-2 cells. Values are mean ± SD of 3 independent experiments.

8.5.6 Effect of *in vitro* digestion on antioxidant activity of Maillard reaction product (MRP)

The *in vitro* digestion of MRP was carried out to determine its stability to gastrointestinal conditions. ABTS radical scavenging activity (ABTS) and ferric reducing antioxidant power (FRAP) of MRP were measured before and after *in vitro* digestion (Table 11). The antioxidant activities of MRP after *in vitro* digestion without and with filtration were also determined. Similar ABTS and FRAP values for MRP before and after *in vitro* digestion (without filtration) were observed. This result suggested that the gastrointestinal enzymes or other digestive conditions (pH) did not affect the antioxidant activity of MRP. It was possible that MRP might be broken down in the gastrointestinal tract potentially, thereby forming novel antioxidant products. However, there was no evidence of enhanced antioxidant activity in the present study. A significant decrease in both ABTS and FRAP was found for MRP after *in vitro* digestion and filtration through a 0.22 μm filter. This decrease indicated

that the antioxidants with high molecular weight in MRP remaining after *in vitro* digestion were removed by filtration. It has been reported that glycated products derived from casein-glucose have resistance to tryptic hydrolysis and high molecular weight compounds were still remained after hydrolysis (Gu *et al.,* 2010). Moscovici *et al.* (2014) found that MRPs derived from a lactoferrin model system possessed a high susceptibility to proteolysis in *in vitro* digestion. Proteomic analyses of digested MRP revealed altered enzymatic cleavage patterns with no pronounced changes in the formation of known bioactive peptides (Moscovici *et al.,* 2014). On the other hand, a reduction in the antioxidant activity of MRPs prepared from casein-glucose model systems was observed after tryptic and peptic digestion (Gu *et al.,* 2010).

Table 11. ABTS radical scavenging activity (ABTS) and ferric reducing antioxidant power (FRAP) of Maillard reaction product (MRP) before and after *in vitro* digestion

Conditions	ABTS µmol TE/g sample	FRAP µmol FE(II) equivalents/g sample
Before *in vitro* digestion	266.37±7.98[a]	10.07±0.02[a]
After *in vitro* digestion		
Without filtration	253.02±2.14[ab]	9.55±0.28[a]
With filtration	250.85±1.15[b]	8.02±0.23[b]

Values represent the mean ± SD for three separated experiment. [a, b, c] indicate significant differences ($p < 0.05$) between values in the same column.

8.6 Conclusion

A Maillard reaction product (MRP) derived from a gelatin hydrolysate of unicorn leatherjacket skin showed cellular antioxidant potential by protecting against oxidant-induced DNA damage and a depletion of intracellular antioxidant enzyme activity. This antioxidant MRP also possessed anti-inflammatory properties as evidenced by a reduction in pro-inflammatory cytokines and nitric oxide production in LPS-stimulated RAW 264.7 cells. MRP demonstrated anticancer activity by inhibiting the proliferation of human colon cancer (Caco-2) cells. In addition, MRP retained its antioxidant activity following an *in vitro* digestion procedure thereby demonstrating a capacity to resist gastro-intestinal digestion.

Therefore, MRP could be a novel source of a natural, fish gelatin-derived bioactive compound with the potential to be exploited for the production of biofunctional foods for human consumption.

8.7 References

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CHAPTER 9

Antioxidative and sensory properties of instant coffee fortified with Maillard reaction products from galactose-fish skin gelatin hydrolysate

9.1 Abstract

Antioxidative Maillard reaction product (MRP) prepared from the mixture of galactose-gelatin hydrolysate from unicorn leatherjacket skin was fortified in instant coffee brew at different levels (0, 0.5, 1.0 mg/mL) and the resulting brew was characterised. Addition of MRP up to 1.0 mg/mL had no effect on browning index, L*, a*, b*, ΔE* and ΔC* of coffee brew (P>0.05). The pH value of resulting instant coffee brew was decreased with increasing MRP level added (P<0.05). ABTS radical scavenging activity and ferric reducing antioxidant power of coffee brew increased as MRP levels increased (P<0.05). Based on sensory evaluation, there were no differences in likeness score between coffee brew fortified with MRP at all levels used and the control (without MRP). Much higher abundances of cyclohexanone and dimethydisulfide were observed in coffee brew added with MRP, compared with the control. Thus, MRP could serve as the rich source of antioxidant, which could be supplemented in coffee brew without negative effect.

