Impact of Endogenous Compounds and Oxidation on Microbial Transglutaminase-induced Gelation of Muscle Protein from Different Fish

Sochaya Chanarat

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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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ผลของสารประกอบในเนื้อปลาและปฏิกิริยาออกซิเดชั่นต่อการเกิดเจลของโปรตีนกล้ามเนื้อจากปลาชนิดต่างๆ ที่เหนี่ยวนำโดยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์

ผู้เขียน นางสาวโสชญา ชนะรัตน์

สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร

ปีการศึกษา 2557

บทคัดย่อ

จากการศึกษาผลของปัจจัยต่างๆ ต่อการเพิ่มความแข็งแรงของเจลในกล้ามเนื้อปลาอันเป็นผลจากเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ เมื่อเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ที่ระดับต่างๆ (0-0.6 ยูนิตต่อกรัม) ในซูริมิจากปลาสามชนิดซึ่งประกอบด้วย ปลาทรายแดง ปลาทูลัง และ ปลาหลังเขียวในสภาวะที่เติมและไม่เติมอีดีทีเอ ที่ความเข้มข้น 10 มิลลิโมลต่อกิโลกรัม พบว่าเจลจากปลาทรายแดงซึ่งมีปริมาณเอปไซลอนอะมิโน (ε-amino group) สูงสุดมีค่าความแข็งแรงเจลสูงสุด ตามด้วยเจลจากปลาทูลัง และปลาหลังเขียวตามลำดับ โดยทั่วไปค่าแรงก่อนเจาะทะลุจากเจลของปลาทุกชนิดมีค่าสูงขึ้นเมื่อปริมาณของทรานส์กลูตามิเนสจากจุลินทรีย์เพิ่มขึ้น ในสภาวะที่มีอีดีทีเอพบว่ายังคงมีความสามารถในการเพิ่มความแข็งแรงแต่พบในระดับที่ต่ำลง แสดงถึงการทำงานร่วมกันระหว่างเอนไซม์ทรานส์กลูตามิเนสจากภายในกล้ามเนื้อและจากจุลินทรีย์มีผลต่อการเพิ่มความแข็งแรงของเจล

เมื่อศึกษาผลของสารประกอบไนโตรเจนต่างๆ ที่มักพบในกล้ามเนื้อปลาต่อสมบัติของเจลซูริมิที่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ ฉลามหนู (*Carcharhinus dussumieri*) ที่ผ่านการเก็บรักษาในน้ำแข็งเป็นเวลา 12 วัน มีปริมาณไนโตรเจนที่ระเหยได้ทั้งหมด (TVB) ไตรเมทิลเอมีน (TMA) และแอมโมเนียมีปริมาณสูงขึ้น ขณะที่ยูเรียมีปริมาณลดลงระหว่างการเก็บรักษา ($P < 0.05$) อย่างไรก็ตามปริมาณไนโตรเจนที่ระเหยได้ทั้งหมด ไตรเมทิลเอมีน ยูเรีย และแอมโมเนียในเนื้อปลาบดที่ผ่านการล้างลดลง บ่งชี้ว่ากระบวนการล้างสามารถกำจัดสารประกอบดังกล่าว การเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์สามารถเพิ่มค่าแรงก่อนเจาะทะลุ ระยะทางก่อนเจาะทะลุ และความสามารถในการอุ้มน้ำของเจลที่ได้จากเนื้อปลาบด และเนื้อปลาบดที่ผ่านการล้าง

จากการศึกษาปริมาณฟอมัลดีไฮด์ในปลาปากคมและผลของฟอร์มัลดีไฮด์ที่ระดับต่างๆ (0-9 มิลลิโมลต่อกิโลกรัม) ต่อสมบัติของเจลซูริมิจากปลาปากคมที่สามารถผลิตฟอร์มัลดีไฮด์

ที่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ พบว่าระหว่างการเก็บรักษาเป็นเวลา 10 วัน ปริมาณฟอร์มัลดีไฮด์ทั้งหมด และฟอร์มัลดีไฮด์อิสระในเนื้อปลาปากคมเพิ่มขึ้นอย่างต่อเนื่อง ($P < 0.05$) การเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ (0.4 ยูนิตต่อกรัม) สามารถพิ่มความแข็งแรงเจล และความสามารถในการอุ้มน้ำของเจล อย่างไรก็ตามสมบัติการเพิ่มความแข็งแรงเจลโดยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ลดลงเมื่อปริมาณฟอร์มัลดีไฮด์สูงขึ้น การลดลงของเอปไซลอนอะมิโน (ε-amino group)ในแอกโตไมโอซินธรรมชาติพบมากขึ้นในสภาวะที่เติมฟอร์มัลดีไฮด์ ปฏิกิริยาการเคลื่อนย้ายหมู่เอซิล (acyl transfer reaction) ในแอกโตไมโอซินธรรมชาติที่มีฟอร์มัลดีไฮด์โดยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ลดลงโดยเฉพาะอย่างยิ่งเมื่อระดับฟอร์มัลดีไฮด์สูงขึ้น

เมื่อศึกษาผลของไบโอเจนิกเอมีน ซึ่งประกอบด้วย พูตรีซีน ฮีสตามีน และไทรามีนที่ระดับต่างๆ (0 2 และ 5 มิลลิโมลต่อกิโลกรัม) ต่อสมบัติของเจลซูริมิจากปลานิลที่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ที่ระดับ 0.4 ยูนิตต่อกรัม พบว่าไบโอเจนิกเอมีนสามารถทำหน้าที่เป็นตัวรับหมู่เอซิลจากการเร่งปฏิกิริยาด้วยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ การเติมไบโอเจนิกเอมีนลงในซูริมิไม่มีผลกระทบต่อสมบัติในการเกิดเจลในสภาวะที่ไม่มีเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ ในกรณีที่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์พบว่าความแข็งแรงเจลและความสามารถในการอุ้มน้ำของเจลเพิ่มมากขึ้น เมื่อเทียบกับตัวอย่างที่ไม่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ อย่างไรก็ตามสมบัติการเพิ่มความแข็งแรงเจลโดยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ลดลงเล็กน้อยเมื่อเติมไบโอเจนิกเอมีนโดยเฉพาะอย่างยิ่งที่ระดับ 5 มิลลิโมลต่อกิโลกรัม

จากการศึกษาผลของการออกซิเดชั่นของไมโอซินจากปลานิลซึ่งเหนี่ยวนำโดยปฏิกิริยา Fenton ที่ใช้ไฮโดรเจนเปอร์ออกไซด์ (0 0.05 0.1 1 และ 5 มิลลิโมลาร์) ต่อสมบัติของเจล พบว่าหลังจากไมโอซินเกิดการออกซิเดชั่น ปริมาณหมู่ซัลฟไฮดริลและกิจกรรมของ Ca^{2+}-ATPase ลดลง ขณะที่ปริมาณคาร์บอนิลและปริมาณหมู่ไฮโดรโฟบิกบริเวณพื้นผิว รวมถึงโมดูลัสสะสม (G') มีค่าเพิ่มขึ้น เอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์สามารถเหนี่ยวนำการเกิดพอลิเมอไรเซชั่นของไมโอซินและเพิ่มโมดูลัสสะสมของไมโอซินทั้งในสภาวะที่ไม่ผ่านและผ่านกระบวนการออกซิเดชั่น ในตัวอย่างเนื้อปลาบดที่ผ่านกระบวนการล้าง เจลมีความแข็งแรงเพิ่มขึ้นเมื่อเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ที่ระดับ 0.3 ยูนิตต่อกรัมโดยไม่คำนึงถึงสภาวะการออกซิเดชั่น อย่างไรก็ตามการจับรวมตัวของโปรตีนที่มากเกินพอดีในตัวอย่างที่ถูกออกซิไดซ์ก่อนกระบวนการเกิดเจลส่งผลให้ความแข็งแรงเจลและความสามารถในการอุ้มน้ำลดลง

ผลของการศึกษาการเตรียมและการปฏิบัติเบื้องต้นของเนื้อปลาบดต่อสมบัติเจลที่เติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ พบว่าการเตรียมโปรตีนไอโซเลตโดยใช้กรรมวิธีการละลายด้วยด่างมีปริมาณไมโอโกลบิน ลิปิด กิจกรรมของ Ca^{2+}-ATPase และโปรตีนที่ละลายได้ลดลง เมื่อเปรียบเทียบกับตัวอย่างจากเนื้อปลาบดที่ไม่ผ่านการล้างและซูริมิ ($P < 0.05$) เมื่อเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ (0-0.6 ยูนิตต่อกรัม) พบว่าค่าแรงก่อนเจาะทะลุระยะทางก่อนเจาะทะลุและความสามารถในการอุ้มน้ำของเจลส่วนใหญ่เพิ่มขึ้นเมื่อปริมาณของเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ที่เติมเพิ่มขึ้น ($P < 0.05$) ที่ระดับเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์เดียวกัน เจลของโปรตีนไอโซเลตที่เตรียมโดยการล้างเบื้องต้นให้ค่าแรงก่อนเจาะทะลุและระยะทางก่อนเจาะทะลุสูงสุด ($P < 0.05$) การเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ไม่ส่งผลต่อการเปลี่ยนแปลงความขาวของเจล ($P > 0.05$) ยกเว้นเจลจากโปรตีนไอโซเลตที่คล้ำขึ้นเมื่อเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ที่ระดับความเข้มข้นสูง โครงข่ายของเจลมีความหนาแน่นขึ้นเล็กน้อยเมื่อเติมเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์

ดังนั้นปัจจัยทั้งจากภายในและภายนอก รวมถึงชนิดของปลา สารประกอบภายในเนื้อปลา การเก็บรักษาภายหลังการตาย กระบวนการปฏิบัติเบื้องต้นและการแปรรูปเนื้อปลา มีผลกระทบต่อความว่องไวในการทำปฏิกิริยาของเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ รวมทั้งต่อคุณสมบัติทางเคมีกายภาพของโปรตีนกล้ามเนื้อที่เกี่ยวข้องกับการเข้าถึงสารตั้งต้นของเอนไซม์ โดยปัจจัยดังกล่าวมีบทบาทสำคัญโดยตรงต่อการเชื่อมประสานของโปรตีนที่เหนี่ยวนำด้วยเอนไซม์ทรานส์กลูตามิเนสจากจุลินทรีย์ รวมถึงสมบัติของเจลของซูริมิจากปลาชนิดต่างๆ

Thesis Title	Impact of Endogenous Compounds and Oxidation on Microbial Transglutaminase-induced Gelation of Muscle Protein from Different Fish
Author	Ms. Sochaya Chanarat
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ABSTRACT

Gel strengthening effect of microbial transglutaminase (MTGase) on fish muscle proteins as influenced by several factors was investigated. When MTGase at different levels (0-0.6 units/g) was added into surimi from three fish species including threadfin bream, Indian mackerel and sardine in the presence and absence of EDTA (10 mmol /kg), gels from threadfin bream which had the highest ε-amino group content showed the highest gel strength, followed by those from Indian mackerel and sardine, respectively. Breaking force of gels from all surimi generally increased as MTGase levels increased. In the presence of EDTA, gel strengthening effect was still achieved with the lower impact, suggesting the combined effect of endogenous transglutaminase with MTGase on gel strengthening.

Different nitrogenous compounds, commonly found in fish muscle, were determined for their effect on properties of surimi gels incorporated with MTGase. When whitecheek shark (*Carcharhinus dussumieri*) stored in ice for 12 days, total volatile base (TVB), trimethylamine (TMA) and ammonia contents increased, whereas urea content decreased during the storage ($P < 0.05$). Nevertheless, TVB, TMA, urea and ammonia contents were lower in washed mince, indicating the removal of those compounds by washing. The addition of MTGase (0.6 units/g) was

able to increase breaking force, deformation and water-holding capacity of both gels from mince and washed mince.

Formaldehyde content in lizardfish was determined and the impact of formaldehyde (FA) at various levels (0-9 mmol/g) on gel properties of surimi from lizardfish, a formaldehyde former, added with MTGase was studied. During iced storage of 10 days, total and free FA in lizardfish flesh increased continuously ($P < 0.05$). The incorporated of MTGase (0.4 units/g) was able to increase gel strength and water holding capacity of gel. Nevertheless, gel strengthening effect of MTGase was lowered when FA at higher level was present. The higher reduction in ε-amino group content was observed in natural actomyosin (NAM) when FA was incorporated. Acyl transfer reaction mediated by MTGase was impeded in NAM containing FA, especially at higher levels.

Additionally, impact of biogenic amines including putrescine, histamine and tyramine at various levels (0, 2 and 5 mmol/kg) on gel properties of surimi from Nile tilapia added with MTGase at 0.4 unit/g was studied. All biogenic amines were able to act as an acyl acceptor in MTGase catalysed reaction. The addition of biogenic amines into surimi has no impact on gel forming ability in the absence of MTGase. In the presence of MTGase (0.4 unit/g), gel strength and water holding capacity of all gels increased, compared to those of gels without MTGase. Nevertheless, gel strengthening effect of MTGase was slightly lowered when biogenic amine, especially at a level of 5 mmol/kg, was incorporated.

Protein oxidation of tilapia myosin induced by Fenton's reaction using H_2O_2 (0, 0.05, 0.1, 1 and 5 mM) on gelling property was also determined. After myosin being subjected to oxidation, sulfhydryl group content and Ca^{2+}-ATPase activity decreased, while carbonyl content and surface hydrophobicity along with storage modulus (G´) increased. MTGase was able to induce polymerisation of myosin in both nonoxidised and oxidised forms and increased G´. A stronger gel was observed in washed mince when 0.3 unit MTGase/g was added, regardless of oxidation process. However, an excessive protein aggregation of oxidised samples prior to gelation resulted in the reduction of gel strength and water-holding capacity.

Effect of mince preparation/pretreatment on properties of gel added with MTGase was investigated. For protein isolate prepared using alkaline solubilisation process, myoglobin content, lipid, Ca^{2+}-ATPase and protein solubility were decreased, compared to those of unwashed mince and surimi ($P < 0.05$). When MTGase (0-0.6 units/g) was incorporated, breaking force, deformation and water holding capacity of most gels increased as MTGase levels increased ($P < 0.05$). At the same MTGase level, gel from protein isolate with prewashing exhibited the highest breaking force and deformation ($P < 0.05$). No change in whiteness of gel was observed with the addition of MTGase ($P > 0.05$), except for protein isolates which were darker as MTGase at high level was added. Gels showed a slightly denser network when MTGase was incorporated.

Therefore, both intrinsic and extrinsic factors including fish species, endogenous compounds, post-mortem storage, pretreatment and processing of fish meat could affect the reactivity of MTGase as well as physicochemical property of muscle proteins associated with substrate availability for the enzyme. Those factors directly played an important role in protein cross-linking induced by MTGase as well as gel properties of resulting gel of surimi from various fish species.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Surimi is concentrated myofibrillar protein obtained from mechanically deboned fish flesh, which is washed with cold water (Park and Morrissey, 2000). Theoretically, any fish can be used to produce surimi but properties of surimi gel vary. Both intrinsic and extrinsic factors including species, freshness, endogenous enzyme, processing parameters, protein concentration, pH, ionic strength, and temperature determine gelling properties of surimi (Niwa, 1992; Shimizu *et al.*, 1992; Benjakul *et al.*, 2003a). Benjakul *et al.* (2005) reported that fish with extended storage drastically undergo deterioration, associated with enzymatic autolysis, oxidation, microbial growth, leading to the inferior surimi quality. In general, lean fish such as threadfin bream, bigeye snapper, croaker, etc. have been used for surimi production in Thailand. Due to insufficient amount of those lean fish as raw material, it is paramount for surimi producers to find profitable uses of by-catch fish species or abundant fish that would otherwise not be suitable to sell in other presentations, and thereby to supply their needs (Martin-Sanchez *et al.*, 2009). Dark-fleshed pelagic fish such as sardine and mackerel have gained attention for surimi production. Due to high content of dark muscle associated with the high content of lipid and myoglobin, it is of difficultly to obtain high quality surimi as evidenced by poor gel forming ability of those species (Chaijan *et al.*, 2004; Arfat and Benjakul, 2012). Surimi from elasmobranch (sharks, rays, and skates) has been also gained interest, since they have been considered as by-catch or underutilised (Turan and Sönmez, 2007). Due to their high content of non-nitrogenous components, especially urea and ammonia, it might affect the physicochemical properties and sensory characteristic, especially offensive odour in shark meat and products (Venugopal *et al.*, 2002). Some fish species such as cod or lizardfish have been known to be formaldehyde former, which can produce the formaldehyde as induced by trimethylamine oxide demethylase (Mizuguchi *et al.*, 2011b; Lanier *et al.*, 2013).

Transglutaminase (TGases, EC 2.3.2.13) is a transferase, which is able to catalyse the protein cross-linking by an acyl transfer reaction (Folk, 1980). The γ-carboxyamide group of glutamine residue in protein serves as an acyl donor, while the amino group of primary amines or ε-amino group of lysine residues acts as an acyl acceptor. The reaction results in the formation of ε-(γ-glutamyl) lysine isopeptide, providing the cross-linked proteins or peptides (Martins *et al.*, 2014; Folk and Finlayson, 1977). The enzyme can be found in various living tissues, such as microorganisms, vertebrates, invertebrates, and plants (Nozawa *et al.*, 2001; Kieliszek and Misiewicz, 2014). The reactions promoted by this enzyme create profound changes in the proteins in food matrices, leading to improved texture and stability (Gaspar and de Góes-Favoni, 2015). TGase plays an important role in cross-linking of fish muscle proteins pre-incubated at 25-40 °C, resulting in the improved gel quality (Benjakul *et al.*, 2003a). Microbial transglutaminase (MTGase) has been widely used to improve the textural property of food proteins due to the availability and effectiveness. (Zhu *et al.*, 2014; Yongsawatdigul *et al.*, 2002). Nevertheless, the efficacy in gel strengthening of surimi by MTGase varies with species containing different compositions. Also, the endogenous compounds (myoglobin, urea, ammonia, formaldehyde, biogenic amines, etc) in fish might affect gel characteristic mediated by MTGase. Those compounds might contribute to the lower transglutaminase activity or changes in physicochemical property of muscle protein associated with availability of transglutaminase's substrate (Gln and Lys). This might be a drawback for surimi industry to achieve the improvement of surimi gel quality by MTGase. Better understanding of gel enhancing effect of MTGase toward surimi from different fish species could lead to the maximised improvement of surimi gel quality. Intrinsic and extrinsic factors including fish species, endogenous compounds, handling and processing, affecting efficacy of MTGase in protein cross-linking should be elucidated. Therefore, the information gained can be of benefit for surimi industry to potentially use MTGase in surimi, especially those from species, which exhibit less reactivity toward cross-linking induced by MTGase.

1.2 Review of Literature

1.2.1 Chemical composition of fish

There are two main groups of fish, bony fish (teleosts) and cartilaginous fish (elasmobranchs). As the common names imply, the skeletons of teleosts are made of bone, while the elasmobranchs have cartilaginous skeletons.

The main constituents of fresh fish are water (65-80%), protein (15-24%), fat (0.1-22%), carbohydrate (1-3%) and inorganic substances (0.8-2%) (Suzuki, 1981). The composition of fish meat varies with species (Table 1). Age, part of body, pre- or post-spawning season and the food condition also affect the fish composition (Suzuki, 1981). The relative amounts of these components are generally within the range found in mammals (Mackie, 1994). Protein is a major composition of fish muscle, ranging from 15 to 20% (wet weight), but protein content is reduced in a spawning period (Almas, 1981). Protein compositions of fish vary depending upon muscle type, feeding period and spawning, etc. Apart from proteins, non-protein nitrogenous compounds such as TMA have a distinctive fishy, amine like odor, which directly affects the sensorial property of fish and fish products. TMA is derived from TMAO, which is abundant in marine fish (Cadwallader, 2000; Debevere and Boskou, 1996). Fat content varies between species, and also between different organs within species. Fish with fat content as low as 0.5% and as high as 18-20% are common. Dark muscle is rich in chromoproteins and contains about 2–5 times more lipids than the ordinary muscles. The white muscle of a typical lean fish contains less than 1% lipids. Based on these variations in fat content, fish are broadly classified as lean (fish that store lipids only to a limited extent) and fatty fishes (fish storing lipids in fat cells distributed in the body tissues) (Tocher, 2003). Sardine meat had high fat content (4.77 %, wet weight.) (Chaijan *et al.*, 2004). Also, dark fleshed fish contained a large amount of pigment protein. Myoglobin content in sardine was 14.27 mg/g (Chaijan *et al,* 2004).

For elasmobranch meat, protein content ranged from 15 to 26% (Kailasapathy and Salampessy, 1999). Shark had a high content of non-protein

nitrogenous compounds, mainly urea, which accounted for more than 25% of total nitrogen in fresh meat (Mathew and Shamasundar, 2002). Urea content in shark meat varies with species. According to Watabe *et al.* (1983), urea in shark meat was in the range of 200–500 mmol/kg. Ray meat also contained high amount of urea (130–200 mmol/kg), contributing to about 47% of non-nitrogenous protein (Kelly and Yancey, 1999).

Table 1. Composition of fish meat

Fish species	Average amounts (%)		
	Moisture	Crude fat	Crude protein
Teleost			
Anchovy	74.4	6.0	17.5
Round herring	71.9	4.6	21.3
Frigate mackerel	62.5	16.5	19.8
Carp	75.4	6.0	18.0
Blak sea bream	75.7	1.7	21.2
Bigeye snapper	78.6	1.3	18.6
Lizardfish	78.7	1.1	18.1
Elasmobranch			
Angel shark (*Squatina* spp.)	75.9	0.1	24.7
Blacktip shark (*Carcharhinus limbatus*)	78.0	0.8	19.7
Catshark (*Scyliorhinus* spp.)	77.7	0.6	20.5
Smooth-hounds (*Mustelus* spp.)	79.6	0.7	18.4
Liveroil shark (*Galeorhinus* spp.)	77.7	0.8	20.4

Source: Adapted from Suzuki (1981); Karakoltsidis *et al.*, (1995); Schubring *et al.,* (2007)

Fish muscle protein can be divided into three major groups on the basis of solubility characteristics including sarcoplasmic protein (water soluble), myofibrillar protein (salt soluble) and stroma protein (insoluble) (Xiong, 1997).

1.2.1.1 Sarcoplasmic proteins

Sarcoplasmic proteins are located inside the sarcolemma and are soluble in low salt concentrations (< 0.1 M KCl). Sarcoplasmic proteins comprise about 30-35% of the total muscle proteins (Xiong, 1997), including myoglobin, enzymes and other albumin (Sikorski, 1994). Sarcoplasmic enzymes influencing the quality of fish include the enzymes of the glycolytic pathway and the hydrolytic

enzymes of lysosomes (Sikorski, 1994). Sarcoplasmic proteins have an adverse effect on the strength and deformability of myofibril protein gels via an interference with myosin cross-linking during gel matrix formation (Sikorski, 1994). However, sarcoplasmic protein also contained TGase, which plays a role in setting of surimi**.** TGase from wash water was recovered from threadfin bream surimi washing process and exhibited protein cross-linking activity, thereby improving surimi gel property (Piyadhammaviboon and Yongsawatdigul, 2009)

1.2.1.2 Myofibrillar proteins

The major proteins in fish muscle are myofibrillar proteins. These proteins account for 65-75% of total proteins in muscle, compared with 52-56% in mammals (Mackie, 1994). The myofibrillar proteins are also mainly responsible for the water holding capacity of fish muscle, for the textural development of fish product, as well as for the functional properties of fish minces and homogenate (Sikorski, 1994; Zayas, 1997). Myofibrillar proteins undergo changes during the rigor mortis and extended frozen storage (Shahidi, 1994). The texture of fish products and the gel-forming ability of fish minces and surimi may also be affected by the changes of myofibrillar proteins (Shahidi, 1994). Myofibrillar proteins are soluble in solution of neutral salts with ionic strength less than 0.5 and are often called the 'salt soluble protein'. Kolakowski *et al.* (1977) recommended that NaCl solution at 2.5-3.0% could be used for extraction of myofibrillar proteins. Myofibrillar proteins can be further divided into two subgroups as follows:

1.2.1.2.1 Contractile proteins

A) Myosin

Myosin is a large fibrous protein with a molecular weight of about 500,000 daltons (Ogawa *et al.*, 1994). It is the most abundant myofibrillar component, constituting approximately 40-60% of total proteins content (Bechtel, 1986). Myosin consists of six polypeptide subunits, two large heavy chains and four light chains arranged into an asymmetrical molecule with two pear-shape globular heads attached to a long α-helical rod-like tail (Xiong, 1997). The two globular heads with ATPase

activity are relatively hydrophobic and are able to bind actin. The rod portion is relatively hydrophilic and is responsible for the assembly of myosin into thick filaments. Cleavage of myosin using trypsin at the hinge region yields two fragments called heavy meromyosin or HMM (head portion) and light meromyosin or LMM (tail portion), respectively. HMM retains all enzymes activity and actin-binding ability. Treatment of HMM with papain results in the formation of two additional fragments termed S-1 (globular head) and S-2 (rod portion) (Xiong, 1997).

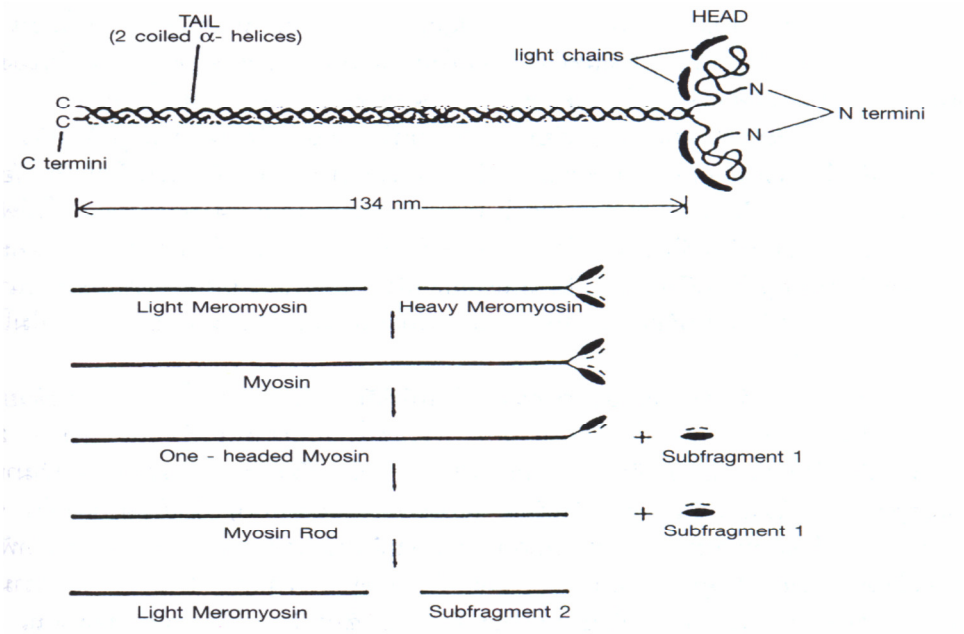


Figure 1. Structure of myosin.
Source: McCormick (1994)

B) Actin

Actin is the second most abundant myofibrillar protein, constituting about 22% of the myofibrillar mass. Actin can exist either as monomer (G-actin) or in a fibrous form (F-actin). Normally, actin exists as double-helical filaments (F-actin) and is composed of polymerised globular monomers. Each monomer has a molecular weight of approximately 43,000 daltons (Xiong, 1997). In muscle tissue, actin is naturally associated with tropomyosin and troponin complex. It also contains a myosin-binding site, which allows myosin to form temporary complex with it during

muscle contraction or the permanent myosin-actin complex during rigor mortis development in postmortem meat (Sikorski, 1994).

C) Actomyosin

When actin and myosin are mixed *in vitro*, a complex, call actomysin, is formed. The complex is bound not by covalent, but by electrostatic bonding with the contribution of phosphate groups (Xiong, 1997). The reconstitute actomyosin produce from both component proteins demonstrates many biochemical and physicochemical features of myosin, however does not exhibit physicochemical and function features of F-actin (Kijowski, 2001). This complex can be dissociated by the addition of ATP or high ionic strengths (Xiong, 1997). Actomyosin is the main state of actin and myosin in postmortem muscle because ATP is depleted by postmortem metabolism (Ochai and Chow, 2000).

1.2.1.2.2 Regulatory proteins

Tropomyosin and troponin constituting 5% of total protein are the main proteins that play an important part in regulation of muscle contraction (Kijowski, 2001). Myofibrils also contain other regulatory protein at lower quantities. They are present in the myofibril filament structure, e.g. A-band, I band, Z-disc, namely, α-, β-, γ-actinin, C-, M-, H-, and X-protein paramyosin (Kijowski, 2001; Xiong, 1997).

A) Tropomyosin

Tropomyosin is a dimeric molecule consisting of two dissimilar subunits designated α- and β- tropomyosin with molecular weights of about 34,000 and 36,000 dalton, respectively. Each tropomyosin molecule is about 385 A° long and associates in head-to-tail fashion to form a filament that follows and associates with the coil of the F-actin filament (McCormick, 1994). Tropomyosin is about 5% of myofibrillar protein. Each tropomyosin molecule consists of 7 molecules of G-actins (Foegeding *et al.*, 1996).

B) Troponin

Troponin is an asymmetrical protein and consists of three subunits. Troponin T (molecular weight of 37,000 dalton), which is also bound to troponin subunits C and I, links the troponin molecule to the tropomyosin molecule in the I-band. Troponin C (molecular weight of 18,000 daltons) binds Ca^{2+} and confers Ca^{2+} sensitivity to the troponin- tropomyosin-actin complex. Troponin I (molecular weight of 23,000 daltons), the inhibitory subunit, binds tightly to troponin C and actin and only slightly to tropomyosin or troponin T (McCormick, 1994)

1.2.1.3 Stroma protein

The stroma is composed of connective tissue proteins, such as collagen and elastin, constitute approximately 3% of total protein content in teleosts and about 10% in elasmobranchs (Suzuki, 1981). The stroma is the residue after extraction of the sarcoplasmic and myofibrillar proteins. Generally, the stroma is insoluble in dilute solutions of hydrochloric acid or sodium hydroxide (Sikorski *et al.*, 1990). Collagen in fish muscle ranges from 1 to 12% of crude protein (Sikorski *et al.,* 1990). Stroma proteins are insoluble in neutral salt solution or in dilute acids or alkalis. Collagen is the major connective tissue protein in fish and is very similar to that present in mammals. However, the collagen in fish is much more thermolabile and contains fewer but more labile cross-links than collagen from warm-blooded vertebrates. Collagen or connective tissue is generally removed using a refiner to concentrate the myofibrillar protein (Park and Lin, 2005).

1.2.2 Post-mortem changes of fish

1.2.2.1 Autolysis

After capture, chemical and biological changes take place in dead fish due to enzymatic breakdown of fish components (FAO, 2005). Hansen *et al*. (1996) stated that autolytic enzymes reduced textural quality during early stages of deterioration but did not produce the characteristic spoilage off-odours and off-flavours. Autolytic degradation can limit shelf-life and product quality even with

relatively low levels of spoilage microorganisms (Ghaly *et al.*, 2010). One of the most important autolytic spoilage changes is the degradation of fish nucleotides (ATP-related compounds) (Haard, 2002; Huss, 1995). During the early stage of storage, nucleotides are degraded owing to endogenous enzymes in fish muscle, and ATP converts to hypoxanthine (Hx), associated with bitter fish flavours, finally through a series of reactions. The intermediates in this transition process include adenosine 5-diphosphate (ADP), adenosine 5-monophosphate (AMP), inosine 5-monophosphate (IMP) and inosine (HxR). As the degradation continues, Hx is converted to xanthine, uric acid and ring cleavage products by the developing spoilage microflora. In a large variety of fish, nucleotide degradation follows a well-defined process: ATP → ADP → AMP → IMP → HxR → Hx → uric acid (Haitula *et al.*, 1993; Song *et al.*, 2012). The rate of nucleotide degradation is usually expressed by the K value, which reflects the formation of Hx and HxR and the decrease in nucleotide levels (Saito *et al.*, 1959). The freshness of yellowfin tuna as measured by K value, is changed in different patterns depending on the storage temperatures (0-10 °C). The higher the temperature of storage, the faster decrease in freshness of yellowfin tuna was observed (Agustini, 2002). Song *et al.* (2012) reported that IMP decreased, while HxR and Hx increased during 10 days under chilled storage and 33 days under partial freezing storage of bream (*Megalobrama amblycephala*). Nucleotide degradation was delayed in bream under partial freezing storage, compared with that stored under chilled storage as indicated by K-value.

Proteolysis is a crucial process taken place in fish after death. Digestive enzymes such as proteinases in ungutted fish, result into a condition referred to as "belly-burst" (Haard, 2002). The digestive enzymes cause extensive autolysis, which results in meat softening, rupture of the belly wall and drain out of the blood water which contains both protein and oil (FAO, 1986). A number of proteolytic enzymes are found in muscle and viscera of the fish after catch (Shahidi and Janak Kamil, 2001). These enzymes contribute to post-mortem degradation in fish muscle and fish products during storage and processing. Several alterations can be contributed by proteolytic enzymes (Engvang and Nielsen, 2001). During improper storage of whole fish, proteolysis is responsible for degradation of proteins and muscle softening (Lin

and Park, 1996). Peptides and free amino acids can be produced as a result of autolysis of fish muscle proteins. During spoilage of fish meat, microbial growth and production of biogenic amines generally occur (Fraser and Sumar, 1998). Martinez and Gildberg (1988) reported that the rate of degradation by proteolytic enzymes was reduced when the fish was kept at 0 °C and a pH of 5. Garrder *et al.* (2012) reported that cathepsin B and L activities of Atlantic salmon muscles increased significantly during post-mortem storage by being super-chilled to a core temperature of −1.5 °C or directly chilled on ice. It was suggested that the principal cause of post-mortem degradation of fish muscle is due to the hydrolytic nature of cathepsins B and L.

1.2.2.2 Lipid and protein oxidation

Fish has a high risk of quality loss due to oxidation (Jeremiah, 2001; Medina *et al.*, 2009). Lipid oxidation in fish and fish-products leads to rancidity and off flavour development. Different substances from lipid oxidation have adverse effects to human health. (Ames *et al.*, 1993). Oxidation is the major cause for quality deterioration during processing and storage of muscle foods (Xiong *et al.*, 2000). Fish rich in polyunsaturated fatty acids (PUFA) is susceptible to peroxidation, resulting in restriction of storage and processing (Gray *et al.*, 1996). Furthermore, peroxidative products, particularly aldehydes, can react with specific amino acids to form carbonyls and protein aggregates, causing additional nutritional losses (Uchida and Stadtman, 1993; Lund *et al.*, 2011).

1.2.2.2.1 Lipid oxidation

Lipid oxidation is a major cause of deterioration for the pelagic fish species such as mackerel and herring with high oil/fat content (Fraser and Sumar, 1998). Fatty fish are very sensitive to oxidation because of their high dark muscle and fat contents. They rapidly lose their freshness and the functional properties. Lipids in fatty fish are rich in long chain PUFA of excellent nutritional value. However, they are very prone to oxidation. Myoglobin and oxidizing enzymes present in dark fish muscle are efficient pro-oxidants (Aydin and Gokoglu, 2014). Lipid oxidation induces

formation of an array of products which decrease the sensory quality of fish and fish products directly or indirectly (Eymand et al., 2005).

Lipid oxidation involves a three-stage free radical mechanism: initiation, propagation and termination (Frankel, 1984; Khayat and Schwall, 1983). Initiation involves the formation of lipid free radicals through catalysts such as heat, metal ions and irradiation. These free radicals react with oxygen to form peroxyl radicals. During propagation, the peroxyl radicals reacting with other lipid molecules to form hydroperoxides and a new free radical (Fraser and Sumar, 1998; Hultin, 1994). Termination occurs when these free radicals interact each other to form non-radical products.

Initiation: The autoxidation of lipid proceeds via typical free radical mechanisms. The process is initiated by abstraction of a hydrogen atom from the central carbon of the pentadiene structure found in most fatty acid acyl chains (LH) containing more than one double bond:

$$\text{-CH=CH-CH}_2\text{-CH=CH-} \longrightarrow \text{-CH=CH-CH-CH=CH-} + H^{\bullet}$$

The formation of lipid radical ($L^{\bullet}$) is usually mediated by trace metals, irradiation, light or heat. Also, the initiation of oxidation may take place by hydroperoxide (LOOH) decomposition, generating a highly reactive alkoxyl lipid radical ($LO^{\bullet}$) and a hydroxyl radical ($HO^{\bullet}$). Lipid hydroperoxides which exist in trace quantities prior to the oxidation can be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase (Nawar, 1996; Sae-leaw *et al.*, 2013). Due to resonance stabilisation of lipid radical ($L^{\bullet}$) species, the reaction sequence is usually accompanied by a shift in position of the double bonds, resulting in the formation of isomeric hydroperoxides that often contain conjugated diene groups (-CH=CH-CH=CH-) (Nawar, 1996). Conjugated diene shows a characteristic UV absorption at 232-234 nm (Nakayama *et al.*, 1994).

Propagation: In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain

reactions that consume oxygen and yield new free-radical species (peroxy radicals, $LOO^{\bullet}$). Lipid peroxy radicals initiate a chain reaction with other molecules (LH), resulting in the formation of lipid hydropeoxides (LOOH) and lipid free radicals ($L^{\bullet}$). This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available. Lipid hydroperoxide, the primary products of autoxidation, are odourless and tasteless (Jadhav *et al.*, 1996).

Termination: A free radical is any atom with unpaired electron in the outermost shell. Owing to the bonding-deficiency and structural unstability, the radicals therefore tend to react with others to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable non-radical compounds. The radicals can also be removed by reaction with an antioxidant (AH) whose resulting radical ($A^{\bullet}$) is much less reactive (Huss, 1995; Jadhav *et al.*, 1996).

Oxidation typically involves the reaction of oxygen with the double bonds of fatty acids. Therefore, fish lipids which consist of polyunsaturated fatty acids are highly susceptible to oxidation. Molecular oxygen needs to be activated in order to allow oxidation to occur. Transition metals are primary activators of molecular oxygen (Hultin, 1994). In fish, lipid oxidation can occur enzymatically or non-enzymatically. The enzymatic hydrolysis of fats by lipases is termed lipolysis (fat deterioration). During this process, lipases split the glycerides, forming free fatty acids which are responsible for: (a) common off flavour, frequently referred to as rancidity and (b) reducing the oil quality (Huis in't Veld, 1996). The lipolytic enzymes could either be endogenous of the food product or derived from psychrotrophic microorganisms (Huis in't Veld, 1996). The enzymes involved are the lipases present in the skin, blood and tissue. The main enzymes in fish lipid hydrolysis are triacyl lipase, phospholipase A2 and phospholipase B (Audley *et al.*, 1978; Yurkowski and Brockerhoff, 1965). Non-enzymatic oxidation is caused by hematin compounds (hemoglobin, myoglobin and cytochrome) catalysis producing hydroperoxides (Fraser and Sumar,1998). The fatty acids formed during hydrolysis of fish lipids interact with

sarcoplasmic and myofibrillar proteins causing denaturation (Anderson and Ravesi, 1969; King *et al.*, 1962). Undeland *et al*. (2005) reported that lipid oxidation can occur in fish muscle due to the highly pro-oxidative Hemoglobin (Hb), specifically if it is deoxygenated and/or oxidised. Lipoxygenase (LOX) is another enzyme causing the formation of highly reactive hydroperoxides. LOX catalyses the oxidation of of polyunsaturated fatty acids containing a cis–cis-1,4-pentadiene unit to the corresponding conjugated cis–trans-dienoic monohydroperoxides. In fish, LOX is generally found in skin of gill (Pan and Kuo, 2000). Unlike lipid autoxidation, oxidation reactions catalysed by LOX are characterised by the common features of enzyme catalysis: substrate and conformation specificity, optimal activity under optimal conditions (temperature and pH) and high susceptibility to heat treatment. The number and position of pentadiene groups in the polyunsaturated fatty acid molecule have the great impact on the end-products of the oxidation (Cadwallader, 2000; Hsieh and Kinsella, 1989).

Chung *et al*. (2013) studied the changes of oxidised lipid products from polyunsaturated fatty acids in the presence of H_2O_2 in medaka fish (*Oryzias latipes)*. Lipids may be oxidised by the action of LOX to produce 5(S)-, 8(S)-, 12(S)- and 15(S)-HETE from arachidonic acid, and resolving D1 from docosahexaenoic acid. All of these products were significantly elevated when exposed to H_2O_2. Levels of 5(S)- and 8(S)- hydroxyeicosatetraenoic acid (HETE) were also elevated with increasing H_2O_2 exposure time to H_2O_2 at 200 and 1000 μM.

The mechanisms of lipid oxidation in fish muscle is very complex, involving not only enzymic initiation systems but also oxidation initiated and promoted by other mechanisms such as photosensitised and haem protein catalysed oxidation (German *et al*., 1985; Richards and Hultin, 2002). The rapid oxidation of fish lipids can be attributed to the cooperative actions of these pro-oxidative systems in fish muscle. By rapid production of hydroperoxides postmortem, LOX may act as an important initiator to activate the process of lipid autoxidation. It is also responsible for the rapid generation of fresh fish odours in some fish species (Josephson *et al.*, 1984). Most volatile compounds (i.e. hexanal, cis-4-heptenal, 2,4-heptadienal and 2,4,7-decadienal) contribute to the characteristic painty, fishy and cod

liver oil-like odours in stored fish and fish oils. The oxidised haem proteins such as met-Hb and met-Mb are the most potent pro-oxidants and the major contributor to lipid oxidation in fish muscle. It was observed that the pro-oxidative activity of Hb remained longer than that of LOXs in herring and sardine (Medina *et al.*, 1999). This may be due to the instability of LOXs. The hydroperoxides generated during lipid oxidation may cause the oxidation or modification of sulphydryl groups of LOX, leading to the inactivation of LOXs at the later stage of oxidation. Similarly, rapid onset of lipid oxidation was found in minced carp model containing LOX, but it had lower PV and TBARS values at the later stage compared to Hb induced oxidation system (Fu *et al.*, 2009).

1.2.2.2.2 Protein oxidation

In fish muscle, proteins are in high concentrations and they are also close to free radical initiators exposing them to oxidative reactions (Eymard *et al.*, 2009). Proteins in muscle can be modified by reactive oxygen species (ROS) that include free radicals but also by non-radical species such as H_2O_2 and lipid hydroperoxides. These reactions can lead to radical formation, formation of amino acid derivatives, protein break down, and polymerisation and negatively impact the functional properties of the product such as its texture and gel forming capacity (Xiong, 2000). Muscle proteins are susceptible to oxidative reactions, in which myosin is the most sensitive, followed by troponin T (Martinaud *et al.*, 1997; Lund *et al.*, 2011). Protein oxidation is dependent on the structure of the protein and peptides. Random coiled proteins are more susceptible to oxidation than globular proteins (Dalsgaard *et al.*, 2007). It is therefore possible that the conformations of globular proteins were more protected against oxidation and consequently were oxidised to a lower extent (Eymard *et al.*, 2009).

For protein oxidation in muscle, the abstraction of a hydrogen atom by an ROS leads to the generation of a protein carbon-centered radical ($P^{\bullet}$), which is consecutively converted into a peroxyl radical ($POO^{\bullet}$) in the presence of oxygen, and an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another molecule.