9.2 Introduction

Maillard reaction products (MRPs) have the wide applications in several foods. MRPs showed antioxidative activity in biscuit (Goya, 2009), cereals (Rufiãn-Henares and Delgado-Andrade, 2009), coffee (Goya *et al*., 2007) and nut (Açar *et al*., 2009). Antioxidative xylan-chitosan MRPs were able to retard lipid oxidation in the refrigerated pork meat (Li *et al*., 2013). Although MRPs have been shown as a potential antioxidant in a variety of foods, the practical use of MRP in food industry has been limited. This is due to the facts that MRPs have their own characteristic dark colour, bitter taste and peculiar flavour, which cause the negative impact on the finished products.

Recently, MRP derived from the mixture of galactose and gelatin hydrolysate of unicorn leatherjacket skin (2:1, w/w) has been reported to show higher ABTS radical scavenging activity and reducing power than mother gelatin hydrolysate (chapter 6). Furthermore, the MRP also had in vitro cellular bioactivities including antioxidant activity, immunomodulatory potential and anticancer effects in different cell lines. MRP demonstrated in vitro suppression of oxidative stress and inflammation and was able to inhibit the proliferation of human colon cancer cells (chapter 7). Therefore, antioxidative MRP derived from fish skin gelatin hydrolysate could be used as an alternative antioxidant or a functional ingredient. Additionally, it can be supplemented or fortified in the foods or drinks with dark colour, e.g. coffee brew, etc.

9.3 Objective

To investigate the effect of the fortification of antioxidative Maillard reaction product (MRP) derived from gelatin hydrolysates of unicorn leatherjacket skin on characteristics, antioxidative and sensory properties of fortified coffee brew.

9.4 Materials and methods

9.4.1 Chemicals

Instant coffee (Nestle, Quality coffee product Ltd., Chachoengsao, Thailand) was purchased from a local market in Hat Yai, Songkhla, Thailand. D-galactose and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were procured from Fluka (Buchs, Switzerland). 2,2′-azinobis (3- thylbenzothiazoline-6-sulfonic acid) (ABTS) and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade.

9.4.2 2.2 Preparation of Maillard reaction product from gelatin hydrolysate of unicorn leatherjacket skin

9.4.2.1 Prepation of fish skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock, Songkhla, Thailand. Skins were pooled and used as a composite sample. The skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2).

The skins were subjected to pretreatment as per the method of Kaewruang *et al*. (2013). Fish skins (0.5×0.5 cm^2) were soaked in 0.05 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v). The pretreatment solution was changed after 2 h and total pretreatment time was 4 h. Skins were then washed with tap water until neutral or faintly basic pH of wash water was obtained. Subsequently, pretreated skins were autolysed following the method of chapter 5.4.2.3. The resulting autolysed skins were used for preparation of gelatin hydrolysate.

9.4.2.2 Preparation of glycyl endopeptidase

Glycyl endopeptidase (GE) was fractionated from papaya latex using the method of chapter 3. Aqueous two phase system (ATPS) with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of GE. The obtained GE was stored at -40 °C until use.

9.4.2.3 Prepartion of gelatin hydrolysates

Autolysed skin suspension (3%, w/v) was mixed with GE (8%, based on solid matter) and incubated at 40 °C for 60 min (chapter 4). After enzyme inactivation by heating at 90 °C for 15 min, the resulting gelatin hydrolysate was centrifuged at 9,000×g at 4 °C for 20 min using a refrigerated centrifuge, model Avanti J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant was collected and lyophilised using Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The gelatin hydrolysate (NS-8GE) was collected and placed in polyethylene bag. NS-8GE was stored at -20 °C until used.

9.4.2.4 Preparation of Maillard reaction product (MRP)

Maillard reaction between NS-8GE and galactose was conducted following the method from chapter 6. The mixture of NS-8GE and galactose (2:1, w/w) was heated at 70 °C and 55% relative humidity for 36 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting galactose-gelatin hydrolysate MRP was collected, analysed and fortified into coffee brew.

9.4.3. Fortification of MRP from gelatin hydrolysate in instant coffee

Instant coffee brew was prepared by adding 2 g of instant coffee powder and MRP in hot water (75 °C) to obtain 100 mL of different final MRP concentrations (0, 50, 100 mg). The resulting coffee brew samples were subjected to analyses.

9.4.4 Analyses

9.4.4.1 Browning Index

Prior to measurement, the samples were appropriately diluted (50-fold) using distilled water and the absorbance was measured at 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). A_{420} was used as the index for browning intensity (Ajandouz *et al*., 2011).

9.4.4.2 Colour

Colour was measured by Hunter Lab (C04-1005-631 colorFlex, Reston, VA, USA). The value was expressed as L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) values. Total difference in colour (ΔE*) and chroma (ΔC*) were calculated according to the following equation (1) and (2), respectively (Gennadios et al., 1996; Jangchud and Chinnan, 1999).

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

$$\Delta C^* = \sqrt{\Delta a^{*2} + \Delta b^{*2}} \quad (2)$$

where ΔL*, Δa*and Δb*are the differences between the corresponding colour parameter of the sample and that of black standard (L*= 0.63, a*= -0.39 and b*= -1.02).

9.4.4.3 pH

The pH value of coffee brew was measured using a pH meter (Sartorius PB-10, Göttingen, Germany).

9.4.4.4 Antioxidative activities

9.4.4.4.1 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan et al. (2008). Sample with approximate dilution (10-fold, 150 μL) was mixed with 2850 μL of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a spectrophotometer (Shimadzu, Kyoto, Japan) (Binsan *et al.*, 2008). A standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalent (TE)/mL sample.

9.4.4.4.2 Ferric reducing antioxidant power (FRAP)

FRAP reagent was prepared by mixing acetate buffer (30 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution at a proportion of 10:1:1 (v/v/v) (Benzie and Strain, 1996). The sample solution (120-fold diluted, 100 μL) was mixed with 3 mL of working FRAP reagent and incubated in the dark at room temperature for 30 min. The absorbance of the reaction mixture was read at 593 nm using a spectrophotometer (Benzie and Strain, 1996). The standard curve was prepared using Trolox ranging from 0 to 500 μM. The activity was expressed as μmol Trolox equivalents (TE)/mL sample.

9.4.4.5 Sensory evaluation

Fifty panellists were randomly recruited from Faculty of Agro-Industry, Prince of Songkla University. Panellists were selected for evaluation of samples when they consumed at least one cup of coffee per day. Panel included 38 % males and 62 % females. Fifty-five percent of the panellists were older than 25 years. Most panellists (97 %) preferred coffee with milk/cream and sugar.

The coffee brews were prepared freshly in a thermostatically controlled flask (75 °C) and the temperature was kept constant within 15 min prior to evaluation in order to minimise aroma loss. Each panellist received one cup of coffee (100 mL containing 2 g instant coffee without and with MRP at various levels) separately. All samples were blind-coded with three-digit randomised numbers. Panellists were instructed to add milk/cream and sugar if preferred. In order to standardise cream and sugar use, a sufficient number of 3 g cream and 6 g sugar sachets, were provided. The panellists expressed their liking of each sample using a 9-point hedonic scale ranging from 1-dislike extremely to 9-like extremely (Meilgaard *et al*., 1999). Attributes including appearance, colour, aroma, flavour, taste and overall likeness were assessed.

9.4.5 The presence of volatile compounds in coffee brews without and with MRP addition

The volatile compounds in coffee brew fortified with 1.0 mg/mL MRP was determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Iglesias and Medina (2008) in comparison with coffee brew without MRP addition and MRP.

9.4.5.1 Extraction of volatile compounds by SPME fibre

The samples were heated at 60 ºC in 20 headspace vial with equilibrium time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC for 15 min before use and then exposed to the headspace. The 20 mL-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

9.4.5.2 GC–MS analysis

GC–MS analysis was performed in a Trace Ultra gas chromatography coupled with a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific Inc., San Jose, CA, USA) and equipped with a splitless injector. Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m ± 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 10 ºC/min to a final temperature of 260 ºC and holding for 5 min. Helium was employed as a carrier gas with a constant flow of 1.5 mL/min. The injector was operated in the splitless mode and its temperature was set at 260 ºC. Transfer line temperature was maintained at 265 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 200 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 10-200 amu and scan rate:

0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

9.4.5.3 Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. The identified volatile compounds were presented in the term of abundance of each identified compound (peak area).