Further reactions with $HO_2^{\bullet}$ lead to the generation of an alkoxyl radical ($PO^{\bullet}$) and its hydroxyl derivative (POH) (Lund *et al.*, 2011).

The nature of the protein oxidation products formed is highly dependent on the amino acids involved and how the oxidation process is initiated (Estévez *et al.*, 2011). Reaction of radicals with proteins and peptides in the presence of oxygen gives rise to alterations of both the backbone and of the amino acid side chains. These oxidative changes include the cleavage of peptide bonds, modification of amino acid side chains, and formation of covalent intermoleular cross-linked protein derivatives (Stadtman and Berlett, 1997). Some of the most general amino acid modifications are the formation of protein carbonyl groups and protein hydroperoxides, while cross-linking is mostly described as the formation of disulfide and dityrosine (Davies *et al.*, 1999; Lund *et al.*, 2011). The side chains of some particular amino acids such as arginine, lysine and proline are oxidised through metal-catalysed reactions into carbonyl residues while others such as cysteine or methionine are involved in cross-linking or yield sulfur-containing derivatives (Lund *et al.*, 2011). Eymand *et al*. (2009) reported that protein oxidation occured rapidly during the storage of horse mackerel mince, leading to the increase in carbonyl content and the loss of sulfhydryl group content upon storage time.

1.2.2.3 Microbial spoilage

Microbial growth is associated with fish spoilage, in which biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with unpleasant and unacceptable off-flavours are produced (Dalgaard *et al.*, 2006; Emborg *et al.*, 2005; Gram and Dalgaard, 2002) (Table 2). For unpreserved fish, spoilage is a result of Gram-negative, fermentative bacteria (such as *Vibrionaceae*), whereas psychrotolerant Gram-negative bacteria (such as *Pseudomonas* spp. and *Shewanella* spp.) tend to spoil chilled fish (Gram and Huss, 2000). It is, therefore, important to distinguish non spoilage microflora from spoilage bacteria as many of the bacteria present do not actually contribute to spoilage (Huss, 1995). Ólafsdóttir *et al*. (2006) reported the proliferation of specific spoilage organisms in haddock fillets stored at 0, 7 and 15 °C. *Photobacterium phosphoreum*

was predominant among spoilage bacteria. *Pseudomonas* spp. appeared responsible for sweet, fruity spoilage odors, while *Shewanella putrefaciens* has been identified as the specific spoilers of marine fish stored in chilled conditions and was responsible for the H_2S production (Li *et al.*, 2013). It is recognised that *S. putrefaciens* is largely responsible for the TMA formation and the amount has been applied as spoilage indicators of chilled fish and fish products (Baixas-Nogueras *et al.*, 2007; Cai *et al.*, 2014).

The major deteriorations in fish caused by microorganisms are shown as the follows:

A) Breakdown of amino acid and formation of primary amines

Deteriorative changes in fish are indicated by several physicochemical characteristics; among them is the production of biogenic amines due to microbial decarboxylation activity on free amino acids or by amination and transamination of aldehydes and ketones (Santos, 1996; Beljaars *et al.*, 1997; Zhai *et al.*, 2012). Biogenic amines including histamine, tyramine, tryptamine, putrescine, and cadaverine, are formed from free amino acids namely histidine, tyrosine, tryptophane, ornithine and lysine, respectively. Spermidine and spermine arise from putrescine (Zarei *et al.*, 2011). Putrescine, cadaverine, spermidine, and spermine have analiphatic structure; histamine, and tryptamine have a heterocyclic structure and tyramine and phenylethylamine have an aromatic structure (Mohamed *et al.*, 2009). Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscle tissue, bacteria may start to grow, resulting in the production of biogenic amines (Visciano *et al.*, 2012).

Factors affecting biogenic amines production are availability of free amino acids, the presence of microorganisms that can decarboxylase amino acids and favourable conditions for the growth of such microorganisms and production of decarboxylase enzyme (Zarei *et al.*, 2011). Storage temperature is the most important factor contributing to biogenic amines formation (Chong *et al.*, 2011). Changes in histamine, cadaverine and putrescine contents take place during storage of Indian

mackerel which were generally higher when the fish was stored at 10 and 23 °C, compared to that stored at 0 and 3 °C and can be an indicator of mishandled or spoilt fish (Zare *et al.*, 2013). Other parameters (i.e., pH, water activity, NaCl concentration, additives) may influence the variation of microorganisms and lead to the differences in biogenic amine content (Suzzi and Gardini, 2003). *Clostridium* spp. and *Enterobacteriaceae*, especially *Proteus morganii*, *Klebsiella pneumoniae* and *Hafina alvei* are the most important histamine producing bacteria (Taylor *et al.*, 1979; Paarup *et al.*, 2002). Kim *et al.* (2009) reported that biogenic amine contents in fish and shellfish increased significantly during the storage, especially at temperatures above 4 °C. *Enterobacter* spp., such as *Enterobacter aerogenes*, are responsible for histamine, putrescine and cadaverine production in fish.

B) Demethylation and reduction of TMAO

In gadoid fish, TMAOase can catalyse the decomposition of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) (Sotelo *et al.*, 1995a). This enzymatic activity has been considered as especially important for the quality of the gadoid species during storage, because the interaction of produced FA with muscle proteins is the principal cause of protein-structure deformation, loss in functionality, and hardening of fish tissue (Careche *et al.*, 1999; Sotelo *et al.*, 1995b). Depending on the source of TMAOase, the activity may be located in the soluble fraction or in the particulate matter of homogenates of organ or tissues (Sotelo and Rehbein, 2000). Rey-Mansilla *et al.* (2002) found that kidney and spleen of hake showed the highest activities of TMAOase while activities in liver, heart, bile and gallbladder were much lower. Moreover, TMAOase has been found in muscle of some fish species, such as red hake (Phillippy and Hultin, 1993) and walleye pollack (Kimura *et al.*, 2002). Additionally, TMAOase is found in lizardfish, where the TMAOase is mainly located in kidney (Leelapongwattana *et al.*, 2010; Benjakul *et al.*, 2004c). Leelapongwattana *et al.* (2010) reported that the addition of partially purified TMAOase from lizardfish kidney into haddock mince resulted in the increase in DMA and FA throughout the storage at -10 °C for 6 weeks. In addition, the loss of protein solubility increased as the result of FA formation, which was associated with the increased aggregation of proteins as evidenced by the increases in

final storage modulus (G′) and loss modulus (G″) (Leelapongwattana *et al.*, 2010). Mizuguchi *et al.* (2011a) reported that DMA was generated more abundantly in dark muscle during frozen storage of walleye pollock, Southern blue whiting, and hoki. The amount of non-haem iron in dark muscle, which catalyses TMAO degradation, was higher than that in ordinary muscle in each species (Mizuguchi *et al.*, 2011a). Moreover, dark muscle in all three fish species had a higher taurine content (known to accelerate DMA formation) than ordinary muscle, indicating that two candidate factors, namely non-haem iron and taurine, may accelerate DMA generation during frozen storage. (Mizuguchi *et al.*, 2011a).

Fish use TMAO as an osmoregulant to avoid dehydration in marine environments and tissue water logging in fresh water. Bacteria such as *Shewanella putrifaciens*, *Aeromonas* spp., psychrotolerant *Enterobacteriacceae*, *P. phosphoreum* and *Vibrio* spp. can obtain energy by reducing TMAO to TMA creating the ammonia-like off-flavours (Gram and Dalgaard, 2002). TMA has been used as spoilage indicator in fish during post-mortem storage. TMA is not produced in a significant amount during the early stage of refrigerated storage of fish, but it appears after 3 or 4 days, in which the rate of production parallels the bacterial proliferation pattern (Ozogul, 2010). *Aeromonas* spp., psychrotolerant *Enterobacteriaceae*, *P. phosphoreum*, *Shewanella putrefaciens*-like organisms and *Vibrio* spp. can all reduce TMAO to TMA (Gram and Dalgaard, 2002. Serio *et al.* (2014) reported that *S. baltica* and *S. putrefaciens*, isolated from tuna and swordfish upon storage showed rapid growth at 4 and 8 °C. The production of TMA and H_2S, amino acids decarboxylation and proteolytic activity were also found at refrigeration temperatures, therefore being potentially able to modify texture and sensory characteristics of finfish. Four strains of *S. baltica* species isolated from iced stored whiting (*Merlangius merlangus*) were able to grow aerobically from 4 to 30 °C and were also able to reduce TMAO at 25 °C. Two strains synthetised an ornithine decarboxylase, being the potential putrescine producers (Dehaut *et al.*, 2014).

C) Formation of ammonia

The formation of ammonia (NH_3) contributes to the fish spoilage off-odours resulting from bacterial degradation of urea in cartilaginous fishes autolysis (Huss, 1988). Ammonia is the major component in the total volatile nitrogen fraction which often used as a quality indicator for fresh fish as TVB (Ozogul, 2010). Spoilage microorganisms convert many nitrogenous compounds into off odour volatile bases. Non-protein nitrogenous compounds present in fish are good substrate for spoilage microorganisms. The free amino acid pool in the muscle of fish is readily utilised by deamination process (Tomiyasu and Zenitani, 1957). This result in the formation of ammonia which is the primary compound produced during decomposition of fish.

In addition, urea presented in elasmobranchs like shark, ray and skate can degrade to ammonia by bacterial action. The formation of large quantities of ammonia and TMA in shark flesh results in the conspicuous difference in spoilage between teleost and shark. Wood (1950) reported that the formation of ammonia in the spoilage shark might be partly due to *Corynebacterium*, *Micrococcus*, *Sarcina*, *Pseudomonas*, *Bacillus*, *Enterobacter*, *Mycoplana* and *Torula*. Kimata and Hata (1953) isolated the urea-splitting bacteria, *Achromobacter*, *Pseudomonas*, *Flavobacterium* and *Micrococcus*, which are considered to play an important role in the production of ammonia in shark muscles. Cultivable microflora of the skin of fresh and stored skate (*Raja Kenojei*) originating from Korea has been described. Numerous bacterial genera were present such as *Vibrionaceae*, *Photobacterim* spp. and *Pseudomonas* amongst other (Cho *et al.*, 2004). Reynisson *et al.* (2012) reported that upon the storage time of skate, *P. phosphoreum* and *Aliivibrio* became dominant in the flesh, *Pseudomonas* and *Oceanisphaera* in the skin. The microorganism in flesh was therefore characterised by organisms that were able to reduce TMAO and probably urea, whereas the skin was mostly dominated by aerobic bacteria such as *Pseudomonas* and *Oceanisphaera*. Both *Oceanisphaera* and *Pseudoalteromonas* had a strong urease activity and were present in high relative quantity in the beginning of the curing process. These species are therefore likely to be the main groups involved in urea hydrolysis and ammonia production during early storage but their numbers

decline as the storage time progresses, either owing to competition to other bacteria or intolerance to their own metabolite, ammonia.

Table 2. Bacterial spoilage compounds

Specific spoilage bacteria	Spoilage compounds
Shewanella putrifaciens	TMA, H_2S, CH_3SH, $(CH_3)_2S$, Hx
Photobacterium phosphoreum	TMA, Hx
Pseudomonas spp.	Ketones, aldehydes, esters, non -H_2S sulphides, NH_3
Vibrionacaea	TMA, H_2S
Aerobic spoilers	NH_3, acetic, butyric and propionic acid

Source: Church, 1998; Ghaly *et al.*, 2010

1.2.2.4 Changes of proteins

1.2.2.4.1 Degradation of proteins

Post mortem tenderisation is one of the most unfavourable quality changes in fish muscle. A proteolytic degradation of myofibrillar and connective tissue components is observed during the extended storage. Myofibrillar protein fraction in muscle of Monterey sardine was unstable during ice storage (Pacheco-Aguilar *et al.*, 2000). The participation of various proteases in autolytic processes of ice-stored fish depends on location of the enzymes in cytosol and/or factors affecting tissue compartmentisation, the presence of activators or inhibitors and the susceptibility of proteins responsible for muscle integrity to cleavage by the respective enzymes (Ladrat *et al.*, 2003). Among post-harvest changes, degradation of fish muscle caused by endogenous proteases is a primary cause of quality losses during cold storage or handling (Haard *et al.*, 1994). The decrease in the relative amount of myosin heavy chain (MHC) and a concomitant increase in the number low MW proteins was found in bigeye snapper during iced storage (Benjakul *et al.*, 2002). Proteases are able to hydrolyse the muscle proteins differently. An *et al.* (1994) reported that among the Pacific whiting muscle proteins, MHC was the most

extensively hydrolysed, followed by troponin-T, α- and β-tropomyosin. Microbial proteases may also be a potential cause of proteolytic degradation. Protease from *Pseudomonas marinoglutinosa* was reported to hydrolyse actomyosin at 0-2 °C and the optimal pH was above 7.0 (Venugopal *et al.*, 1983). Benjakul *et al.* (1997) showed that MHC of Pacific whiting muscle was hydrolysed continuously throughout iced storage. MHC decreased to 45% of the original content within 8 days, whereas no changes in actin were observed on SDS-PAGE. The initial steps in deterioration of raw fish during its storage on ice consist of hydrolytic reactions catalysed by endogenous enzymes, which produce nutrients that allow bacteria proliferation (Busconi *et al.*, 1989).

1.2.2.4.2 Denaturation of proteins

Denaturation of muscle proteins during postmortem storage is another phenomenon causing the changes of fish quality and the functional properties. Pacific whiting muscle protein underwent denaturation during iced storage (Benjakul *et al.*, 1997). ATPase (E.C.3.6.1.8, ATP pyrophosphohydrolase) is associated with the postmortem disappearance of ATP in fish muscle, leading to rigor mortis (Nambudiri and Gopakumar, 1992). Ca^{2+}-ATPase activity is a good indicator of the integrity of the myosin molecule (Roura and Crupkin, 1995). Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex in the presence of endogenous or exogenous Ca^{2+} ions, respectively. Mg^{2+}-EGTA-ATPase activity indicates the integrity of the tropomyosin-troponin complex (Ouali and Valin, 1981; Watabe *et al.*, 1989). Kamal *et al.* (1991) reported that myofibrillar ATPase activities of sardine ordinary and dark muscles decreased during extended iced storage of 10 days. During iced storage of Pacific whiting muscle, no changes in Ca^{2+}-ATPase, Mg^{2+},-Ca^{2+}-ATPase or Mg^{2+}-ATPase were observed, but Mg^{2+}-EGTA-ATPase activity gradually increased during iced storage (Benjakul *et al.*, 1997). Roura and Crupkin (1995) reported the enzymatic activities of myofibrils from pre and post-spawned hake during iced storage. Seki *et al.* (1979) found that EGTA-ATPase activity of the myofibrils from minced carp decreased rapidly, while Ca^{2+}-ATPase and Mg^{2+}-ATPase activities decreased gradually during iced storage with the exception that Mg^{2+}-ATPase activity in the presence of EGTA increased. The increase in this

ATPase activity was accompanied with a loss of Ca^{2+}-sensitivity of myofibrils during iced storage for 16 days. Reza *et al.* (2009) reported Ca^{2+}-ATPase activity of actomyosin isolated from silver jewfish decreased gradually with the increase in storage period. The loss of ATPase activity may be explained by the weaker interaction of myosin with actin. Some lysosomal proteases might be responsible for degradation of certain muscle proteins, thus decreasing myofibrillar ATPase activity during long time of storage (Reza *et al.*, 2009).

Ca^{2+}-sensitivity was reported to be a good indicator of Ca^{2+} regulation of myofibrillar proteins (Roura and Crupkin, 1995) and was dependent upon the affinity of the troponin molecule for Ca^{2+} ion (Ebashi and Endo, 1968). The decrease in both Ca^{2+}-binding capacity and Ca^{2+} sensitivity was shown to be caused by proteolysis (Tokiwa and Matsumiya, 1969). Additionally, oxidation of thiol groups of myosin was shown to reduce Ca^{2+}- sensitivity and modify actin-myosin interactions (Seki *et al.*, 1979). Therefore, loss of Ca^{2+}- sensitivity in myofibrils from pre-spawned fish could be related to an increment in proteinase activity, which selectively degrades myosin during gonadal maturity (Roura and Crupkin, 1995).

The denaturation of muscle proteins is caused by the oxidation of thiol (SH) groups of muscle. Hamada *et al.* (Hamada *et al.*, 1977) reported the oxidation of carp and rabbit actomyosin during 14 days of storage at 4 °C. The rabbit actomyosin had little changes in SH content and disulfide bond contents. On the other hand, carp actomyosin had the marked decreased in SH content with an increase in disulfide bond. Benjakul *et al.* (1997) found that total SH content of actomyosin increased slightly after 2 days of iced storage, followed by a gradual continued decrease up to 8 days. A decrease in total SH group was reported to be due to formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). Chan *et al.* (1995) reported that myosin contained 42 SH groups. Two types of SH groups on the myosin head portion (SH1 and SH2) have been reported to be involved in ATPase activities of myosin (Yamaguchi and Sekine, 1966). Another SH group (SH_a) was localised in the light meromyosin region of myosin molecule and was responsible for oxidation of MHC (myosin heavy chain) and its dimer formation

resulted in an increase in Mg^{2+}-EGTA-ATPase activity of carp actomyosin during iced storage (Sompongse *et al.*, 1996).

1.2.2.4.3 Cross-linking of proteins

During storage of fish, quality is lost due to deterioration of texture, flavour and colour, especially after long period of storage, and when poor freezing practices are employed or when the initial quality of fish is low (Matsumoto, 1980; Shenouda, 1980). The development of drier and firmer texture of fish muscle after frozen storage has been frequently attributed to protein denaturation (Bass *et al.*, 1997). Both formaldehyde (FA) and dimethylamine (DMA) are products of the enzyme-catalysed reaction, which has trimethylamine oxide (TMAO) as its substrate (Benjakul *et al.*, 2004c). TMAOase is located in the viscera (Benjakul *et al.*, 2003b) and is commonly found in certain gadoid species (Krueger and Fennema, 1989). The effect of FA on the loss of protein functionality was studied (Careche and Tejada, 1990; Tejada *et al.*, 2002). Formaldehyde is a reactive substance that can rapidly bind free amino groups of protein. One formaldehyde molecule is theoretically able to react with two amino groups to form cross-links between protein chains and thus reduce the solubility of the protein in aqueous salt solutions (Badii and Howell, 2002; Leelapongwattana *et al.*, 2005). Addition of formaldehyde resulted in changes in the secondary structure of cod myosin, causing the exposure of the hydrophobic aliphatic groups, eventually leading to the appearance of covalent cross-links (Careche and Li Chan, 1997).

1.2.3. Surimi

Surimi is minced fish, washed with water to remove strong flavouring compounds, pigments, and nonfunctional proteins and subsequently dewatered to reduce the moisture content to approximately that of intact fish muscle (Suzuki, 1981). When fish flesh is separated from bones and skin (usually mechanically), it is called “minced fish” According to Pigott and Tucker (1990), after the minced fish is water-washed to remove fat and water-soluble components, it becomes “raw surimi”. This raw surimi is a wet concentrate of myofibrillar proteins and possesses enhanced

gel-forming, water-holding, fat-binding, and other functional properties relative to minced fish (Okada, 1992). For frozen surimi, fresh surimi is blended with cryoprotectants to provide a better frozen shelf life (Lanier, 1992). It is an intermediate product, which is used for a variety of foods from the traditional kamaboko products of Japanese to the recent shellfish substitutes (Kumazawa *et al.*, 1995)

1.2.3.1 Gelation of surimi

1.2.3.1.1 Protein gelation

In surimi paste preparation, surimi (myofibrillar proteins) is comminuted with salt and water. Addition of salt is needed to solubilise and destabilise the native structure of myofibrillar proteins prior to thermal denaturation (Park and Lanier, 1990). Thermal unfolding of these proteins, especially of myosin and natural actomyosin (NAM), is important for gelation. Ziegler and Acton (1984) demonstrated the mechanisms of gel formation by muscle proteins. Thermal denaturation of NAM begins at 30-35 °C and native tropomyosin and troponin dissociated from F-actin. Subsequently, helical structure of F-actin underwent a single chain at approximately 38 °C. LC subunits started to dissociate from the globular head of MHC when temperature reached at 40-45 °C and conformational changes of globular head also occurred. Actin-myosin complex started to dissociate from each other at 45-50 °C and helical regions of MHC then unfolded to the random coil structure. The denaturation of G-actin occurred at temperature greater than 70 °C. Thermal denaturation mechanism of NAM from carp was also proposed by Sano *et al.* (1994). The solubilised NAM began to unfold at temperature about 30 °C and aggregation started at this temperuature. Aggregation of proteins was formed extensively around 40-60 °C. Myosin molecule dissociated from F-actin at temperature greater than 40 °C. Taguchi *et al.* (1987) reported that myosin gelation was initiated by interaction of HMM at 30-40 °C followed by thermal unfolding as well as interaction of LMM at 50 °C. Gill and Conway (1989) also reported that the tail portion of myosin participated in aggregation at 40-50 °C. In contrast, Sano *et al.* (1990) reported that gel formation of carp myosin was started to form through the

interaction of LMM at temperature about 30-45 °C. Chan *et al*., (1993) suggested that myosin initially aggregated through the interaction of S-2 at 30-40 °C. Further aggregation was from interaction of LMM at 40- 50 °C. The results of these studies indicated that the discrete regions of myosin subfragment play important role in gel network formation. The role of actin in gelation of myofibrillar proteins was also observed as synergistic effect. Gelation of fish protein generally involves denaturation and aggregation. Denaturation is a process, in which proteins undergo conformational changes, primarily unfolding without alteration of the amino acid sequence. Then, protein-protein interactions, known as association, aggregation and polymerisation, take place and a three-dimensional network can be formed. Normally, gel is formed when partially unfolded proteins developed uncoiled polypeptide segments that interact at specific points to form a three dimensional cross-linked network (Zayas, 1997) and capable of holding water. Table 3 gives a summary of changes, which may occur during the heat denaturation of natural actomyosin.

Table 3. Conformational change occurring during the thermal denaturation of natural actomyosin

Temperature (°C)	Protein (s) or segment involved	Description of events
30-35	Native tropomyosin	Thermal dissociation from the F-actin backbone
38	F-actin	Super helix dissociates into single chain
40-45	Myosin	Dissociated into light and heavy chains Head possibly some conformational change hinge helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

Source: Ziegler and Aton (1984)

1.2.3.1.2 Setting (Suwari)

A) Protein-protein interaction during setting

Setting is a phenomenon explaining the increased textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C for a specific period of time prior to cooking (Lanier *et al.*, 2013). A gel with higher rigidity and elasticity is obtained when setting is followed by cooking (Roussel and Cheftel, 1990). This phenomenon involves gel network formation of muscle proteins triggered by protein unfolding. Setting temperature can be varied, depending on fish species, and setting phenomenon is related to habitat temperature of fish species. The optimum temperature for setting among species may be determined by the heat stability of myosin (Morales *et al.*, 2001). Generally, setting can be carried out at low (0-4 °C), medium (25 °C), and high (40 °C) temperatures (Lanier, 1992). Low and medium temperatures are applied for setting of surimi from cold and temperate habitats. Setting of Alaska pollock surimi from Bering sea was achieved at 4-5 °C (Kim *et al.*, 1993). Hossain *et al.* (2011) reported that optimum setting temperature for Pacific whiting surimi was 30 °C which shown a fine and uniform network structure together with the highest polymerisation of myosin heavy chain (MHC) and the highest gel strength. High temperature at 40 °C has been applied for setting of surimi from tropical or warm water fish species such as Atlantic croaker, Mexican flounder, Northern kingfish, threadfin bream, bigeye snapper, barracuda, and white croaker (Lee and Park, 1998; Morales *et al.*, 2001; Yongsawatdigul *et al.*, 2002; Benjakul *et al.*, 2004a; Phu *et al.*, 2010). It has been documented that various types of protein–protein interactions contributed to textural changes during setting. However, setting can be varied with species (Table 4).

Disulfide bond formation was found to participate in setting. Chan *et al.* (1995) observed that disulfide bond of surimi from herring increased after setting at 10 °C for 24 h. Such increase resulted in polymerisation of MHC during setting. Gel network formation of myosin was also initiated by disulfide formation between myosin head (Samejima *et al.*, 1981).

Table 4. Suwari forming capacity of different fish species

Suwari forming capacity	Fish species
Easy	Sardine (*Sardinops melanosticta*) Anchovy (*Engraulis japonica*) Alaska pollack (*Theragra chalcogramma*) Lizardfish (*Saurida undosquamis*) Cutlass fish (*Trichiurus lepturus*) Mackerel (*Scomber japonicus*)
Normal	Sea bass (*Lateolabrax japonicus*) Skipjack (*Katsuwonus pelamis*) Cod (*Gadus morhua macrocephalus*) Bigeye tuna (*Parathunnus sibi*)
Difficult	Sharks Carp (*Cyprinus carpio*) Black marlin (*Makaira mazara*) Sharp-toothed eel (*Muraenesox cinereus*) Croaker (*Argyrosomus argentatus*) Squids (*Todarodes pacificus*) etc.

Source: Okada (1992)

Disulfide bond formation was noticed during setting of herring myosin at 40 °C (Gill *et al.*, 1992). The role of hydrophobic interactions on setting phenomenon at 35-40 °C in the presence of NaCl was also suggested by Chan *et al.* (1995). Sano *et al.* (1994) suggested that hydrophobic interactions of carp NAM occurred extensively at 30–50 °C. Nowsad *et al.* (1996) reported that hydrophobic interactions play an important role in gelation of fish proteins at high temperature. Thermal aggregation of cod myosin was also formed by hydrophobic interactions of the rod portion (Chan *et al.*, 1993). In addition, Gill and Conway (1989) reported that the tail region of cod myosin was involved in non-covalent interactions at about 40–50 °C. Thus, hydrophobic interactions would play a role in setting of fish proteins.

B) Protein cross-linking catalysed by endogenous TGase

Isopeptide bond catalysed by TGase has been reported to be the major force involving polymerisation of myosin in the setting (Kamath *et al.*, 1992). TGase is an enzyme that catalyses acyl transfer reaction between the γ-carboxyl amide group of glutamine residues within protein to the side chain of lysine, resulting in protein cross-linking. Tissue or endogenous TGases require Ca^{2+} for catalytic reaction (Folk,

1980). Cross-linking of MHC was also the Ca^{2+}-dependent reaction in the setting and such cross-links play a crucial role in gel strengthening (Kumazawa *et al.*, 1995). The effect of Ca^{2+} on setting at 25 °C (3 h) or 5 °C (20 h) in surimi from Pacific whiting and Alaska pollock was reported (Lee and Park, 1998). Addition of Ca^{2+} significantly improved textural properties of surimi from threadfin bream after setting at either low or high temperatures but breaking force value of gels set at 40 °C was higher than at 25 °C (Yongsawatdigul *et al.*, 2002). Tsukamasa *et al.* (1993) found that the isopeptide bond formation of sardine sol was found at temperature below 30 °C. An increase in gel strength was correlated with the amount of isopeptide bond. Setting phenomenon, gel strength, cross-linking of MHC, and the content of isopeptide bond increased with setting time (Kumazawa *et al.*, 1995). Takeda and Seki (1996) reported that complete suppression of myosin cross-linking of walleye pollock surimi gel was associated with the inhibition of endogenous TGase. These results were in agreement with the results from Kumazawa *et al.* (1995). They suggested that setting phenomenon was suppressed in the presence of TGase inhibitors, such as NH_4Cl and EDTA.

NAM and myosin are also the major components responsible for setting and their molecules were aligned into three-dimensional network of gel. Different fish species, perhaps different individuals within species, could vary in natural content of the endogenous TGase, possibly affected by habitat, feed, and physiological condition (Lanier *et al.*, 2013). Maruyama *et al.* (1995) found that rate of dimerisation of MHC from carp was slower than that from rainbow trout and atka mackerel. They also reported that glutamine residues within NAM from mackerel were more reactive than those from others (Maruyama *et al.*, 1995). Rate of MHC polymerisation from fish species were walleye pollock > rainbow trout > chum salmon > atka mackerel > white croaker > carp (Araki and Seki, 1993). Kishi *et al.* (1991) observed that the extent of monodansylcadaverine (MDC) incorporation appeared to be 2.4 folds higher in soluble NAM than that in insoluble NAM or myofibril at the identical conditions. They also suggested that salt soluble NAM exposed glutamine residues to be more reactive for TGase catalysed reaction. In addition, the reactivity of TGase on protein cross-linking was also varied by

conformational changes of the protein substrates. Ogawa *et al.* (1995) suggested that unfolding of myosin rod is the prerequisite for setting of fish proteins. Thus, type and conformation of proteins directly affect setting induced by TGase-catalysed reaction. Incorporation of MDC and monobiotincadaverine into HMM from rabbit using guinea pig liver TGase were also performed by Kunioka and Ando (1996). Incorporation sites were at S-2 near the HMM and LMM junction. In addition, dimerisation sites on carp MHC catalysed by indigenous TGase were also at S-2 when the reaction was studied under the condition similar to setting. The results from these studies suggested that the rod portion of myosin participated in cross-linking rather than S-1. Although MHC is the preferred substrate for TGase, its cross-linking is also depended on myosin subfragments. Therefore, conformation of each myosin subfragment would also affect TGase catalysed reaction and setting phenomenon. Due to the differences in the optimal temperature of TGase, it is necessary to take into account that setting temperature may vary among species. Benjakul *et al.* (2004a) studied the setting temperature, medium (25 °C) and high (40 °C), of several tropical fish species. Good quality gels were obtained, but longer times were required to increase the gel quality in the medium-temperature setting by endogenous TGase, whereas for high-temperature setting, an extended setting would decrease surimi gel strength. Moreover, the addition of TGase inhibitors, such as *N*-methylmaleimide (NEM), ammonium chloride and EDTA, resulted in a marked decrease in breaking force and deformation. These indicate that endogenous TGase played an essential role in setting at high temperature (40 °C), of surimi from tropical fish (Benjakul *et al.*, 2004b).

1.2.3.1.3 Gel weakening (modori)

Gel softening or gel weakening termed "modori" is a problem found in surimi, especially for some species. This is associated with degradation of muscle proteins caused by the endogenous heat-activated proteinase (An *et al.*, 1996; Benjakul *et al.*, 1997; Jiang *et al.*, 2000). Proteolytic activity in muscle is high at temperature above 50 °C and causes the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson *et al.*, 1992). Such proteolytic degradation of myofibrillar proteins has a detrimental effect on surimi quality, and substantially lowers the gel strength (Morrissey *et al.*, 1993). Proteinases associated with gel

weakening can be categorised into two major groups: cathepsin (Seymour *et al.*, 1994) and heat-stable alkaline proteinase (Wasson *et al.,* 1992). High level of cysteine proteinase activity mediated by cathepsin B, H, and L was found in Pacific whiting and arrowtooth flounder (An *et al.*, 1994; Wasson *et al.,* 1992), chum salmon and mackerel (Lee *et al.*, 1993). Softening of arrowtooth flounder gel is due to a cysteine proteinase that has maximum autolytic activity at 50-60 °C (Greene and Babbitt, 1990). When Pacific whiting muscle was incubated at 60 °C for 30 min before cooking at 90 °C, most of MHC was degraded; the resultant surimi gel did not have measurable gel strength (Morrissey *et al.*, 1993). Modori is attributed to proteolysis by heat stable alkaline proteinases (Lanier *et al.*, 1981; Kinoshita *et al.*, 1990). Benjakul *et al.* (2004a) reported that suwari gel from some tropical fish, prepared by setting at 25 °C showed lower degradation although the setting time increased up to 8 h, compared with setting at 40 °C. Kamath *et al.* (1992) found that proteolysis in croaker paste increased with increasing temperature of setting, especially in the temperature range of 40-50 °C. Proteolytic degradation of surimi gels is increased at temperatures above 50 °C with the rapid and severe degradation of myofibrillar proteins, particularly myosin (Jiang *et al.*, 2000). Klomklao *et al.* (2008) also reported the presence of proteinases in sardine (*Sardinop*s *melanosticus*). Cathepsins B and H are easily washed off during surimi processing, whereas cathepsin L remains in the muscle tissue (An *et al.*, 1994; Park and Morrissey, 2000). Cathepsin L has an optimum temperature of 55 °C and causes textural deterioration when the Pacific whiting surimi paste is slowly heated (An *et al.*, 1994). Benjakul *et al.* (2003b) compared the autolysis from two species of bigeye snapper and found that *P. macracanthus* had higher degradation of myosin heavy chain in both mince and washed mince than those from *P. tayenus*, especially when the incubation time was increased. Autolysis of washed mince from both species was inhibited by soybean trypsin inhibitor, suggesting that myofibril-associated proteases were serine proteases, while *P. tayenus* contained various proteases. The protease extracted from bigeye snapper muscle had an optimum pH and temperature of 8.5 and 60 ºC which was capable of hydrolysing MHC effectively (Benjakul *et al.*, 2003c).

1.2.3.2 Factors affecting gelling properties

In general, the characteristics of gel are governed by many factors including temperature (Sano *et al.*, 1994), heating rate (Yongsawatdigul and Park, 1999), pH (Shikha *et al.*, 2006) and type of actomyosin used (Lefevre *et al.*, 2007). Muscle protein gel network is influenced by a number of factors as shown in table 5.

Table 5. Factors influencing heat-induced gelation of muscle protein

Factors	Effects
Myosin	Myosin can form excellent gels.
Actin	F-actin cannot form gels; it becomes a curdy sol on heating. Combination of low levels of F-actin with myosin can enhance gel elasticity above that of myosin alone, but actin decreases gel elasticity at higher (6.1%) levels.
Types of muscles	White muscle generally forms stronger gels than red muscle.
Source of muscles	Gel forming ability of muscles from different species is complex, and is influenced by different processing conditions.
Protein concentration	A "minimal gelation concentration" or critical protein concentration (CPC) is needed to form a gel. A myofibrillar protein content of about 0.5% is sufficient to produce a heat-induced gel. Regardless of protein source, gel hardness increases with increasing protein concentration.
pH	Gelation properties of myofibrillar protein are strongly pH-dependent. At the isoelectric point (pI) of myofibrillar protein (pH 5.3), either only poor gels are formed or gel formation is inhibited. At pH 6, the optimum pH value for heat-induced gelation of myosin is reached. Myofibrillar protein can form a gel at lower pH without heating.
Ionic strength	It is generally accepted that a high concentration (2% to 3%) of sodium chloride is required to solubilise the muscle protein to form a good gel. The microstructure of myofibrillar gels is also affected by ionic strength. At low ionic strength (0.25 M KCl), fine-stranded gel structures were formed; at high ionic strength (0.6 M KCl) coarsely aggregated gel structures were formed. The fine-stranded structure had higher rigidity than the coarsely aggregated structure.

Table 5. Factors influencing heat-induced gelation of muscle protein (cont.)

Factors	Effects
Heating rate	A slow heating rate may allow more favourable protein-protein interactions to occur, producing a stronger, better-ordered 3-dimensional gel.
Fat content	Fat content has an impact on gelation properties of meat products, but the changes in gel hardness observed are inconsistent, due to different processing approaches.
Protein additives (egg white, whey protein, soy protein, blood globin, plasma, gluten hydrolysate)	Egg white, whey protein, soy protein, and sodium caseinate can enhance myofibrillar protein gel strength. However, egg white and whey protein weaken fish myofibrillar protein gels. Blood plasma and globin can increase or decrease myofibrillar protein gel strength, and this differs with different meat products.
Transglutaminase	Transglutaminase can significantly increase myofibrillar protein gel strength.

Source: Adapted from Sun and Holley (2011)

1.2.3.3 Factors affecting surimi quality

1.2.3.3.1 Fish species

Depending on the species used, the functional and compositional properties of the surimi vary. Chan *et al.* (1992) studied the cross-linking of muscle protein extracted from cod, herring, and silver hake. The differences in gel-forming abilities of 3 fish species were determined by the relative cross-linking abilities of the myosin helical tail or MHC. Cod and silver hake MHC molecules could be polymerised to form large protein aggregates, whereas herring MHC could not. Cod and silver hake MHC formed both small and large polymers ($n \geq 6$), but herring MHC only formed small polymers ($n \leq 3$). Muscle proteins from two species of bigeye snapper showed the different gelling property. *Priacanthus tayenus* protein exhibited the larger aggregation stabilised by hydrophobic interaction and disulfide bond than *P. macracanthus* (Benjakul *et al.*, 2001; Benjakul *et al.*, 2002). Surimi from tropical species had the different gel properties when set at medium and high temperature prior to heating (Benjakul *et al.*, 2003a; Benjakul *et al.*, 2004a). The gel-forming ability of dark muscle fish meat has been known to be lower than that of ordinary

muscle (Chen, 2002; Ochiai *et al.*, 2001). Chen (1995) found that gel-forming ability of silvertip, hammer-head and thresher shark increased with increasing NaCl concentration. The three species had different sensitivities to ionic strength. The highest NaCl-induced gel-forming ability of surimi was achieved at 0.5 M salt concentration for silvertip and thresher; at 1.0 M for hammer-head shark.

1.2.3.3.2 Freshness and handling

During handling, leakage of digestive enzymes into the muscle also results in subsequent hydrolysis of muscle proteins. Therefore, pretreatment of fish, including beheading and evisceration prior to handling, can be another means to retard the deterioration caused by proteolysis (Benjakul *et al.*, 2002). Surimi gel quality can be influenced by protein structure. Freshness of fish is primarily time/temperature-dependent. Generally, surimi produced from fish stored in ice for a longer time showed the decrease in gel-forming ability. Bigeye snapper and lizardfish kept in ice with extended storage time had the continuous decrease in breaking force and deformation (Benjakul *et al.*, 2002; Benjakul *et al.*, 2003d). The length of time that fish can be held in ice or refrigeration before processing varies, depending on the species. Time and temperature of the fish between capture and processing can be considered as the most important factors in the final surimi quality. Degradation of MHC was also affected by storage temperatures. Fish kept at 5 °C showed higher degradation than those stored at 0 °C, suggesting that ice water was more efficient than refrigeration in controlling proteolysis (Park and Morrissey, 2000). Cathepsins B and H contribute to the degradation occurring at low-temperature storage (An *et al.,* 1994). Consequently, to minimise proteolysis, fish should be processed promptly on landing or kept at 0 °C if holding is necessary. According to Hu *et al.* (2015), the short term storage of the carp fish in ice only slightly affected the activity of cathepsin L. As a result, the enzyme still retained high activity during 4 days of storage in ice, suggesting its strong potential hydrolysis of fish meat protein during the storage. However, the activity of the trypsin-like protease increased with the iced-storage time. This enzyme may have undergone activation during the short term storage in ice. The gel strength of carp surimi added with cathepsin L decreased significantly by 24.33%, compared to the control, while that of surimi gels added with cathepsin L together

with inhibitors, E-64 and leupeptin, increased by 13.7% and 21.6%, respectively. Cathepsin L could hydrolyse surimi protein and was proved to be one of the enzymes contributing to the modori phenomenon (Hu *et al.*, 2012).

The reduction of TMAO to TMA, dimethylamine (DMA) and formaldehyde (FA) is caused by endogenous enzymes in fish and exogenous enzymes produced by bacteria during fish spoilage. The produced FA has been suggested to cause a decrease in solubility and extractability of the myofibrillar proteins. This was resulted in the detrimental changes in texture and functional properties, including viscosity, emulsifying ability, and gel-forming ability (Abe *et al.*, 2003). In the presence of FA, the gels could become rigid, since FA can form cross-links between myofibrillar proteins (Martın-Sanchez *et al*, 2009). Leelapongwattana *et al*. (2005); Badii and Howell (2002) also reported that FA can rapidly bind to free amino groups of protein to form a cross-links between protein chains leading to the reduction of protein solubility in salt solution. Kongpun *et al*. (2001) reported that the addition of FA into dorab mince and washed mince caused the reduction in gel strength. Benjakul *et al.* (2003) reported that the loss in gel-forming ability of iced storage lizardfish was associated with the formation of FA. Oka and Wang (2011) reported that TMAOase in the lizardfish fillets stored at 5 °C acts sufficiently under oxygen-deficient storage, and the produced FA is rapidly combined with fish protein, causing the deterioration of the gel-forming ability.

Elasmobranchs including sharks, rays and skates contain urea and trimethylamine in the blood and tissues to maintain their osmotic balance. Urea is converted by bacteria to ammonia and lodges in the tissues. Improper handling causes a strong ammonia odor and taste. Due to the formation of urea and ammonia, the shelf-life of fresh products is limited to a few days. Shelf-life of 8–12 days was reported for spiny dogfish (*Squalus acanthias*) (Bilinski *et al.*, 1983; Ravesi *et al.*, 1985). Mathew and Shamasundar (2002) studied the effect of iced storage on physicochemical and functional properties of shark meat (*Scoliodon laticaudus*). The gel forming ability of the shark meat showed marginal decrease during 12 days of iced storage, despite decline in solubility of total proteins in high-ionic-strength

buffer. The increase in TVBN content during iced storage is mainly correlated to reduction in urea content (Mathew and Shamasundar, 2002).

1.2.4. Transglutaminase

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) catalyses an acyl transfer reaction between the γ-carboxyamide group of a peptide bound glutaminyl residue (acyl donors) and a variety of primary amines (acyl acceptors), including the amino group of lysine. In the absence of amine substrates, TGase catalyses the hydrolysis of the γ-carboxyamide group of the glutaminyl residue, resulting in deamidation (Figure 2). When the ε-amino group of a peptide-bound lysyl residue is the substrate, peptide chains are covalently connected through ε-(γ-glutamyl)lysine (GL) bonds. TGase is able to introduce covalent cross-links in proteinaceous systems by catalysing acyl transfer reactions between the γ-carboxyamide group of peptide or protein bound glutamine (acyl donor) and primary amines (acyl acceptor) including the ε-amino group of lysine residues (Yokoyama *et al.*, 2004).

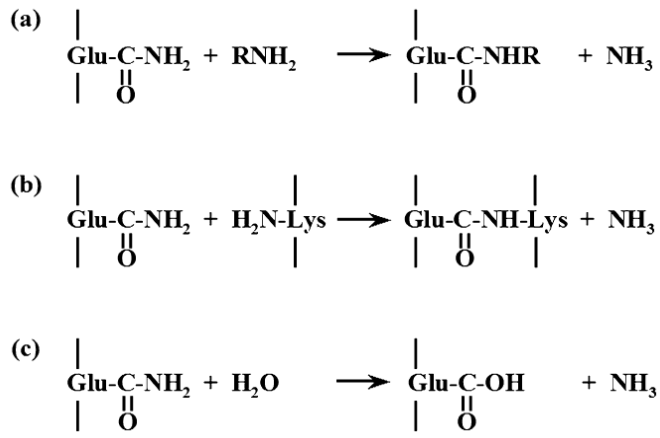


Figure 2. Reactions catalysed by TGase. (a) acyl transfer. (b) cross-linking of Gln and Lys residues in proteins or peptides. The resulting bridge is called an ε-(γ-glutamyl)lysine (GL) bond. (c) deamidation

Source: Yokoyama *et al.* (2004)

TGases are widely distributed in most animal tissues and body fluids, and are involved in several biological phenomena, such as blood clotting, wound healing, epidermal keratinisation, and stiffening of the erythrocyte membrane (Aeschlimann and Thomazy, 2000). Cross-linking of proteins, resulting in the formation of high molecular weight polymers, seems to be the most dominant reaction in nature for this enzyme (DeJong and Koppelman, 2002). 3D structure of substrate proteins are also important for increasing the reactivity of transglutaminase, because denatured proteins are usually better substrates than native ones (Beninati *et al.*, 2009).

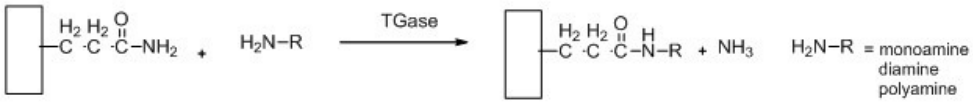
Reactions catalysed by various TGases have been demonstrated by Cooper and Kim (2007) (Figure 3). Protein and peptide substrates are depicted as rectangles and ovals, respectively. The carboxamide group of glutaminyl residue is the acyl donor. The acyl accptor maybe: (1) lysyl residue of protein or peptide; (2) an amine, diamine or polyamine; (3) water; (4) polyamine already attached to a glutaminyl residue; or (5) an ester. Quantitatively, the more important reactions are 1 and 2. Hydrolysis occurs only under conditions of limiting amine substrate. Esterification has only been shown to be important with TGase found in mammalian tissue which catalyses the esterification of carboxamide residue of skin protein involucrin with the ω-hydroxy group of an artificial ω-hydroxy ceramide (Nemes *et al.*, 2001).

1.2.4.1 Isoforms of transglutaminase

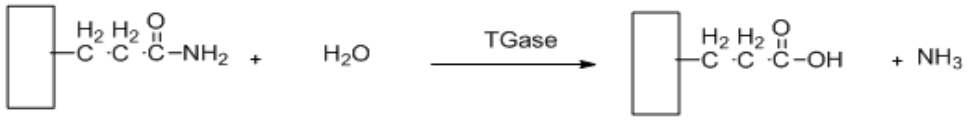
Transglutaminases (TGase) have been found in mammals (Folk and Chung, 1985), plants (Icekson and Apelbaum, 1987), fish (Yasueda *et al.*, 1994) and bacteria (Ando *et al.*, 1989). The physiological role of TGase isolated from these various sources has been investigated and appears to be diverse. TGase from plants are believed to be involved in the formation of cytoskeletal and cell wall structures (Serafini-Fracassini *et al.*, 1995), while the microbial TGase (MTGase) that is produced by sporulating cells of *Bacillus subtilus* is supposed to cross-link proteins during coat assembly (Kobayashi *et al.*, 1998).

1. **Cross-linking**

2. **Amine Incorporation**



3. **Hydrolysis**



4. **Polyamine Bridging**

5. **Esterification**

Figure 3. Reactions catalysed by various TGase
Source: Cooper and Kim (2007)

1.2.4.2 Microbial transglutaminase (MTGase)

1.2.4.2.1 Characteristic of MTGase

Microbial transglutaminase (MTGase) have been found in some microorganisms. Ando *et al.* (1989) isolated microorganism (*Streptoverticillium mobaraense*) that produced a MTGase, which did not require calcium ions for activity. MTGase is a monomeric enzyme consisting of a single polypeptide chain (Ando *et al.,* 1989). Compared to mammalian tissue TGase, also a monomeric enzyme, which has a molecular weight of 85,000 Da and pI of 4.5, MTGase has approximately half the molecular weight and has a substantially different pI of 8.9. However, even at pH 4 or

9, MTGase still expresses some enzymatic activity. MTGase is thus considered to be stable over a wide pH range. The optimum temperature for enzymatic activity was 50 °C, and MTGase fully sustained its activity even at 50 °C for 10 min.

Table 6 Isoforms of transglutaminase

Enzyme	Synonyms	Source for enzyme
Secretory transglutaminase	Dorsal prostate protein-1 (DP1)	Coagulating gland, dorsal prostate (guinea pig, rat)
Tissue Transglutaminase	Liver-, cytosolic-, endothelial-, erythrocyte transglutaminase, transglutaminase type II	Liver (guinea pig, rat), testis (rat), erythrocytes (human), A431 tumor cells (human)
Factor XIII	Plasma transglutaminase, fibrin stabilizing factor, Laki-Lorand factor	Platelets, placenta, plasma (human)
Keratinocyte transglutaminase	Particulate transglutaminase, transglutaminase type I	Liver (rat), chondrosarcroma tumor (rat),squamous carcinoma cell line (human)
Epidermal transglutaminase	Bovine snout-, hair follicle transglutaminase, transglutaminase type III	Cow snout, skin (human, guinea pig)
Hemocyte transglutaminase	Limulus transglutaminase	Limulus hemocytes
Microbial transglutaminase		Yeast and bacteria (eg. *Streptoverticillium* sp., *Bacillus subtilis*., *Streptoverticillium ladakanum*)

Source: Modified from Wilhelm *et al.* (1996)

On the other hand, it lost activity within a few minutes on heating at 70 °C. MTGase still shows activity at 10 °C, and still retains some activity at temperatures just above the freezing-point. Kanaji *et al.* (1993) determined the complete amino acid sequence of MTGase produced by S-8112, a variant of *Streptoverticilliurn mobaraense*. It comprised 331 amino acid residues with a single Cys residue at position 64 and had a molecular weight 37,863 Da. Protein sequencing by the automated Edman method and mass spectrometry also revealed that MTGase

consisted of 331 amino acid residues (Kanaji *et al.*, 1993). The results from cDNA sequencing of the gene from the producing microorganism coincided well with the result of protein sequencing. It further suggested that MTGase possibly has a signal peptide of 18 amino acid residues at its amino terminal (Washizu *et al.*, 1994). The overall sequence data indicate that MTGase has a single cysteine residue. The molecular weight calculated from the amino acid composition (331 residues) is 37,842, which is similar to the experimentally obtained value of 38,000. MTGase is, therefore, considered to be a monomeric and simple protein (not a glycoprotein or lipoprotein), although there are two potential glycosylation sites (-Thr-Xxx-Asn-) in the primary structure (Motoki and Seguro, 1998).

MTGase from a variant of *Streptoverticillium mobaraense* is totally independent of Ca^{2+}. In this aspect, MTGase is quite unique from other mammalian enzymes. Such a property is very useful in the modification of functional properties of food proteins, because many food proteins, such as milk caseins, soybean globulins and myosin, are susceptible to Ca^{2+}. They are easily precipitated in the presence of Ca^{2+} and become less sensitive to MTGase. The sensitivity of MTGase toward other cations in the absence of reducing agents has also been investigated (Seguro *et al.*, 1996): Cu^{2+}, Zn^{2+}, Pb^{2+} and Li^{+} significantly inhibited MTGase. Because heavy metals such as Cu^{2+}, Zn^{2+} and Pb^{2+} bind the thiol group of the single cysteine residue, this strongly supports the idea that the cysteine residue could be part of the active site of MTGase (Yokoyama *et al.*, 2004).

1.2.4.2.2 Structure and activity

Although the overall structures of TGases from various sources are widely different with respect to molar mass, amino acid sequence and conformation, the secondary structure arrangements around the active site are very similar (Kashiwagi *et al.*, 2002). Factor XIII-like TGases (FTGase) possess a catalytic triad similar to those of cysteine proteases, consisting of one cysteine^{272}, one histidine^{332} and one aspartic acid^{355} residue (Kashiwagi *et al.*, 2002). The active site cysteine^{272} is located near the N-terminus of an α-helix, which is flanked by a four stranded β-sheet, containing histidine^{332} and aspartic acid^{335}. In the MTGase molecule, cysteine^{64},

aspartic acid^{255} and histidine^{274} occupy the corresponding positions. Since the relative positions of histidine and aspartic acid seem to be interchanged, Kashiwagi *et al.* (2002) proposed a cysteine protease-like catalytic mechanism for MTGase in which aspartic acid^{255} plays the role of histidine in Factor XIII-like TGases.

Kashiwagi *et al.* (2002) proposed the mechanism for MTGase action as shown in Figure 4. Initially, the thiolate ion of cysteine^{64} nucleophilically attacks the side chain of the glutamine residue (acyl donor), where a binary complex between the glutamine donor protein and MTGase is formed (oxyanion intermediate). At this step, the acceptance of a substrate is determined by the conformation and the amino acid sequence adjacent to glutamine (Armbrust *et al.*, 2003). In steps 2 and 3, aspartic acid^{255} donates a proton to the oxyanion intermediate, one molecule of ammonium is released and an acyl/enzyme intermediate is formed. Thereafter, the negatively charged aspartic acid^{255} nucleophilically attacks a proton of the side chain of a lysine residue (acyl acceptor), which now reacts with the acyl/enzyme intermediate (step 4). In steps 5 and 6, the catalytic reaction is completed by releasing the product from the oxy anion intermediate. The histidine^{274} residue in the catalytic mechanism may have positive and negative effect. Nevertheless, the preferable conformation of the active site is retained by forming a hydrogen bond between histidine^{274} and aspartic acid^{255}. On the other hand, this hydrogen bond seems to decrease the nucleophilicity of aspartic acid^{255} in step 4, leading to a reduction of the catalytic efficiency. As the catalytic importance of histidine^{274} is reduced, Kashiwagi *et al.* (2002) suggested that histidine^{274} is not essential for the catalytic mechanism, because MTGase mutant, in which histidine^{274} was replaced by alanine, showed about half of the initial enzymatic activity.

1.2.4.2.3 Substrate specificities

TGase catalysed reacton is known to occur via a modified ping-pong mechanism (Folk, 1969) in which a glutamine-containing protein or peptide, the acyl-donor substrate, reacts with the enzyme's catalytic cysteine residue to form a thioester bond which generates the covalent acyl enzyme intermediate with concomitant release of ammonia. This intermediate then reacts with a second substrate, the acyl-acceptor,

which can be almost any primary amine (Aeschlimann and Paulsson, 1994), to yield the amide product and free enzyme. The acyl-enzyme intermediate can also be hydrolysed in the absence of primary amine but at a slower rate.

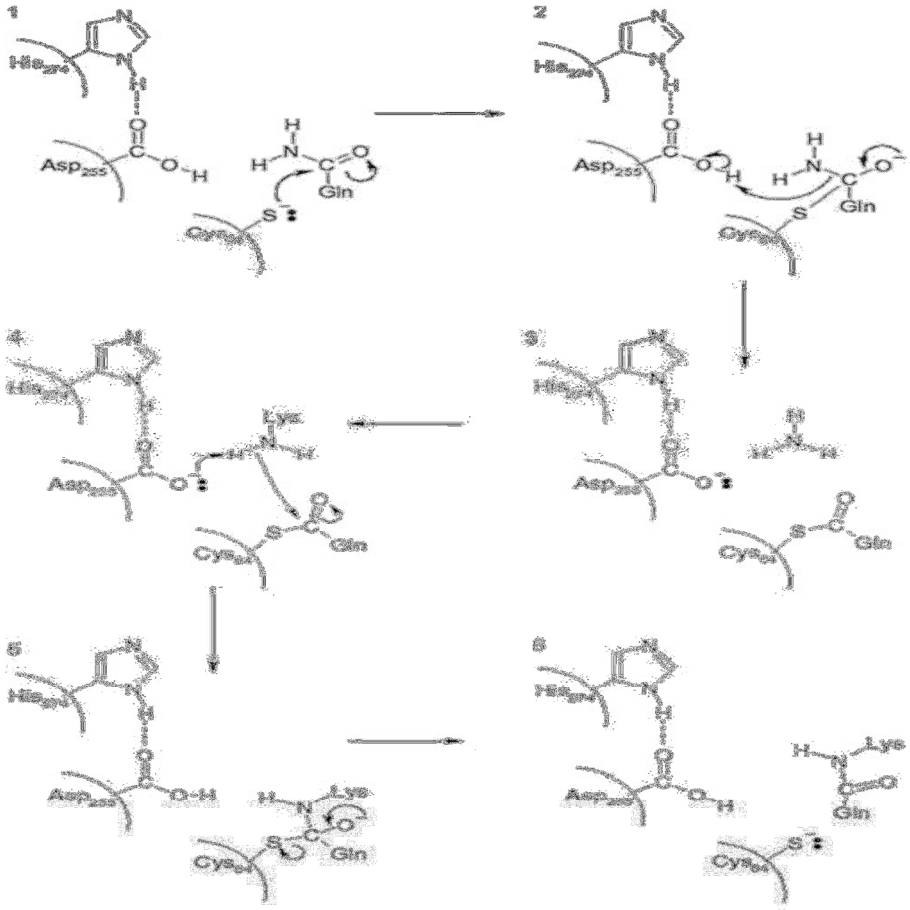


Figure 4. Proposed catalytic mechanism of the reaction of MTGase
Source: Kashiwagi *et al.* (2002)

A conserved Cys-His-Asp catalytic triad that is similar to that of cysteine proteases catalyses these reactions. Although TGases exhibit high specificity towards the side chain of L-Gln as the acyl-donor substrate (Asn and D-Gln are not recognised), their specificity towards the acyl-acceptor is lower, and many primary amines can be recognised (Clarke *et al.*, 1959; Folk, 1983). Both primary structure and local conformation around Gln involved in the TGase-catalysed reaction contribute to determine the enzyme specificity (Coussons *et al.*, 1992) for globular

proteins, the reactive Gln must be located in protein segments characterised by enhanced chain flexibility (Fontana *et al.*, 2008).

A) Acyl donor

Folk and Cole (1965) reported that the free glutamine (Q) cannot be catalysed by guinea pig liver TGase (GTG) and the C- and N- terminal side of Gln must be blocked. The Gln must be located at least in the third position from the N-terminus and at least the second position from the C- terminus. Ohtsuka, *et al.* (2000) suggested that TGases did not only recognise the L-Gln but also D-Gln. The factors governing specificity of TGase were proposed by Coussons *et al.* (1992). They reported that the minimal requirements for modification of Gln side chain in the proteins are: (i) The Q must be in the satisfied accessibility, either by being in a highly flexible region of polypeptide chain or by being clearly exposed to solvent in a more open structure. (ii) The feature surrounding Q must not be discouraged enzyme-substrate interactions. Ohtsuka *et al*. (2000) reported that substitution of C-terminal side of Q with phenylalanine (F) had a strong activation effect for GTG and FTG, while activity suppression was found in MTGase. In addition, substitution of that site by leucine (L) showed the similar results. Effect of amino acids surrounding reactive Q on TGase activity was investigated by Ohtsuka *et al*. (2000). Glycine (G) and alanine (A) were used to represent amino acids with small hydrophobic side chain, valine (V) and F as the bulky hydrophobic side chain, serine (S) and glutamic acid (E) as the small hydrophilic side chains, and Y and arginine (N) as bulky hydrophilic side chains. Replacement of large hydrophobic amino acid at the N-terminal side of Q accelerated the reactivity of MTGase, while that of the C-terminal side accelerated the reactivity of guinea pig liver TGase. These results suggested that enzyme-substrate interactions on both sides are important for optimum catalysis.

In addition to the primary sequence specificity surrounding the reactive glutamine residue, it has been suggested that a local secondary structure also plays an important role in determining whether a particular glutamine on the protein surface is a substrate of MTGase (Fontana *et al.*, 2008). Furthermore, it has been postulated that local unfolding and peptide chain flexibility improve reactivity (Spolaore *et al.*,

2012). MTGase specificity is governed by a combination of primary sequence, secondary structure, as well as flexibility around the glutamine, and accessibility of the glutamine to MTGase (Strop, 2014).

B) Acyl acceptor

TGases showed the broader substrate specificity toward primary amines, mainly natural compounds or their analogues, and they had no stereo specificity for lysine molecules (Ohtsuka *et al.*, 2000; Strop, 2014). TGase could use D-lysine as an acyl acceptor in the TGase-catalysed reaction but the reactivity toward L-lysine was higher than that of D-lysine. TGases required the primary amines with more than four carbon atoms and did not recognise the α-amino groups of amino acids. It speculated that the space around the amine-binding site is narrow and sensitive to the steric hindrance and amine containing negatively charged moieties. Grootjans *et al.*(1995) reported that G or D residues before the reactive lysine has the strongest adverse effects on substrate reactivity for TGase. The proline (P), H, and W residues are less favourable. The V, N, and W residues had an enhancing effect on the reactivity. The preferential feature of glutamyl substrates for guinea pig liver TGase on gliadin peptides was reported to be the consensus sequence Q-X-P (Mamone *et al.*, 2004), while other peptides or proteins showed different preferred sequences (Ruoppolo *et al.*, 2003). This consensus sequence was also catalysed slowly by MTGase (Piersma *et al.*, 2002). Gundersen *et al.* (2014) reported that long alkylamines can substitute for lysine as acyl-acceptor substrates, to link molecules of interest onto peptides or proteins. Also, the esterified α-amino acids Thr, Ser, Cys, and Trp have shown reactivity for MTGase.

Moreover, Punakivi *et al.* (2006) studied the uses of biogenic amine as MTGase substrate by having CBZ-Gln-Gly as a donor and found that cadaverine was the best acceptor substrate for MTGase and thus the highest concentration of ammonia was produced from cadaverine. With tyramine and histamine, the production of ammonia was about the same. The amino groups of biogenic amines can function as acceptor groups, and also other amines such as ethylenediamine, hexamethylenediamine, spermine, spermidine and 3,3′-iminobispropylamine have

been reported to function as acceptors. These polyamines function *in vitro* as acceptor substrates due to their protonated amine groups (Schrode and Folk, 1978; Folk, 1970). Ohtsuku *et al.* (2000) reported that the primary amines could be incorporated into Z-Gln-Gly. Those amines were required to have more than four carbon chains without side chains between the functional groups.

1.2.4.2.4 Application of MTGase

Cross-linking of proteins results in formation of dimers, trimers, and larger protein polymers. Without any interference, the cross-linking reaction will continue until no more glutamines or lysines are available to the enzyme. When incubation protein with TGase over longer periods, the formation of a protein polymer network will eventually limit the accessibility of the amino acids and the mobility of the TGase. However, only limited cross-linking is needed for optimal results, when TGase are applied for changes in protein functionality (Dejong and Koppleman, 2002). Because the ε-(γ-glutamyl)lysine (inter- and intramolecular) are covalent bonds, they are more stable than ionic or hydrophobic bonds. These connections, even in small amounts, can have a significant effect in food properties (Kuraishi et al. 2001). Functionality of the proteins can be drastically altered, causing dramatic changes in the size, organisation, stability, and other protein properties (Truong *et al*., 2004).

According to Gaspar *et al.* (2014), MTGase has the influence on some properties of foods as follow:

Gelation capacity: A protein that is initially unable to form gel by itself can form gel and become firmer. The use of MTGase, through the cross-links, enables highly elastic and irreversible gels to be obtained in different substrates, even at relatively low protein concentrations (Motoki and Kumazawa, 2000).

Emulsifacation and foam formation: The use of MTGase leads to the formation of high molecular weight peptides which are adsorbed on the surface of the oil droplets and promote electrostatic repulsion, preventing the approximation of these droplets. Thus, their flocculation, coalescence and phase separation can be prevented,

thereby increasing the stability of the emulsion (Agyare *et al.*, 2009; Babiker, 2000; Hong *et al.*, 2012). For foaming ability, MTGase increased the foaming capacity due to the rapid adsorption of peptides at the air-water interface at their isoelectric point, since electrostatic repulsion in proteins with neutral net charge is minimal. This improvement in peptide adsorption causes a rapid reduction in surface tension and increases the protein–protein interaction thus, improving the capacity to form foam, as well as its stabilisation. According to Renzetti *et al*. (2008), deamidation is the mechanism responsible for the increased adsorption of protein on the surface, since it causes a decrease in pH and an increase in polar groups that facilitate the unfolding of proteins on the water surface.

Viscosity: Viscosity of protein solution increases. proportionally to the increase in MTGase content. This polymerisation results in the formation of high molecular weight polymers that can reduce water mobility in the protein network, providing greater flow resistance and giving the product a suitable consistency. According to Bönisch *et al*. (2007), the higher the concentration of enzyme, the more numerous cross-links are formed, and hence, the greater the degree of polymerisation and viscosity in the end-product are achieved.

Water holding capacity: With appropriate concentrations, MTGase yields stable gels with higher porosity that are able to immobilise water more efficiently. The increase in WHC renders the better textural properties (Han *et al.*, 2009; Min and Green, 2008)

Most food proteins, such as legume globulins, wheat gluten, egg yolk and egg white proteins, actins, myosins, fibrins, milk caseins, α-lactalbumin and β-lactoglobulin, as well as many other albumins, could be cross-linked by MTGase (Kang *et al.*, 1994; Nonaka *et al.*, 1992; Seguro *et al.*, 1995)

A) Myofibrillar protein

The addition of MTGase could apparently induce a setting effect in the thermal gelation of carp actomyosin sol and markedly strengthened the two-step heated gel (Ni *et al.*, 1999). Hemung *et al.* (2008) studied the effect of MTGase and

fish TGase reactivity toward fish NAM from Pacific whiting and threadfin bream. NAM conformation and type of TGase were important for MHC cross-linking reactions. Based on Ca^{2+}-ATPase activity results, cross-linking sites by MTGase might not be located at the myosin head. Nakahara *et al.* (1999) compared the cross-linking of fish myofibrillar protein by endogenous TGase and MTGase. They reported that MTGase rapidly cross-linked fish MHC at different sites from those with TGase extracted from carp muscle. MTGase preferentially cross-linked connectin than MHC in soluble and aggregated states but could not cross-link actin molecule due to a lack of reactive lysyl residue. In addition to the polymerisation of myosin, MTGase produced highly polymerised forms of myofibrillar proteins, such as heteropolymers between connectin and actin. Huang *et al.* (1992) studied the cross-linking of contractile proteins from skeletal muscle by MTGase and found that myosin rod was quickly cross-linked, while S-1 was not. ATPase activity of myosin, HMM, and S-1 were not affected by the presence of MHC cross-linking. Nozawa and Ezou (2009) reported that the Gln residue that serves as the major amine incorporation site in both carp and walleye pollack myosins is the Gln residue at the 520^{th} position of the S-2 domain, and that it is a conserved part of the sequence that is critical for dimerisation.

B) Surimi

MTGase is used in surimi to increase the number of cross-links, thereby augmenting its functional properties (Motoki and Kumazawa, 2000; de Góes-Favoni and Bueno, 2014). Since MTGase is not calcium dependent; neither chelating agents nor calcium salt have any marked effect on its activity. MTGase and endogenous TGase in fish exhibited different substrate specificity and reactivity. MTGase catalyses the cross-linking reaction to a greater extent than endogenous TGase (Lanier *et al.*, 2000).

Addition of MTGase to surimi significantly increases its gel strength, particularly when the surimi has lower natural setting ability, presumably due to lower endogenous TGase activity (Kumazawa *et al.*, 1995; Lee and Park, 1998). An increase in non-disulfide polymerisation and formation of ε-(γ-glutamyl) lysine isopeptides was found with increasing setting time and MTGase concentration (Tsukamasa *et al.*,

1993). The effect of MTGase on breaking strength and deformation of gels from MTGase-treated surimi Alaska pollock with and without setting at 30 °C was studied by monitoring the formation of ε-(γ-glutamyl)lysine (GL) crosslink. In set gels, breaking strength and GL cross-link increased, and MHC decreased correspondingly with MTGase concentration. These changes were smaller in gels prepared without setting. Thus, surimi gel could be improved through the formation of GL crosslinks by added MTGase in surimi (Sakamoto *et al.*, 1995). Vácha *et al.* (2006) evaluated the influence of the addition of MTGase (0.5%) and NaCl (1%) to fish tissues and concluded that, regardless of the salt content, the enzyme increased the product's firmness by 52%. When MTGase was added together with NaCl, firmness was increased by 71% compared with the control (without the addition of salt and MTGase). Water-binding capacity was another property assessed by investigators, who found that the addition of MTGase combined with NaCl augmented this property from 53% to 64.4%. Benjakul *et al.* (2008) studied the effect of MTGase at different levels (0 to 0.8 units/g samples) on the properties of gels from lizardfish (*Saurida undosquamis*) mince set at 25 °C for 2 h or 40 °C for 30 min prior to heating at 90 °C for 20 min. MTGase showed the gel strengthening effect on lizardfish mince, particularly when high amounts of MTGase were used. For the gels added with MTGase at 0.8 units/g and set at 25 and 40 °C, the highest breaking force 93.1% and 90.7% was obtained, respectively. Karayannakidis *et al.* (2008) studied the effect of MTGase and Ca^{2+} ions on the textural characteristics of heat induced surimi gels from sardines (*Sardina pilchardus*). Incorporation of 2% MTGase (w/w) and 0.2 % Ca^{2+} ions (w/w) in surimi significantly affected the textural characteristics of heat-induced surimi gels. Fish gels with MTGase and $CaCl_2$ added were firmer and more cohesive, compared with the untreated gels. The former catalyses the cross-linking reaction of myosin, while the latter activates indigenous TGase, which also leads to the formation of covalent non-disulfide cross-links. However, MTGase-containing fish gels exhibited a more elastic texture, compared with the untreated fish pastes and those containing $CaCl_2$. Heat-induced gels prepared without any washing operation from the mince of sea bass trimmings with MTGase addition had a favourable effect on texture, gel strength and force at rupture were improved, and salt incorporation also improved texture and WHC. MTGase addition enabled to reduce salt content to 1.0%

(w/w), without significant loss of textural and overall quality, thereby producing a low salt fish product (Cardoso *et al.*, 2010). Kudre and Benjakul (2014) reported that the addition of MTGase at 0.6 units/g with bambara groundnut protein isolates in sardine surimi increased the breaking force, deformation and WHC of gels. MTGase could effectively induce the interaction between surimi and bambara groundnut proteins, thereby improving the gel properties. Zhu *et al* (2014) reported that a high pressure processing treatment at 25 °C in combination with MTGase increased gel strength and deformability with more dense and fibrous structure in Alaska pollack surimi, compared to those gels without MTGase addition.

C) Egg white

Egg white has been used in restructured meat due to the elevated crude protein concentration, the higher gel strength and its ability to stabilise batters. However, ovalbumin has been proved to be a poor substrate for MTGase because of its compact structure which limits the accessibility of MTGase to the target glutamine and lysine residues (Motoki and Nio, 1983; Dickinson and Yamamoto, 1996; Matsumura *et al.*, 1996). The susceptibility of these globular proteins to MTGase can be enhanced by partially unfolding the proteins using various techniques. Partial denaturation of egg white proteins by a preheating treatment at pH 10.5 could make them more susceptible to MTGase. Polymerisation reaction increased with increasing preheating temperature (from 60 to 80 °C) before the enzyme treatment. The egg white protein solutions exhibited near-zero storage moduli (G′, indicative of gel elasticity) during the onset of MTGase treatment but progressively increased with incubation time. In addition, the rate of development of the gel rigidity increased with increasing MTGase concentrations (Lim *et al.*, 1998). Giosafatto *et al.* (2012) reported that heat-treated ovalbumin was modified by MTGase to form both intramolecular and intermolecular crosslinks. The addition of MTGase reinforced the formation of covalent bonds with a well-developed viscoelastic gel network, with higher modulus and lower phase angle values, providing a better developed structure in the MTGase-induced gels.

D) Whey protein

Whey proteins (β-lactoglobulin and α-lactalbumin) have a globular structure. Thus they tend to have weaker cross-linking reactions with TGase, compared to other proteins, such as caseins (DeJong and Koppelman 2002; Jaros *et al.*, 2006). With respect to isolated milk proteins, it was shown that the cross-linking decreased in the order of sodium caseinate > ultrafiltered skim milk powder > skim milk powder > whey protein isolate (WPI) (Lorenzen, 2000). The globular whey proteins are hardly susceptible to MTGase, but the accessibility of β-lactoglobulin and α-lactalbumin to MTGase-catalysed reaction can be enhanced by various treatments to unfold protein (Coussons *et al.*, 1992; DeJong and Koppelman 2002; Lee *et al.,* 2002). β-Lactoglobulin is partially unfolded at pH 8.5–9.0; at this particular pH range, MTGase is still active, and the protein can be polymerised (Faergemand and Qvist, 1999). Truong *et al.* (2004) examined the impact of MTGase (10–25 units/g protein at 40 ºC for 0.5–8 h) on the rheological properties of heat-induced gels from WPI (4 and 8% (w/w)) containing 10 mM DTT at pH 7.5. Electrophoresis revealed large polymers after prolonged incubation time (>1 h), and significant higher gel point for 4% WPI solutions after 30 min of MTGase treatment, indicating increased heat stability upon extensive cross-linking. Further experiments using 8% WPI solutions and 10 units/g protein showed increasing apparent viscosity upon increasing incubation time. The gel point temperature of the untreated sample (67.6 ºC) was markedly increased to 78 and 94 ºC after 30 min and 4 h of incubation with MTGase, respectively, and led to significantly lower values of G´ of the gels.

1.2.4.2.5 Food Safety of MTGase

The Novel Food Regulation (EC) No. 258/97 and the Food Safety for Additive (89/107 EEC) of the European Commission, as well as, the U.S. Food and Drug Administration in the division of the Center for Food Safety and Applied Nutrition, Office of Food Additive Safety (2002) published the GRAS Notice No. GRN 000095 to designate MTGase from *Streptoverticillium mobaraense* produced by Ajinomoto as a substance generally recognised as safe.

1.3 Objectives

1.3.1 To investigate the impact of MTGase on protein cross-linking and gel forming ability of surimi from different fish species.

1.3.2 To elucidate the effect of ammonia and washing on the gel properties of shark mince added with MTGase

1.3.3 To study the effect of formaldehyde on protein cross-linking and gel forming ability of lizardfish surimi mediated by MTGase.

1.3.4 To investigate the impact of some biogenic amines on gel forming ability of Nile tilapia surimi containing MTGase

1.3.5 To study the effect of protein oxidation on protein cross-linking and gelling properties of Nile tilapia mince containing MTGase.

1.3.6 To comparatively investigate the effect of MTGase on the gel properties of protein isolates, mince and surimi obtained from conventional process from Indian mackerel.

1.4 References

Abe, E., Hayakawa, K., Kimura, M., Kimura, I., and Seki, N. 2003. Preventive effects of amino acids and glutathione on the formaldehyde-induced denaturation of myofibrillar proteins. Fish. Sci. 69: 605-614.

Aeschlimann, D., and Paulsson, M. 1994. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. Thromb. Haemost. 71: 402-415.

Aeschlimann, D., and Thomazy, V. 2000. Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. Connect. tissue Res. 41: 1-27.

Agustini, T. W. 2002. Freshness changes of yellowfin tuna (*Thunnus albacares*) during storage at low temperatures. J. Coast. Dev. 5: 143-149.

Agyare, K. K., Addo, K., and Xiong, Y. L. 2009. Emulsifying and foaming properties of transglutaminase-treated wheat gluten hydrolysate as influenced by pH, temperature and salt. Food Hydrocolloid. 23: 72-81.

Almas, K.A. 1981. Chemistry and microbiology of fish proceeding. Department of Biochemistry, Norwegian Institue of Technology, University of Trondheim. Norway.

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. P. Natl. Acad. Sci. USA. 90: 7915-7922.

An, H., Peters, M. Y., and Seymour, T. A. 1996. Roles of endogenous enzymes in surimi gelation. Trends. Food Sci. Technol. 7: 321-327.

An, H., Weerasinghe, V., Seymour, T. A., and Morrissey, M. T. 1994. Cathepsin degradation of Pacific whiting surimi proteins. J. Food Sci. 59: 1013-1017.

Anderson, M. L., and Ravesi, E. M. 1969. Reaction of free fatty acids with protein in cod muscle frozen and stored at -29 °C after aging in ice. J. Fish Res. Board Can. 26: 2727-2736.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613-2617.

Araki, H., and Seki, N. 1993. Comparison of reactivity of transglutaminase to various fish actomyosins. Nippon Suisan Gakkaishi. 59: 711-716.

Arfat, Y. A., and Benjakul, S. 2012. Gelling characteristics of surimi from yellow stripe trevally (*Selaroides leptolepis*). Int. Aqua. Res. 4: 1-13.

Armbrust, C., Werlein, H. D., and Watkinson, B. M. 2003. Transglutaminase-Application and Properties in Fish Products. Deut. Lebensm-Rundsch. 99: 181-187.

Audley, M., Shetty, K., and Kinsella, J. 1978. Isolation and properties of phospholipase A from pollock muscle. J. Food Sci. 43: 1771-1775.

Aydin, I., and Gokoglu, N. 2014. Effects of temperature and time of freezing on lipid oxidation in anchovy (*Engraulis encrasicholus*) during frozen storage. Eur. J. Lipid Sci. Technol. 116: 996-1001.

Babiker, E. E. 2000. Effect of transglutaminase treatment on the functional properties of native and chymotrypsin-digested soy protein. Food Chem. 70: 139-145.

Badii, F., and Howell, N. K. 2002. Changes in the texture and structure of cod and haddock fillets during frozen storage. Food Hydrocolloid. 16: 313-319.

Baixas-Nogueras, S., Bover-Cid, S., Veciana-Nogués, M. T., and Vidal-Carou, M. C. 2007. Effects of previous frozen storage on chemical, microbiological and sensory changes during chilled storage of Mediterranean hake (*Merluccius merluccius*) after thawing. Eur. Food Res. Technol. 226: 287-293.

Bass, E. C., Duncan, N. A., Hariharan, J. S., Dusick, J., Bueff, H. U., and Lotz, J. C. 1997. Frozen storage affects the compressive creep behavior of the porcine intervertebral disc. Spine. 22: 2867-2876.

Bechtel, J. P. 1986. Muscle development and contractile proteins. *In* Muscles as foods. (Bechtel, J. P. eds.). p. 1-35. Academic Press. London.

Beljaars, P. R., Van Dijk, R., Jonker, K. M., and Schout, L. J. 1997. Liquid chromatographic determination of histamine in fish, sauerkraut, and wine: interlaboratory study. J. AOAC Int. 81: 991-998.

Beninati, S., Bergamini, C. M., and Piacentini, M. 2009. An overview of the first 50 years of transglutaminase research. Amino Acids. 36: 591-598.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003a. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Leelapongwattana, K., and Visessanguan, W. 2003b. Comparative study on proteolysis of two species of bigeye snapper, *Priacanthus macracanthus* and *Priacanthus tayenus*. J. Sci. Food Agric. 83: 871-879.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Benjakul, S., Visessanguan, W., and Chantarasuwan, C. 2004a. Effect of high temperature setting on gelling characteristic of surimi from some tropical fish. Int. J. Food Sci. Technol. 39: 671-680.

Benjakul, S., Visessanguan, W., Ishizaki, S., and Tanaka, M. 2001. Differences in gelation characteristics of natural actomyosin from two species of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. J. Food Sci. 66: 1311-1318.

Benjakul, S., Visessanguan, W., and Leelapongwattana, K. 2003c. Purification and characterization of heat-stable alkaline proteinase from bigeye snapper (*Priacanthus macracanthus*) muscle. Comp. Biochem. Phys. B. 134: 579-591.

Benjakul, S., Visessanguan, W., and Pecharat, S. 2004b. Suwari gel properties as affected by transglutaminase activator and inhibitors. Food Chem. 85: 91-99.

Benjakul, S., Visessanguan, W., Riebroy, S., Ishizaki, S., and Tanaka, M. 2002. Gel forming properties of surimi produced from bigeye snapper, *Priacanthus tayenus* and *P. macracanthus*, stored in ice. J. Sci. Food Agric. 82: 1442-1451.

Benjakul, S., Visessanguan, W., and Tanaka, M. 2004c. Induced formation of dimethylamine and formaldehyde by lizardfish (*Saurida micropectoralis*) kidney trimethylamine-*N*-oxide demethylase. Food Chem. 84: 297-305.

Benjakul, S., Visessanguan, W., Thongkaew, C., and Tanaka, M. 2005. Effect of frozen storage on chemical and gel-forming properties of fish commonly used for surimi production in Thailand. Food Hydrocolloid. 19: 197-207.

Benjakul, S., Visessanguan, W., and Tueksuban, J. 2003d. Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during post-mortem storage in ice. Food Chem. 80: 535-544.

Bilinski, E., Jonas, R. E. E., and Peters, M. D. 1983. Factors controlling the deterioration of the spiny dogfish (*Squalus acanthias*) during iced storage. J. Food Sci. 48: 808-812.

Bönisch, M. P., Huss, M., Weitl, K., and Kulozik, U. 2007. Transglutaminase cross-linking of milk proteins and impact on yoghurt gel properties. Int. Dairy J. 17: 1360-1371.

Busconi, L., Folco, E. J., Martone, C. B., and Sμnchez, J. J. 1989. Postmortem changes in cytoskeletal elements of fish muscle. J. Food Biochem. 13: 443-451.

Cadwallader, K. R. 2000. Enzymes and flavor biogenesis in fish. *In* Seafood enzymes: utilization and influence on postharvest seafood quality. (Haard, N. F. Haard and Simpson, B. K. eds.). p. 365-383. Marcel Dekker. New York.

Cai, L., Wu, X., Li, X., Zhong, K., Li, Y., and Li, J. 2014. Effects of different freezing treatments on physicochemical responses and microbial characteristics of Japanese sea bass (*Lateolabrax japonicas*) fillets during refrigerated storage. LWT-Food Sci. Technol. 59: 122-129.

Cardoso, C., Mendes, R., Vaz-Pires, P., and Nunes, M. L. 2010. Effect of salt and MTGase on the production of high quality gels from farmed sea bass. J. Food Eng. 101: 98-105.

Careche, M., Herrero, A., Rodriguez-Casado, A., Del Mazo, M., and Carmona, P. 1999. Structural changes of hake (*Merluccius merluccius* L.) fillets: Effects of freezing and frozen storage. J. Agric. Food Chem. 47: 952-959.

Careche, M., and Li Chan, E. C. Y. 1997. Structural changes in cod myosin after modification with formaldehyde or frozen storage. J. Food Sci. 62: 717-723.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2004. Characteristics and gel properties of muscles from sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) caught in Thailand. Food Res. Int. 37: 1021-1030.

Chan, J. K., Gill, T. A., and Paulson, A. T. 1992. Cross linking of myosin heavy chains from cod, herring and silver hake during thermal setting. J. Food Sci. 57: 906-912.

Chan, J. K., Gill, T. A., and Paulson, A. T. 1993. Thermal aggregation of myosin subfragments from cod and herring. J. Food Sci. 58: 1057-1061.

Chan, J. K., Gill, T. O. M. A., Thompson, J. W., and Singer, D. S. 1995. Herring surimi during low temperature setting, physicochemical and textural properties. J. Food Sci. 60: 1248-1253.

Chen, H. H. 1995. Thermal stability and gel forming ability of shark muscle as related to ionic strength. J. Food Sci. 60: 1237-1240.

Chen, H. H. 2002. Decoloration and gel forming ability of horse mackerel mince by air flotation washing. J. Food Sci. 67: 2970-2975.

Cho, S. H., Jahncke, M. L., and Eun, J. B. 2004. Nutritional composition and microflora of the fresh and fermented skate (*Raja Kenojei*) skins. Int. J. Food Sci. Nutr. 55: 45-51.

Choi, Y. J., Lanier, T. C., and Park, J. W. 2005. Proteolytic enzymes and control in surimi. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. eds.). p. 227-277. CRC Press. Boca Raton, Florida.

Chong, C., Abu Bakar, F., Russly, A., Jamilah, B., and Mahyudin, N. 2011. The effects of food processing on biogenic amines formation. Int. Food Res. J. 18: 867-876.

Chung, M. L. S., Lee, K. Y. E., and Lee, C.-Y. J. 2013. Profiling of oxidized lipid products of marine fish under acute oxidative stress. Food Chem. Toxicol. 53: 205-213.

Church, N. 1998. MAP fish and crustacean-sensory enhancement. Food Sci. Technol. Today. 12: 73-83.

Clarke, D., Mycek, M., Neidle, A., and Waelsch, H. 1959. The incorporation of amines into protein. Arch. Biochem. Biophys. 79: 338-354.

Cooper, A. J. L., and Kim, S. Y. 2007. Transglutaminase. *In* Handbook of neurochemistry and molecular neurobiology: Neural protein metabolism and function (Lajtha, A. and Banik, N. L. eds.). p. 243-258. Springer Science and Business Media. New York.

Coussons, P. J., Price, N. C., Kelly, S. M., Smith, B., and Sawyer, L. 1992. Factors that govern the specificity of transglutaminase-catalysed modification of proteins and peptides. Biochem. J. 282: 929.

Dalgaard, P., Madsen, H., Samieian, N., and Emborg, J. 2006. Biogenic amine formation and microbial spoilage in chilled garfish (*Belone belone belone*)– effect of modified atmosphere packaging and previous frozen storage. J. Appl. Microbiol. 101: 80-95.

Dalsgaard, T. K., Otzen, D., Nielsen, J. H., and Larsen, L. B. 2007. Changes in structures of milk proteins upon photo-oxidation. J. Agric. Food Chem. 55: 10968-10976.

Davies, M. J., Fu, S., Wang, H., and Dean, R. T. 1999. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Rad. Biol. Med. 27: 1151-1163.

de Góes-Favoni, S. P., and Bueno, F. R. 2014. Microbial transglutaminase: general characteristics and performance in food processing technology. Food Biotechnol. 28: 1-24.

Debevere, J., and Boskou, G. 1996. Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillets. Int. J. Food Microbiol. 31: 221-229.

Dehaut, A., Midelet-Bourdin, G., Brisabois, A., and Duflos, G. 2014. Phenotypic and genotypic characterization of H_2S-positive and H_2S-negative strains of *Shewanella baltica* isolated from spoiled whiting (*Merlangius merlangus*). Lett. Appl. Microbiol. 59: 542-548.

DeJong, G. A. H., and Koppelman, S. J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

Dickinson, E., and Yamamoto, Y. 1996. Rheology of milk protein gels and protein-stabilized emulsion gels cross-linked with transglutaminase. J. Agric. Food Chem. 44: 1371-1377.

Ebashi, S., and Endo, M. 1968. Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. 18: 123-183.

Emborg, J., Laursen, B. G., and Dalgaard, P. 2005. Significant histamine formation in tuna (*Thunnus albacares*) at 2 °C—effect of vacuum-and modified atmosphere-packaging on psychrotolerant bacteria. Int. J. Food Microbiol. 101: 263-279.

Engvang, K., and Nielsen, H. 2001. Proteolysis in fresh and cold-smoked salmon during cold storage: Effects of storage time and smoking process. J. Food Biochem. 25: 379-395.

Estévez, M., Ventanas, S., Ganhão, R., and Morcuende, D. 2011. Oxidation. *In* Handbook of analysis of edible animal by-products. (Nollet, L. M. L. and Toldra, F. eds.). p. 65-83. CRC Press. Boca Raton, Florida.

Eymard, S., Baron, C. P., and Jacobsen, C. 2009. Oxidation of lipid and protein in horse mackerel (*Trachurus trachurus*) mince and washed minces during processing and storage. Food Chem. 114: 57-65.

Faergemand, M., and Qvist, K. B. 1999. On the importance of using Ca^{2+} independent transglutaminase for cross-linking of β-lactoglobulin. Food Hydrocolloid. 13: 199-201.

FAO 1986. The production of fish meal and oil. FAO fisheries technical paper. Fisheries Industries Division, Food and Agriculture Organization. Rome.

FAO. 2005. Post-harvest changes in fish. Topics Fact Sheets. Fisheries Industries Division, Food and Agriculture Organization. Rome.

Foegeding, E. A., Lanier, T. C., and Hultin, H. O. 1996. Characteristics of edible muscle tissues. *In* Food Chemistry (Fennema, O. R. eds.). p. 879-942. Marcel Dekker. New York.