9.4.6 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Ducan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

9.5 Results and discussion

9.5.1 Browning index, colour, pH and antioxidative activity of coffee brew fortified with MRP

Browning index, colour and pH values of coffee brew added with MRP at different concentrations (0.0-1.0 mg/mL) are shown in Table 12. In general, there was no difference in A_{420}, L*, a*, b*, ΔE* and ΔC* values ($P>0.05$) between the coffee brew without and with MRP at various levels added. The result suggested that the fortification of MRP in coffee brew at the tested concentrations did not affect the colour of resulting coffee brew. Nevertheless, the decreases in pH of coffee brew added with MRP were found with increasing MRP level used ($P<0.05$). It was reported that galactose-gelatin hydrolysate MRP had the acidic pH due to the production of formic and acetic acids from the reducing sugar, which was partially

degraded into these compounds via Maillard reaction (Rufiãn-Henares *et al*., 2006). Therefore, the addition of MRP more likely led to the decrease in pH of resulting coffee brew.

Antioxidative activities of coffee brew fortified with MRP at different concentrations were evaluated and expressed as ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) as shown in Figure 36 (a) and (b), respectively. ABTS radical scavenging activity and FRAP increased with increasing MRP levels ($P<0.05$). From chapter 6, we found that the highest antioxidative activity was obtained when MRP was prepared from the mixture of unicorn leatherjacket skin gelatine hydrolysate and galactose (2:1) at 70 °C and 55% RH for 36 h. The resulting MRPs had the increase in ABTS radical scavenging activity and FRAP by 15 and 150-fold, respectively, compared with those of galactose-NS-8GE before heating (chapter 6). The fortification of MRPs could therefore increase the antioxidative activity of coffee brew.

Table 12 Browning index, colour and pH of coffee brew added with galactose-fish skin gelatin hydrolysate MRP at different concentrations

Samples	Browning index $(A_{420})^{ns}$	Colour					pH
		L^{*ns}	a^{*ns}	b^{*ns}	ΔE^{*ns}	ΔC^{*ns}	
Control	12.00±0.52	5.54±0.23	4.61±0.63	5.59±0.27	9.55±0.19	6.18±0.34	4.85±0.02[a]
0.5 mg/mL	12.86±1.97	5.36±0.13	4.57±0.48	5.54±1.17	9.55±0.62	6.16±0.66	4.80±0.02[b]
1.0 mg/mL	13.53±0.74	5.63±0.19	4.70±0.87	5.32±1.34	9.84±0.74	6.13±0.62	4.75±0.02[c]

Values represent the mean ± SD (n=3). A_{420} indicate absorbance at 420 nm.
[ns] indicate non-significant differences ($p< 0.05$) between values in the same column. Different lowercase letters in the same column indicate significant differences ($p<0.05$).