Folk, J. 1969. Mechanism of action of guinea pig liver transglutaminase VI. Order of substrate addition. J. Biol. Chem. 244: 3707-3713.

Folk, J., and Cole, P. 1965. Structural requirements of specific substrates for guinea pig liver transglutaminase. J. Biol. Chem. 240: 2951-2960.

Folk, J. E. 1970. Transglutaminase. Method Enzymol. 17: 889-894.

Folk, J. E. 1980. Transglutaminases. Annu. Rev. Biochem. 49: 517-531.

Folk, J. E. 1983. Mechanism and basis for specificity of transglutaminase-catalyzed ε-(γ-glutamyl) lysine bond formation. *In* Advances in enzymology and related areas of molecular biology. (Meister, A. eds.). p. 1-56. John Wiley and Sons Inc. New Jersey.

Folk, J. E., and Chung, S. I. 1985. Transglutaminases. Methods Enzymol. 113: 358-375.

Folk, J. E., and Finlayson, J. S. 1977. The ε-(γ-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv. Protein Chem. 31: 1-133.

Fontana, A., Spolaore, B., Mero, A., and Veronese, F. M. 2008. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. Adv. Drug Delivery Rev. 60: 13-28.

Frankel, E. 1984. Chemistry of free radical and singlet oxidation of lipids. Prog. Lipid Res. 23: 197-221.

Fraser, O., and Sumar, S. 1998. Compositional changes and spoilage in fish-an introduction. Nutr. Food Sci. 98: 275-279.

Fu, X., Xu, S., and Wang, Z. 2009. Kinetics of lipid oxidation and off-odor formation in silver carp mince: The effect of lipoxygenase and hemoglobin. Food Res. Int. 42: 85-90.

Gaarder, M. Ø., Bahuaud, D., Veiseth-Kent, E., Mørkøre, T., and Thomassen, M. S. 2012. Relevance of calpain and calpastatin activity for texture in super-chilled and ice-stored Atlantic salmon (*Salmo salar L.*) fillets. Food Chem. 132: 9-17.

Gaspar, A. L. C., and de Góes-Favoni, S. P. 2015. Action of microbial transglutaminase (MTGase) in the modification of food proteins: a review. Food Chem. 171: 315-322.

German, J. B., Chen, S. E., and Kinsella, J. E. 1985. Lipid oxidation in fish tissue. Enzymic initiation via lipoxygenase. J. Agric. Food Chem. 33: 680-683.

Ghaly, A. E., Dave, D., Budge, S., and Brooks, M. 2010. Fish spoilage mechanisms and preservation techniques: review. Am. J. Appl. Sci. 7: 859.

Gill, T. A., Chan, J. K., Phonchareon, K. F., and Paulson, A. T. 1992. Effect of salt concentration and temperature on heat-induced aggregation and gelation of fish myosin. Food Res. Int. 25: 333-341.

Gill, T. A., and Conway, J. T. 1989. Thermal aggregation of cod (*Gadus morhua*) muscle proteins using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a zero length cross-linker (biological chemistry). Agric. Biol. Chem. 53: 2553-2562.

Giosafatto, C. V. L., Rigby, N. M., Wellner, N., Ridout, M., Husband, F., and Mackie, A. R. 2012. Microbial transglutaminase-mediated modification of ovalbumin. Food Hydrocolloid. 26: 261-267.

Gram, L., and Dalgaard, P. 2002. Fish spoilage bacteria–problems and solutions. Curr. Opin. Biotechnol. 13: 262-266.

Gram, L., and Huss, H. H. 2000. Fresh and processed fish and shellfish. *In* The microbiological safety and quality of foods (Lund, B. M., Baird-Parker, A. C. and Gould, G. W. eds.). p. 472-506. Chapman and Hall. London.

Gray, J., Gomaa, E., and Buckley, D. 1996. Oxidative quality and shelf life of meats. Meat Sci. 43: 111-123.

Greene, D. H., and Babbitt, J. K. 1990. Control of muscle softening and protease parasite Interactions in arrowtooth flounder (*Atheresthes stomias*). J. Food Sci. 55: 579-580.

Grootjans, J. J., Groenen, P. J., and de Jong, W. W. 1995. Substrate requirements for transglutaminases: Influence of the amino acid residue preceding the amine donor lysine in a native protein. J. Biol. Chem. 270: 22855-22858.

Haard, N. 2002. The role of enzymes in determining seafood color, flavor and texture. *In* Safety and quality issues in fish processing. (Bremner, H. A. ed). p. 220-253. CRC Press. Boca Rato, Florida.

Haard, N. F., Simpson, B. K., and Sikorski, Z. E. 1994. Biotechnological applications of seafood proteins and other nitrogenous compounds. *In* Seafood Proteins. (Sikorski, Z. E., Pan, B. S., and Shahidi, F. eds.). p. 194-216. Chapman and Hall. New York.

Haitula, T., Kiesvaara, M., and Moran, M. 1993. Freshness evaluation in European whitefish (*Coregonus wartmanni*) during chill storage. J. Food Sci. 58: 1212-1215.

Hamada, I., Tsuji, K., Nakayama, T., and Niwa, E. 1977. Oxidative denaturation of actomyosin. Nippon Suisan Gakkaishi. 43: 1105–1109.

Han, M., Zhang, Y., Fei, Y., Xu, X., and Zhou, G. 2009. Effect of microbial transglutaminase on NMR relaxometry and microstructure of pork myofibrillar protein gel. Eur. Food Res. Technol. 228: 665-670.

Hansen, L. T., Gill, T., Røntved, S. D., and Huss, H. H. 1996. Importance of autolysis and microbiological activity on quality of cold-smoked salmon. Food Res. Int. 29: 181-188.

Hayakawa, S., and Nakai, S. 1985. Contribution of hydrophobicity, net charge and sulfhydryl groups to thermal properties of ovalbumin. Can. Inst. Food Sci. Technol. J. 18: 290-295.

Hemung, B. O., Li-Chan, E. C. Y., and Yongsawatdigul, J. 2008. Thermal stability of fish natural actomyosin affects reactivity to cross-linking by microbial and fish transglutaminases. Food Chem. 111: 439-446.

Hong, G. P., Min, S. G., and Chin, K. B. 2012. Emulsion properties of pork myofibrillar protein in combination with microbial transglutaminase and calcium alginate under various pH conditions. Meat Sci. 90: 185-193.

Hossain, M. I., Morioka, K., Shikha, F. H., and Itoh, Y. 2011. Effect of preheating temperature on the microstructure of walleye pollack surimi gels under the inhibition of the polymerisation and degradation of myosin heavy chain. J. Sci. Food Agric. 91: 247-252.

Hsieh, R. J., and Kinsella, J. E. 1989. Lipoxygenase generation of specific volatile flavor carbonyl compounds in fish tissues. J. Agric. Food Chem. 37: 279-286.

Hu, Y., Ji, R., Jiang, H., Zhang, J., Chen, J., and Ye, X. 2012. Participation of cathepsin L in modori phenomenon in carp (*Cyprinus carpio*) surimi gel. Food Chem. 134: 2014-2020.

Hu, Y., Morioka, K., Chen, S., Liu, D., and Ye, X. 2015. Effect of iced-storage on the activity of cathepsin L and trypsin-like protease in carp dorsal muscle. LWT-Food Sci. Technol. 60: 1249-1253.

Huang, Y. P., Seguro, K., Motoki, M., and Tawada, K. 1992. Cross-linking of contractile proteins from skeletal muscle by treatment with microbial transglutaminase. J. Biochem. 112: 229-234.

Huis in't Veld, J. H. 1996. Microbial and biochemical spoilage of foods: an overview. Int. J. Food Microbiol. 33: 1-18.

Hultin, H. 1994. Oxidation of lipids in seafoods. *In* Seafoods: chemistry, processing technology and quality. (Shahidi, F. and Botta, J. R. eds.). p. 49-74. Blackie Academic and Professional. London.

Huss, H. H. 1988. Post motem changes in fish. *In* Fresh fish quality and quality changes: a training manual prepared for the FAO/DANIDA. Training programme on fish technology and quality control. p. 27-61. Food and Agriculture Organization. Rome.

Huss, H. H. 1995. Quality and quality changes in fresh fish. FAO fisheries technical paper. Food and Agriculture Organization. Rome.

Icekson, I., and Apelbaum, A. 1987. Evidence for transglutaminase activity in plant tissue. Plant phys. 84: 972-974.

Jadhav, S. J., Nimbalkar, S. S., Kulkarni, A. D., and Madhavi, D. L. 1996. Lipid oxidation in biological and food systems. *In* Antioxidant: technological, toxicological, and heath perspective. (Despande, S. S., Salunkhe, D. K., and Madhavi, D. L. eds.). Marcel Dekker. New York.

Jaros, D., Partschefeld, C., Henle, T., and Rohm, H. 2006. Transglutaminase in dairy products: chemistry, physics, applications. J. Texture Stud. 37: 113-155.

Jeremiah, L. 2001. Packaging alternatives to deliver fresh meats using short- or long-term distribution. Food Res. Int. 34: 749-772.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000. Combination effects of microbial transglutaminase, reducing agent, and protease inhibitor on the quality of hairtail surimi. J. Food Sci. 65: 241-245.

Josephson, D. B., Lindsay, R. C., and Stuiber, D. A. 1984. Biogenesis of lipid-derived volatile aroma compounds in the emerald shiner (*Notropis atherinoides*). J. Agric. Food Chem. 32: 1347-1352.

Kailasapathy, K., and Salampessy, J. 1999. Suitability of shark flesh for surimi production. Food Aus. 51: 110-115.

Kamal, M., Watabe, S., and Hashimoto, K. 1991. Postmortem changes in myofibrillar ATPase of sardine ordinary and dark muscles. Nippon Suisan Gakkaishi. 57: 1177-1184.

Kamath, G. G., Lanier, T. C., Foegeding, E. A., and Hamann, D. D. 1992. Nondisulfide covalent cross-linking of myosin heavy chain in "setting" of Alaska pollock and Atlantic croaker surimi. J. Food Biochem. 16: 151-172.

Kanaji, T., Ozaki, H., Takao, T., Kawajiri, H., Ide, H., Motoki, M., and Shimonishi, Y. 1993. Primary structure of microbial transglutaminase from *Streptoverticillium* sp. strain S-8112. J. Biol. Chem. 268: 11565.

Kang, I. J., Matsumura, Y., Ikura, K., Motoki, M., Sakamoto, H., and Mori, T. 1994. Gelation and gel properties of soybean glycinin in a transglutaminase-catalyzed system. J. Agric. Food Chem. 42: 159-165.

Karakoltsidis, P. A., Zotos, A., and Constantinides, S. M. 1995. Composition of the commercially important Mediterranean finfish, crustaceans, and molluscs. J. Food Compos. Anal. 8: 258-273.

Karayannakidis, P. D., Zotos, A., Petridis, D., and Taylor, K. D. A. 2008. Physicochemical changes of sardines (*Sardina pilchardus*) at -18°C and functional properties of kamaboko gels enhanced with Ca^{2+} ions and MTGase. J. Food Process. Eng. 31: 372-397.

Kashiwagi, T., Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H., and Suzuki, E. 2002. Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. J. Biol. Chem. 277: 44252-44260.

Kelly, R. H., and Yancey, P. H. 1999. High contents of trimethylamine oxide correlating with depth in deep-sea teleost fishes, skates, and decapod crustaceans. Biol. Bull. 196: 18-25.

Khayat, A., and Schwall, D. 1983. Lipid oxidation in seafood. Food Technol. 37: 30-140.

Kieliszek, M., and Misiewicz, A. 2014. Microbial transglutaminase and its application in the food industry: a review. Folia Microbiol. 59: 241-250.

Kijowski, J. M. 2001. Muscle protein. *In* Chemical and functional properties of food proteins. (Sikorski, Z. E. eds.). p. 233-270. Techomic publishing. Pennsylvania.

Kim, M. K., Mah, J. H., and Hwang, H. J. 2009. Biogenic amine formation and bacterial contribution in fish, squid and shellfish. Food Chem. 116: 87-95.

Kim, S., Carpenter, J. A., Lanier, T. C., and Wicker, L. 1993. Setting response of Alaska pollock surimi compared with beef myofibrils. J. Food Sci. 58: 531-534.

Kimata, M., and Hata, Y. 1953. On the urea-splitting bacteria causing the spoilage of fresh fish. Mems. Res. Inst. Food Sci. Kyoto Univ. 5: 55-64.

Kimura, M., Seki, N., and Kimura, I. 2002. Enzymatic and nonenzymatic cleavage of trimethylamine-*N*-oxide *In vitro* at subzero temperatures. Nippon Suisan Gakkaishi. 68: 85-91.

King, F. J., Anderson, M. L., and Steinberg, M. A. 1962. Reaction of cod actomyosin with linoleic and linolenic acids. J. Food Sci. 27: 363-366.

Kishi, H., Nozawa, H., and Seki, N. 1991. Reactivity of muscle transglutaminase on carp myofibrils and myosin B. Nippon Suisan Gakkaishi. 57: 1203-1210.

Klomklao, S., Kishimura, H., and Benjakul, S. 2008. Endogenous proteinases in true sardine (*Sardinops melanostictus*). Food Chem. 107: 213-220.

Kobayashi, K., Hashiguchi, K., Yokozeki, K., and Yamanaka, S. 1998. Molecular cloning of the transglutaminase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. Biosci. Biotechnol. Biochem. 62: 1109-1114.

Kolakowski, E., Szybowicz, Z., and Raczek, K. 1977. Extractability of muscle protein as criterion for technological evaluation of sea fish Part 1. Changes of protein fraction of Baltic herring and Baltic cod during storage in ice. Food/Nahrung. 21: 485-490.

Kongpun, O., Suwansakornkul, P., and Predalumpabut, P. 2001. Effect of formaldehyde on the gel forming ability of fish meat. Kasetsart J. Nat. Sci. 35: 318-325.

Krueger, D. J., and Fennema, O. R. 1989. Effect of chemical additives on toughening of fillets of frozen Alaska pollack (*Theragra chalcogramma*). J. Food Sci. 54: 1101-1106.

Kudre, T., and Benjakul, S. 2014. Effects of bambara groundnut protein isolates and microbial transglutaminase on textural and sensorial properties of surmi gel from sardine (*Sardinella albella*). Food Bioprocess. Technol. 7: 1570-1580.

Kumazawa, Y., Numazawa, T., Seguro, K., and Motoki, M. 1995. Suppression of surimi gel setting by transglutaminase inhibitors. J. Food Sci. 60: 715-717.

Kunioka, Y., and Ando, T. 1996. Innocuous labeling of the subfragment-2 region of skeletal muscle heavy meromyosin with a fluorescent polyacrylamide nanobead and visualization of individual heavy meromyosin molecules. J. Biochem. 119: 1024.

Ladrat, C., Verrez-Bagnis, V., and Fleurence, J. 2003. *In vitro* proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax L.*): effects of cathepsins B, D and L. Food Chem. 81: 517-525.

Lanier, T. C. 1992. Measurement of surimi composition and functional properties. *In* Surimi technology (Lanier, T. C. and Lee, C. M. eds.). p. 123-166. Marcel Dekker. New York.

Lanier, T. C., Carvajal, P., and Yongsawatdigul, J. 2000. Surimi gelation chemistry. *In* Surimi and surimi seafood. (Park, J. W. eds.). p. 237-265. CRC Press. Boca Raton, Florida.

Lanier, T. C., Carvajal, P., and Yongsawatdigul, J. 2013. Surimi gelation chemistry. *In* Surimi and surimi seafood. 3rd ed. (Park, J. W. eds.). p. 101-140. CRC Press. Boca Raton, Florida.

Lanier, T. C., Lin, T. S., Hamann, D. D., and Thomas, F. B. 1981. Effect of alkaline protease in minced fish on texture of heat-induced gel. J. Food Sci. 46: 1643–1645.

Lee, J. J., Chen, H. C., and Jiang, S. T. 1993. Purification and characterization of proteinases identified as cathepsins L and L-like (58 kDa) proteinase from mackerel (*Scomber australasicus*). Biosci. Biotechnol. Biochem. 57: 1470-1476.

Lee, N., and Park, J. W. 1998. Calcium compounds to improve gel functionality of Pacific whiting and Alaska pollock surimi. J. Food Sci. 63: 969-974.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N. K. 2005. Physicochemical and biochemical changes during frozen storage of minced flesh of lizardfish (*Saurida micropectoralis*). Food Chem. 90: 141-150.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N. K. 2010. Effects of trimethylamine-*N*-oxide demethylase (TMAOase) inhibitors and antioxidants on physicochemical and biochemical changes of haddock muscle induced by lizardfish TMAoase during frozen storage. J. Food Biochem. 34: 1032-1048.

Lefevre, F., Fauconneau, B., Thompson, J. W., and Gill, T. A. 2007. Thermal denaturation and aggregation properties of atlantic salmon myofibrils and myosin from white and red muscles. J. Agric. Food Chem. 55: 4761-4770.

Li, T., Li, J., Hu, W., and Li, X. 2013. Quality enhancement in refrigerated red drum (*Sciaenops ocellatus*) fillets using chitosan coatings containing natural preservatives. Food Chem. 138: 821-826.

Lim, L. T., Mine, Y., and Tung, M. A. 1998. Transglutaminase cross-linked egg white protein films: tensile properties and oxygen permeability. J. Agric. Food Chem. 46: 4022-4029.

Lin, T. M., and Park, J. W. 1996. Protein solubility in Pacific whiting affected by proteolysis during storage. J. Food Sci. 61: 536-539.

Lorenzen, P. C. 2000. Techno-functional properties of transglutaminase-treated milk proteins. Milchwissenschaft. 55: 667-670.

Lund, M. N., Heinonen, M., Baron, C. P., and Estévez, M. 2011. Protein oxidation in muscle foods: a review. Mol. Nutr. Food Res. 55: 83-95.

Mackie, I. M. 1994. Fish protein. *In* New and developing sources of food proteins. (Hudson, B. J. G. ed.). p. 95-143 Champman and Hall. New York.

Mamone, G., Ferranti, P., Melck, D., Tafuro, F., Longobardo, L., Chianese, L., and Addeo, F. 2004. Susceptibility to transglutaminase of gliadin peptides predicted by a mass spectrometry-based assay. FEBS Lett. 562: 177-182.

Martin-Sanchez, A. M., Navarro, C., Perez-Alvarez, J. A., and Kuri, V. 2009. Alternatives for efficient and sustainable production of surimi: a review. Comp. Rev. Food Sci. F. 8: 359-374.

Martinaud, A., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P., and Renerre, M. 1997. Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. J. Agric. Food Chem. 45: 2481-2487.

Martins, I. M., Matos, M., Costa, R., Silva, F., Pascoal, A., Estevinho, L. M., and Choupina, A. B. 2014. Transglutaminases: Recent achievements and new sources. Appl. Microbiol. Biotechnol. 98: 6957-6964.

Maruyama, N., Nozawa, H., Kimura, I., Satake, M., and Seki, N. 1995. Transglutaminase-induced polymerization of a mixture of different fish myosins. Fish. Sci. 61: 495-500

Mathew, S., and Shamasundar, B. A. 2002. Effect of ice storage on the functional properties of proteins from shark (*Scoliodon laticaudus*) meat. Food/Nahrung. 46: 220-226.

Matsumoto, J. J. 1980. Chemical deterioration of muscle proteins during frozen storage. *In* Chemical deterioration of proteins. (Whitaker, J. K. and Fujimaki, J. R. eds.). p. 95-124. ACS Symposium Series. Washington, DC.

Matsumura, Y., Chanyongvorakul, Y., Kumazawa, Y., Ohtsuka, T., and Mori, T. 1996. Enhanced susceptibility to transglutaminase reaction of α-lactalbumin in the molten globule state. BBA. Protein Sruct. M. 1292: 69-76.

McCormick, R. J. 1994. Structure and properties of tissue. *In* Muscle foods meat poultry and seafood technology. (Kinsman, D. M., Kotula, A. W., and Breidenstein, B. C. eds.). p. 25-62. Champman and Hall. New York.

Medina, I., Gallardo, J. M., and Aubourg, S. P. 2009. Quality preservation in chilled and frozen fish products by employment of slurry ice and natural antioxidants. Int. J. Food Sci. Technol. 44: 1467-1479.

Medina, I., Saeed, S., and Howell, N. 1999. Enzymatic oxidative activity in sardine (*Sardina pilchardus*) and herring (*Clupea harengus*) during chilling and correlation with quality. Eur. Food Res. Technol. 210: 34-38.

Min, B., and Green, B. 2008. Use of microbial transglutaminase and nonmeat proteins to improve functional properties of low NaCl, phosphate-free patties made

from channel catfish (*Ictalurus punctatus*) belly flap meat. J. Food Sci. 73: 218-226.

Mizuguchi, T., Kumazawa, K., Yamashita, S., and Safey, J. 2011a. Factors that accelerate dimethylamine formation in dark muscle of three gadoid species during frozen storage. Fish. Sci. 77: 143-149.

Mizuguchi, T., Kumazawa, K., Yamashita, S., and Stuart, G. 2011b. Effect of surimi processing on dimethylamine formation in fish meat during frozen storage. Fish. Sci. 77: 271-277.

Mohamed, R., Livia, S.-S., Hassan, S., Soher, E.-s., and Ahmed-Adel, E. B. 2009. Changes in free amino acids and biogenic amines of Egyptian salted-fermented fish (Feseekh) during ripening and storage. Food Chem. 115: 635-638.

Morales, O. G., Ramírez, J. A., Vivanco, D. I., and Vázquez, M. 2001. Surimi of fish species from the Gulf of Mexico: evaluation of the setting phenomenon. Food Chem. 75: 43-48.

Morrissey, M. T., Wu, J. W., Lin, D., and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting surimi. J. Food Sci. 58: 1050-1054.

Motoki, M., and Kumazawa, Y. 2000. Recent research trends in transglutaminase technology for food processing. Food Sci. Technol. Res. 6: 151-160.

Motoki, M., and Nio, N. 1983. Crosslinking between different food proteins by transglutaminase. J. Food Sci. 48: 561-566.

Motoki, M., and Seguro, K. 1998. Transglutaminase and its use for food processing. Trends Food Sci. Technol. 9: 204-210.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

Nakayama, T., Osawa, T., Mendosa, E. N. T., Laurena, A. C., and Kawakishi, S. 1994. Comparative study of antioxidative assays of plant materials. *In* Posthavest biochemistry of plant food-materials in the tropics. (Uritani, I. ed.). p. 241-251. Japan Scientific Societies Press. Tokyo.

Nambudiri, D. D., and Gopakumar, K. 1992. ATPase and lactate dehydrogenase activities in frozen stored fish muscle as indices of cold storage deterioration. J. Food Sci. 57: 72-76.

Nawar, W. W. 1996. Lipids. *In* Food Chemistry. 3rd ed. (Fennema, O. R. ed.). p. 226-318. Marcel Dekker. New York.

Nemes, Z., Fésüs, L., Égerházi, A., Keszthelyi, A., and Degrell, I. M. 2001. N-$^{\varepsilon}$(γ-glutamyl) lysine in cerebrospinal fluid marks alzheimer type and vascular dementia. Neurobiol. Aging. 22: 403-406.

Ni, S., Nozawa, H., and Seki, N. 1999. The combined effect of transglutaminase and protease inhibitors on the thermal gelation of actomyosin sol from carp and salmon muscles. Fish. Sci. 65: 606-612.

Niwa, E. 1992. Chemistry of surimi gelation. *In* Surimi technology. (Lanier, T. C., and Lee, C. eds.). p. 389-427. Marcel Dekker. New York.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., and Motoki, M. 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J. Food Sci. 57: 1214-1241.

Nowsad, A. A. K. M., Katoh, E., Kanoh, S., and Niwa, E. 1996. Contribution of transglutaminase to the setting of fish pastes at various temperatures. Fish. Sci. 62: 94-97.

Nozawa, H., Cho, S. Y., and Seki, N. 2001. Purification and characterization of transglutaminase from squid gill. Fish. Sci. 67: 912-919.

Nozawa, H., and Ezou, M. 2009. Identification of the glutamine residue that may be involved in the transglutaminase-mediated intramolecular crosslinking of carp and walleye pollack myosin. Fish. Sci. 75: 1445-1452.

Ochai, Y., and Chow, C. J. 2000. Myosin ATPase. *In* Seafood enzyme: Utilization and influence on postharvest seafood quality. (Haard, N. F. and Simpson, B. K. eds.). p. 69-90. Marcel Dekker. New York.

Ochiai, Y., Ochiai, L., Hashimoto, K., and Watabe, S. 2001. Quantitative estimation of dark muscle content in the mackerel meat paste and its products using antisera against myosin light chains. J. Food Sci. 66: 1301-1305.

Ogawa, M., Kanamaru, J. U. N., Miyashita, H., Tamiya, T., and Tsuchiya, T. 1995. Alpha-helical structure of fish actomyosin: Changes during setting. J. Food Sci. 60: 297-299.

Ogawa, M., Tamiya, T., and Tsuchiya, T. 1994. Structural changes of carp myosin during heating. Fish. Sci. 60: 723-727.

Ohtsuka, T., Ota, M., Nio, N., and Motoki, M. 2000. Comparison of substrate specificities of transglutaminases using synthetic peptides as acyl donors. Biosci. Biotechnol. Biochem. 64: 2608-2613.

Oka, H., and Wang, X.-C. 2011. Effect of modified atmosphere packaging for keeping gel-forming ability of lizardfish. Tran. Jap. Soc. Refrig. 20: 49-56.

Okada, M. 1992. History of surimi technology in Japan. *In* Surimi technology. (Lanier, T. C. and Lee, C. M. eds.). p. 3-21. Marcel Dekker. New York.

Ólafsdóttir, G., Lauzon, H., Martinsdottir, E., and Kristbergsson, K. 2006. Influence of storage temperature on microbial spoilage characteristics of haddock fillets (*Melanogrammus aeglefinus*) evaluated by multivariate quality prediction. Int. J. Food Microbiol. 111: 112-125.

Ouali, A., and Valin, C. 1981. Effect of muscle lysosomal enzymes and calcium activated neutral proteinase on myofibrillar ATPase activity: Relationship with ageing changes. Meat Sci. 5: 233-245.

Ozogul, Y. 2010. Methods for freshness quality and deterioration. *In* Handbook of seafood and seafood products analysis (Nollet, L. and Toldrá, F. T. eds.). p. 189-241. CRC Press. New York.

Paarup, T., Sanchez, J. A., Peláez, C., and Moral, A. 2002. Sensory, chemical and bacteriological changes in vacuum-packed pressurised squid mantle (*Todaropsis eblanae*) stored at 4° C. Int. J. Food Microbiol. 74: 1-12.

Pacheco-Aguilar, R., M.E. Lugo-Sánchez, and Robles-Burgueño, M. R. 2000. Postmortem biochemical and functional characteristic of Monterey sardine muscle stored at 0 °C. J. Food Sci. 65: 40-47.

Park, J. W., and Lin, T. M. J. 2005. Surimi: manufacturing and evaluation. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. ed.). p. 33-106. CRC Press. Boca Raton, Florida.

Park, J. W., and Morrissey, M. T. 2000. Manufacturing of surimi from light muscle fish. *In* Surimi and surimi seafood. (Park, J. W. ed.). p. 23-58. CRC Press. Boca Raton, Florida.

Phillippy, B. Q., and Hultin, H. O. 1993. Distribution and some characteristics of trimethylamine-*N*-oxide (TMAO) demethylase activity of red hake muscle. J. Food Biochem. 17: 235-250.

Phu, N. V., Morioka, K., and Itoh, Y. 2010. Gel-forming characteristics of surimi from white croaker under the inhibition of the polymerization and degradation of protein. J. Biol Sci. 10: 432-439.

Piersma, S. R., van de Pijpekamp, A., Wijngaards, G., Gruppen, H., and Boumans, H. 2002. Quantitation and localisation of (*In vitro*) transglutaminase-catalysed

glutamine hydroxylation using mass spectrometry. Enzyme Microb. Technol. 30: 266-272.

Pigott, G. M., and Tucker, B. W. 1990. Seafood: effects of technology on nutrition. Marcel Dekker. New York.

Piyadhammaviboon, P., and Yongsawatdigul, J. 2009. Protein cross-linking ability of sarcoplasmic proteins extracted from threadfin bream. LWT-Food Sci. Technol. 42: 37-43.

Ravesi, E., Licciardello, J., Tuhkunen, B., and Lundstrom, R. 1985. The effect of handling or processing treatments on storage characteristics of fresh spiny dogfish. Mar. Fish Rev. 47: 48–67.

Renzetti, S., Dal Bello, F., and Arendt, E. K. 2008. Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. J. Cereal Sci. 48: 33-45.

Rey-Mansilla, M., Sotelo, C. G., and Pérez-Martín, R. I. 2002. TMAOase activity of European hake (*Merluccius merluccius*) organs: Influence of biological condition and season. J. Food Sci. 67: 3242-3251.

Reynisson, E., Þór Marteinsson, V., Jónsdóttir, R., Magnússon, S. H., and Hreggvidsson, G. O. 2012. Bacterial succession during curing process of a skate (*Dipturus batis*) and isolation of novel strains. J. Appl. Microbiol. 113: 329-338.

Reza, M. S., Bapary, M. A. J., Ahasan, C. T., Islam, M. N., and Kamal, M. 2009. Shelf life of several marine fish species of Bangladesh during ice storage. Int. J. Food Sci. Technol. 44: 1485-1494.

Richards, M. P., and Hultin, H. O. 2002. Contributions of blood and blood components to lipid oxidation in fish muscle. J. Agric. Food Chem. 50: 555-564.

Roura, S. I., and Crupkin, M. 1995. Biochemical and functional properties of myofibrils from preand post spawned hake (*Merluccius hubbsi Marini*) stored on ice. J. Food Sci. 60: 269-272.

Roussel, H., and Cheftel, J. C. 1990. Mechanisms of gelation of sardine proteins: Influence of thermal processing and of various additives on the texture and protein solubility of kamaboko gels. Int. J. Food Sci. Technol. 25: 260-280.

Ruoppolo, M., Orrù, S., D'Amato, A., Francese, S., Rovero, P., Marino, G., and Esposito, C. 2003. Analysis of transglutaminase protein substrates by functional proteomics. Protein Sci. 12: 1290-1297.

Sae-leaw, T., Benjakul, S., Gokoglu, N., and Nalinanon, S. 2013. Changes in lipids and fishy odour development in skin from Nile tilapia (*Oreochromis niloticus*) stored in ice. Food Chem. 141: 2466-2472.

Saito, T., Arai, K., and Matsuyoshi, M. 1959. A new method for estimating the freshness of fish. Nippon Suisan Gakkaishi. 24: 749-750.

Sakamoto, H., Kumazawa, Y., Toiguchi, S., Seguro, K., Soeda, T., and Motoki, M. 1995. Gel strength enhancement by addition of microbial transglutaminase during onshore surimi manufacture. J. Food Sci. 60: 300-304.

Samejima, K., Ishioroshi, M., and Yasui, T. 1981. Relative roles of the head and tail portions of the molecule in heat induced gelation of myosin. J. Food Sci. 46: 1412-1418.

Sano, T., Noguchi, S. F., Matsumoto, J. J., and Tsuchiya, T. 1990. Thermal gelation characteristics of myosin subfragments. J. Food Sci. 55: 55-58.

Sano, T., Ohno, T., Otsuka Fuchino, H., Matsumoto, J. J., and Tsuchiya, T. 1994. Carp natural actomyosin: thermal denaturation mechanism. J. Food Sci. 59: 1002-1008.

Santos, M. 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29: 213-231.

Schrode, J., and Folk, J. 1978. Transglutaminase-catalyzed cross-linking through diamines and polyamines. J. Biol. Chem. 253: 4837-4840.

Schubring, R. 2007. DSC measurements on sharks. Thermochim. Acta. 458: 124-131.

Seguro, K., Kumazawa, Y., Ohtsuka, T., Toiguchi, S., and Motoki, M. 1995. Microbial transglutaminase and ε-(γ-glutamyl) lysine crosslink effects on elastic properties of kamaboko gels. J. Food Sci. 60: 305-311.

Seguro, K., Nio, N., and Motoki, M. 1996. Some characteristics of a microbial protein cross-Linking enzyme: transglutaminase. *In* Macromolecular Interactions in food technology. p. 271-280. American Chemical Society. Washington, DC.

Seki, N., Ikeda, M., and Narita, N. 1979. Changes in ATPase activities of carp myofibril during ice-storage. Nippon Suisan Gakkaishi. 45: 791-799.

Serafini-Fracassini, D., Duca, S. D., and Beninati, S. 1995. Plant transglutaminases. Phytochemistry. 40: 355-365.

Serio, A., Fusella, G. C., Chaves López, C., Sacchetti, G., and Paparella, A. 2014. A survey on bacteria isolated as hydrogen sulfide-producers from marine fish. Food Control. 39: 111-118.

Seymour, T. A., Morrissey, M. T., Peters, M. Y., and An, H. 1994. Purification and characterization of Pacific whiting proteases. J. Agric. Food Chem. 42: 2421-2427.

Shahidi, F. 1994. Seafood proteins and preparation of protein concentrates. *In* Seafoods: Chemistry, processing technology and quality. (Shahidi, F. and Botta, J. R. eds.). p. 3-9. Chapman and Hall. London.

Shahidi, F., and Janak Kamil, Y. V. A. 2001. Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci. Technol. 12: 435-464.

Shenouda, S. Y. K. 1980. Theories of protein denaturation during frozen storage of fish flesh. Adv. Food Res. 26: 275-311.

Shikha, F. H., Hossain, M. I., Morioka, K., Kubota, S., and Itoh, Y. 2006. Effect of pH shifting on the gel forming characteristics of salt ground meat from walleye pollack. Fish. Sci. 72: 870-876.

Shimizu, Y., Toyohara, H., and Lanier, T. C. 1992. Surimi production from fatty and dark-fleshed fish species. Marcel Dekker. New York.

Sikorski, Z. E. 1994. The contents of proteins and other nitrogenous compounds in marine animals. *In* Seafood Proteins. (Sikorski, Z. E., Pan, B. S. and Shahidi, F. eds.). p. 6-12. Champman and Hall. New York.

Sikorski, Z. E., Kolakowski, E., and Pan, B. S. 1990. The nutritive compositionof the major groupd of marine food organisms. *In* Seafood: Resources, nutritional composition and preservation (Sikorski, Z. E. ed.). p. 29-54. CRC Press. Florida.

Sompongse, W., Itoh, Y., and Obatake, A. 1996. Role of SH_a in the polymerization of myosin heavy chain during ice storage of carp actomyosin. Fish. Sci. 62: 110-113.

Song, Y., Luo, Y., You, J., Shen, H., and Hu, S. 2012. Biochemical, sensory and microbiological attributes of bream (*Megalobrama amblycephala*) during partial freezing and chilled storage. J. Sci. Food Agric. 92: 197-202.

Sotelo, C., Gallardo, J. M., Piñeiro, C., and Pérez-Martin, R. 1995a. Trimethylamine oxide and derived compounds' changes during frozen storage of hake (*Merluccius merluccius*). Food Chem. 53: 61-65.

Sotelo, C. G., and Rehbein, H. 2000. TMAO-degrading enzymes. *In* Seafood enzymes: Utilization and influence on postharvest seafood quality. (Haard, N. F. and Simpson, B. K. eds.). p. 167-190. Marcel Dekker. New York.

Sotelo, G. C., Pineiro, C., and Perez-Martin, R. 1995b. Denaturation of fish proteins during frozen storage: Role of formaldehyde. Z. Lebensm. Untersuch. Forsch. 200: 14-23.

Spolaore, B., Raboni, S., Ramos Molina, A., Satwekar, A., Damiano, N., and Fontana, A. 2012. Local unfolding is required for the site-specific protein modification by transglutaminase. Biochem. 51: 8679-8689.

Stadtman, E. R., and Berlett, B. S. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem. Res. Toxicol. 10: 485-494.

Strop, P. 2014. Versatility of microbial transglutaminase. Bioconjugate Chem. 25: 855-862.

Sun, X. D., and Holley, R. A. 2011. Factors influencing gel formation by myofibrillar proteins in muscle foods. Comp. Rev. Food Sci. F. 10: 33-51.

Suzuki, T. 1981. Fish and Krill Protein. *In* Protein Technology. p. 1-61. Applied Science Publishers Ltd. London.

Suzzi, G., and Gardini, F. 2003. Biogenic amines in dry fermented sausages: a review. Int. J. Food Microbiol. 88: 41-54.

Taguchi, T., Ishizaka, H., Tanaka, M., Nagashima, Y., and Amano, K. 1987. Protein-protein interaction of fish myosin fragments. J. Food Sci. 52: 1103-1104.

Takeda, H., and Seki, N. 1996. Enzyme-catalyzed cross-linking and degradation of myosin heavy chain in walleye pollack surimi paste during setting. Fish. Sci. 62: 462-467.

Taylor, S., Guthertz, L., Leatherwood, M., and Lieber, E. 1979. Histamine production by *Klebsiella pneumoniae* and an incident of scombroid fish poisoning. Appl. Environ. Microbiol. 37: 274-278.

Tejada, M., Mohamed, G. F., and Huidobro, A. 2002. Addition of sardine to hake minces and subsequent effect on dimethylamine and formaldehyde formation. J. Sci. Food Agric. 82: 351-359.

Tocher, D. R. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11: 107-184.

Tokiwa, T., and Matsumiya, H. 1969. Fragmentation of fish myofibril: Effect of storage condition and muscle cathepsin. Nippon Suisan Gakkaishi. 35: 1099-1109.

Tomiyasu, Y., and Zenitani, B. 1957. Spoilage of fish and its preservation by chemical agents. *In* Advance in food research. (Mrak, E. M. and Stewart, G. F. eds.). p. 42-74. Academic Press. Massachusetts.

Truong, V. D., Clare, D. A., Catignani, G. L., and Swaisgood, H. E. 2004. Cross-linking and rheological changes of whey proteins treated with microbial transglutaminase. J. Agric. Food Chem. 52: 1170-1176.

Tsukamasa, Y., Sato, K., Shimizu, Y., Imai, C., Sugiyama, M., Minegishi, Y., and Kawabata, M. 1993. ε-(γ-glutamyl)-lysine crosslink formation in sardine myofibril sol during setting at 25 °C. J. Food Sci. 58: 785-787.

Turan, H., and Sönmez, G. 2007. Changes in the quality of surimi made from thornback ray (*Raja clavata*, L. 1758) during frozen storage. Int. J. Food Sci. Nutr. 58: 557-566.

Uchida, K., and Stadtman, E. 1993. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra-and intermolecular cross-linking reaction. J. Biol. Chem. 268: 6388-6393.

Undeland, I., Hall, G., Wendin, K., Gangby, I., and Rutgersson, A. 2005. Preventing lipid oxidation during recovery of functional proteins from herring (*Clupea*

harengus) fillets by an acid solubilization process. J. Agric. Food Chem. 53: 5625-5634.

Vácha, F., Novik, I., Spicka, J., and Podola, M. 2006. Determination of the effect of microbial transglutaminase on technological properties of common carp (*Cyprinus carpio L.*) meat. Czech J. Anim. Sci. 51: 535.

Venugopal, V., Alur, M. D., and Lewis, N. F. 1983. Extracellular protease from Pseudomonas marinoglutinosa: some properties and its action on fish actomyosin. J. Food Sci. 48: 671-674.

Venugopal, V., Kakatkar, A., Bongirwar, D., Karthikeyan, M., Mathew, S., and Shamasundar, B. 2002. Gelation of shark meat under mild acidic conditions: physicochemical and rheological characterization of the gel. J. Food Sci. 67: 2681-2686.

Visciano, P., Schirone, M., Tofalo, R., and Suzzi, G. 2012. Biogenic amines in raw and processed seafood. Front. Microbiol. 3: 1-10.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H., Motoki, M., and Takeuchi, K. 1994. Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci. Biotechnol. Biochem. 58: 82-87.

Wasson, D. H., Babbitt, J. K., and French, J. S. 1992. Characterization of a heat stable protease from arrowtooth flounder; *Atheresthes stomias*. J. Aquat. Food Prod. Technol. 1: 167-182

Watabe, S., Ochiai, Y., Kanoh, S., and Hashimoto, K. 1983. Proximate and protein compositions of requiem shark muscle. Nippon Suisan Gakkaishi. 49: 265-268.

Watabe, S., Ushio, H., Iwamoto, M., Yamanaka, H., and Hashimoto, K. 1989. Temperature dependency of rigor mortis of fish muscle: Myofibrillar Mg^{2+}

ATPase activity and Ca^{2+} Uptake by sarcoplasmic reticulum. J. Food Sci. 54: 1107-1107.

Wilhelm, B., Meinhardt, A., and Seitz, J. 1996. Transglutaminases: purification and actiivity assays. J. Chromatogr. B. 684: 163-177.

Wood, E. F. 1950. The bacteriology of shark spoilage. Mar. Freshwater Res. 1: 129-138.

Xiong, Y. L. 1997. Structure-function relationships of muscle proteins. *In* Food proteins and their applications. (Damodaran, S. and Paraf, A. eds.). p. 341-392. Marcel Decker. New York.

Xiong, Y. L., Decker, E., Faustman, C., and Lopez-Bote, C. 2000. Protein oxidation and implications for muscle food quality. *In* Antioxidants in muscle foods: nutritional strategies to improve quality. (Decker, E., Faustman, C. and Lopez-Bote, C. J. eds.). p. 85-111. John Wiley and Sons. New York.

Yamaguchi, M., and Sekine, T. 1966. Sulfhydryl groups involved in the active site of myosin A adenosine triphosphatase. I. Specific blocking of the SH group responsible for the inhibitory phase in" B phasic response" of the catalytic activity. J. Biochem, 59: 24-33.

Yasueda, H., Kumazawa, Y., and Motoki, M. 1994. Purification and characterization of a tissue-type transglutaminase from red sea bream (*Pagrus major*). Biosci. Biotechnol. Biochem. 58: 2041-2045.

Yokoyama, K., Nio, N., and Kikuchi, Y. 2004. Properties and applications of microbial transglutaminase. Appl. Microbiol. Biotechnol. 64: 447-454.

Yongsawatdigul, J., Worratao, A., and Park, J. W. 2002. Effect of endogenous transglutaminase on threadfin bream surimi gelation. J. Food Sci. 67: 3258-3263.

Yurkowski, M., and Brockerhoff, H. 1965. Lysolecithinase of cod muscle. J. Fish Res. Board Can. 22: 643-652.

Zare, D., Muhammad, K., Bejo, M. H., and Ghazali, H. M. 2013. Changes in urocanic acid, histamine, putrescine and cadaverine levels in Indian mackerel (*Rastrelliger kanagurta*) during storage at different temperatures. Food Chem. 139: 320-325.

Zarei, M., Najafzadeh, H., Enayati, A., and Pashmforoush, M. 2011. Biogenic amines content of canned tuna fish marketed in Iran. Am. Eura. J. Toxicol. Sci. 3: 190-193.

Zayas, J. F. 1997. Gelling properties of proteins. *In* Functionality of proteins in food. (Zayas, J. F. ed.). p. 310-355. Springer Berlin Heidelberg. New York.

Zhai, H., Yang, X., Li, L., Xia, G., Cen, J., Huang, H., and Hao, S. 2012. Biogenic amines in commercial fish and fish products sold in southern China. Food Control. 25: 303-308.

Zhu, Z., Lanier, T. C., Farkas, B. E., and Li, B. 2014. Transglutaminase and high pressure effects on heat-induced gelation of Alaska pollock (*Theragra chalcogramma*) surimi. J. Food Eng. 131: 154-160.

Ziegler, G. R., and Acton, J. C. 1984. Mechanisms of gel formation by proteins of muscle tissue. Food Technol. 38: 77-82.

CHAPTER 2

COMPARATIVE STUDY ON PROTEIN CROSS-LINKING AND GEL ENHANCING EFFECT OF MICROBIAL TRANSGLUTAMINASE ON MUSCLE PROTEINS FROM DIFFERENT FISH

2.1 Abstract

Effect of microbial transglutaminase (MTGase) at different levels (0-0.6 units/g) on gel property of surimi from three fish species including threadfin bream, Indian mackerel and sardine in the presence and absence of EDTA (10 mmol /kg) was investigated. Without EDTA addition, breaking force of gel from all surimi prepared by 2 step-heating (40 ºC for 30 min, followed by 90 ºC for 20 min) increased as MTGase levels increased, except for threadfin bream surimi gel, where breaking force decreased at 0.6 units/g ($P < 0.05$). Deformation of threadfin bream surimi decreased continuously as the MTGase increased ($P < 0.05$), while there was no change in deformation for surimi from other two species ($P > 0.05$). In the presence of EDTA, gel strengthening effect was still achieved, but with the lower impact, suggesting the combined effect of endogenous transglutaminase with MTGase on gel strengthening. With the addition of MTGase, the highest increase in breaking force with the coincidentally highest decrease in myosin heavy chain was found in surimi from threadfin bream, followed by those from mackerel and sardine, respectively. When cross-linking activity of MTGase on natural actomyosin (NAM) was determined, the highest decreasing rate in ε-amino group content with the concomitant increased formation of cross-linked proteins was found in NAM from threadfin bream, followed by NAM from mackerel and sardine, respectively. The reactivity of muscle proteins toward MTGase-induced cross-linking was in agreement with surimi gel enhancement. Thus, the composition and property of muscle proteins of varying fish species more likely determined protein cross-linking induced by MTGase, thereby affecting their gel properties.

2.2 Introduction

Transglutaminase (TGases, EC 2.3.2.13) is a transferase, which is able to catalyse the protein cross-linking by an acyl transfer reaction (1980). The γ-carboxyamide group of glutamine residue in protein serves as an acyl donor, while the amino group of primary amines or ε-amino group of lysine residues acts as an acyl acceptor. The reaction results in the formation of ε-(γ-glutamyl) lysine isopeptide, providing the crosslinked proteins or peptides (Folk and Finlayson, 1977). The enzyme can be found in various living tissues, such as microorganisms, vertebrates, invertebrates, and plants (Nozawa *et al.*, 2001). TGase-catalysed reaction can be used to modify the functional properties of food proteins. TGase plays an important role in cross-linking of fish muscle proteins pre-incubated at 25-40 °C, resulting in the improved gel quality (Benjakul *et al.*, 2003). Seki *et al.*(1990) reported that endogenous TGase contributes to the polymerisation of myosin heavy chain of pollack surimi. Increases in ε-(γ-glutamyl) lysine bond, which is a product of TGase reaction, in salted fish meat paste during setting have been reported (Tsukamasa *et al.*, 1993; Kumazawa *et al.*, 1995).

Surimi is concentrated myofibrillar protein obtained from mechanically deboned fish flesh, which is washed with cold water. Theoretically, any fish can be used to produce surimi but properties of surimi gel vary, depending on fish species. Both intrinsic and extrinsic factors including species, freshness, endogenous enzyme, processing parameters, protein concentration, pH, ionic strength, and temperature determine gelling properties of surimi (Benjakul *et al.*, 2003; Niwa, 1992; Shimizu *et al.*, 1992). In general, lean fish have been used for surimi production in Thailand. Threadfin bream has been widely used for surimi production because of it white colour, good flavor and strong gel-forming ability. Its myofibrillar proteins are highly stable in frozen storage (Guenneugues *et al.*, 2005). Owing to insufficient amount of these lean fish as raw material, dark-fleshed pelagic fish such as sardine and mackerel have gained attention for surimi production. Although they are cheaper, their high content of lipid and myoglobin makes them difficult to yield high-quality surimi as evidenced by poor gel forming ability of those species (Chaijan *et al.*, 2004). Since about 40% of total fish catch in the world is dark-muscle pelagic species,

there is great interest in developing methods to make use of those species for human consumption (Martin-Sanchez *et al.*, 2009). To improve the setting of surimi, microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins, thereby increasing the gel strength of surimi (Jiang *et al.*, 2000a; Yongsawatdigul *et al.*, 2002). Asagami *et al*. (1995) found that the amount of MTGase added highly depended on fish species and some other factors such as freshness, protein quality, and harvesting season. The gel strengthening effect of MTGase was lower when produced from lizardfish that was not fresh (Benjakul *et al.*, 2008). This might be a drawback for the surimi industry to achieve gel improvement of surimi by MTGase. A better understanding of gel enhancing effect of MTGase toward surimi from different fish species could lead to the maximised improvement of surimi gel quality. Thus, the objective of this study was to investigate the impact of MTGase on protein cross-linking and gel forming ability of surimi from different fish species including threadfin bream, Indian mackerel, and sardine.

2.3 Materials and Methods

2.3.1 Chemicals

All chemicals for gel preparation and analyses were of analytical grade. 2,4,6-trinitrobenzenesulphonic acid (TNBS), Sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, high molecular weight marker, and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). *N, N, N', N'*-tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Sodium hydrogen carbonate ($NaHCO_3$) was obtained from Merck (Darmstadt, Germany). Microbial transglutaminase (MTGase) from *Streptoverticilium mobaraense* (TG-K) containing 1% of pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

2.3.2 Fish samples

Fresh threadfin bream (*Nemipterus furcosus*), Indian mackerel (*Rastrelliger kanagurta*), and sardine (*Sardinella gibbosa*) with the sizes of 100-120, 140-160 and 50-60 g/fish, respectively, were purchased from a dock in Songkhla,

Thailand. The fish, off-loaded approximately 36–48 h after capture, were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 3 h. The fish were immediately washed and drained before using for the preparation of surimi.

2.3.3 Surimi preparation

Fish were subjected to beheading, eviscerating, followed by washing. Fish skin and bone were removed manually and the flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then washed with cold water (0-4 °C), using a mince/water ratio of 1:3 (w/v). The mixture was stirred gently for 3 min and washed mince was filtered with a layer of nylon screen. The washing process was carried out for three times. Finally, the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 xg for 15 min. Washed mince was mixed thoroughly with 4% sorbitol and 4% sucrose. The mixture (500 g) was packed in a polyethylene bag, kept at -18 °C and was referred to as 'frozen surimi'. The frozen surimi was used within 2 weeks of frozen storage.

2.3.4 Preparation of crude MTGase

Crude MTGase was prepared according to the method of Visessanguan *et al.* (2003) with a slight modification. Commercial enzyme powder (4 g) was dissolved with 20 ml of 20 mM Tris-HCl, pH 7.0 at 4 °C. The mixture was stirred gradually for 20 min, followed by centrifugation at 17500 xg at 4 °C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti JE Centrifuge, Fullerton, CA, USA). Supernatant was filtered through a Whatman filter paper (Whatman Ltd., Maidstone, UK) and then through a 0.20 mm nylon syringe filter. The filtrate was dialysed against 15 volumes of 20 mM Tris-HCl, pH 7.0, at 4 °C for 36 h, with four changes of dialysis buffer. The dialysate was centrifuged at 17500 xg for 30 min at 4 °C. The resulting supernatant was analysed for TGase activity.

2.3.4.1 Determination of MTGase activity

MTGase activity was measured by the hydroxamate method (Folk, 1970). Freshly prepared substrate mixture containing 350 µl of 0.1 M Tris-acetate, pH 6.0, 25 µl of 2.0 M hydroxylamine, 75 µl of 0.1 M N-ε-CBZ-L-glutamidylglycine and 25 µl of deionised water was used. To initiate the reaction, 25 µl of crude extract were added and reaction was performed for 10 min at 37 °C. The reaction was terminated by adding 500 µl of 15% TCA containing 5% $FeCl_3$. The resulting suspension was centrifuged at 9000 x g for 5 min and the absorbance was measured at 525 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). The calibration was performed using L-glutamic acid-γ- monohydroxamic acid as standard. One unit of MTGase was defined as the amount of enzyme required to catalyse the formation of 1 µmole hydroxamic acid.min^{-1} at pH 6.0 and 37 °C.

2.3.5 Effect of MTGase on gel properties of surimi from different fish species

Surimi was added with 2.5% NaCl and the moisture content was adjusted to 80%. After grinding for 3 min, the paste was added with various amounts of MTGase (0, 0.2, 0.4, and 0.6 units/g). The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min. The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analysis. To study the role of only MTGase (without endogenous TGase) on gel properties, 10 mmol/kg EDTA was added into the surimi paste to inactivate endogenous TGase (Benjakul *et al.*, 2004; Van Phu *et al.*, 2010). The gel were then prepared in the same manner. All gels were subjected to analyses.

2.3.5.1 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (20 to 25 °C) before analysis. Five cylindrical samples (2.5 cm in length)

were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

2.3.5.2 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (Park, 1994).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

2.3.5.3 Determination of expressible moisture content.

Expressible moisture content was measured according to the method of Benjakul *et al.* (2003). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*), and placed between 2 pieces of Whatman paper No. 1 at the bottom and 1 piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y)*. Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content } (\%) = [(X - Y)/X] \times 100$$

2.3.5.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% SDS solution heated to 85 °C were added to the sample (3 g). The mixture was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) at a speed of 11000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500 xg for 20 min to remove undissolved debris. Supernatant was determined for protein content using the Biuret method (Robinson and Hogden, 1940). The samples (10 μg protein) were loaded onto

the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.2% Comassie Brilliant Blue R-250 in 5% methanol and 7.5% acetic acid and destained with 50% methanol and 7.5% acetic acid, followed by 5% methanol and 7.5% acetic acid.

2.3.6 Effect of MTGase on cross-linking of natural actomyosin from different fish species

2.3.6.1 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul *et al.* (1997). Fish mince (10 g) was homogenised in 100 ml of chilled 0.6 M KCl, pH 7.0 for 4 min at a speed of 11000 rpm using a homogeniser. Overheating during extraction was avoided by keeping the sample in an iced container and each 20 s of homogenisation was followed by a 20 s rest interval. The homogenate was centrifuged at 5000 xg for 30 min at 4 °C. Three volumes of chilled water (0–4 °C) were added to precipitate NAM, which was then collected by centrifuging at 5000 xg for 20 min at 4 °C using a refrigerated centrifuge. The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.6 M KCl, pH 7.0 for 30 min at 4 °C.

2.3.6.2 Study on cross-linking of NAM by MTGase

For cross-linking reaction, NAM solution (5 mg/ml) was preheated at 40 °C for 5 min and MTGase (0, 10 and 20 units/g protein) was added. The reaction was performed for different times (0, 1, 3, 5, 10, 20 and 30 min). To terminate the reaction, the assay mixtures were heated at 100 °C for 5 min. All samples were subjected to analyses of ε-amino group content and protein pattern.

2.3.6.2.1 Determination of ε-amino group content

The ε-amino group content was measured according to the method of Bubnis and Ofner (1992) with a slight modification. Sample (1 ml) was placed in 50 ml screw cap test tube. One millilitre of 4% $NaHCO_3$ and 1 ml of 0.50%

TNBS were added. The reaction mixture was heated at 40 °C for 4 h with continuous shaking (SV1422, Memmert, Schwabach, Germany). Three millilitres of 6 N HCl was added and the mixture was autoclaved at 120 °C for 1 h using an autoclave (SX-500, Tomy, Tokyo, Japan). The hydrolysate was then diluted with 5 ml of deionised-water and extracted with 3 volumes of ethyl ether 3 times. The aqueous phase was removed and heated for 15 min in a temperature controlled water bath (W350, Memmert, Schwabach, Germany) to evaporate the residual ethyl ether. The aliquot was diluted with 3 volumes of deionised water and the absorbance was measured at 346 nm using a spectrophotometer. All samples were read against a reagent blank, which was prepared in the same manner with the sample but HCl was added before the addition of TNBS. ε-amino group content was calculated as follows (Bubnis and Ofner, 1992):

$$\text{ε-amino group content } (10^{-5} \text{ mol Lys/g protein}) = \frac{2(A_{346})(0.020)}{(1.46 \times 10^{4})(b)(x)}$$

where b: cell path length (cm); X: protein content (mg)

2.3.6.2.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns were analysed by SDS-PAGE according to the method of Laemmli (1970) as mentioned above.

2.3.5 Statistical analysis

Experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests. T-test was used for pair comparison (Steel and Torrie, 1980). Analysis was performed using the Statistical Package for Social Science package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and Discussion

2.4.1 Effect of MTGase on gel properties of different surimi in the presence and absence of EDTA

2.4.1.1 Breaking force and deformation

Breaking force and deformation of gels from threadfin bream, Indian mackerel, and sardine added with various levels of MTGase in the presence and absence of EDTA at a level of 10 mmol/kg are shown in Figure 5. Without EDTA, breaking force of gel from all surimi increased as MTGase levels increased up to the particular level ($P < 0.05$). For gel from threadfin bream surimi, the addition of MTGase at 0.2 units/g yielded the highest breaking force, which was 2.3-fold higher than that of the control. When MTGase higher than 0.4 units/g was added, the decrease in breaking force was observed ($P < 0.05$). For surimi from Indian mackerel and sardine, breaking force of gel added with MTGase increased with increasing MTGase levels ($P < 0.05$). At the same level of MTGase added, gels from threadfin bream surimi had the highest breaking force, followed by mackerel and sardine respectively. Breaking force of surimi from threadfin bream, Indian mackerel and sardine increased by 133.38, 351.9 and 48.9% when MTGase at level of 0.2, 0.6 and 0.6 units/g was incorporated, respectively. It was noted that the increase in breaking force was lowest in sardine surimi gel when MTGase at all levels was added ($P < 0.05$). This indicated that the strong gel development was not achieved by addition of MTGase. The extent of setting response depends on not only TGase activity level but also the conformation of actomyosin, which varies among fish species (Araki and Seki, 1993). Similar results were obtained for deformation. Deformation of threadfin bream surimi added with MTGase more than 0.2 units/g decreased continuously as MTGase level increased ($P < 0.05$), while there was no change in deformation for surimi from other two species ($P > 0.05$). Higher amount of MTGase added might induce the formation of non-disulphide covalent bond to a greater extent. As a result, the strength of gel matrix was enhanced (Benjakul *et al.*, 2008). Nevertheless, breaking force and deformation of surimi from threadfin bream decreased as the excessive amount of MTGase was added. This phenomenon was also observed in

several previous studies (Benjakul *et al.*, 2008; Jiang *et al.*, 2000b; Sakamoto *et al.*, 1995; Tsai *et al.*, 1996). Gels of casein and collagen added with MTGase could be divided into two types based on the concentration of MTGase incorporated. The first one was difficult to break by axial compression (incubated with enzyme at low concentration) and the second one was more fragile under axial compression, (incubated with enzyme at high concentration) (Nonaka *et al*., 1992; Erwanto *et al*., 2003). The decrease in breaking force in threadfin bream surimi, one of the good gel formers (Guenneugues *et al.*, 2005), might be due to the excessive and rapid aggregation of protein caused by both endogenous TGase and MTGase, particularly at the high level. The gel with random aggregate generally shows the poorer mechanical property (Ju and Kilara, 1998; Renkema *et al.*, 2001).

In the presence of EDTA, all surimi gels also had the increases in breaking force and deformation with increasing MTGase levels up to the particular level ($P < 0.05$). In the presence of EDTA, the lower breaking force and deformation were found in all surimi gels ($P < 0.05$). The result indicated that endogenous TGase played a role in setting, in which non-disulphide covalent bonds were formed. The higher breaking force and deformation were observed when EDTA was excluded. No differences in breaking force were found in surimi from Indian mackerel and sardine when MTGase greater than 0.2 and 0.4 units/g was added, respectively. Also, there was no difference in deformation between sardine surimi with and without EDTA when MTGase at levels of 0.2-0.6 units/g was added ($P > 0.05$). Thus, MTGase showed its efficacy in protein cross-linking, even when endogenous TGase was omitted. EDTA was able to chelate Ca^{2+}, which is required for activation of Ca-dependent endogenous TGase. The combined effect of endogenous TGase with MTGase added in surimi on gel strengthening was therefore obtained, but varied with species. Gel formation of Alaska pollack suirmi was totally inhibited in the presence of 5 mmol/kg EDTA (Kumazawa *et al.*, 1995). Van Phu *et al.* (2010) reported that the addition of 10 mmol/kg EDTA in surimi from white croaker resulted in the decrease in gel strength approximately 2.5-fold compared with that of gels without EDTA. Seki *et al.* (1990) found that the endogenous TGase is essentially involved in the setting of surimi and plays a major role in the strengthening surimi gel. Both endogenous TGase and exogenous MTGase enable to improve the functionality of surimi by increasing

protein cross-linking (Jiang *et al.*, 2000a). Asagami *et al.* (1995) reported that the amount of MTGase added for strengthening surimi gel highly depended on fish species and other factors such as freshness, protein quality, and harvesting season.

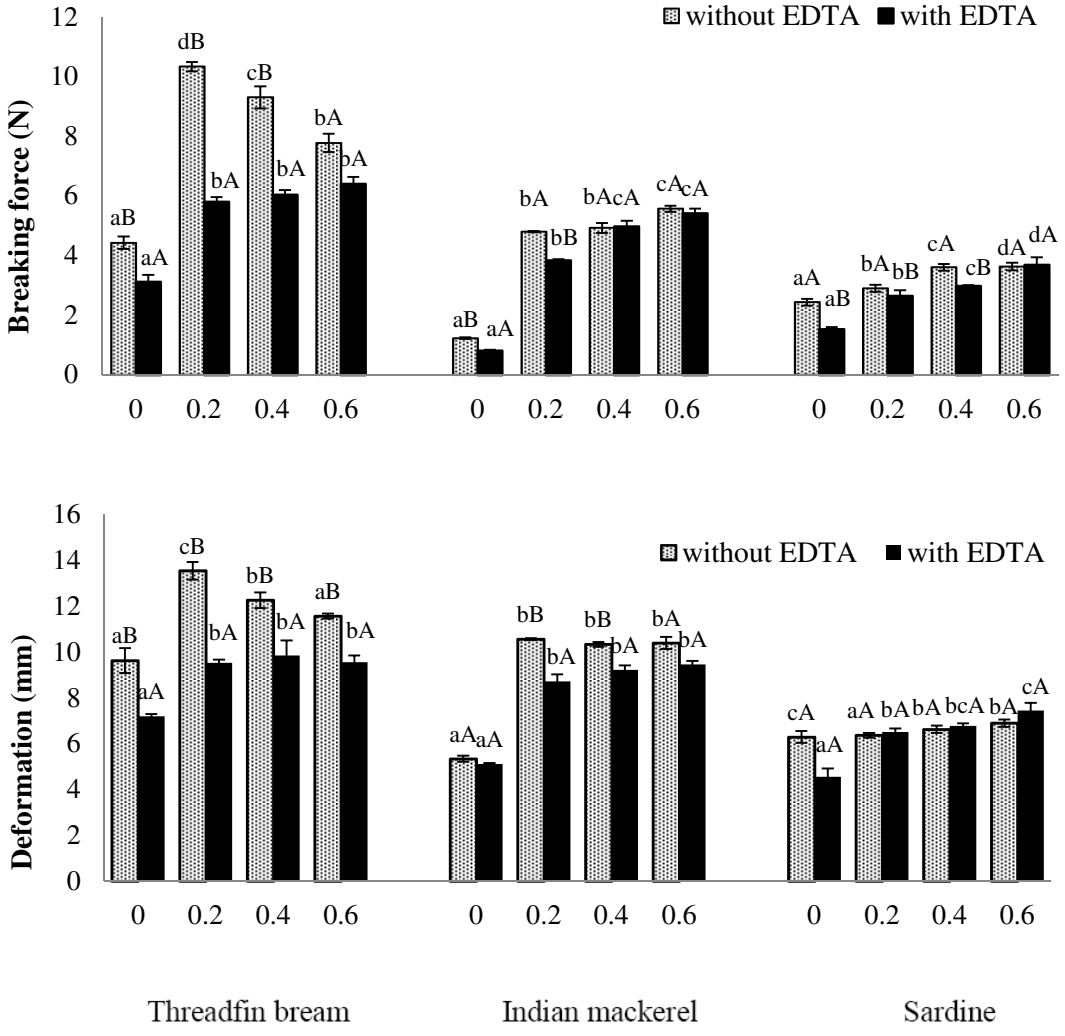


Figure 5. Breaking force and deformation of gels from threadfin bream, Indian mackerel, and sardine added with various levels of MTGase (0-0.6 units/g) in the absence and presence of EDTA. Bars represent the standard deviation (n=3). Different letters within same level of EDTA and the same fish species indicate significant differences ($P < 0.05$). Different capital letters within the same level of MTGase and the same fish species indicate significant differences ($P < 0.05$). Numbers designate the level of MTGase added (units/g).

2.4.1.2 Expressible moisture content

The expressible moisture content of surimi gel added with MTGase at various levels without and with EDTA is shown in Table 7. The decreases in expressible moisture content were observed with increasing MTGase levels for all surimi gels, regardless of EDTA addition. Addition of MTGase induced some increase in WHC of the samples and also a more porous microstructure (Han *et al.*, 2009; Moreno *et al.*, 2008). At the same levels of MTGase, the lowest expressible moisture content was observed in surimi gels from threadfin bream, a lean fish, indicating that a higher water amount was retained in the gel matrix. Different expressible moisture content between surimi gels from different species suggested the differences in water holding capacity of gel network. It has been reported that fatty and medium fatty fish do not respond to MTGase as well as lean fish, because fat has an adverse effect on the binding properties of the fish (Ju and Kilara, 1998). Higher fat content in surimi from Indian mackerel and sardine, dark-fleshed fish, might impede the aggregation of proteins in the gel matrix. Also, hydrophobicity of those lipids might lower the water binding capacity of gel. During setting at 40 °C, proteins underwent some denaturation and aligned themselves gradually to form the network, which can imbibe water (Benjakul *et al.*, 2003). When EDTA was added into gel, the increase in expressible moisture content was noticed. In the presence of EDTA, less calcium ion was available for TGase activation. As a consequence, a lower TGase activity was assumed, leading to a lower cross-linking of protein. The weakened gel matrix might have the lower water holding capacity. In general, lower expressible moisture content of gels suggested more water retained in the gel network (Niwa, 1992). The decrease in expressible moisture was in agreement with the higher breaking force and deformation for surimi gel from threadfin bream (Figure 5). However, this phenomenon was not found in surimi from Indian mackerel and sardine. There are several factors involving in water holding capacity of gels. Indian mackerel and sardine are pelagic dark-fleshed fish containing a higher amount of fats in comparison with threadfin bream. The fat remained in surimi might lower or have the influence on water binding of surimi gel. Additionally, the amino acid composition in different surimi varied, thereby affecting the water holding capacity

differently. When MTGase was added, the cross-linking of proteins could be enhanced, resulting in the formation of stronger network with the greater water holding capacity.

2.4.1.3 Whiteness

Slight increases in whiteness were found in surimi gels added with MTGase at higher levels in the absence of EDTA ($P < 0.05$). However, there was no difference in whiteness of surimi gels added with increasing levels of MTGase in the presence of EDTA ($P > 0.05$). Among surimi gels from three fish species, that from Indian mackerel had the lowest whiteness, followed by those from sardine and threadfin bream. Dark-fleshed fish such as mackerel and sardine has high content of dark muscle associated with high content of myoglobin, resulting in natural dark colour of surimi (Chaijan *et al.*, 2004; Chen, 2002). The whiteness of surimi gels increased when EDTA was incorporated. The increase in whiteness might be associated with the light scattering effect from released water of gel matrix, which was higher in gel added with EDTA. Thus, the addition of MTGase had generally no profound impact on resulting surimi gel.

2.4.1.4 Protein patterns

Protein patterns of surimi gels added with different levels of MTGase in the absence and presence of EDTA are depicted in Figure 6. Surimi paste contained myosin heavy chain (MHC) and actin as the major proteins. Nevertheless, MHC band intensity varied with species. The paste from sardine surimi contained the lower MHC band intensity than those from threadfin bream and Indian mackerel. Decrease in MHC band intensity was observed in the gel without MTGase addition, compared to that found in paste. The result suggested the formation of cross-linking stabilised by non-disulphide covalent bond mediated by endogenous TGase, especially during setting. Among all samples, no MHC band was retained in surimi gel from sardine which possibly resulted from endogenous TGase. Less initial amount of MHC might undergo cross-linking almost completely during setting, resulting in the negligible

Table 7 Expressible moisture content and whiteness of gels from threadfin bream, Indian mackerel and sardine surimi added with different levels of MTGase (0-0.6 units/g) in the absence and presence of EDTA (10 mmol/kg)

Parameters	MTGase levels	Threadfin bream		Indian mackerel		Sardine	
		Without EDTA	With EDTA	Without EDTA	With EDTA	Without EDTA	With EDTA
Expressible moisture content (%)	0	2.98 ± 0.08^{bA}	3.43 ± 0.10^{aB}	5.51 ± 0.28^{cA}	5.68 ± 0.06^{bA}	4.83 ± 0.13^{bA}	4.90 ± 0.05^{bB}
	0.2	2.89 ± 0.53^{bA}	3.40 ± 0.33^{aB}	4.83 ± 0.18^{bA}	5.55 ± 0.26^{abB}	4.76 ± 0.17^{bA}	4.81 ± 0.56^{bA}
	0.4	2.66 ± 0.22^{abA}	3.30 ± 0.51^{aB}	4.79 ± 0.26^{aA}	5.44 ± 0.22^{abB}	4.67 ± 0.29^{bA}	4.76 ± 0.30^{abA}
	0.6	2.35 ± 0.33^{aA}	3.24 ± 0.41^{aB}	4.60 ± 0.47^{aA}	5.24 ± 0.10^{aB}	4.40 ± 0.31^{aA}	4.58 ± 0.26^{aA}
Whiteness	0	79.59 ± 0.53^{abA}	83.82 ± 0.31^{aB}	67.38 ± 0.36^{aA}	70.37 ± 0.41^{aB}	71.72 ± 0.32^{aA}	75.55 ± 0.44^{aB}
	0.2	79.25 ± 0.14^{aA}	83.88 ± 0.52^{aB}	68.33 ± 0.36^{bA}	70.74 ± 0.47^{aB}	72.39 ± 0.79^{abA}	75.88 ± 0.75^{aB}
	0.4	79.59 ± 0.61^{bA}	83.96 ± 0.25^{aB}	68.89 ± 0.11^{cA}	70.80 ± 0.08^{aB}	72.62 ± 0.07^{bA}	75.98 ± 0.15^{aB}
	0.6	80.13 ± 0.26^{bA}	83.98 ± 0.26^{aB}	68.30 ± 0.23^{bA}	70.79 ± 0.50^{aB}	72.74 ± 0.32^{bA}	76.22 ± 0.26^{aB}

Values are given as mean ± SD (n=3)

* Different letters within the same column under the same parameter tested indicate the significant differences ($P < 0.05$).

** Different capital letters within the same row under the same fish species indicate the significant differences ($P < 0.05$).

MHC retained. Montero and Gomez-Guillen (1996) found that kamaboko gel of sardine (preset at 35 °C and then cooked at 90 °C) had no MHC remaining as a consequence of setting, in which more covalent bonds could be formed. Furthermore, proteolysis of MHC stilled occurred to some extent during setting (Montero and Gómez-Guillén, 1996). The highest MHC band intensity in gel without MTGase addition was observed in surimi from Indian mackerel, suggesting the poorest setting phenomenon in this species. This was in accordance with the lowest breaking force and deformation of this species (Figure 5). MHC was most susceptible to cross-linking during setting (Benjakul *et al.*, 2003). MHC band disappeared when 0.2 units/g was added in all gel samples evidenced the occurrence of MHC cross-linking via non-disulphide covalent bond (Jiang *et al.*, 2000b). No marked changes in actin were obtained in the presence and absence of MTGase. MHC appeared to be a preferable substrate for cross-linking induced by either endogenous TGase or MTGase. Nevertheless, actin could not serve as a substrate for TGase. Nakahara *et al.* (1999) reported that MTGase and carp TGase could not cross-link actin molecules. The decrease in MHC band intensity was concomitant with the increased breaking force and deformation (Figure 5). Thus, MTGase together with endogenous TGase effectively induced polymerisation of MHC, a protein contributing to the gel network formation. As a result, the stronger gel network was formed. The result was in accordance with Jiang *et al.* (1998) who reported that cross-linking of MHC occurred rapidly in mackerel surimi added with MTGase.

In the presence of EDTA, MHC band was more retained in all samples, especially when MTGase at level of 0 and 0.2 units/g was added. In the presence of EDTA, less calcium ion was available for endogenous TGase activation. As a consequence, a lower TGase activity was assumed, leading to a lower cross-linking of protein, especially MHC. Although there was no MHC retained in all surimi when MTGase particularly at levels higher than 0.2 units/g was added, gel property was different with species. Therefore, not only degree of cross-linking, but also configuration of gel matrix as well as other intrinsic factors determined gel properties of surimi.

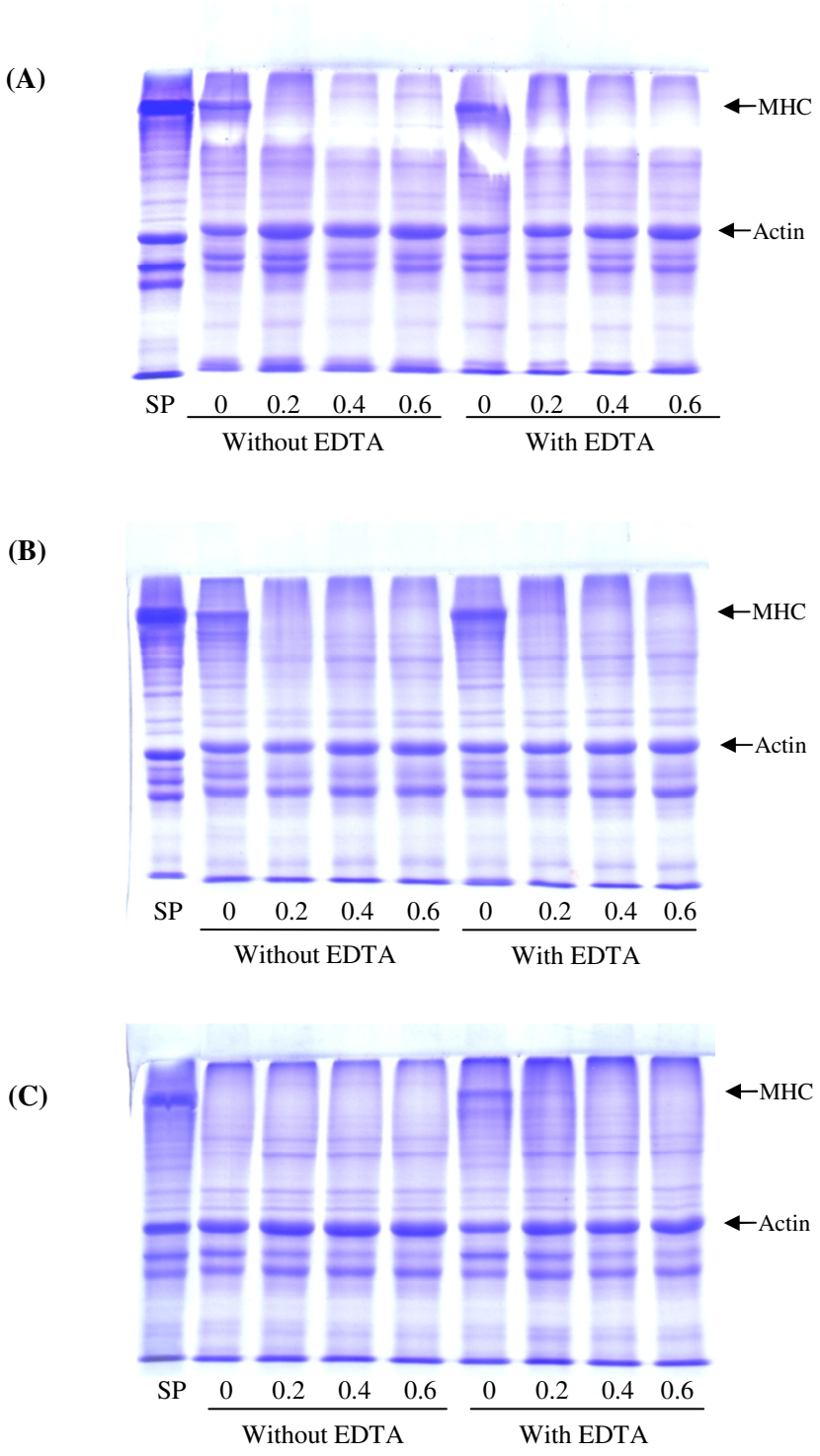


Figure 6. Protein pattern of surimi gels from threadfin bream (A), Indian mackerel (B), and sardine (C) added with various levels of MTGase (0-0.6 units/g) in the absence and presence of EDTA. MHC: myosin heavy chain. Numbers designate the level of MTGase added (units/g). SP: surimi paste; MHC: myosin heavy chain

2.4.2 Effect of MTGase on protein cross-linking of NAM from different fish species

2.4.2.1 ε-amino group content

Changes in ε-amino group contents of natural actomyosin (NAM) from threadfin bream, Indian mackerel, and sardine added with 2 levels of MTGase are demonstrated in Figure 7. Addition of MTGase into NAM resulted in the decrease in ε-amino group content, especially within the first 10 min ($P < 0.05$). During setting at 40 °C for 10 min, MTGase catalysed an acyl transfer reaction between the γ-carboxyamide group of a peptide-bound glutaminyl residue (acyl donors) and ε-amino group of a peptide-bound lysyl residue. Peptide chains are covalently connected through ε-(γ-glutamyl)lysine (GL) bonds. This resulted in the reduction of ε-amino group content in NAM. NAM extracted from threadfin bream with the highest amount of ε-amino group content (32.46×10^{-5} mol Lys/g) had the highest reduction rate, followed by NAM from sardine and mackerel, respectively. After 10 min of incubation, threadfin bream NAM had the lower ε-amino group content, especially as the amount of MTGase increased. On the other hand, no further changes in ε-amino group content were obtained after 10 min. Thus, cross-linking of proteins mainly occurred within the first 10 min. ε-amino group contents of threadfin bream NAM at 10 min of incubation added with 10 or 20 units/g protein decreased by 15.8% or 21.4%, respectively, compared with those of initial sample. At the same incubation time (10 min), ε-amino group content of NAM from Indian mackerel and sardine decreased by 13.1 and 16.4%, compared with that observed at time 0 min. Jiang *et al.* (2000) found that MTGase-containing hairtail actomyosin incubated at 45 °C decreased greatly at the first 5 min of incubation and cross-linking of MHC also became prominent within a short period. MTGase acts as an intermediate factor in coupling glutamyl and lysine residues in one strong complex by affinity relation through covalent bonds between 3-glutamyl and 5-lysine. The lowest amount of ε-amino group content in sardine was in accordance with the lowest improvement of gel strength by MTGase (Figure 5). The result suggested that the limited ε-amino group content also affected protein cross-linking by MTGase. Ahhmed *et al.* (2009) suggested that there are remarkable differences in the myofibrillar protein within the

same muscle type in different species. Although TGase is known to catalyse the cross-linking between glutamine and lysine residues in proteins, structure of substrate containing glutamine and lysine residues had the direct impact on cross-linking induced by TGase. The compact globular structures of the 11S and 7S soybean proteins, for instance, make them rather poor substrates for TGase, despite their relatively high glutamine contents (Larre *et al.*, 1992). Thus, the decreasing rate of ε-amino group content might be another factor affecting the cross-linking as well as gel-forming ability of proteins in surimi.

2.4.2.2 Protein patterns

NAM from threadfin bream, Indian mackerel and sardine were incubated with 2 levels of MTGase at 40 °C for up to 30 min and their protein patterns were analysed by SDS-PAGE (Figure 8). The intensity of MHC band decreased as the incubation time increased. The disappearance of MHC indicated the formation of MHC cross-links mainly via the formation the ε-(γ-glutamyl) lysine isopeptide induced by MTGase. Crosslinks were not be dissociated by the mixture of SDS and mercaptoethanol used for electrophoresis (Jiang *et al.*, 1998). When MTGase at a level of 10 units/g protein was used, MHC band intensity of threadfin bream and sardine NAM rapidly decreased within the first 5-10 min of incubation, which was correlated with the reduction of ε-amino group contents (Figure 7). Threadfin bream NAM showed the highest MHC band intensity, followed by Indian mackerel and sardine respectively. MHC has been known to serve as the major contributor for gel formation and determine gel property of fish protein (Nakahara *et al.*, 1999). With the higher content of preferable substrate, MHC, MTGase could induce the cross-linking of protein substrate to a higher extent. Jiang *et al.*(2000) reported that polymerisation of MHC caused by MTGase in pollack surimi was much faster in golden threadfin bream surimi. At the same incubation time, the less MHC band was retained as the higher amount of MTGase (20 units/g protein) was added. Although MHC band intensity decreased markedly, actin band was rarely changed in NAM from sardine and Indian mackerel. However, actin band intensity of NAM from threadfin bream slightly decreased with increasing incubation time, especially at higher MTGase concentration.

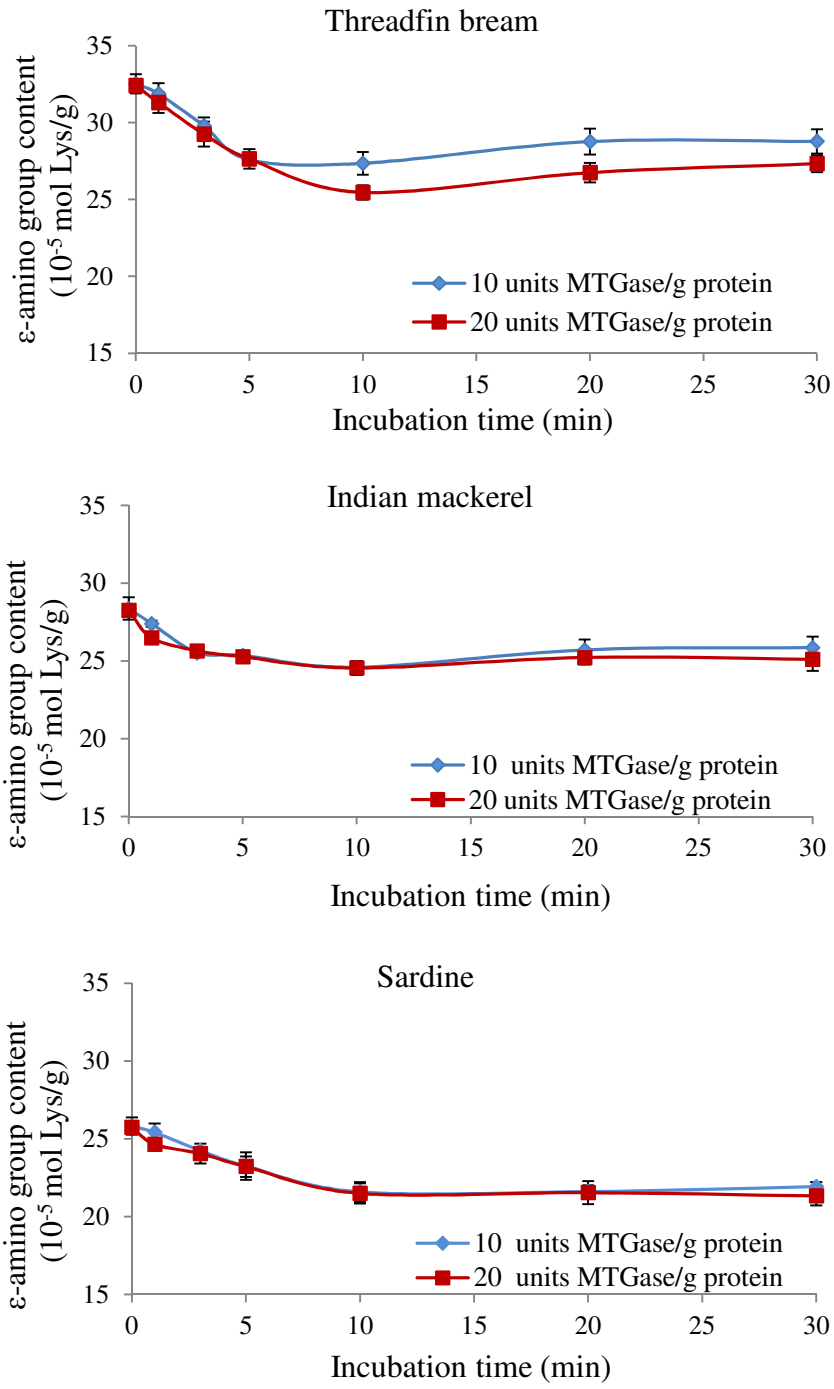


Figure 7. Changes in ε-amino group contents of natural actomyosin from bream, Indian mackerel, and sardine added with MTGase (10 and 20 units/g protein) as a function of incubation time at 40 °C. Bars represent the standard deviation (n=3).

Araki and Seki (1997) reported that the rates of changes in MHC from various fish actomyosin incubated with carp TGase at 25 °C were significantly different and concluded that the rate of TGase-mediated cross-linking depends upon the substrate specificity. For NAM from Indian mackerel and sardine, it was found that protein with MW of 35 kDa, which was more likely to be Troponin-T, decreased in band intensity when incubation time was longer than 5 min. That protein was decreased in threadfin bream NAM as the incubation time was longer than 10-20 min. Thus, troponin-T was also served as substrate for cross-linking reaction induced by MTGase, but reactivity for reaction was governed by species. Coincidentally, the protein with MW approximately 70 kDa was found in all samples especially for NAM from sardine. This might be the dimer of tropomyosin induced by MTGase. Nakahara *et al.* (1999) found that MTGase preferentially cross-linked connectin, followed by MHC, troponin-T and actin respectively. Thus, the cross-linking of NAM from different species varied and substrate specificity was also different.

2.5 Conclusion

The addition of MTGase enhanced the gel-forming ability of surimi from three fish species, but the strengthening effect varied. Gels from threadfin bream which had the highest ε-amino group content showed the highest gel strength, when MTGase (0.2-0.6 units/g) was added, followed by Indian mackerel and sardine, respectively. The reactivity of muscle proteins toward MTGase induced cross-linking and MHC proportion were the key factors determining the gel strength of surimi or fish muscle proteins. Thus, the composition and property of muscle proteins of varying fish species, which more likely determined the ability of MTGase in protein cross-linking, should be further investigated.