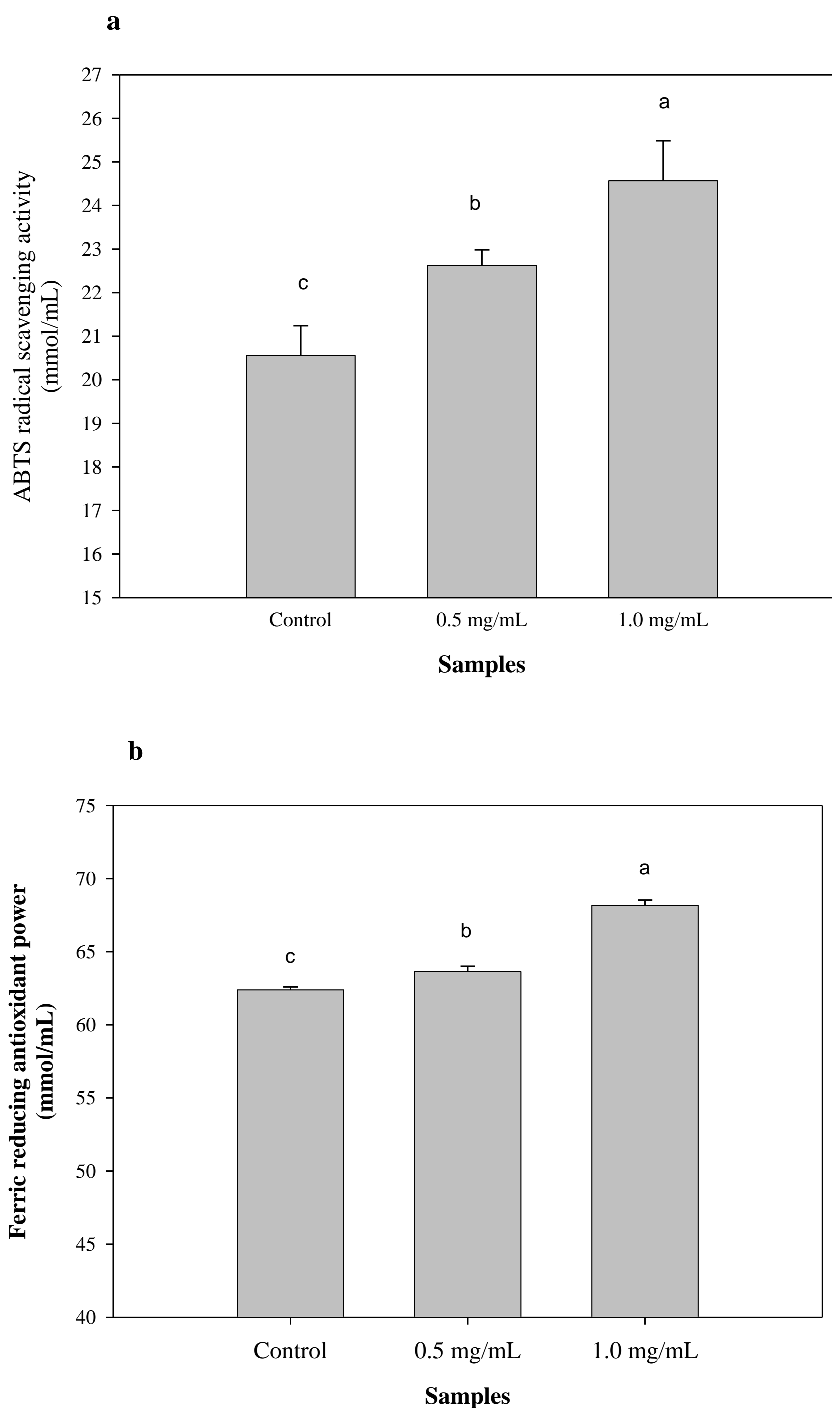

Figure 36 ABTS radical scavenging activity (a) and ferric reducing antioxidant power (b) of coffee brew added with galactose-fish gelatine hydrolysate MRP at different concentrations. Bars represent standard deviation (n=3). Different lowercase letters on the bars indicate the significant differences ($p < 0.05$).

9.5.2 Sensory property of instant coffee brew fortified with MRP

Likeness score of coffee brew added with MRP at various levels is shown in Table 13. There was no difference in likeness score for all attributes including appearance, colour, aroma, flavour, taste and overall amongst all samples ($P>0.05$). This result indicated that the addition of MRP up to 1.0 mg/mL had no affect on the likeness of coffee brew. All samples had likeness score of approximately 7 for all attributes, suggesting the moderate likeness of coffee brew, regardless of MRP fortification ($P>0.05$). Volatile compounds present in aromatic oil from roasted coffee did not have the impact on sensory property of instant coffee brew (Oliveira *et al.*, 2009). Thus, the incorporation of instant coffee with MRP could enhance antioxidative activity without the negative effect on sensory property of resulting coffee brew. This was plausibly due to the strong odour or smell of coffee brew, which could mask the typical flavour or odour of MRP. From our previous study, the mild cytotoxic activity of MRP was demonstrated in human hepatoma (HepG2) and human histiocytic lymphoma (U937) cell lines with IC_{50} of 1.0 and 1.25 mg/mL, respectively. Moreover, MRP (0-1.50 mg/mL) had a very low cytotoxicity in mouse macrophages (RAW264.7) cells, in which an IC_{50} value for MRP could not be determined (chapter 7.5.1). Nevertheless, MRP did not cause DNA damage to U937 cell lines under tested concentrations (750-1500 μg/mL) (chapter 7.5.2). The similar results were reported for cytotoxicity and genotoxicity of traditional medicines Agaricus blazei, Grifola frondosa and Hericium erinaceus extracts which showed weak cytotoxic effect without DNA damage effect against Chinese fibroblast cell line (Shahirah *et al.*, 2014). The result suggested that MRP used was safe for consumption.