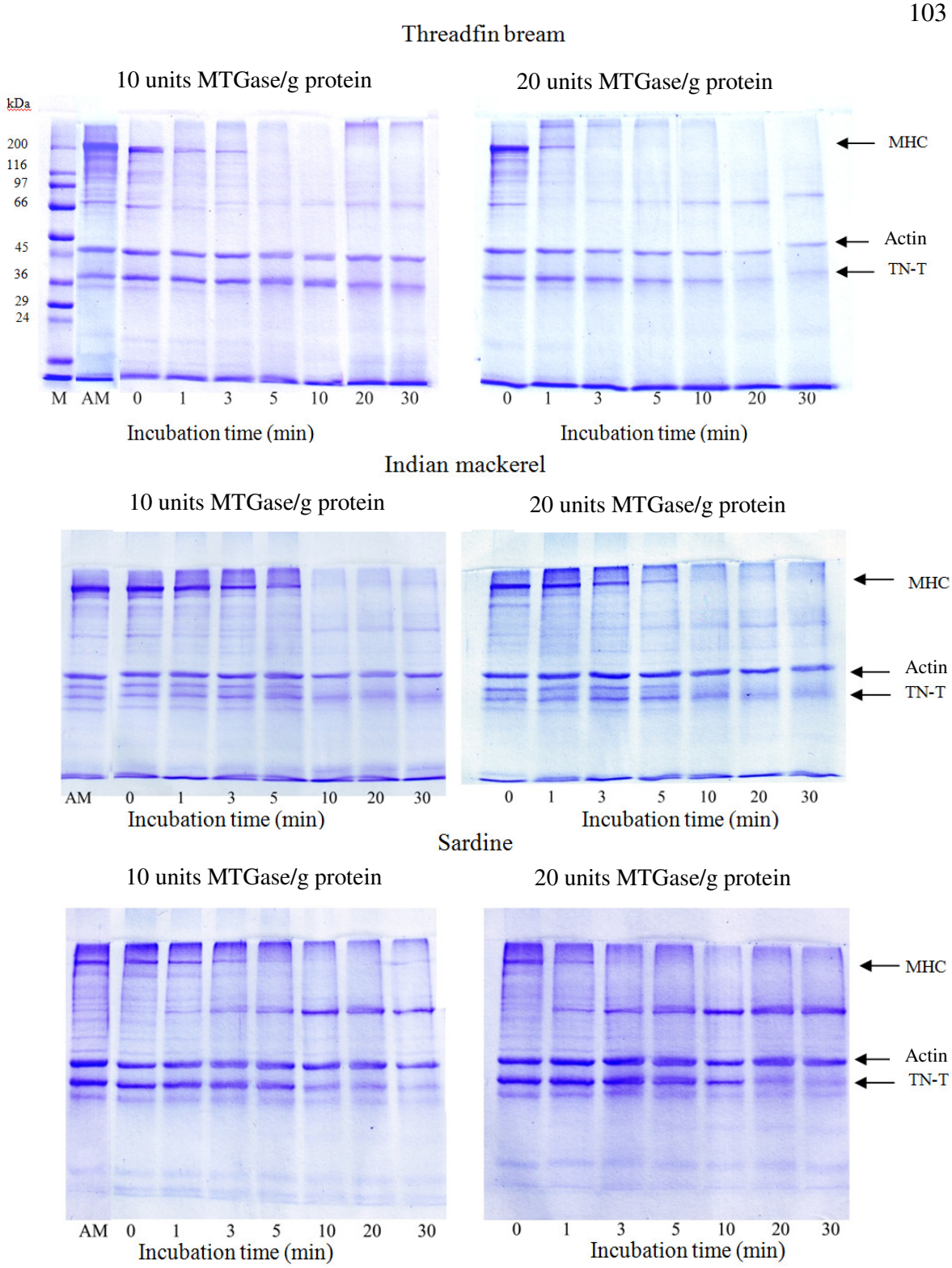


Figure 8. Protein patterns of natural actomyosin from threadfin bream, Indian mackerel, and sardine added with MTGase at 10 and 20 units/g protein as a function of incubation time at 40 °C. AM: actomyosin; MHC: myosin heavy chain; TN-T: troponin-T. Numbers designate the incubation time (min).

2.6 References

Ahhmed, A. M., Kuroda, R., Kawahara, S., Ohta, K., Nakade, K., Aoki, T., and Muguruma, M. 2009. Dependence of microbial transglutaminase on meat type in myofibrillar proteins cross-linking. Food Chem. 112: 354-361.

Araki, H., and Seki, N. 1993. Comparison of reactivity of transglutaminase to various fish actomyosins. Nippon Suisan Gakkaishi. 59: 711-716.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Benjakul, S., Visessanguan, W., and Pecharat, S. 2004. Suwari gel properties as affected by transglutaminase activator and inhibitors. Food Chem. 85: 91-99.

Bubnis, W. A., and Ofner, C. M. 1992. The determination of ε-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. Anal. Biochem. 207: 129-133.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2004. Characteristics and gel properties of muscles from sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) caught in Thailand. Food Res. Int. 37: 1021-1030.

Chen, H. H. 2002. Decoloration and gel forming ability of horse mackerel mince by air flotation washing. J. Food Sci. 67: 2970-2975.

Erwanto, Y., Kawahara, S., Katayama, K., Takenoyama, S., Fujino, H., Yamauchi, K., Morishita, T., Kai, Y., Watanabe, S., and Muguruma, M. 2003. Microbial transglutaminase modifies gel properties of porcine collagen. Asian-Aust. J. Anim. Sci. 16: 269-276.

Folk, J. E. 1970. Transglutaminase. Methods Enzymol. 17: 889-894.

Folk, J. E. 1980. Transglutaminases. Annu. Rev. Biochem. 49: 517-531.

Folk, J. E., and Finlayson, J. S. 1977. The ε-(γ-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv. Protein Chem. 31: 1-133.

Guenneugues, P., Morrissey, M. T., and Park, J. W. 2005. Surimi resources. *In* Surimi and surimi seafood (Park, J. W. eds.). p. 3-32. Taylor and Francis. Boca Raton, Florida.

Han, M., Zhang, Y., Fei, Y., Xu, X., and Zhou, G. 2009. Effect of microbial transglutaminase on NMR relaxometry and microstructure of pork myofibrillar protein gel. Eur. Food Res. Technol. 228: 665-670.

Jiang, S. T., Leu, S. Z., and Tsai, G. J. 1998. Cross-linking of mackerel surimi actomyosin by microbial transglutaminase and ultraviolet irradiation. J. Agric. Food Chem. 46: 5278-5282.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000a. Combination effects of microbial transglutaminase, reducing agent, and protease inhibitor on the quality of hairtail surimi. J. Food Sci. 65: 241-245.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000b. Microbial transglutaminase affects gel properties of golden threadfin bream and pollack Surimi. J. Food Sci. 65: 694-699.

Ju, Z., and Kilara, A. 1998. Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. J. Dairy Sci. 81: 925-931.

Kumazawa, Y., Numazawa, T., Seguro, K., and Motoki, M. 1995. Suppression of surimi gel setting by transglutaminase inhibitors. J. Food Sci. 60: 715-717.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Larre, C., Kedzior, Z. M., Chenu, M. G., Viroben, G., and Gueguen, J. 1992. Action of transglutaminase on an 11 S seed protein (pea legumin): influence of the substrate conformation. J. Agric. Food Chem. 40: 1121-1126.

Martin-Sanchez, A. M., Navarro, C., Perez-Alvarez, J. A., and Kuri, V. 2009. Alternatives for efficient and sustainable production of surimi: a review. Comp. Rev. Food Sci. F. 8: 359-374.

Montero, P., and Gómez-Guillén, C. 1996. Thermal aggregation of sardine muscle proteins during processing. J. Agric. Food Chem. 44: 3625-3630.

Moreno, H. M., Carballo, J., and Borderias, A. J. 2008. Influence of alginate and microbial transglutaminase as binding ingredients on restructured fish muscle processed at low temperature. J. Sci. Food Agric. 88: 1529-1536.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

Niwa, E. 1992. Chemistry of surimi gelation. *In* Surimi Technology (Lanier, T. C., and Lee, C. eds.). p. 389-427. Marcel Dekker. New York.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., and Motoki, M. 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J. Food Sci. 57: 1214-1241.

Nozawa, H., Cho, S. Y., and Seki, N. 2001. Purification and characterization of transglutaminase from squid gill. Fish. Sci. 67: 912-919.

Park, J. W. 1994. Functional protein additives in surimi gels. J. Food Sci. 59: 525-527.

Renkema, J., Knabben, J., and Van Vliet, T. 2001. Gel formation by β-conglycinin and glycinin and their mixtures. Food Hydrocolloid. 15: 407-414.

Robinson, H. W., and Hogden, C. G. 1940. The biuret reaction in the determination of serum proteins. 1. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135: 707-725.

Sakamoto, H., Kumazawa, Y., Toiguchi, S., Seguro, K., Soeda, T., and Motoki, M. 1995. Gel strength enhancement by addition of microbial transglutaminase during onshore surimi manufacture. J. Food Sci. 60: 300-304.

Seki, N., Uno, H., Lee, N. H., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in Alaska pollack muscle and surimi and its reaction with myosin B. Nippon Suisan Gakkaishi. 56: 125–132.

Shimizu, Y., Toyohara, H., and Lanier, T. C. 1992. Surimi production from fatty and dark-fleshed fish species. *In* Surimi Technology (Lanier, T. C. and Lee, C eds.). p. 181-207. Marcel Dekker. New York.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

Tsai, G. J., Lin, S. M., and Jiang, S. T. 1996. Transglutaminase from *Streptoverticillium ladakanum* and application to minced fish product. J. Food Sci. 61: 1234-1238.

Tsukamasa, Y., Sato, K., Shimizu, Y., Imai, C., Sugiyama, M., Minegishi, Y., and Kawabata, M. 1993. ε-(γ-glutamyl)-lysine crosslink formation in sardine myofibril sol during setting at 25 °C. J. Food Sci. 58: 785-787.

Van Phu, N., Morioka, K., and Itoh, Y. 2010. Gel-forming characteristics of surimi from white croaker under the inhibition of the polymerization and degradation of protein. J. Biol. Sci. 10: 432-439.

Yongsawatdigul, J., Worratao, A., and Park, J. W. 2002. Effect of endogenous transglutaminase on threadfin bream surimi gelation. J. Food Sci. 67: 3258-3263.

CHAPTER 3

NON-PROTEIN NITROGENOUS COMPOUNDS AND GELLING PROPERTY OF WHITECHEECK SHARK (*CARCHARHINUS DUSSUMIERI*) MINCE AS AFFECTED BY WASHING AND MICROBIAL TRANSGLUTAMINASE

3.1 Abstract

Changes in non-protein nitrogenous compounds and gelling properties of whitecheek shark (*Carcharhinus dussumieri*) meat during iced storage for 12 days were monitored. Total volatile base (TVB), trimethylamine (TMA) and ammonia contents increased, whereas urea content decreased during the storage ($P < 0.05$). TVB, TMA, urea and ammonia contents were lower in washed mince, indicating the removal of those compounds by washing. The addition of microbial transglutaminase (MTGase, 0.6 units/g) was able to increase breaking force, deformation and water-holding capacity of both gels from mince and washed mince. Nevertheless, gel strengthening effect of MTGase was lowered when mince or washed mince were obtained from shark stored for an extended time. Polymerisation of myosin heavy chain was enhanced when MTGase was incorporated but took place at a lower degree for mince and washed mince produced from unfresh shark. Washing process in combination with MTGase addition could therefore improve gel quality of shark meat.

3.2 Introduction

Sharks have been used as human food, especially for Chinese cuisine. Among their products, shark fins are in demand. However, some shark species have been considered as by-catch or underutilised species. Shark flesh has low market value, despite being a good source of protein. This may be associated with the peculiar characteristics such as appearance, meat odour and taste, especially their ammonia like smell, which is caused by the decomposition of urea during extended handling and storage (Venugopal *et al.*, 1994). Because of a large amount of urea and

ammonia, the shelf-life of flesh is limited. To better exploit shark flesh, the conversion to surimi and surimi products by an appropriate washing, in which those offensive undesirable compounds can be eliminated, is promising. Simultaneously, myofibrillar proteins can be concentrated, and soluble proteins, blood, colour pigments and fat in muscle could be reduced (Park and Morrissey, 2000). Generally, washing of fish mince results in an increase in gel strength (Lanier *et al.*, 2004; Phatcharat *et al.*, 2006). Surimi is also considered an ingredient of many products such as kamaboko, fish sausages and fish balls. Surimi can be used as a binder in restructured meat products or as a carrier in high omega-3 fatty acid-containing seafood products (Tolasa *et al.*, 2010; Ramírez *et al.*, 2011). Theoretically, any fish can be used for surimi production, but properties of surimi gel could be varied, depending on fish species. Both intrinsic and extrinsic factors, including species, freshness, endogenous enzyme, processing parameters, protein concentration, ionic strength, and temperature, determine the gelling properties of surimi (Niwa, 1992; Shimizu *et al.*, 1992; Benjakul *et al.*, 2003a). In general, lean fish such as threadfin bream, bigeye snapper and croaker have been used for surimi production in Thailand Benjakul *et al.*, 2003a). Due to the awareness on the insufficient fish resources, the underutilised and by-catch species have gained attention for surimi production (Martin-Sanchez *et al.*, 2009). Elasmobranch (sharks, rays and skates), considered underutilised, was reported to possess gel-forming ability (Venugopal *et al.*, 1994).

To improve the gel properties of surimi, microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins via the formation of ε-(γ- glutamyl) lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and γ-carboxyamide group of a glutamine residue (DeJong and Koppelman, 2002). However, the efficiency of MTGase in improving the gel property of proteins depends on many factors, e.g., amount of MTGase, type of fish and fat content (Asagami *et al.*, 1995; DeJong and Koppelman, 2002; Visessanguan *et al.*, 2003). Owing to the high content of non-protein nitrogenous compounds in shark meat, poor gel is generally obtained with undesirable smell. Appropriate washing to remove those compounds, along with the use of MTGase, can be a means to improve the gel properties of shark meat. Therefore, the

objective of the study was to investigate the effect of washing and MTGase on the gel properties of shark stored in ice for various times.

3.3 Materials and Methods

3.3.1 Chemicals

All chemicals were of analytical grade. Urease, α-ketoglutarate, sodium dodecyl sulfate (SDS), β- mercaptoethanol (β-ME), Coomassie Brilliant Blue R-250, glutamate dehydrogenase and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). *N*,*N*,*N'*,*N'*-tetramethylethylenediamine, acrylamide and bis-acrylamide were procured from Fluka (Buchs, Switzerland). Microbial transglutaminase (MTGase) from *Streptoverticillium mobaraense* (TG-K) containing 1% pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand). Enzyme powder contained MTGase at 100 units/g, as determined by hydroxamate assay (Folk, 1970).

3.3.2 Shark samples

Whitecheek sharks (*Carcharhinus dussumieri*), with an average weight of 1–1.2 kg, were purchased from a dock in Songkhla, Thailand. Sharks, off-loaded approximately 24–36 h after capture, were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand, within 3 h.

3.3.3 Effect of iced storage on chemical changes of shark meat

3.3.3.1 Preparation of ice-stored shark.

Upon arrival, sharks were washed and kept in the polystyrene box containing crushed ice with a fish/ice ratio 1:2 (w/w). The sharks were placed between the layers of ice. The box containing sharks was kept at room temperature (28–30 °C). To maintain the ice content, molten ice was removed and replaced with an equal amount of ice every 2 days. The temperature of sharks was 4–10 °C throughout the 12 days of storage. Sharks were randomly taken at days 0, 4, 8 and 12

for analyses and gel preparation. At the time designated, sharks were washed and flesh was collected manually. The flesh was minced using a mincer with a hole diameter of 5 mm. Shark mince obtained was divided into two portions: (1) mince and (2) washed mince. To prepare washed mince, the conventional washing process was implemented. Mince was washed with cold water (0–4 °C) using a water/mince ratio of 3:1 (v/w). The mixture was gently stirred for 10 min in a cold room (4 °C) and the washed mince was filtered with a layer of nylon screen (3-mm mesh screen). Washing was performed three times. Finally, the washed mince was centrifuged at 700xg for 15 min using a basket centrifuge (model CE 21K, Grandiumpiant, Belluno, Italy). Both mince and washed mince were subjected to analyses.

3.3.3.2 Total Volatile Base (TVB) and Trimethylamine (TMA) contents.

TVB and TMA contents were determined using the Conway microdiffusion assay, according to the method of Conway and Byrne (1936). The sample (2 g) was mixed with 8 ml of 4% trichloroacetic acid. The mixture was homogenised at 6,500 rpm using a homogeniser (IKA Labortechnik, Selangor, Malaysia) for 1 min. The homogenate was filtered using a Whatman No. 41 filter paper (Whatman Ltd., Maidstone, UK) and the filtrate was. used for analysis. To determine TMA content, formaldehyde was added to the filtrate to fix ammonia present in the sample. TVB and TMA were released after the addition of saturated K_2CO_3 and diffused into the boric acid solution. The titration of solution was performed and the contents of TVB or TMA were calculated.

3.3.3.3 Urea and ammonia content

Urea and ammonia contents were determined using an enzymatic determination according to the method of Cheuk and Finne (1984), with a slight modification. Ten grams of mince or washed mince was extracted with 20 ml of 1 N perchloric acid. The mixtures were homogenised using a homogeniser for 5 min at a speed of 11,000 rpm. The homogenate was then centrifuged at 6,500 xg for 15 min using a refrigerated centrifuge (Beckman Coulter, Avanti JE Centrifuge, Fullerton,

CA, USA).Ten milliliters of supernatant was neutralised with 2 M potassium hydroxide solution. The mixture was filtered through a Whatman No.1 filter paper. For ammonia determination, the filtrate (0.1 ml) was mixed thoroughly with 1 ml of buffer (0.5 M triethanolamine and 35 mM α-ketoglutarate, pH 8.6), 0.1 ml of 6 mM NADH solution and 1.9 ml of distilled water. After 3 min, the absorbance was read at 340 nm ($A1$). The reaction was started by the addition of 0.02 ml of 10 mM glutamate dehydrogenase. The reaction mixture was kept at room temperature (24–28 °C) for 25 min. Absorbance at 340 nm was read ($A2$). To determine urea, 0.02 ml of urease (2.5 g/l) was added into the reaction mixture. Reaction was extended for another 25 min at room temperature (24–28 °C). The final absorbance ($A3$) was recorded and the urea and ammonia concentrations were calculated as follows:

$$C = \frac{V \times EW \times \Delta A}{W \times \varepsilon \times d \times v \times 10}$$

where C: concentration of ammonia or urea (mg/100 g); V: final volume (ml); v: sample volume (ml); EW: equivalent weight of ammonia (17.03 g/mol); w: sample weight (g); d: light path (cm); ε: extinction coefficient of NADH at 340 nm (6.3 $M^{-1}cm^{-1}$); ΔA for ammonia is difference between A_1 and A_2, whilst that for urea is the difference between A_2 and A_3.

3.3.4 Effect of MTGase on gel properties of mince and washed mince from shark stored in ice for various times

3.3.4.1 Preparation of washed mince

Sharks stored in ice for 0, 4, 8, and 12 days were used and washing was performed in the same manner as previously described.

3.3.4.2 Preparation of gel

Mince and washed mince from shark stored for 0, 4, 8, and 12 days were used for gel preparation. The samples were ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). Moisture content was adjusted to 80% and NaCl (2.5% w/w) was added to the samples. After grinding for 2 min, the paste was

added with MTGase (0.6 units/g). The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature-controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

3.3.4.2.1 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

3.3.4.2.2 Determination of expressible moisture content.

Expressible moisture content (%) was determined according to the method of Benjakul *et al*. (2003a). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between two pieces of Whatman no. 1 filter paper at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y)*. Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = [(X - Y)/X]\text{x}100$$

3.3.4.2.3 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (NFI, 1991).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

3.3.4.2.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution (85 °C) were added to the sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 × g for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

3.3.4.2.5 Microstructure

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100%. Samples were subjected to critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

3.3.5 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. T-test was used for pair comparison (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).

3.4 Results and Discussion

3.4.1 TVB, TMA, urea and ammonia contents of mince and washed mince from whitecheek shark stored in ice

Changes in TVB and TMA contents of mince obtained from shark during iced storage are shown in Figure 9A and 9B, respectively. When storage time increased, both TVB and TMA contents in mince increased ($P < 0.05$). The increases in TVB and TMA contents in mince indicated that the decomposition of nitrogenous compounds became more pronounced with increasing storage time. The formation of TVB is generally associated with the growth of microorganisms and it can be used as a spoilage indicator (Benjakul *et al.*, 2003b). Generally, TVB include trimethylamine, dimethylamine, ammonia and other compounds, produced by both microbial and endogenous enzymes (Benjakul *et al.*, 2002). A number of specific spoilage bacteria, such as *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Vibrionaceae*, typically use trimethylamine oxide as an electron acceptor in anaerobic respiration, resulting in the formation of off-odour and off-flavour (Gram and Huss, 1996; Ocaño-Higuera *et al.*, 2009). As the fish samples were kept in ice, the formation of TVB and TMA was probably mediated by psychrotrophic bacteria (Sasajima, 1973). Additionally, other non-protein nitrogenous compounds such as urea can be decomposed into low molecular weight compounds by microorganisms (Tomiyasu and Zenitani, 1957). Mathew *et al*. (2002) reported that the reduction of urea in icestored shark (*Scoliodon laticaudus*) was coincidental with the increase in TVB content. TVB content of 30–35 mg N/100 g is regarded as the acceptability limit for different fish species. However, this limit is 50 mg N/100 g for sharks and rays, which

contain high amount of urea (Sikorski *et al.*, 1990; Dalgaard, 2000). At the end of iced storage (12 days), TVB content of mince was lower than the limit (30.65 mg N/100 g), whereas TMA content in mince (6.47 mg N/100 g) was higher than 5 mg N/100 g, which was proposed as a rejection limit by Sikorski *et al*. (1990). After washing, washed mince had the lower TVB and TMA contents, compared with mince. The result indicated that washing process could remove volatile-based compounds to a high degree. TVB and TMA contents were decreased by 59.8–63.6 and 72.7–73.1%, respectively, after washing. This could reduce offensive odour in the resulting washed mince.

Urea and ammonia contents of mince and washed mince obtained from shark during iced storage are depicted in Figure 9C and 9D, respectively. At day 0, mince had urea content of 89.72 mg/100 g. Urea content in washed mince was decreased by 46.7% compared with that found in mince. Upon 12 days of iced storage, urea content in mince and washed mince decreased by 83.3 and 92.5%, respectively, compared with those found at the initial storage time (day 0). On the contrary, ammonia content in both mince and washed mince increased as the storage time increased ($P < 0.05$). At day 0, mince had ammonia content of 3.24 mg/100 g, which was higher than washed mince by 86.7%. Generally, urea and ammonia contents in washed mince were lower than those found in mince. Urea and ammonia are water-soluble and could be leached out by washing. The result was in accordance with Mathew *et al*. (2002) who reported that urea and ammonia could be easily removed from shark meat during water washing process of surimi production. The degradation of urea and the accumulation of ammonia were correlated with the increases of TVB in both samples (Figure 9A). The increase in TVB content can be explained as a consequence of microbial decomposition of urea to ammonia. Elasmobranches (sharks and rays) contain high amount of urea in blood and tissue to maintain their osmotic balance (Hazon *et al.*, 2003). Urea should be removed immediately by bleeding. Furthermore, dressing and icing are recommended after capture of shark (Schubring, 2007). Urea is broken down to ammonia by urease in muscle and bacteria. Improper handling caused a strong ammonia odour and taste, which limited the shelf-life of shark meat to a few days (Kailasapathy and

Salampessy, 1999). Sengör *et al*. (2007) reported that the maximum shelf-life of spiny dogfish stored in ice was 8–12 days. Therefore, storage time in ice should be minimised and washing process could be implemented to reduce urea and ammonia content presented in muscle.

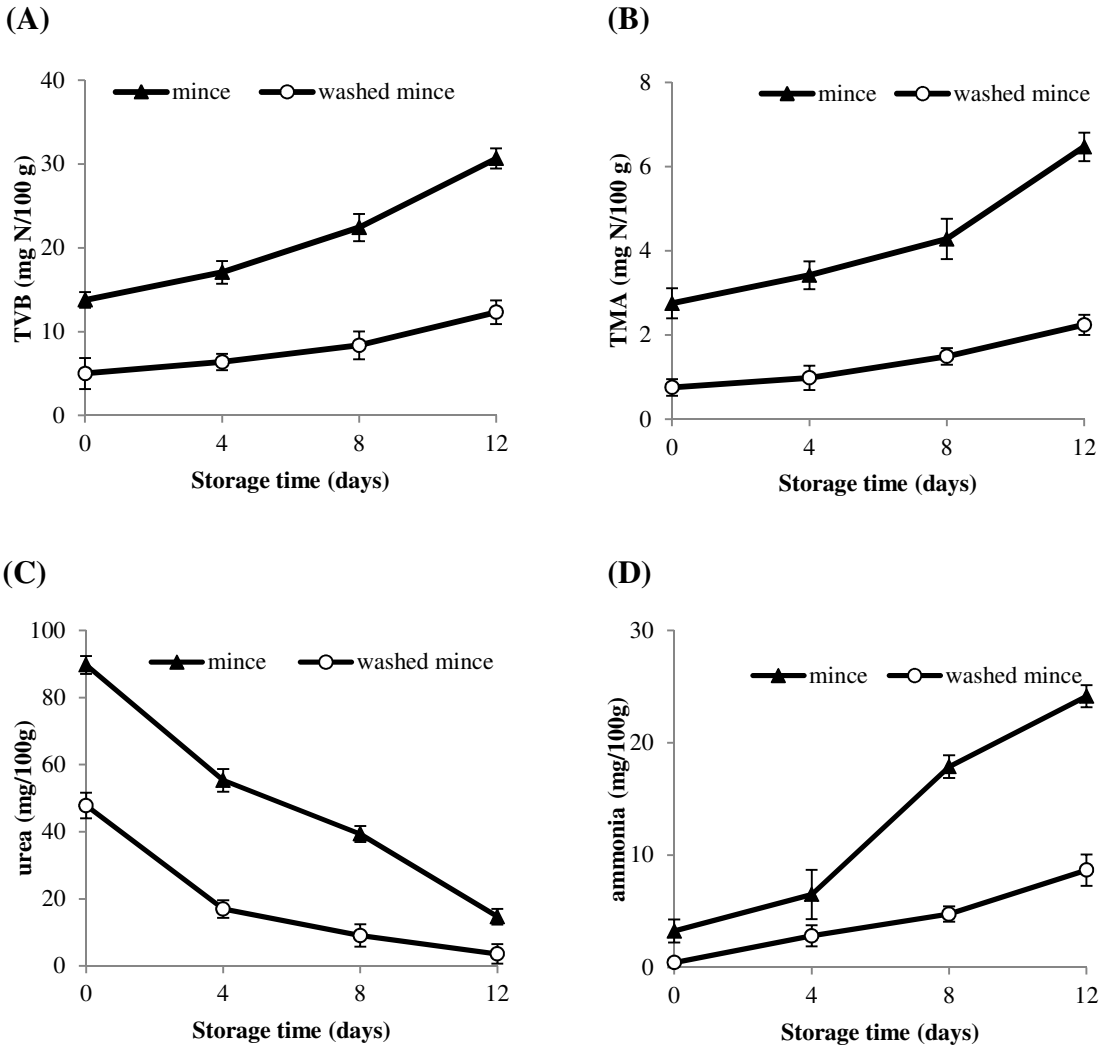


Figure 9. Changes in TVB (A), TMA (B), urea (C) and ammonia (D) of mince and washed mince from whitecheeck shark stored in ice for different times. Bars represent the standard deviation (n=3).

3.4.2 Effect of MTGase on gel properties of mince and washed mince from whitecheek shark stored in ice

3.4.2.1 Breaking force and deformation

Breaking force and deformation of gels from mince and washed mince obtained from shark during iced storage of 12 days in the absence and presence of MTGase (0.6 units/g) are depicted in Figure 10. At day 0, gels from mince showed the lower breaking force and deformation compared with those from washed mince ($P < 0.05$). Generally, breaking force of the gel was positively correlated with hardness, whereas the deformation represented the elasticity of gels (Kim *et al.*, 2000; Rawdkuen *et al.*, 2004). The increases in breaking force and deformation of gels prepared from washed mince were most likely due to the removal of sarcoplasmic proteins such as enzymes and heam proteins, pigments, odorous compounds and other impurities during washing process. Simultaneously, the myofibrillar proteins became concentrated (Hultin *et al.*, 2005; Martin-Sanchez *et al.*, 2009). Appropriate washing resulted in the concentrated myofibrillar proteins, and consequently increased breaking force of surimi gel (Park and Lin, 2005; Jin *et al.*, 2007). Moreover, shark mince at day 0 contained urea at high concentration. Urea is a well-known protein denaturant, which might change the structure of most proteins including myofibrillar protein in muscle (Kanoh *et al.*, 2001). Mathew *et al.* (2002) reported that the addition of urea at levels of 250–1,000 mM into shark myofibrillar protein lowered its gelling ability. Therefore, the washing process is a crucial method for production of surimi from shark mince. Washing could reduce urea effectively (Figure 9C), thereby improving the gel strength. Apart from urea, the amounts of sarcoplasmic proteins were also decreased in shark muscle via washing (Mathew *et al.* 2002). From the previous study, breaking force and deformation of gels produced from shark were comparable with those from sardine and Indian mackerel surimi gels. Nevertheless, the higher whiteness was obtained for gel from shark meat (Chanarat *et al.*, 2012). When the storage time increased, breaking force and deformation of gels produced from both mince and washed mince decreased up to 12 days of storage ($P < 0.05$). After 12 days of storage, breaking force and deformation of gel prepared from mince and washed mince decreased by 43.1–45.0% and 22.8–23.6%, respectively. Freshness

of fish is one of the most important factors determining the gel-forming ability of surimi (Park and Morrissey, 2000). The freshness primarily affects the denaturation and integrity of muscle protein. Basically, autolysis impairs the gel forming ability (Choi *et al*., 2005). Moreover, ammonia content in shark increased with increasing storage time. Ammonia generated by the degradation of urea in elasmobranch was presumed to inhibit endogenous transglutaminase activity. Ammonia generated during the acyl transfer reaction between γ-carboxyamide groups of glutamine residues and primary amines at the excessive amount prevents further progress of the reaction (Takagi *et al.*, 1986; Ashie and Lanier, 2000). Benjakul *et al*. (2004) reported that the addition of ammonium chloride (0.1 mol/kg) suppressed the gel formation by inactivation of endogenous transglutaminase in suwari gel. Kumazawa *et al*. (1995) reported that the addition of ammonium chloride resulted in the suppression of ε-(γ-glutamyl) lysine formation of Alaska pollock surimi gel. When MTGase (0.6 units/g) was incorporated, breaking force and deformation increased ($P < 0.05$) for both gels from mince and washed mince. However, the strengthening effect of MTGase was lowered with increasing storage time, especially at day 12. MTGase catalyses an acyl transfer between lysine and glutamine residues of proteins. As a result, ε-(γ-glutamyl) lysine cross-links between protein chains could be formed. The ammonia accumulated in mince and retained in washed mince, especially in the sample kept in ice for a longer time, more likely lowered the reactivity of MTGase and endogenous transglutaminase. It was noted that gel strengthening effect of MTGase toward washed mince was higher than mince. Mathew *et al*. (2002) reported that fat and non protein nitrogenous compounds in shark mince were decreased by 23 and 62%, respectively, after washing. Washing also led to the increased intensity of myosin heavy chain (MHC). When sarcoplasmic proteins, urea or fat was removed by washing, myofibrillar proteins became more concentrated and available for cross-linking (Park and Morrissey 2000; Chaijan *et al.*, 2004; Hultin *et al*. 2005). Myosin has been known as the good substrate for MTGase cross-linking (Nakahara *et al.*, 1999). At day 0, MTGase could increase breaking force of mince and washed mince by 25.7 and 70.6%, respectively. With increasing storage time, ammonia generated might contribute to the retardation of cross-linking mediated by MTGase to some degree. As a result, gel enhancing effect of MTGase was lowered as evidenced by the

lower efficacy in improving gel strength. At day 12, breaking force of gel from washed mince added with MTGase increased by 21.8%. However, no significant increase in breaking force was found for mince gel ($P > 0.05$). Therefore, washing, in combination with MTGase addition, was able to improve the gel-forming ability of shark mince.

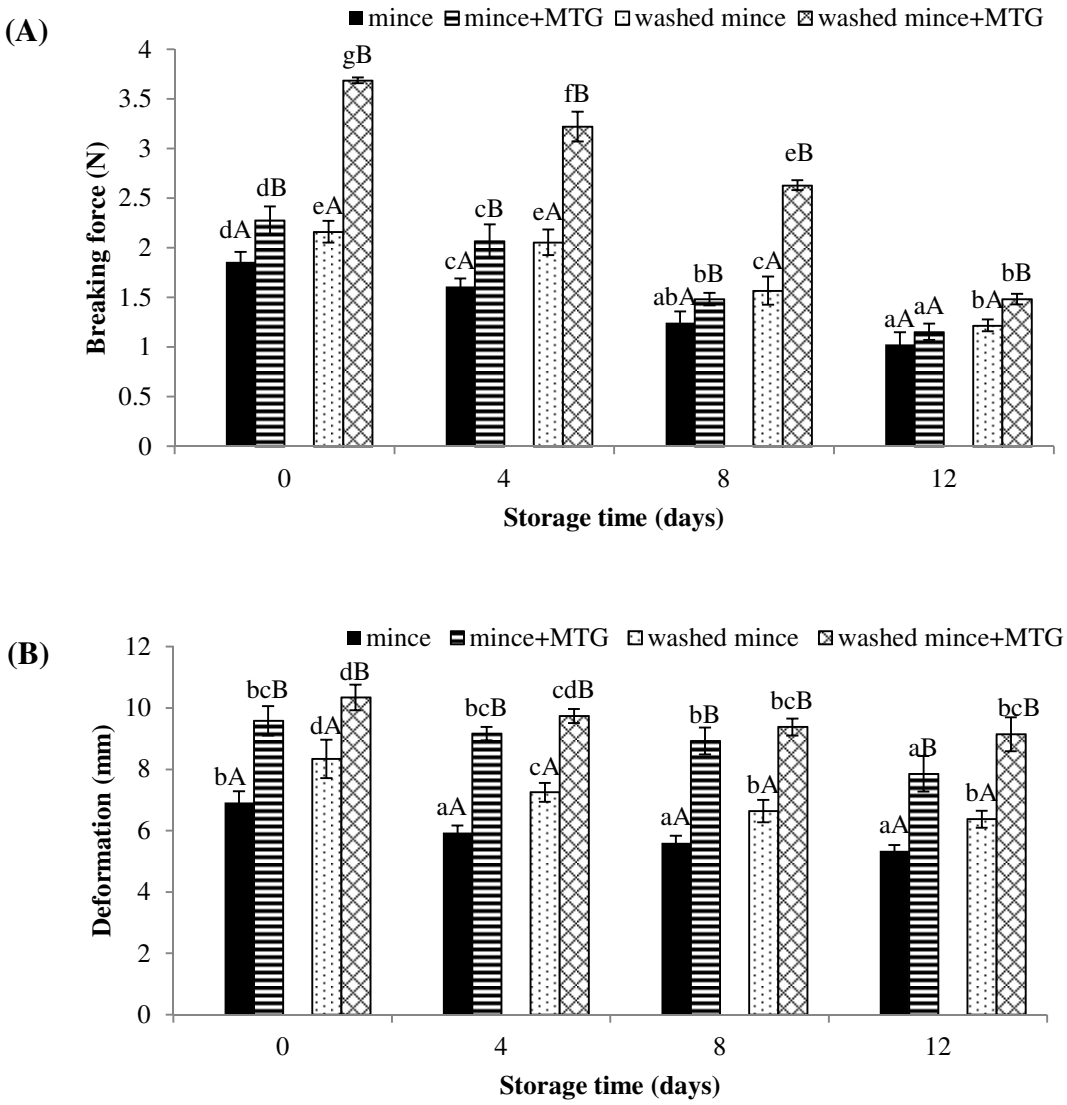


Figure 10. Breaking force and deformation of gels from mince and washed mince from whitecheeck shark stored in ice for different times in the absence and presence of MTGase (0.6 units/g). Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same level of MTGase indicate significant differences ($P < 0.05$). Different uppercase letters on the bars within the same storage time and washing condition indicate significant differences ($P < 0.05$).

3.4.2.2 Expressible moisture content

Expressible moisture content of gels of mince and washed mince from ice-stored shark in the absence and presence of MTGase (0.6 units/g) is shown in Table 8. Without MTGase addition, expressible moisture content of gels from mince and washed mince increased as storage time increased ($P < 0.05$). Lower expressible moisture content was generally found in gels from washed mince compared with those of mince. The lower expressible moisture content of gels suggested more water retained in the gel network (Niwa, 1992). In general, the lower expressible moisture was coincidental with the increased breaking force. Gel network with ordered structure could imbibe water effectively. When MTGase was added, the increase in water-holding capacity of gels was noticeable as evidenced by the lowered expressible moisture content. The decreases in expressible moisture content were noticeable as MTGase was added in both mince and washed mince from shark stored in ice at all times ($P < 0.05$). However, no difference in water-holding capacity was found in gels from both mince and washed mince obtained from shark stored in ice for 12 days when MTGase was added ($P > 0.05$). The lowered efficiency in improving water-holding capacity of gel from shark stored in ice for extended time was in agreement with the decreased gel strengthening effect of MTGase. This was more likely due to the presence of ammonia, MTGase inhibitor, to a higher extent. Thus, the addition of MTGase could enhance the cross-linking of proteins in both mince and washed mince to some degree, resulting in the formation of stronger network with the greater water-holding capacity.

3.4.2.3 Whiteness

Whiteness of gels from mince and washed mince from ice-stored shark in the absence and presence of MTGase (0.6 units/g) is shown in Table 8. At the same storage time, slightly higher whiteness was found in gels from washed mince compared with those from mince ($P > 0.05$). The colour of surimi gels can be affected by the presence of sarcoplasmic proteins, blood and pigment, etc. Washing process could remove those components to some extent, leading to whiter gels. However, no

differences in whiteness were found in gels when MTGase was incorporated, regardless of washing or storage time ($P > 0.05$).

Table 8. Expressible moisture content and whiteness of gels from mince and washed mince from whitecheeck shark stored in ice for different times in the absence or presence of MTGase (0.6 units/g).

Storage time (days)		Expressible moisture (%)		Whiteness	
		Without MTGase	With MTGase	Without MTGase	With MTGase
0	Mince	5.86 ± 0.38^{cA}	3.76 ± 0.48^{bB}	77.91 ± 0.56^{abA}	78.20 ± 0.51^{aA}
	Washed mince	3.79 ± 0.30^{aA}	3.25 ± 0.13^{aB}	79.26 ± 0.69^{bA}	79.45 ± 0.73^{aA}
4	Mince	6.57 ± 0.36^{deA}	6.07 ± 0.18^{cB}	78.58 ± 0.87^{abA}	78.27 ± 0.63^{aA}
	Washed mince	4.68 ± 0.09^{bA}	4.13 ± 0.20^{bB}	78.70 ± 0.58^{abA}	79.46 ± 0.69^{aA}
8	Mince	7.00 ± 0.20^{eA}	6.51 ± 0.27^{cB}	78.65 ± 0.78^{abA}	78.72 ± 0.53^{aA}
	Washed mince	5.80 ± 0.15^{cA}	4.00 ± 0.16^{bB}	79.17 ± 0.81^{abA}	79.94 ± 0.55^{aA}
12	Mince	7.59 ± 0.38^{fA}	7.25 ± 0.23^{dA}	77.85 ± 0.56^{aA}	77.49 ± 0.54^{aA}
	Washed mince	6.32 ± 0.23^{cdA}	6.41 ± 0.39^{cA}	78.83 ± 0.63^{abA}	78.92 ± 0.77^{aA}

Values are given as mean ± SD (n=3)

* Different lowercase superscripts within the same column indicate the significant differences ($P < 0.05$).

** Different uppercase superscripts within the same row under the same parameter indicate the significant differences ($P < 0.05$).

3.4.2.4 Protein pattern

Protein patterns of gels from mince and washed mince from shark stored in ice for different times in the absence and presence of MTGase (0.6 units/g) are shown in Figure 11. All gels contained MHC and actin as the major proteins. Higher band intensity of MHC and actin of gels from washed mince were noticeable compared with that of mince. Washing process could remove water-soluble matters include sarcoplasmic proteins, nonprotein nitrogenous compounds (TVB, TMA, urea and ammonia), leading to the increased concentration of myofibrillar proteins. Highly retained myofibrillar proteins, particularly MHC, in gels indicated directly that gel from shark mince and washed mince had a negligible setting phenomenon mediated

by endogenous transglutaminase. The gel formed was more likely stabilised by other weak bonds such as hydrogen bond and hydrophobic interaction. Those bonds were destroyed by denaturants used in sample buffer. When MTGase was added into mince or washed mince from shark kept in ice for various times, the intensity of MHC band decreased markedly compared with those without MTGase addition. The decreases of MHC band intensity indicated the formation of MHC cross-links mainly via the formation of the ε-(γ-glutamyl) lysine isopeptide induced by MTGase. MHC has been known to serve as an important protein substrate for MTGase (Nakahara *et al.*, 1999). Although MHC band intensity decreased markedly, actin band was rarely changed in all samples. The decrease in MHC band intensity was concomitant with the increased breaking force and deformation (Figure 10), indicating that MTGase effectively induced polymerisation of MHC, a protein contributing to the gel network formation. With increasing storage time, cross-linking of MHC induced by MTGase was decreased. This was obvious in gel from unwashed mince. Ammonia at high level most likely inhibited the gelation of shark mince induced by MTGase. As a result, MHC underwent less cross-linking. The result suggested that the lowered gel strengthening effect of MTGase was obtained for mince or washed mince from shark kept in ice for an extended time. For washed mince, the less cross-linking of MHC was observed in sample from shark stored in ice for 12 days. The lower ammonia content might lower the inhibitory effect toward MTGase. As a result, the cross-linking could take place more effectively than that found in unwashed mince.

3.4.2.5 Microstructures

Microstructures of gel from mince and washed mince from shark stored in ice in the absence and presence of MTGase (0.6 units/g) are illustrated in Figure 12. At the same storage time, gel of mince displayed a coarser gel matrix, whereas that from washed mince had a finer three-dimensional protein network with smaller void. Washing could remove interfering components for gelation, especially sarcoplasmic proteins, urea and ammonia. After 12 days of storage, mince and washed mince yielded the gel with less compactness and larger void. Irregular structure with larger voids of gel from unfresh shark mince might contribute to the

poorer gel properties with lower water holding capacity. The addition of MTGase could lead to the more compact network of aggregated proteins with smaller voids.

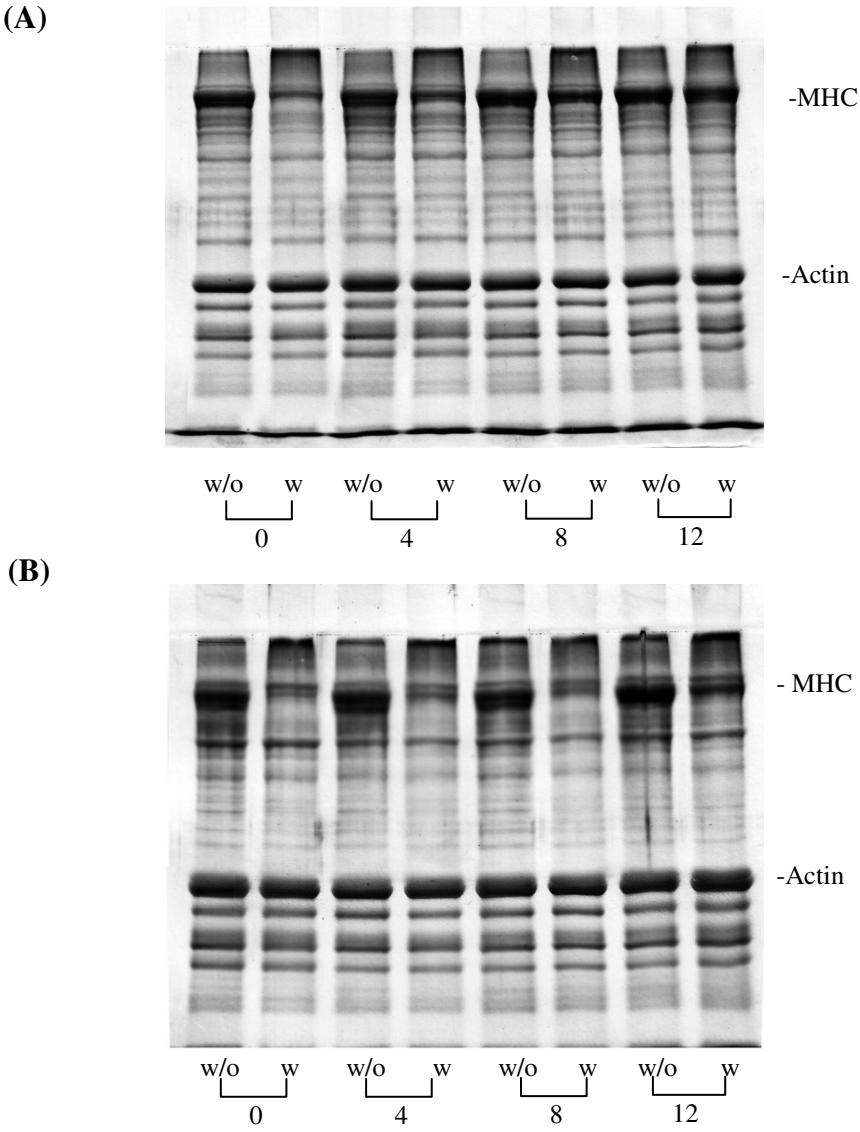


Figure 11 Protein patterns of gels from mince (A) and washed mince (B) from whitecheeck shark stored in ice for different times without (w/o) and with (w) MTGase addition (0.6 units/g). MHC: myosin heavy chain. Numbers designate the storage time (days).

The denser and more ordered structure of MTGase added gel was in accordance with higher breaking force (Figure 10) along with lowered expressible moisture content (Table 8). MTGase likely built up the network through intermolecular ε-(γ-glutamyl) lysine cross-linking in cooperation with protein aggregation via hydrophobic

interaction, disulfide bonds and/or other interactions during heating process (Benjakul *et al.*, 2008). However, no marked difference was observed in gels from unwashed mince obtained from shark stored in ice for 12 days despite the MTGase addition. This might be due to the increase in ammonia content, which might inactivate MTGase. Hence, the extended storage of shark, in which high content of ammonia was produced, had a negative impact on gel formation and lowered the cross-linking activity of MTGase in gel.

3.5 Conclusion

The extended storage time caused a negative impact on the gelling properties of shark mince. The addition of MTGase enhanced the gel-forming ability of resulting gels from fresh shark effectively, but gel enhancing effect of MTGase was decreased as the storage time increased. The washing process, which reduced urea and ammonia contents, was an effective means to lower the inactivation of both endogenous transglutaminase and MTGase caused by ammonia. Therefore, washing in conjunction with MTG addition could effectively improve the properties of gels from shark mince, resulting in the better exploitation of shark.

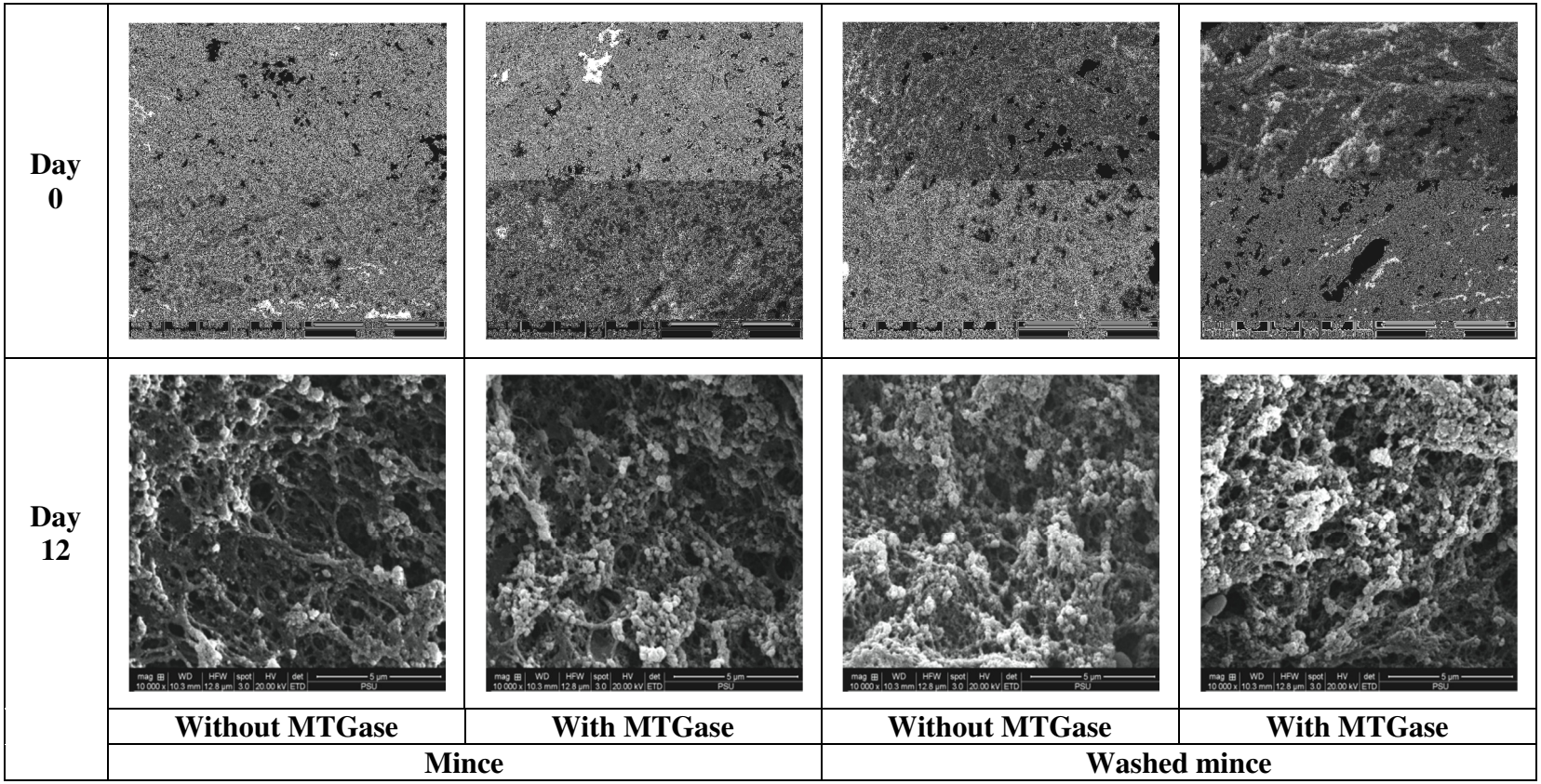


Figure 12 Electron microscopic image of gels from mince and washed mince from whitecheeck shark stored for 0 and 12 days in the absence and presence of MTGase (0.6 units/g) (magnification: 10,000x)

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3.6 References

Asagami, T., Ogiwara, M., Wakameda, A., and Noguchi, S. F. 1995. Effect of microbial transglutaminase on the quality of frozen surimi made from various kinds of fish species. Fish. Sci. 61: 267-272.

Ashie, I., and Lanier, T. C. 2000. Transglutaminases in seafood processing. *In* Seafood enzyme:utilization and influence on postharvest seafood quality (Haard, N. F., and Simpson, B. K. eds.). p. 147-166. Marcel Dekker. New York.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003a. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

Benjakul, S., Visessanguan, W., and Pecharat, S. 2004. Suwari gel properties as affected by transglutaminase activator and inhibitors. Food Chem. 85: 91-99.

Benjakul, S., Visessanguan, W., Riebroy, S., Ishizaki, S., and Tanaka, M. 2002. Gel forming properties of surimi produced from bigeye snapper, *Priacanthus tayenus* and *P macracanthus*, stored in ice. J. Sci. Food Agric. 82: 1442-1451.

Benjakul, S., Visessanguan, W., and Tueksuban, J. 2003b. Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during post-mortem storage in ice. Food Chem. 80: 535-544.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2004. Characteristics and gel properties of muscles from sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) caught in Thailand. Food Res. Int. 37: 1021-1030.

Chanarat, S., Benjakul, S., and H-Kittikun, A. 2012. Comparative study on protein cross-linking and gel enhancing effect of microbial transglutaminase on surimi from different fish. J. Sci. Food Agric. 92: 844-852.

Cheuk, W. L., and Finne, G. 1984. Enzymatic determination of urea and ammonia in refrigerated seafood products. J. Agric. Food Chem. 32: 14-18.

Choi, Y. J., Lanier, T. C., and Park, J. W. 2005. Proteolytic enzymes and control in surimi. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. eds.). p. 227-277. CRC Press. Boca Raton, Florida.

Conway, E. J., and Byrne, A. 1936. An absorption apparatus for the micro-determination of certain volatile substances: The micro-determination of ammonia. Biochem J. 27: 419-429.

Dalgaard, P. 2000. Fresh and lightly preserved seafood. *In* Shelf life evaluation of foods (Man, C. M. D. and Jones, A. A. eds.). p. 110-139. Aspen Publishing Inc. Gaithersburg, Maryland.

DeJong, G. A. H., and Koppelman, S. J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

Folk, J. E. 1970. Transglutaminase. Methods Enzymol. 17: 889-894.

Gram, L., and Huss, H. H. 1996. Microbiological spoilage of fish and fish products. International J. Food Microbiol. 33: 121-137.

Hazon, N., Wells, A., Pillans, R. D., Good, J. P., Gary Anderson, W., and Franklin, C. E. 2003. Urea based osmoregulation and endocrine control in elasmobranch fish with special reference to euryhalinity. Comp. Biochem. Phys. B. 136: 685-700.

Hultin, H. O., Kristinsson, H. G., Lanier, T. C., and Park, J. W. 2005. Process for recovery of functional proteins by pH shifts. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. ed.). p. 107-139. CRC Press. Boca Raton, Florida.

Jin, S. K., Kim, I. S., Kim, S. J., Jeong, K. J., Choi, Y. J., and Hur, S. J. 2007. Effect of muscle type and washing times on physico-chemical characteristics and qualities of surimi. J. Food Eng. 81: 618-623.

Kailasapathy, K., and Salampessy, J. 1999. Suitability of shark flesh for surimi production. Food Aus. 51: 110-115.

Kanoh, S., Kitamura, M., Horie, Y., Kasama, Y., and Niwa, E. 2001. Effects of urea and trimethylamine-*N*-oxide on ATPase of requiem shark myofibril and its constituents. Fish. Sci. 67: 943-947.

Kim, B. Y., Park, J. W., and Yoon, W. 2000. Rheology and texture properties of surimi gels. *In* Surimi and Surimi Seafood (Park, J. W. ed.). p. 267-324. CRC Press. Boca Raton, Florida.

Kumazawa, Y., Numazawa, T., Seguro, K., and Motoki, M. 1995. Suppression of surimi gel setting by transglutaminase inhibitors. J. Food Sci. 60: 715-717.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lanier, T. C., Carvajal, P., and Yongsawatdigul, J. 2005. Surimi gelation chemistry. *In* Surimi and surimi seafood, (Park, J. W. ed.). p. 237-265. CRC Press. Boca Raton, Florida.

Martin-Sanchez, A. M., Navarro, C., Perez-Alvarez, J. A., and Kuri, V. 2009. Alternatives for efficient and sustainable production of surimi: a review. Comp. Rev. Food Sci. F. 8: 359-374.

Mathew, S., Karthikeyan, M., and Shamasundar, B. 2002. Effect of water washing of shark (*Scoliodon laticaudus*) meat on the properties of proteins with special reference to gelation. Food/Nahrung. 46: 78-82.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

NFI 1991. A manual of standard methods for measuring and specifying the properties of surimi. National Fisheries Institute. Washington, DC.

Niwa, E. 1992. Chemistry of surimi gelation. *In* Surimi technology. (Lanier, T. C., and Lee, C. eds.). p. 389-427. Marcel Dekker. New York.

Ocaño-Higuera, V. M., Marquez-Ríos, E., Canizales-Dávila, M., Castillo-Yáñez, F. J., Pacheco-Aguilar, R., Lugo-Sánchez, M. E., García-Orozco, K. D., and Graciano-Verdugo, A. Z. 2009. Postmortem changes in cazon fish muscle stored on ice. Food Chem. 116: 933-938.

Park, J., and Lin, T. 2005. Surimi: manufacturing and evaluation. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. ed.). p. 33-106. CRC Press. Boca Raton, Florida.

Park, J. W., and Morrissey, M. T. 2000. Manufacturing of surimi from light muscle fish. *In* Surimi and surimi seafood (Park, J. W. ed.). p. 23-58. CRC Press. Boca Raton, Florida.

Phatcharat, S., Benjakul, S., and Visessanguan, W. 2006. Effects of washing with oxidising agents on the gel-forming ability and physicochemical properties of surimi produced from bigeye snapper (*Priacanthus tayenus*). Food Chem. 98: 431-439.

Ramírez, J. A., Uresti, R. M., Velazquez, G., and Vázquez, M. 2011. Food hydrocolloids as additives to improve the mechanical and functional properties of fish products: a review. Food Hydrocolloid. 25: 1842-1852.

Rawdkuen, S., Benjakul, S., Visessanguan, W., and Lanier, T. C. 2004. Chicken plasma protein affects gelation of surimi from bigeye snapper (*Priacanthus tayenus*). Food Hydrocolloid. 18: 259-270.

Sasajima, M. 1973. Studies on the psychrotolerant bacteria in fish and shellfish. IV. Relation between the number of trimethylamine oxide-reducing

psychrotrophic bacteria and their activity. Nippon Suisan Gakkaishi. 39: 511-518.

Schubring, R. 2007. DSC measurements on sharks. Thermochim Acta. 458: 124-131

Şengör, G. F., Mol, S., and Üçok, D. 2007. The effect of ascorbic acid, citric acid and salt on the quality of spiny dogfish (*Squalus acanthias*) fillet. J. Aquat. Food Prod. Technol. 16: 103-113.

Shimizu, Y., Toyohara, H., and Lanier, T. C. 1992. Surimi production from fatty and dark-fleshed fish species. *In* SurimiTechnology (Lanier, T. C., and Lee, C. eds.). p. 181-207. Marcel Dekker. New York.

Sikorski, Z. E., Kolakowska, A., and Burt, J. R. 1990. Postharvest biochemical and microbial changes. *In* Seafood: Resources, nutritional composition and preservation (Sikorski, Z. E. ed.). p. 55-76. CRC Press Boca Raton, Florida.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

Takagi, J., Saito, Y., Kikuchi, T., and Inada, Y. 1986. Modification of transglutaminase assay: use of ammonium sulfate to stop the reaction. Anal. Biochem. 153: 295-298.

Tolasa, S., Lee, C. M., and Cakli, S. 2010. Physical and oxidative stabilization of omega-3 fatty acids in surimi gels. J. Food Sci 75: 305-310.

Tomiyasu, Y., and Zenitani, B. 1957. Spoilage of fish and its preservation by chemical agents. Adv. Food Res. 7: 41-82.

Venugopal, V., Doke, S., and Nair, P. 1994. Gelation of shark myofibrillar proteins by weak organic acids. Food Chem. 50: 185-190.

Visessanguan, W., Benjakul, S., and Tanaka, M. 2003. Effect of microbial transglutaminase on rheological properties of oxidised and non oxidised

natural actomyosin from two species of bigeye snapper. J. Sci. Food Agric. 83: 105-112.

CHAPTER 4

EFFECT OF FORMALDEHYDE ON PROTEIN CROSS-LINKING AND GEL FORMING ABILITY OF SURIMI FROM LIZARDFISH INDUCED BY MICROBIAL TRANSGLUTAMINASE

4.1 Abstract

Impact of formaldehyde (FA) at various levels (0-9 mmol/g) on gel properties of surimi from lizardfish added with microbial transglutaminase (MTGase) was studied. During iced storage of 10 days, total and free FA in lizardfish flesh increased continuously ($P < 0.05$). In the presence of FA, breaking force of gels slightly increased, whilst the deformation decreased ($P < 0.05$). The addition of MTGase (0.4 units/g) was able to increase gel strength and water holding capacity of resulting gel. Nevertheless, gel strengthening effect of MTGase was lowered when FA at higher level was present. Myosin heavy chain (MHC) dominantly underwent polymerisation to a higher extent when either MTGase or FA was added. The higher reduction in ε-amino group content was observed in natural actomyosin (NAM) when FA at higher levels (0-30 mmol/g protein) was incorporated. Acyl transfer reaction mediated by MTGase was impeded in NAM containing FA, especially at higher levels. Generally, FA had an adverse effect on cross-linking ability towards surimi proteins induced by MTGase. Therefore, cross-linking and gel-forming ability of lizardfish surimi could be maximised by MTGase when surimi contained no FA.

4.2 Introduction

Formaldehyde (FA) is a reactive substance that has the ability of reacting with many functional groups of proteins, especially free amino groups. One FA molecule is theoretically able to react with two amino groups to form intra- and intermolecular crosslinks via methylene bridges of adjacent polypeptides (Shenouda, 1980; Sikorski, 1978). This causes the decreased solubility of proteins in aqueous salt solutions (Badii and Howell, 2002; Leelapongwattana *et al.*, 2005). Lysine and tyrosine undergo cross-linking via intra- and inter-molecular irreversible methylene

linkage in the presence of FA (Fraenkel-Conrat and Olcott, 1948; Marquié, 2001).

Lizardfish (*Saurida* spp.) are tropical fish that have been commonly used as a potential raw material for surimi production due to their availability, white colour, good flavour, and good gel forming ability (Benjakul *et al.*, 2003b; Morrissey and Tan, 2000). However, the freshness and gel-forming ability decreased during iced storage (Benjakul *et al.*, 2005; Holmes *et al.*, 1992). This phenomenon was mainly caused by the proteolysis as well as FA formation (Benjakul *et al.*, 2003b). Both FA and dimethylamine (DMA) can be produced enzymatically from trimethylamine oxide (TMAO), a compound found in several marine fish such as cod, hake, lizardfish, pollack and whiting (Benjakul *et al.*, 2004; Hebard *et al.*, 1982). Benjakul *et al.* (2003b) reported the increase in FA in flesh of whole lizardfish during frozen storage. FA is believed to contribute to the toughening of fish muscle, caused by the aggregation of proteins and also induces changes in protein molecules (Ang and Hultin, 1989; Ragnarsson and Regenstein, 1989). The decrease in gel forming ability of frozen stored lizardfish was mainly a result of FA formation (Benjakul *et al.*, 2005).

Microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins, thereby increasing the gel strength of surimi (Benjakul *et al.*, 2008; Duangmal and Taluengphol, 2010; Jiang *et al.*, 2000). MTGase induces the formation of an ε-(γ-glutamyl) lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and γ -amide group of a glutamine residue (Benjakul *et al.*, 2008).However, cross-linking ability of MTGase for surimi from unfresh lizardfish became lowered in comparison with surimi from very fresh fish (Benjakul *et al.*, 2008). FA found in lizardfish might lower the availability of amino group, especially from lysine. As a consequence, the cross-linking activity of MTGase in surimi from this species can be impeded. Nevertheless, there is no information regarding the adverse effect of FA found in lizardfish on cross-linking activity of muscle proteins by MTGase. Therefore, the objective of this study was to investigate the effect of FA on protein cross-linking and gel forming ability of lizardfish surimi mediated by MTGase.

4.3 Materials and Methods

4.3.1 Chemicals

2,4,6-trinitrobenzenesulphonic acid (TNBS), sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, glutaraldehyde, and formaldehyde solution were purchased from Sigma (St. Louis, MO, USA). *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Sodium hydrogen carbonate ($NaHCO_3$) was obtained from Merck (Darmstadt, Germany). Microbial transglutaminase (MTGase) from *Streptoverticillium mobaraense* (TG-K) containing 1% of pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

4.3.2 Fish samples

Lizardfish (*Suarida tumbil*) were purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 36-48 h after capture, were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 3 h.

4.3.3 Formaldehyde formation in lizardfish during iced storage

4.3.3.1 Preparation and collection of fish

Upon arrival, lizardfish were washed and kept in styrene foam box containing crushed ice with a fish/ice ration of 1:2 (w/w). The fish were placed between the layers of ice. The box containing fish was kept at room temperature (28-30 °C). To maintain the ice content, molten ice was removed and replaced with an equal amount of ice every 2 days. The fish were stored for up to 10 days in ice and FA formation was monitored.

4.3.3.2 Determination of FA content

FA content in lizardfish flesh was determined using Nash's reagent, as described by Bechmann (1998). To five grams of mince, 2.5 ml of 10% phosphoric acid and 50 ml of distilled water were added. The mixture was homogenised with a

homogeniser (IKA Labortechnik, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate was transferred to distillation flask and the distillation was conducted for 30 min until the distillate of 50 ml was obtained. Distillate was used for measurement of total FA content.

For free FA determination, sample (5 g) was mixed with 20 ml of 5% TCA, followed homogenisation at a speed of 11,000 rpm for 2 min. The homogenate was filtered with a Whatman no. 41 filter paper (Whatman Ltd., Maidstone, UK). The residue was added with 10 ml of 5% TCA, homogenised and filtered as previous described. The filtrates were combined and adjusted to pH of 6.0–6.5 using 0.1 N KOH or 1 N KOH. The resulting filtrate was made up to a final volume of 50 ml with distilled water.

To determine the FA content, an aliquot (3 ml) of distillate or filtrate was mixed with 3 ml of Nash reagent (0.2% 2,4-pentanedione; 0.1 M acetic acid; 3.89 M ammonium acetate). The mixture was kept at 60 °C for 15 min and cooled with running water. The absorbance was measured at 412 nm and the FA content was calculated from a standard curve prepared using FA ranging from 0 to 10 mM. FA content was expressed as μmol FA/g sample.

4.3.4 Effect of MTGase on gel properties of surimi added with FA at different levels

4.3.4.1 Surimi preparation

Fish skin and bones were removed manually and the flesh was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then washed with cold water (5-8 °C) at a mince/water ratio of 1:3 (w/v). The mixture was stirred gently for 3 min and washed mince was filtered with a layer of nilon screen. The washing process was carried out three times. Finally, the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700xg for 15 min. Washed mince was mixed thoroughly with 4% sorbitol and 4% sucrose. The mixture was kept at -18 °C and was

referred to as ‘frozen surimi’. The frozen surimi was used for study within a week of frozen storage.

To determine the effect of FA on gel forming ability, FA was added to the surimi to obtain the final concentration of 0, 3, 6, and 9 μmol/g and chopped for 3 min at 4 °C. The mixture was then added with 2.5% salt and the moisture content was adjusted to 80% in the absence and presence of MTGase (0.4 units/g). The mixture was chopped for another 3 min to obtain the homogenous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the sol at 40 °C for 30 min, followed by heating at 90 °C for 20 min. The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

4.3.4.2 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

4.3.4.3 Determination of expressible moisture content.

Expressible moisture content (%) was determined according to the method of Benjakul *et al*. (2003a). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between two pieces of Whatman no. 1 filter paper at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y)*. Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = [(X - Y)/X]\text{x}100$$

4.3.4.4 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (NFI, 1991).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

4.3.4.5 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution (85 °C) were added to the sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500xg for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

4.3.4.6 Microstructure

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of

50, 70, 80, 90 and 100%. Samples were subjected to critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

4.5 Effect of MTGase on the cross-linking of natural actomyosin in the presence of FA at different levels

4.5.1 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul *et al*. (1997). Fish mince (10 g) was homogenised in 100 ml of chilled 0.6 M KCl, pH 7.0 using a homogeniser for 4 min at a speed of 11,000 rpm. Overheating during extraction was avoided by keeping the sample in an iced container and each 20s of homogenisation was followed by a 20s rest interval. The homogenate was centrifuged at 5000xg for 30 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Three volumes of chilled water (0–4 °C) were added to precipitate NAM, which was then collected by centrifuging at 5,000xg for 20 min at 4 °C using a refrigerated centrifuge. The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.45 M NaCl, pH 7.0 for 30 min at 4 °C.

For cross-linking reaction, NAM solution (5 mg/ml) containing FA at different levels (0, 10, 20 and 30 μmol/g protein) was allowed to stand in ice for 1 h. The prepared solutions were preheated at 40 °C for 10 min in a temperature-controlled water bath. Thereafter, MTGase (0, 10, 20 and 40 units/g protein) was added and incubated at 40 °C for 30 min. All samples were subjected to the determination of ε-amino group content.

4.5.2 Determination of ε-amino group content

The ε-amino group content was measured as per the method of Bubnis and Ofner (1992) with a slight modification. NAM sample was placed in 50 ml screw cap test tube. One milliliter of 4% $NaHCO_3$ and 1 ml of 0.50% TNBS were added. The reaction mixture was incubated at 40°C for 4 h with mild shaking using an incubator shaker (SV1422, Memmert, Schwabach, Germany). Three milliliters of 6N HCl were added and the mixture was autoclaved at 120 °C for 1 h. The hydrolysate was then diluted with 5 ml of distilled water and extracted with 3 volumes of ethyl ether 3 times. The aqueous phase was removed and heated for 15 min in a boiling water bath to evaporate the residual ethyl ether. The aliquot was diluted with 3 volumes of distilled water and the absorbance was measured at 346 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). All samples were read against a reagent blank, which was prepared by the same manner with the sample but HCl was added before the addition of TNBS. ε-amino group content was calculated as follows (Bubnis and Ofner, 1992):

$$\text{ε-amino group content } (10^{-5} \text{ mole Lys/g protein}) = \frac{2(A_{346})(0.020)}{(1.46 \times 10^{4})(b)(x)}$$

where b: cell path length (cm); X: protein content (mg)

4.6 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests. T-test was used for pair comparison (Steel and Torrie, 1980). Analysis was performed using the Statistical Package for Social Science package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and Discussion

4.4.1 Formaldehyde (FA) formation in lizardfish during iced storage

Both free and total FA contents in lizardfish flesh increased continuously as the storage time increased up to 10 days ($P < 0.05$) (Figure 13). This result confirmed the work carried out by Benjakul *et al.* (2003b) who reported the increase in free FA in lizardfish during the extended iced storage. trimethylamine-*N*-oxide demethylase (TMAOase) was capable of catalysing the conversion of trimethylamine oxide (TMAO) to dimethylamine (DMA) and FA (Leelapongwattana *et al.*, 2005). When comparing free and total FA contents at all storage times, the former was much lower than the latter ($P < 0.05$). The result indicated that the large amount of FA formed was bound tightly to muscle proteins and the lower amount was present in the free form. FA bound with proteins was more likely involved in the aggregation of protein, resulting in the insolubilisation, and the changes in conformational and functional properties (Benjakul *et al.*, 2004; Careche *et al.*, 2002). Those changes were dependent on fish species and storage conditions (Hebard *et al.*, 1982). FA induced the conformational changes of myosin, especially the globular head region, leading to the decrease in Ca^{2+}-ATPase activity (Benjakul & Sutthipan, 2009). At day 10 of storage, free and total FA contents in lizardfish flesh were 1.23 and 5.15 mmol/g mince, respectively. Thus, FA formed in lizardfish during the extended iced storage might contribute to changes in quality as well as functional properties of lizardfish.

4.4.2 Effect of MTGase on gel properties of surimi in the presence of FA at different levels

4.4.2.1 Breaking force and deformation

Breaking force and deformation of gels from lizardfish surimi in the presence of FA at different levels with and without the addition of MTGase (0.4 units/g) were depicted in Figure 14. Without MTGase addition, breaking force of gels slightly increased as FA content increased ($P < 0.05$). FA has been known as protein

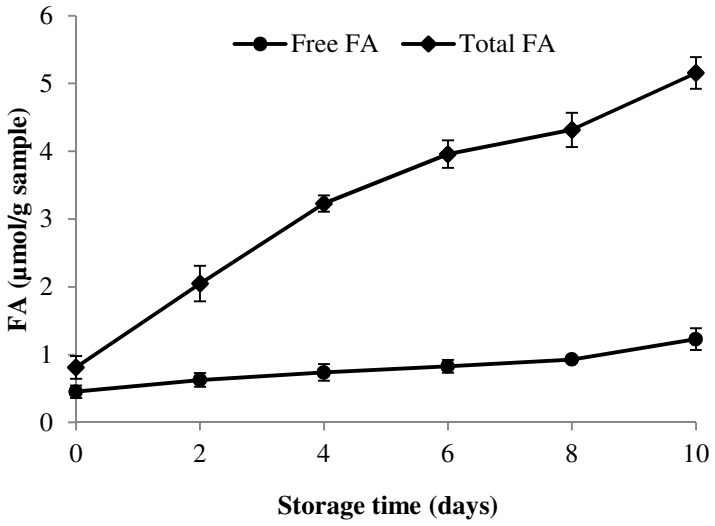


Figure 13. Changes in free and total formaldehyde contents in lizardfish during iced storage of 10 days. Bars represent the standard deviation (n=3). Different letters within the same parameter indicate significant differences ($P < 0.05$).

cross-linker via methylene bridge, in which intra- and inter-molecular cross-links of adjacent polypeptides could be formed (Sikorski, 1978). Those bondings more likely contributed to the stronger gel as indicated by the slight increase in breaking force. Abe *et al.* (2003) reported that the addition of 1 mM FA into Alaska pollack surimi resulted in a strong gel formation due to the accelerated aggregation of surimi proteins. When MTGase at a level of 0.4 units/g was incorporated, the increase in breaking force by 60.25% was observed. However, the strengthening effect of MTGase was lowered as FA content in surimi gel increased. MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins. As a result, ε-(γ-glutamyl) lysine crosslinks between protein chains could be formed. In the presence of FA, ε-amino groups of lysine might interact with FA. Furthermore, Metz *et al.* (2004) found that FA could react with the amino group of the N-terminal amino acid residue and the side chains of lysine, arginine, cysteine, and histidine residues via methylol groups, Schiff-bases, or methylene bridges. In the presence of FA, proteins became aggregated and insoluble in salt solution. As a consequence, free amino groups were not available for acyl transfer reaction induced by MTGase. Therefore,

gel enhancing effect of MTGase was lowered. FA has been reported to result in detrimental changes in texture and functional properties (Abe et *al.*, 2003). Although MTGase is known to catalyse protein cross-linking, glutamine and lysine residues should be located in a flexible region to allow the covalent cross-linking of adjacent proteins (Ramírez *et al.*, 2011). Larre *et al.* (1992) reported that the 11s and 7s soybean proteins with compact globular structures are poor substrates for TGase, despite their relatively high glutamine contents. Therefore, the presence of FA at higher levels directly lowered gel strengthening effect of MTGase added into surimi. Nevertheless, similar breaking force was found between surimi gel containing FA at 6 and 9 mmol/g ($P > 0.05$).

Deformation of all gels decreased with increasing FA ($P < 0.05$), regardless of MTGase addition. Strong and rigid gels were formed in the presence of both FA and MTGase due to the excess aggregation or severe denaturation of protein in surimi. These resulted in the loss of elasticity of surimi gel. Benjakul *et al*. (2005) reported that frozen lizardfish showed a poorer gel-forming ability, compared with other fish species, predominantly due to the formation of FA in this species. The results suggested that MTGase had no impact on deformation of surimi gel, except for surimi gel containing 3 mmol FA/g where MTGase increased the deformation slightly ($P < 0.05$).

4.4.2.2. Expressible moisture content

Expressible moisture content of gels from lizardfish surimi containing FA at different levels in the absence and presence of MTGase (0.4 units/g) is shown in Table 9. When FA was present in surimi, the increases in expressible moisture content were noticed, especially when FA concentration increased ($P < 0.05$). In the presence of FA, enhanced cross-linkings were formed between proteins, thereby lowering the binding sites of proteins with water. As a result, free water became more available and appeared as exudates. Decreases in expressible moisture content were observed in gels containing FA at all levels, when MTGase was added ($P < 0.05$). The lower expressible moisture content of gels suggested more water retained in the gel

network (Niwa, 1992). This property depends on the structure of the muscle proteins that bind and interact with the water molecules in the gel network.

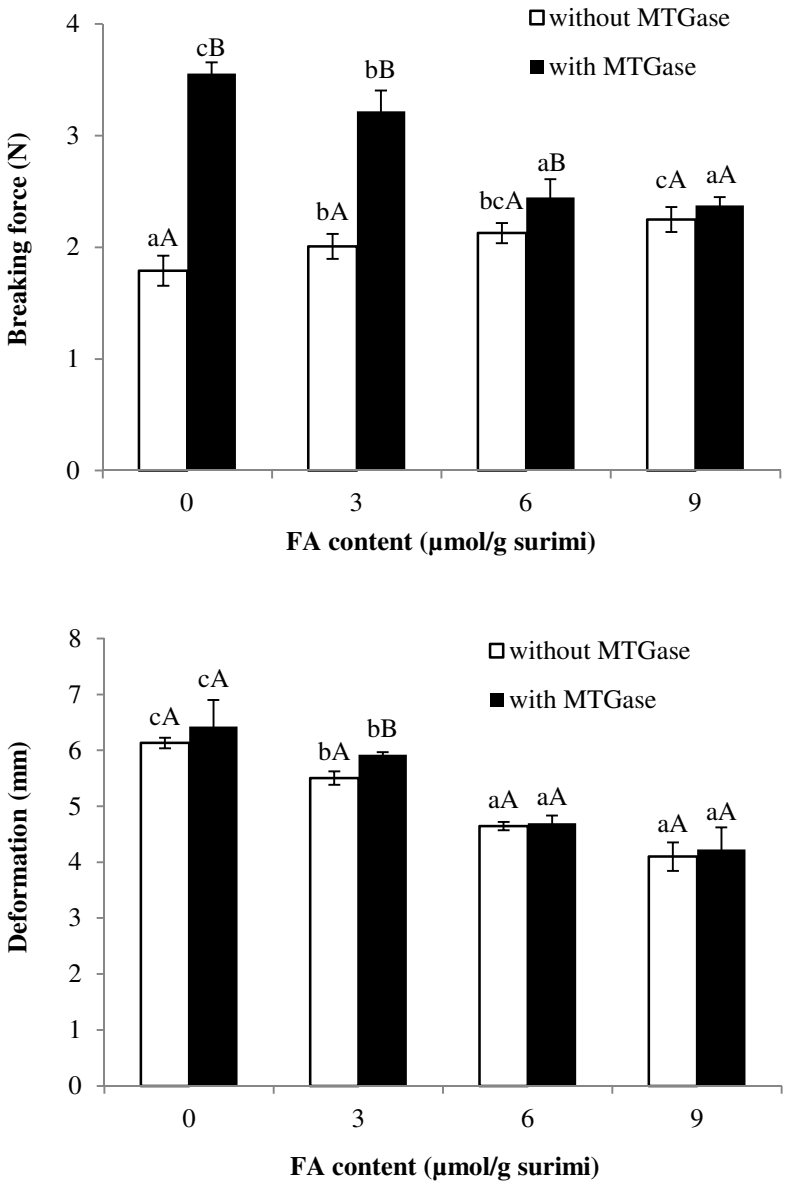


Figure 14. Breaking force and deformation of gels from lizardfish surimi containing formaldehyde at different levels FA (0-9 µmol/g) in the absence and presence of MTGase (0.4 units/g). Bars represent the standard deviation (n=3). Different lowercase letters within the same level of MTGase indicate significant differences ($P < 0.05$). Different uppercase letters within the same level of formaldehyde indicate significant differences ($P < 0.05$).

Connell (1975) also reported that the poor water holding capacity of surimi gel from lizardfish was associated with the increased FA, which caused the toughening in fish muscle by inducing the cross-linking of protein. It was noted that the addition of MTGase could increase the ability of gel in water holding as evidenced by the lowered expressible moisture content. In general, the higher expressible moisture content of surimi gel from lizardfish was closely associated with the poor gel forming ability as shown in Figure 14. However the addition of MTGase could enhance the cross-linking of proteins to some degree, resulting in the formation of stronger network with the greater water holding capacity. Moreno *et al.* (2008) also reported that the addition of MTGase increased WHC of restructured fish muscle.

4.4.2.3 Whiteness

Whiteness of gels from lizardfish surimi containing different levels of FA in the absence and presence of MTGase is shown in Table 9. No differences in whiteness were found in lizardfish surimi gel when FA at different levels was present ($P > 0.05$). Also, gels containing FA ranging from 3 to 9 µmol/g had the similar whiteness ($P > 0.05$), when MTGase was added. However, MTGase addition resulted in the slight increase in whiteness of gels containing no FA ($P < 0.05$). This might be caused by other compounds in commercial MTGase powder, which might cause the light scattering effect. Nevertheless, such an effect might be negligible as FA was present. Increased free water or exudates might decrease the light scattering effect of those compounds. Karayannakidis *et al.* (2008) reported that the addition of MTGase had a significant effect on whiteness index of heat-induced sardine surimi gels.

4.4.2.4 Protein patterns

Protein patterns of surimi gels containing FA at different levels in the absence and presence of MTGase are shown in Figure 15. Surimi contained myosin heavy chain (MHC) and actin as the major proteins. For surimi gel, the decreases in MHC and actin band intensity were noticeable, indicating that some covalent bonds were formed and contributed to cross-linking of those proteins.

Table 9 Expressible moisture content and whiteness of gels from lizardfish surimi containing formaldehyde at different levels (0-9 μmol/g) in the absence and presence of MTGase (0.4 units/g)

FA levels (μmol/g)	Expressible moisture (%)		Whiteness	
	Without MTGase	With MTGase	Without MTGase	With MTGase
0	8.286 ± 0.247^{aB}	3.666 ± 0.165^{aA}	78.871 ± 0.245^{aA}	79.803 ± 0.219^{aB}
3	8.967 ± 0.112^{bB}	5.759 ± 0.211^{bA}	78.836 ± 0.437^{aA}	78.522 ± 0.208^{bA}
6	10.070 ± 0.484^{cB}	6.540 ± 0.192^{cA}	78.244 ± 0.039^{aA}	78.836 ± 0.437^{bA}
9	11.816 ± 0.273^{dB}	7.172 ± 0.312^{dA}	78.521 ± 0.763^{aA}	78.588 ± 0.121^{bA}

Values are given as mean ± SD (n=3)
* Different lowercase superscripts within the same column indicate the significant differences ($P < 0.05$)
** Different uppercase superscripts within the same row indicate the significant differences ($P < 0.05$)

In the presence of FA at higher level, MHC and actin of gels became lowered. This confirmed the cross-linking activity of FA towards fish muscle proteins. Ang and Hultin (1989) reported that FA accelerated the formation of high-molecular weight polymer in cod myosin. Some protein-protein interactions responsible for myosin insolubility by FA were more pronounced with increasing FA content. FA preferentially cross-linked myosin, followed by actin, troponin, myosin light chain, and tropomyosin, respectively, depending on the amount of FA and the reaction time (Del Mazo *et al.*, 1994). Aggregates of high molecular mass were found probably as a result of covalent binding of myosin molecules. When high amount of FA was added, these protein aggregations became insoluble, forming high-molecular mass structures (Del Mazo *et al.*, 1994). When MTGase was added into surimi, the intensity of MHC band decreased in all samples, compared to those without MTGase addition, irrespective of FA present in surimi. The disappearance of MHC indicated the formation of MHC cross-links mainly via the formation the ε-(γ-glutamyl) lysine isopeptide induced by MTGase. MHC has been known to serve as the major contributor for gel formation and determine gel property of fish protein (Nakahara *et al.*, 1999). Although MHC band intensity decreased markedly, actin and tropomyosin rarely changed in all samples. Thus, MHC appeared to be a preferable substrate for cross-linking induced by either MTGase or FA. As FA levels increased, especially at

6 and 9 mmol/g, MHC in gel was more remained in spite of MTGase addition. FA at high level might induce aggregation of proteins, in which the reactive sites, especially ε-amino group of lysine, was hindered. As a result, MHC underwent less cross-linking induced by MTGase. Furthermore, Abe *et al.* (2003) demonstrated that FA inhibited the thermal gelation and setting effect of surimi paste from Alaska pollack through the inactivation of TGase mediated myosin cross-linking. The result reconfirmed the negative effect of FA on MHC cross-linking induced by MTGase. This led to the lowered gel strengthening effect of MTGase in surimi containing FA.

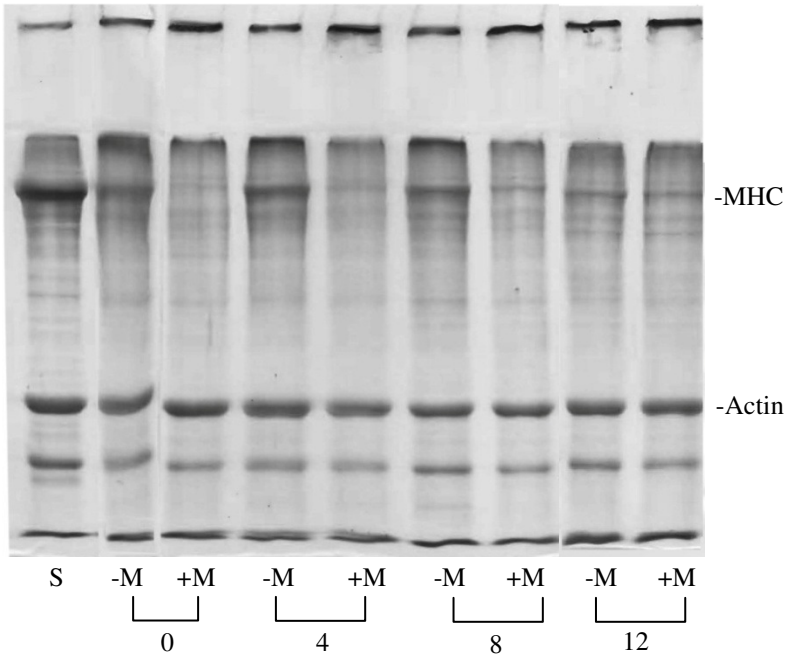


Figure 15. Protein patterns of gels from lizardfish surimi containing formaldehyde at different levels (0-9 µmol/g) in the absence (-M) and presence (+M) of MTGase (0.4 units/g). S: surimi; MHC: myosin heavy chain. Numbers designate the level of formaldehyde added (µmol/g).