Table 13 Liking score of instant coffee brew added with galactose-fish skin gelatin hydrolysate MRP at different concentrations

Samples	Appearance^{ns}	Colour^{ns}	Aroma^{ns}	Flavour^{ns}	Taste^{ns}	Overall^{ns}
Control	7.62±0.82	7.65±0.92	6.68±1.43	6.97±1.29	7.0±1.30	7.0±1.26
0.5 mg/mL	7.74±0.75	7.65±0.81	6.88±1.47	6.94±1.28	6.79±1.53	6.85±1.41
1.0 mg/mL	7.41±0.92	7.47±0.90	6.91±1.44	7.12±1.59	7.0±1.65	7.06±1.30

Values represent the mean ± SD (n=3). ^{ns} indicate not significant differences ($p<0.05$) between values in the same column.

9.5.3 Volatile compounds of instant coffee fortified with MRP

Volatile compounds present in coffee brew without and with MRP addition are shown in Table 14. Volatile compounds of MRP were also determined. Thirty volatile compounds were indentified and quantified in coffee brew (without MRP). Those included aldehydes, ketones, furans, pyrroles, pyrazines and others, which were commonly found in coffee brew (Petisca *et al*., 2013; Lopez-Galilea *et al*., 2006; Sanz *et al*., 2002). 2-Methylbutanal, pyridine, methylpyrazine, ethylpyrazine, furfural and furfuryl alcohol were found as the major volatile compounds in instant coffee. Lopez-Galilea *et al*. (2006) reported that 2-methylbutanal was responsible for chocolate-like and fruity note. Pyridine and pyrazines are well-known as Maillard reaction products and give characteristic roasted or toasted flavours to coffee (Moon and Shibamoto, 2009). On the other hand, furfural and furfuryl alcohol do not have a high odorant impact (Lopez-Galilea *et al*., 2006). Similar volatiles were found in instant coffee brew added with MRP. However, some compounds including cyclohexanone and dimethyldisulfide were much higher in abundance, compared with the control. It was noted that methyleugenol, pyrrole, 2-ethyl hexanol, acetol acetate, diethyl-2,6-pyrazine, 2-methyl-2-cyclopentenone, 3-heptanone, 2-methylfuran and dimethyldisulfide were found only in coffee brew added with MRP. Moreover, other volatile compounds were found in both coffee brew (control) and MRP added sample at similar levels. When considering volatiles in MRP, cyclohexanone and dimethyldisulfide were found as the major constituents. Those compounds were detected as dominant volatiles in coffee brew when MRP was fortified. Volatile compounds from MRP were reported in coffee such as 2-ethyl hexanol, diethyl-2,6-pyrazine, 2-methyl-2-cyclopentenone, 2-methylfuran, dimethyldisulfide and cyclohexanal (Petisca *et al*., 2013; Lopez-Galilea *et al*., 2006; Sanz *et al*., 2002). In general, the Maillard reaction was responsible for the generation of roasted, toasted or caramel-like aromas as well as the development of brown colour in foods (Sanz *et al*., 2002). Nevertheless, sensory test revealed that the volatiles in MRP at levels tested had no impact on likeness of coffee brew.

Table 14 Volatile compounds in coffee brew without and with galactose-fish skin gelatin hydrolsate MRP

Compounds	Peak area×10^8		
	Instant coffee		MRP
	without MRP	with MRP*	
2-Methylbutanal	377.56	327.56	nd
1-Methylpyrrole	42.30	55.15	nd
Pyridine	212.46	144.88	nd
Furfuryl methyl ether	25.81	30.79	nd
p-Cymene	13.83	4.28	nd
Methylpyrazine	152.70	153.22	4.55
Cyclohexanone	1.68	95.25	135.41
2,5-Dimethylpyrazine	51.86	46.01	nd
Ethylpyrazine	135.90	69.53	nd
2,3-Dimethylpyrazine	24.22	17.75	nd
2-Ethyl-6-methylpyrazine	44.14	49.94	nd
2-Methyl-6-methylpyrazine	32.45	31.13	nd
2-Ethyl-3-methylpyrazine	35.24	36.40	nd
2-Ethyl-3,5-dimethylpyrazine	24.03	24.28	nd
Furfural	110.08	110.16	39.97
2-Furfurylmethylsulfide	37.14	45.47	nd
2-Acetylfuran	22.12	26.26	7.94
Benzaldehyde	30.09	28.67	15.60
Furfuryl acetate	44.28	60.55	nd
5-Methyl-2-furfural	56.09	58.18	5.79
2-Furfurylfuran	30.26	35.12	nd
2-Formyl-1-methylpyrrole	24.69	36.21	nd
Furfuryl alcohol	116.32	94.00	nd
5-Methyl-2-furfurylfuran	18.95	20.63	nd
1-(2-furfuryl)pyrrole	24.12	28.27	nd
2-Methoxyphenol	22.13	21.67	nd
2-Acetylpyrrole	7.33	7.03	2.91
2-Formylpyrrole	9.37	8.60	nd
p-Ethylguaiacol	7.76	8.20	nd
4-Vinyl-2-methoxy-phenol	5.69	5.75	nd
2,4-Ditert-butylphenol	3.29	5.05	12.79
Methyleugenol	nd	13.19	2.90
Pyrrole	nd	12.90	5.76

Table 14 Volatile compounds in coffee brew without and with galactose-fish skin gelatin hydrolsate MRP (Continue)

Compounds	Peak area×10^8		
	Instant coffee		MRP
	without MRP	with MRP*	
2-Ethyl hexanol	nd	14.95	11.98
Acetol acetate	nd	16.31	0.38
Diethyl-2,6-pyrazine	nd	14.34	1.50
2-Methyl-2-cyclopentenone	nd	5.96	5.53
3-Heptanone	nd	7.40	7.16
2-Methylfuran	nd	4.14	0.26
Dimethyldisulfide	nd	228.74	302.80
Dimethyltrisulfide	nd	nd	5.53
5-Nonanone	nd	nd	1.19
Nonanal	nd	nd	2.85
Cyclohexanol	nd	nd	5.91
Benzeneacetaldehyde	nd	nd	3.21
Benzeneacetonitrile	nd	nd	3.46

nd: not detected.
MRP: Maillard reaction product.
*MRP at 1 mg/mL was used.

9.6 Conclusion

Fortification of galactose-fish skin gelatin hydrolysate MRP in coffee brew could increase ABTS radical scavenging activity and FRAP. Nevertheless, addition of MRP had no effect on colour and sensory property of resulting coffee brew, though MRP was rich in 2-methylbutanal and dimethyldisulfide. Therefore, MRP could be used as an alternative antioxidant which was used as supplement in food or drinks, particularly.

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CHAPTER 10
CONCLUSION AND SUGGESTION

10.1 Conclusions

1. Autolysis assisted process mediated by indigenouse protease from unicorn leatherjacket skin in combination with thermal hydrolysis and hydrolysis using papain was able to enhance antioxidative activity of gelatin hydrolysate.

2. The partially purified glycyl endopeptidase from papaya latex partitioned using aqueous two-phase system (10% PEG 6000-10% $(NH_4)_2SO_4$) in combination with ammonium sulphate precipitation (40-60% saturation) showed the potential in production of antioxidative gelatin hydrolysates with negligible odourous compounds from papaya latex.

3. Gelatin hydrolysate from autolysed non-swollen and swollen skin of unicorn leatherjacket skin had different modes of action. Gelatin hydrolysates from autolysed non-swollen skin was effective in preventing protein denaturation and lipid oxidation of mackerel washed mince model system induced by freeze-thawing process.

4. Maillard reaction was an effective tool to improve antioxidative activity of gelatin hydrolysate from autolysed non-swollen skin. Maillard reaction between gelatin hydrolysate from autolysed non-swollen skin and galactose (2:1, w/w) under dry condition (55% RH) at 70°C for 36 h could effectively increase antioxidative activity of gelatin hydrolysate.

5. The gelatin hydrolysate of autolysed non-swollen unicorn leatherjacket skin and its resulting Maillard reaction product (MRP) showed cellular antioxidant potential by protecting against oxidant-induced DNA damage and a depletion of intracellular antioxidant enzyme activity. Gelatin hydrolysate and its MRP also reduced the pro-inflammatory cytokines and nitric oxide production in LPS-stimulated RAW 264.7 cells and showed anticancer activity against proliferation of human colon cancer (Caco-2) cells.

6. Fortification of MRP derived from gelatin hydrolysate in instant coffee could increase antioxidative activity without negative effect on sensory property.

10.2 Suggestions

1. Identification of antioxidative peptide from gelatin hydrolysate should be studied.

2. Purification and identification of antioxidative compound in MRP should be further carried out.

3. Mode of action of gelatin hydrolysate and MRP at molecular level should be investigated.

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