4.4.2.5 Microstructure

Gels from lizardfish surimi in the absence of FA showed the well-structured and fine matrix with highly interconnected strands. Similar structure was found between surimi gel without and with the addition of MTGase. However, the addition of MTGase could induce the formation of stronger gel network as evidenced by a higher breaking force (Figure 14) along with lowered expressible moisture

content (Table 9). In the presence of FA, a discontinuous network with the larger voids was observed in the gel. It was noted that the addition of MTGase had no marked impact on microstructure of surimi gel containing FA. This result was coincidental with the negligible gel strengthening effect of MTGase in surimi added with FA at high level (9 mmol/g) (Figure 14). FA formed in lizardfish might cross-link proteins via methylene bridge. As a result, proteins could not be fully solubilised when salt was added. Protein aggregates or bundles were not able to form the fine and continuous network as evidenced by the formation of large voids (Figure 16). Irregular structure with larger voids might contribute to the poorer gel properties of surimi gel with lower water holding capacity. Thus, FA had the negative impact on gel formation and lowered the cross-linking activity of MTGase in gel.

4.4.3 Effect of MTGase on the cross-linking of natural actomyosin in the presence of FA at different levels

Changes in ε-amino group content of NAM from lizardfish added with FA (0, 10, 20 and 30 mmol/g protein) as influenced by MTGase at different levels (0, 10, 20 and 40 units/g protein) are shown in Table 10. In the absence of MTGase, ε-amino group content of NAM decreased as FA concentration increased, especially when FA was higher than 10 mmol/g protein ($P < 0.05$). It was suggested that FA could react with lysine in NAM, thereby lowering available ε-amino groups. FA is known to react with a wide spectrum of amino side chain in proteins (Ang and Hultin, 1989; Tome *et al.*, 1985). Means and Feeney (1995) reported that the reaction between FA and lysine is a two-step process. Firstly, ε-NH_2 residues and FA form amino methylol derivatives. Secondly, an irreversible reaction leads to the formation of inter- and intra-molecular methylene linkages. Marquié (2001) also found that the percentage of lysine in protein from cottonseed was decreased as FA at level up to 0.05 mol FA/100 g protein was added and then remained unchanged when higher concentration was used.

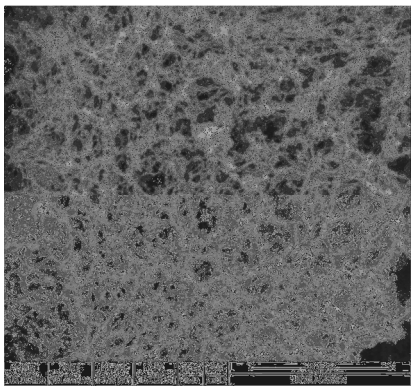
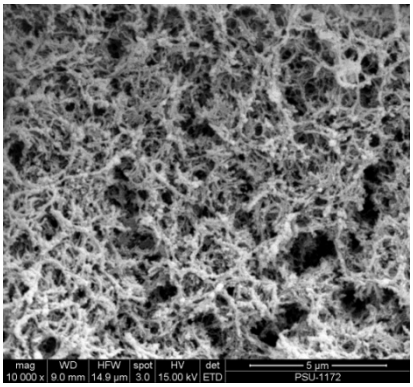
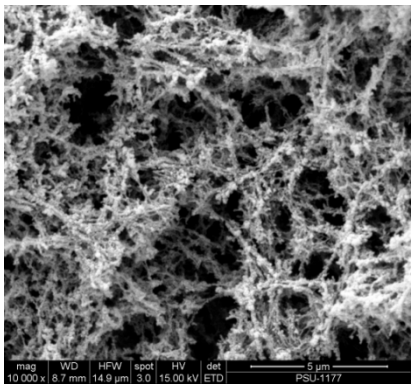
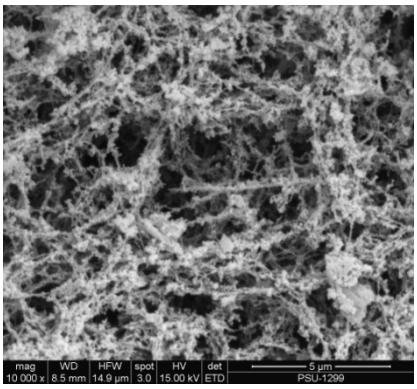
FA level (µmol/g)	Without MTGase	With MTGase (0.4 units/g)
0		
9		

Figure 16. Electron microscopic image of surimi gel in the absence and presence of FA (9 µmol/g) with and without MTGase (0.4 units/g) addition. (Magnification: 10,000x).

In the absence of FA, the decrease in ε-amino group content was observed in NAM when MTGase at 10 units/g protein was incorporated ($P < 0.05$). However, no further changes in ε-amino group content were noticeable when MTGase above 10 units/g protein was added ($P > 0.05$). In the presence of FA, a higher level of MTGase (20-40 units/g protein) was required to decrease ε-amino group content of NAM. For NAM containing 30 mmol FA/g protein, MTGase at a level of 40 units/g protein resulted in the decrease in ε-amino group content ($P < 0.05$). In general, the decreasing rate of ε-amino group of NAM meditated by MTGase was governed by FA concentration. When MTGase at a level of 10 units MTGase/g protein was incorporated, the decreasing rates of ε-amino groups were 8.13%, 4.21%, 3.72%, and 3.53% for NAM

containing 0, 10, 20, and 30 mmol FA/g protein, respectively. As ε-amino group of lysine became less available in the presence of FA, acyl transfer reaction was lowered as evidenced by the lower decreasing rate of ε-amino group. The result indicated that the presence of FA directly lowered the amount of ε-amino group of lysine in protein, thereby impeding protein cross-linking activity of MTGase. The result was in accordance with the lower gel enhancing effect of MTGase in surimi containing FA (Figure 14).

Table 10 Changes in ε-amino group contents (10^{-5} mol Lys/g protein) of natural actomyosin from lizardfish containing formaldehyde at different levels (0-30 μmol/g protein) as influenced by MTGase at various levels (0-40 units/g protein).

MTGase (units/g protein)	FA levels (μmol/g protein)			
	0	10	20	30
0	30.95 ± 1.05^{aA}	29.49 ± 0.80^{aAB}	29.02 ± 1.13^{aB}	28.75 ± 0.49^{aB}
10	28.44 ± 1.13^{bA}	28.25 ± 0.64^{abA}	27.99 ± 0.53^{abA}	27.68 ± 1.63^{abA}
20	27.73 ± 1.31^{bA}	27.52 ± 0.76^{bA}	27.30 ± 0.77^{bcA}	27.12 ± 0.76^{abA}
40	27.04 ± 0.44^{bA}	26.89 ± 1.11^{bA}	26.22 ± 0.27^{cA}	25.94 ± 0.89^{bA}

Values are given as mean ± SD (n=3)
* Different lowercase superscripts within the same row indicate the significant differences ($P< 0.05$)
** Different uppercase superscripts within the same column indicate the significant differences ($P < 0.05$)

4.5 Conclusion

FA content in lizardfish flesh increased continuously during iced storage. MTGase was able to increase the gel strength of surimi from lizardfish. However, the addition of FA in surimi had a negative impact on gel improvement and cross-linking ability induced by MTGase. FA presented in lizardfish could lower the availability of reactive group of Lys (ε-amino group), thereby affecting the protein cross-linking induced by MTGase as well as gel characteristic. Surimi should be

prepared from fresh lizardfish to avoid FA formation, in which the gel strengthening effect of MTGase could be maximised.

4.6 References

Ang, J. F., and Hultin, H. O. 1989. Denaturation of cod myosin during freezing after modification with formaldehyde. J. Food Sci. 54: 814-818.

Badii, F., and Howell, N. K. 2002. Changes in the texture and structure of cod and haddock fillets during frozen storage. Food Hydrocolloid. 16: 313-319.

Bechmann, I. E. 1998. Comparison of the formaldehyde content found in boiled and raw mince of frozen saithe using different analytical methods. LWT-Food Sci. Technol. 31: 449-453.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003a. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Benjakul, S., Visessanguan, W., Thongkaew, C., and Tanaka, M. 2005. Effect of frozen storage on chemical and gel-forming properties of fish commonly used for surimi production in Thailand. Food Hydrocolloid. 19: 197-207.

Benjakul, S., Visessanguan, W., and Tueksuban, J. 2003b. Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during post-mortem storage in ice. Food Chem. 80: 535-544.

Benjakul, S., Visessanguan, W., Tueksuban, J., and Tanaka, M. 2004. Effect of some protein additives on proteolysis and gel-forming ability of lizardfish (*Saurida tumbil*). Food Hydrocolloid. 18: 395-401.

Bubnis, W. A., and Ofner, C. M. 1992. The determination of ε-amino groups in soluble and poorly soluble proteinaceous materials by a spectrophotometric method using trinitrobenzenesulfonic acid. Anal. Biochem. 207: 129-133.

Careche, M., Garcia, M., Herrero, A., Solas, M., and Carmona, P. 2002. Structural properties of aggregates from frozen stored hake muscle proteins. J. Food Sci. 67: 2827-2832.

Connell, J. J. 1975. The role of formaldehyde as a protein crosslinking agent acting during the frozen storage of cod. J. Sci. Food Agric. 26: 1925-1929.

Del Mazo, M. L., Huidobro, A., Torrejón, P., Tejada, M., and Careche, M. 1994. Role of formaldehyde in formation of natural actomyosin aggregates in hake during frozen storage. Z. Lebensm unters F. A. 198: 459-464.

Duangmal, K., and Taluengphol, A. 2010. Effect of protein additives, sodium ascorbate, and microbial transglutaminase on the texture and colour of red tilapia surimi gel. Int. J. Food Sci. Technol. 45: 48-55.

Fraenkel-Conrat, H., and Olcott, H. S. 1948. The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups. J. Am. Chem. Soc. 70: 2673-2684.

Hebard, C., Flick, G., and Martin, R. 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. *In* Chemistry and biochemistry of marine food products (Martin, R. E., Flick, G. J., Hebard, C. E., and Ward, D. R. eds.). p. 149-304. AVI Publishing. Connecticut.

Holmes, K. L., Noguchi, S. F., and MacDonald, G. A. 1992. The Alaska pollock resource and other species used for surimi. *In* Surimi technology (Lanier, T. C. and Lee, C. M. eds.). p. 41-76. Marcel Dekker. New York.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000. Combination effects of microbial transglutaminase, reducing agent, and protease inhibitor on the quality of hairtail surimi. J. Food Sci. 65: 241-245.

Karayannakidis, P. D., Zotos, A., Petridis, D., and Taylor, K. D. A. 2008. Physicochemical changes of sardines (*Sardina pilchardus*) at –18°C and functional properties of kamaboko gels enhanced with Ca^{2+} ions and MTGase. J. Food Process Eng. 31: 372-397.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Larre, C., Kedzior, Z. M., Chenu, M. G., Viroben, G., and Gueguen, J. 1992. Action of transglutaminase on an 11 S seed protein (pea legumin): influence of the substrate conformation. J. Agric. Food Chem. 40: 1121-1126.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., and Howell, N. K. 2005. Physicochemical and biochemical changes in whole lizardfish (*Saurida micropectoralis*) muscles and fillets during frozen storage. J. Food Biochem. 29: 547-569.

Marquié, C. 2001. Chemical reactions in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal for the formation of protein films with enhanced mechanical properties. J. Agric. Food Chem. 49: 4676-4681.

Means, G. E., and Feeney, R. E. 1995. Reductive alkylation of proteins. Anal. Biochem. 224: 1-16.

Moreno, H. M., Carballo, J., and Borderias, A. J. 2008. Influence of alginate and microbial transglutaminase as binding ingredients on restructured fish muscle processed at low temperature. J. Sci. Food Agric. 88: 1529-1536.

Morrissey, M. T., and Tan, S. M. 2000. World resources for surimi. *In* Suirmi and Surimi Seafood (Park, J. W. ed.). p. 1-22. Marcel Dekker. New York.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

NFI 1991. A Manual of Standard Methods for Measuring and Specifying the Properties of Surimi. National Fisheries Institute. Washington, DC.

Ragnarsson, K., and Regenstein, J. M. 1989. Changes in electrophoretic patterns of gadoid and non-gadoid fish muscle during frozen storage. J. Food Sci. 54: 819-823.

Ramírez, J. A., Uresti, R. M., Velazquez, G., and Vázquez, M. 2011. Food hydrocolloids as additives to improve the mechanical and functional properties of fish products: a review. Food Hydrocolloid. 25: 1842-1852.

Shenouda, S. Y. K. 1980. Theories of protein denaturation during frozen storage of fish flesh. Adv. Food Res. 26: 275-311.

Sikorski, Z. 1978. Protein changes in muscle foods due to freezing and frozen storage. Int. J. Refrig. 1: 173-180.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

Tome, D., Naulet, N., and Kozlowski, A. 1985. ^{13}C-NMR characterization of formaldehyde bonds in model mixtures and proteins containing lysine. Int. J. Pept. Prot. Res. 25: 258-266.

CHAPTER 5

EFFECT OF SOME BIOGENIC AMINES ON PROTEIN CROSS-LINKING AND GEL FORMING ABILITY OF SURIMI FROM NILE TILAPIA INDUCED BY MICROBIAL TRANSGLUTAMINASE

5.1 Abstract

Impact of different biogenic amines including putrescine, histamine and tyramine at various levels (0, 2 and 5 mmol/kg) on gel properties of surimi from Nile tilapia added with microbial transglutaminase (MTGase) at 0.4 units/g was studied. All biogenic amines were able to act as an acyl acceptor in MTGase catalysed reaction, in which ammonia was released as by-product. The addition of biogenic amines into surimi had no impact on gel forming ability in the absence of MTGase. In the presence of MTGase (0.4 units/g), breaking force and water holding capacity of all gels increased, compared to those of gels without MTGase. Nevertheless, breaking force of gel containing MTGase was lowered when biogenic amine at a level of 5 mmol/kg was incorporated. Polymerisation of myosin heavy chain was enhanced when MTGase was added, but took place at a slightly lower degree in gel containing biogenic amines. Thus, biogenic amines at high concentrations decreased gel strengthening effect of MTGase in surimi.

5.2 Introduction

Biogenic amines have been found in various foods, particularly fish and fish products, cheese, meat and fermented foods (Ruiz-Capillas and Jiménez-Colmenero, 2005). They are low molecular-weight organic compounds, which are derived from the corresponding amino acids when the carboxylic group is removed by enzymatic reactions (Visciano *et al.*, 2012; Zhai *et al.*, 2012). During storage and processing, food proteins can be degraded into free amino acids. When food is contaminated with bacteria containing decarboxylase, these free amino acids undergo decarboxylation and biogenic amines are produced (Ruiz-Capillas and Jiménez-Colmenero, 2005). In addition to the availability of free amino acids as precursors,

biogenic amines accumulation requires the presence of microorganisms with decarboxylases and favourable conditions for their growth and decarboxylation activity (Zarei *et al.*, 2011; Zhai *et al.*, 2012).

The concentrations of some biogenic amines (tyramine, putrescine, and histamine) nomally increase during processing and storage of meat and fish (Ruiz-Capillas and Jiménez-Colmenero, 2005; Visciano *et al.*, 2012). Özogul and Özogul (2006) reported that biogenic amine contents in sardines (*Sardina pilchardus*) increased with increasing storage time. Putrescine and cardaverine reached 122 mg/kg and 100 mg/kg at 15 days of storage at 4 °C. It was reported that the accumulation of histamine in humans causes health risks when the level is above 500 mg/kg. US Food and Drug Administration (FDA) has also recommended that 100 mg/kg of tyramine and 1000 mg/kg of total biogenic amines should be the tolerance levels in fish.

Microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins, thereby increasing the gel strength of surimi (Benjakul *et al.*, 2008; Duangmal and Taluengphol, 2010; Jiang *et al.*, 2000). MTGase induces the formation of an ε-(γ-glutamyl)lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and γ-carboxamide group of a glutamine residue (Motoki and Kumazawa, 2000). MTGase is also capable of catalysing acyl transfer reactions between the γ-carboxyamide of glutaminyl residues and various primary amines, diamine or polyamine in peptides or protein (acyl acceptors) (Motoki and Kumazawa, 2000). The amino groups of biogenic amines can function as acyl acceptor, and also other amines such as putrescine, ethylenediamine, spermine, spermidine and 3,3-iminobispropylamine have been reported to function as acceptors (Lai *et al.*, 2004; Punakivi *et al.*, 2006; Schrode and Folk, 1978). Nile tilapia is one of the important economic freshwater fish, contributing more than 40% to total aquaculture production in Thailand (Rawdkuen *et al.*, 2009; Department of Fisheries, 2010). Moreover, low levels of biogenic amines in tilapia has been reported even after a long storage period (Sil *et al.*, 2008; Kulawik *et al.*, 2013). Due to the availability of this species in market and low biogenic amines content, Nile tilapia was used as a model in this study. Since biogenic amines can serve as an acyl acceptor in acyl transfer reaction mediated by MTGase, the cross-linking between

protein and protein by MTGase in surimi, especially produced from unfreshed fish with high biogenic amines can be impeded. Nevertheless, there is no information regarding the effect of biogenic amines on gelling properties of surimi added with MTGase. Therefore, the aim of this study was to investigate the impact of some biogenic amines on gel forming ability of Nile tilapia surimi mediated by MTGase.

5.3 Materials and Methods

5.3.1 Chemicals

Sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, glutaraldehyde, histamine, tyramine and putrescine were purchased from Sigma (St. Louis, MO, USA). *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Microbial transglutaminase (MTGase) from *Streptoverticillium mobaraense* (TG-K) containing 1% of pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

5.3.2 Fish samples

Live nile tilapia (*Oreochromis niloticus*) with the average size of 800-1000 g were obtained from a local market in Songkhla province, Thailand. After head-blowing, fish were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Fish were then washed and filleted manually.

5.3.3 Effect of different biogenic amines on acyl transfer reaction in natural actomyosin mediated by MTGase

5.3.3.1 Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul *et al*. (1997). Fish mince (10 g) was homogenised in 100 ml of chilled 0.5 M NaCl, pH 7.0 using a homogeniser for 4 min at a speed of 11,000 rpm (IKA Labortechnik, Selangor, Malaysia). Overheating during extraction was avoided by keeping the sample in an

iced container and each 20s of homogenisation was followed by a 20s rest interval. The homogenate was centrifuged at 5,000xg for 30 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Three volumes of chilled water (0–4 °C) were added to precipitate NAM, which was then collected by centrifuging at 5,000xg for 20 min at 4 °C using a refrigerated centrifuge. The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.5 M NaCl, pH 7.0 for 30 min at 4 °C.

5.3.3.2 MTGase mediated reaction in the presence of various biogenic amines

Ten ml of NAM solution (5 mg/ml) containing biogenic amines at different levels (0, 2, and 5 mM) was allowed to stand in ice for 1 h. The prepared solutions were preheated at 40 °C for 10 min in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany). Thereafter, 100 µl of MTGase was added into NAM to obtain a level of 20 units/g protein. The reaction mixtures were incubated at 40 °C for 30 min. The reaction was terminated by adding 10 ml of 15% (w/v) TCA. The resulting reaction mixtures were then subjected to the determination of ammonia content.

5.3.3.3 Determination of ammonia content

Ammonia content in reaction mixtures was determined according to the Standard Methods (APHA-AWWA/WPCF, 1998). Appropriately diluted sample (500 ml) was placed in 800-ml Borosilicate glass flask containing 25 ml of 0.125 M $Na_2B_4O_7$ and pH was adjusted to 9.5 using 6 N NaOH. The mixture was distilled and the distillate was collected in 50 ml of 2% boric acid consisting of the indicator (methyl red: methylene blue). The solution was then titrated with 0.02 N H_2SO_4 to reach the end-point. Ammonia content was expressed as mg /l using the following equation:

$$\text{Ammonia content (mg/l)} = (\text{ml } H_2SO_4 \times 280)/\text{ml of sample}$$

5.3.4 Effect of MTGase on gel properties of surimi containing different biogenic amines at various levels

5.3.4.1 Surimi preparation

Fillet was minced to uniformity using a mincer with a hole diameter of 5 mm. The mince was then washed with cold water (5-8 °C) at a mince/water ratio of 1:3 (w/v). The mixture was stirred gently for 3 min and washed mince was filtered with a layer of nilon screen. The washing process was carried out three times. Finally, the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 xg for 15 min. Washed mince was mixed thoroughly with 4% sorbitol and 4% sucrose as cryoprotectants. The resulting mixture was frozen at -18°C using an air-blast freezer. The frozen surimi was kept at -20 °C until used. Before use, frozen surimi was tempered using a running water until temperature reached 0-2 °C

To determine the effect of different biogenic amines (putrescine, tyramine and histidine) on gel forming ability of surimi, biogenic amines were added to the surimi to obtain the final concentrations of 0, 2, 5 mmol/kg and chopped for 3 min at 4°C. The resulting mixture was allowed to stand for 1 h at 4°C. The mixture was then added with 2.5% salt and the moisture content was adjusted to 82% in the absence and presence of MTGase (0.4 units/g). The mixture was chopped for another 3 min to obtain the homogenous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min. The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

5.3.4.2 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (28 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation

(cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

5.3.4.3 Determination of expressible moisture content.

Expressible moisture content (%) was determined according to the method of Benjakul *et al*. (2003). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between two pieces of Whatman no. 1 filter paper (Whatman Ltd., Maidstone, UK) at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y)*. Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = [(X - Y)/X]\text{x}100$$

5.3.4.4 Determination of whiteness

Gel samples were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (NFI, 1991).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

5.3.4.5 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the sample, 27 ml of 5% (w/v) SDS solution (85 °C) were added to the gel sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500xg for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4%

stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

5.3.4.6 Microstructure analysis

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100%. Samples were subjected to critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA,USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

5.3.5 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 Duncan'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).

5.4 Results and Discussion

5.4.1 Effect of different biogenic amines on acyl transfer reaction in natural actomyosin (NAM) mediated by MTGase

The effect of different biogenic amines including putrescine, histamine and tyramine at a concentration of 2 mM in NAM (5 mg/ml) as acyl acceptor in the

MTGase catalysed reaction was determined by monitoring the formation of ammonia as shown in Table 11. MTGase can induce acyl transfer reaction, in which the acyl of γ-carboxamide groups of peptides is transferred to the ε-amino group of lysine residues and a variety of primary amines (acyl acceptor) (Folk and Finlayson, 1977; Gaspar and de Góes-Favoni, 2015; Motoki and Seguro, 1998). As all reactions mediated by MTGase result in the formation of free ammonia, the measurement of ammonia can be used as a tool to monitor the overall transglutaminase reaction (Punakivi *et al.*, 2006).

When MTGase was added into NAM, ammonia was produced, indicating that cross-linking of NAM occurred intermolecularly or intramolecularly. Therefore, NAM could serve as the substrate for transglutaminase. Several studies have been reported that myofibrillar protein, especially myosin, is a good substrate for MTGase mediated reaction (Ahhmed *et al.*, 2009; Chin *et al.*, 2009; Ramirez-Suarez and Xiong, 2002). Ahhmed *et al*. (2009) reported that MTGase was able to catalyse the interconnections of myofibrillar protein extracted from beef and chicken, resulting in the significant increase in ε-(γ-glutamyl)lysine content. However, no ammonia was found in NAM in the absence of MTGase, indicating that no acyl transfer reaction took place. In the presence of biogenic amines at a level of 2 mM, the concentration of ammonia increased, compared to that without biogenic amines ($P < 0.05$). Nevertheless, no difference in ammonia content was found between NAM added with different types of biogenic amines ($P > 0.05$). Punakivi *et al.* (2006) reported the formation of ammonia in MTGase catalysed reaction using *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) as donor substrate and biogenic amines as acceptor. Cadaverine was the best acceptor substrate for MTGase with the highest concentration of ammonia produced (Punakivi *et al.*, 2006). The similar concentration of ammonia produced from MTGase reaction having tyramine and histmine as acyl acceptor was found. Those biogenic amines were smaller in size in comparison with muscle proteins, where lysine was located. As a result, those biogenic amines could expose or localise themselves for acyl reaction more effectively than protein. This was indicated by the higher ammonia content (Table 11). Therefore, putrescine,

histamine and tyramine were shown to be the better acyl acceptor for MTGase reaction than NAM.

Table 11 Ammonia content in NAM formed by MTGase catalysed reaction in the absence and presence of different biogenic amines

Samples	Ammonia content (mg/l)
NAM	ND**
NAM with MTGase	7.60±0.10[a]
NAM+2 mM putrescine with MTGase	10.27±0.81[b]
NAM+2 mM histamine with MTGase	10.73±0.81[b]
NAM+2 mM tyramine with MTGase	9.80±0.81[b]

Values are given as mean ± SD (n=3)
* Different lowercase superscripts indicate the significant differences ($P < 0.05$)
** ND: not detectable

5.4.2 Effect of MTGase on gel properties of surimi in the presence of biogenic amines at different levels

5.4.2.1 Breaking force and deformation

Breaking force and deformation of gels from Nile tilapia surimi in the presence of biogenic amines (putrescine, histamine and tyramine) at 0, 2, and 5 mmol/kg without and with the addition of MTGase (0.4 units/g) were depicted in Figure 17. Without MTGase addition, no difference in breaking force was found in gels without and with the addition of biogenic amines ($P > 0.05$). Thus, biogenic amine ranging from 0 to 5 mmol/kg had no effect on gel forming ability of surimi from Nile tilapia.

When MTGase at a level of 0.4 units/g was incorporated into surimi gel, the increase in breaking force by 53.9% was observed, compared with the control gel (without MTGase and biogenic amines). MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins, in which ε-(γ-glutamyl)lysine cross-links between protein chains could be formed. As a result, the strength of gel matrix was increased. In the presence of biogenic amines at 2 mmol/kg, similar breaking force was noticeable, compared with the control gel. It was suggested that

the strong gel development was still achieved by addition of MTGase when biogenic amines were present at low level. These protein cross-links via covalent bonds altered in their functional properties and enhanced textural characteristics (Gaspar and de Góes-Favoni, 2015). However, the strengthening effect of MTGase on surimi gel was lowered as biogenic amines at 5 mmol/kg were incorporated, regardless of type of biogenic amines. With the addition of MTGase, breaking force of gel from surimi added with tyramine at 5 mmol/kg had the increase by 19.7%, compared to that without MTGase, followed gels containing putrescine and histamine, respectively. Apart from the cross-linking reaction, the other two reactions catalysed by transglutaminase are deamidation and amine incorporation (Motoki and Seguro, 1998). At high concentration, biogenic amines could function as a competitive substrate in the cross-linking reaction. Therefore, the presence of biogenic amines including histamine, tyramine and putrescine in surimi might prevent some intermolecular cross-linking between myofibrillar protein mediated by MTGase. Those biogenic amines could be found in unfreshed fish used for fish mince or surimi production. This might be related with inferior setting phenomenon in poor quality surimi or the lower gel strengthening effect of MTGase for poor quality surimi.

For deformation, there was no marked difference amongst gels without and with biogenic amines ($P > 0.05$). However, it was noted that deformation of gel slightly increased when MTGase was added ($P < 0.05$). MTGase could induce the cross-linking of muscle protein, in which the longer chains could be formed. The interaction between those chains might yield the gel network with more elasticity as evidenced by the increased deformation. Although biogenic amines, particularly at higher level, lowered breaking force, they did not show the adverse effect on deformation of the resulting gels.

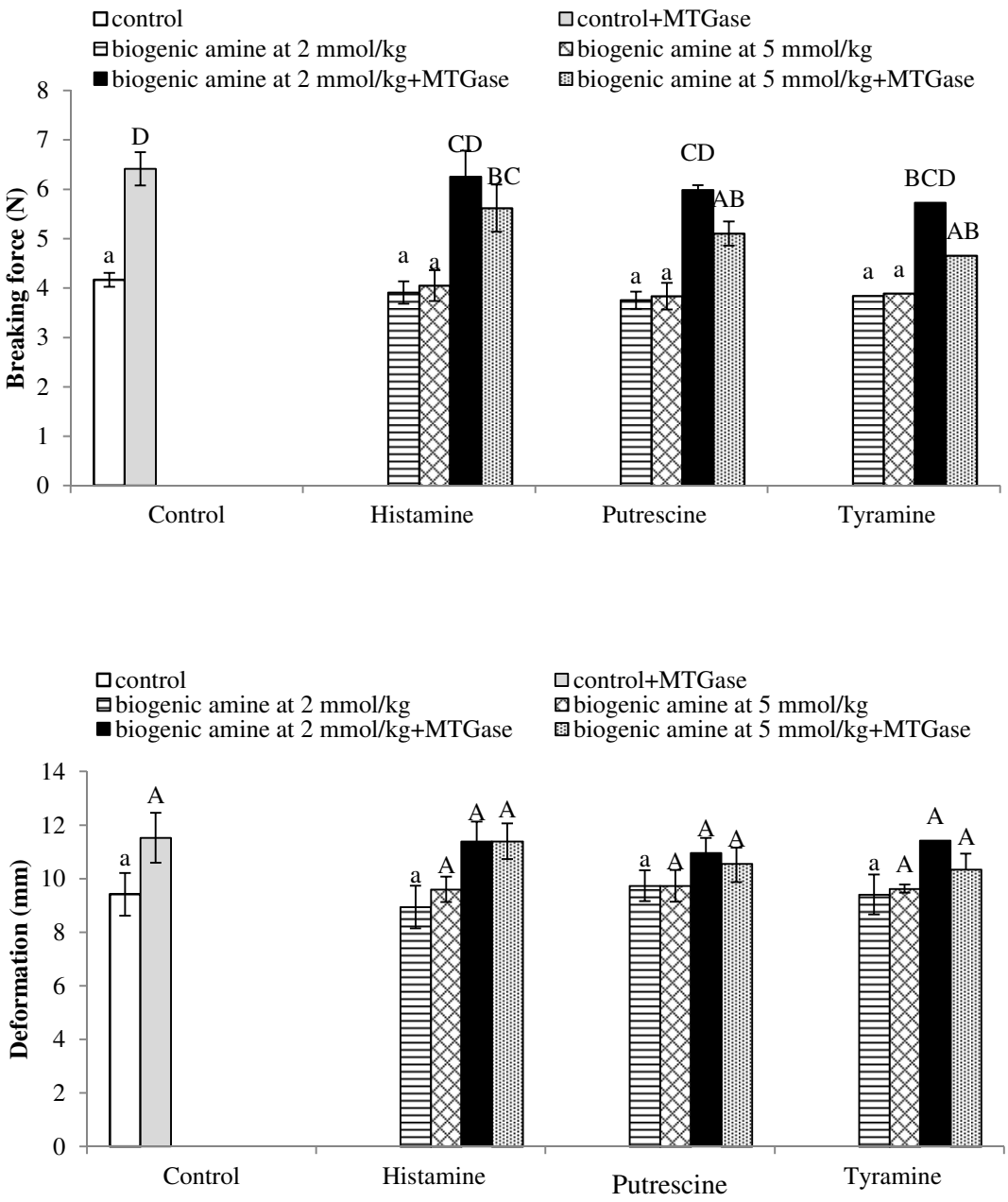


Figure 17. Breaking force and deformation of gels from surimi containing different amines at various levels in the absence and presence of MTGase (0.4 units/g). Bars represent the standard deviation (n=3). Different uppercase or lowercase letters on the bars within the same MTGase level indicate significant difference ($P < 0.05$).

5.4.2.2 Expressible moisture content

Expressible moisture content of gels from surimi containing biogenic amines at different levels in the absence and presence of MTGase (0.4 units/g) is shown in Table 12. No difference in expressible moisture content of gels was noticeable when biogenic amines were incorporated in surimi, regardless of the types of biogenic amines. Non-significant decreases in expressible moisture content were observed in all gels, when MTGase was added ($P > 0.05$). The lower expressible moisture content of gels suggested more water retained in the gel network (Niwa, 1992). This property depends on the structure of the muscle proteins that bind and interact with the water molecules in the gel network. It was noted that the addition of MTGase could increase the ability of gel in water holding as evidenced by the lowered expressible moisture content. The addition of MTGase could enhance the cross-linking of proteins to some degree, resulting in the formation of stronger network with the greater water holding capacity (Benjakul *et al.*, 2008). With increasing water holding capacity, the better textural properties including stiffness, cohesion, chewability and elasticity of protein gels were obtained (Han *et al.*, 2009; Min and Green, 2008; Gaspar and Góes-Favoni, 2015). Although biogenic amines were able to serve as acyl acceptor for MTGase mediated reaction, the interconnected network between the main proteins, myosin heavy chain or actin, still took place. As a consequence, the network could still imbibe water effectively. Therefore, biogenic amines at 2 or 5 mmol/kg showed no mark effect on water holding capacity of the resulting gels.

5.4.2.3 Whiteness

Whiteness of gels from surimi containing biogenic amines at different levels in the absence and presence of MTGase is shown in Table 12. Similar whiteness was observed amongst gels containing biogenic amines, irrespective of types and levels ($P > 0.05$). Also, no differences in whiteness were found between gel added without and with MTGase addition. Kaewudom *et al.* (2012) also reported that the addition of MTGase at 1.2 units/g had no impact on whiteness of surimi gels from

threadfin bream . Thus, the presence of putrescine, histamine and tyramine had no influence on the whiteness of gels from Nile tilapia, regardless of MTGase addition.

Table 12 Expressible moisture content and whiteness of gels from surimi containing different biogenic amines at various levels in the absence and presence of MTGase (0.4 units/g).

	Biogenic amine concentration (mmol/kg)	Expressible moisture content		Whiteness	
		Without MTGase	With MTGase	Without MTGase	With MTGase
Control		5.042±0.415[a]	4.470±0.286[a]	85.154±0.272[a]	85.365±0.440[a]
Putrescine	2	5.128±0.205[a]	4.966±0.364[a]	85.174±0.529[a]	85.530±0.602[a]
	5	5.175±0.233[a]	4.967±0.242[a]	85.351±0.381[a]	85.527±0.285[a]
Histamine	2	5.158±0.487[a]	4.664±0.300[a]	85.418±0.592[a]	85.629±0.259[a]
	5	5.176±0.382[a]	4.679±0.281[a]	85.080±0.732[a]	85.297±0.855[a]
Tyramine	2	5.154±0.373[a]	4.635±0.373[a]	85.096±0.747[a]	85.339±0.240[a]
	5	5.184±0.326[a]	4.712±0.252[a]	84.872±0.310[a]	85.271±0.497[a]

Values are given as mean ± SD (n=3)
* Different lowercase superscripts within the same column indicate the significant differences ($P < 0.05$)

5.4.2.4 Protein pattern

Protein patterns of surimi gels containing biogenic amines at different levels in the absence and presence of MTGase are shown in Figure 18. Gels contained myosin heavy chain (MHC) and actin as the major proteins. Similar pattern was noticeable between the control gel and those containing biogenic amines (2 and 5 mmol/kg). The result showed that biogenic amines had no pronounced impact on protein cross-linking.

When MTGase was added into the control gel, the intensity of MHC band decreased drastically, compared to that without MTGase addition. The disappearance of MHC indicated the formation of MHC cross-links mainly via the formation the ε-(γ-glutamyl)lysine isopeptide induced by MTGase. MHC has been known to serve as the major contributor for gel formation and determine gel property of fish protein (Nakahara *et al.*, 1999). Although MHC band intensity decreased markedly, actin and tropomyosin were rarely changed in all samples. Thus, MHC appeared to be a preferable substrate for cross-linking induced by MTGase. Herrero *et al*. (2008) reported that the cross-links introduced by MTGase altered the structure of

MHC, with a significant reduction in the content of the α-helix structure, and an increase in the β-sheet and percentage of pleats/folds, allowing for the formation of high molecular weight polymers. These modifications improved textural properties, resulting in strong gels with a compact and ordered structural conformation (Gaspar and Góes-Favoni, 2015).

When biogenic amine was incorporated into surimi in the presence of MTGase, MHC in gel was remained to some degree, compared to that found in the control gel. This confirmed that the cross-linking of muscle proteins was lowered when biogenic amines was present, resulting in the lowered protein-protein interaction. Lai *et al.* (2004) reported that primary amine including putrescine and biotinylated pentylamine antagonised transglutaminase ability to cross-link glutamine monomers into high molecular weight complexes. The results were in accordance with the ability of biogenic amines as acyl acceptor in MTGase mediated reaction (Table 11).

5.4.3 Microstructure

Gels from Nile tilapia surimi showed the well-structured and fine matrix with highly interconnected strands. Similar structure was found between surimi gels without and with the addition of biogenic amines. It was noted that biogenic amines in surimi had no marked impact on microstructure of surimi gel. In the presence of MTGase, protein could undergo the cross linking more effectively. Gel became slightly more compact, with a denser gel network and smaller voids as MTGase was added. Therefore, the addition of MTGase could induce the formation of stronger gel network as evidenced by a higher breaking force (Figure 17) along with lowered expressible moisture content (Table 12). However, the control gel in the presence of MTGase showed the most compact network with the smallest voids, compared to gels containing biogenic amines, especially at 5 mmol/kg. MTGase induced the formation of isopeptide bonds between glutamine and lysine residues in proteins, thus introducing both inter- and intramolecular covalent cross-links (Motoki and Seguro, 1998; DeJong and Koppelman, 2002; Gaspar and Góes-Favoni, 2015).

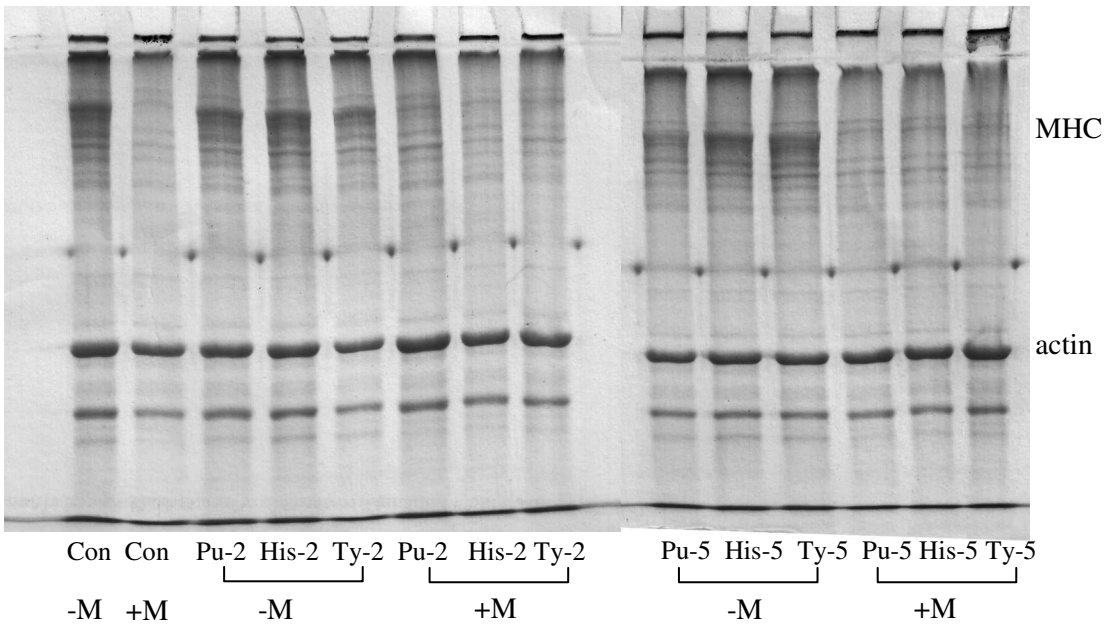


Figure 18 Protein patterns of gels from surimi containing different biogenic amines at various levels in the absence (-M) and presence (+M) of MTGase (0.4 units/g). Con: control (without amine); Pu: putrescine; His: histamine; Ty: tyramine; MHC: myosin heavy chain. Numbers designate the levels of amines added (mmol/kg); M: MTGase

However, the control gel in the presence of MTGase showed the most compact network with the smallest voids, compared to gels containing biogenic amines, especially at 5 mmol/kg. MTGase induced the formation of isopeptide bonds between glutamine and lysine residues in proteins, thus introducing both inter- and intramolecular covalent cross-links (Motoki and Seguro, 1998; DeJong and Koppelman, 2002; Gaspar and Góes-Favoni, 2015). In the presence of biogenic amines, MTGase could induce cross-linking between glutamine and amines to a lower extent. Thus, protein-protein cross-linking was decreased. Nevertheless, in the presence of MTGase, there was no difference in microstructure of gel containing different biogenic amines.

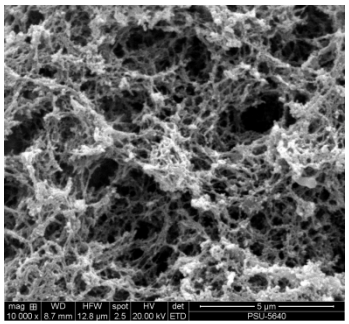
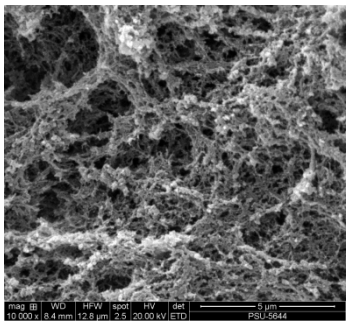
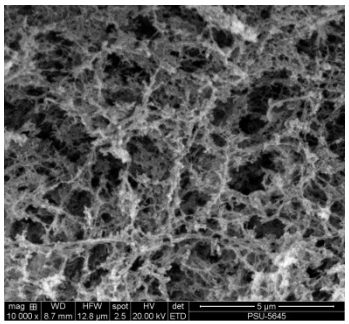
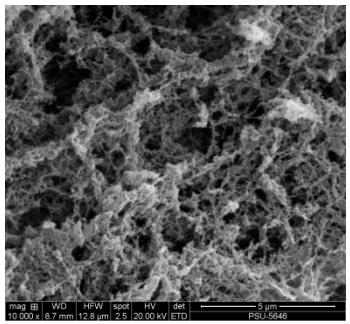
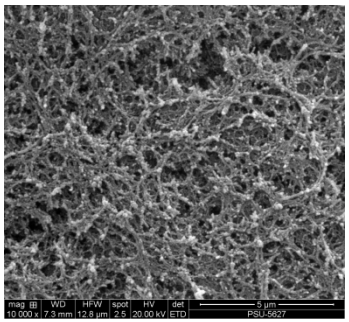
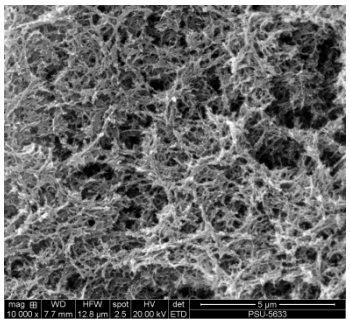
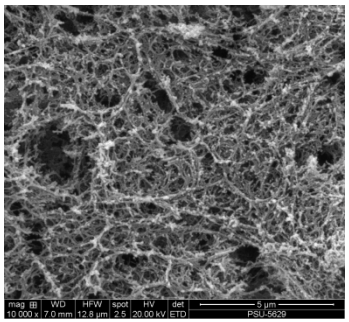
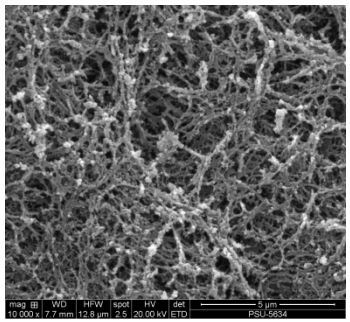
Without MTGase				
With MTGase				
	Control (no amine)	Putrescine (5 mmol/kg)	Histamine (5 mmol/kg)	Tyramine (5 mmol/kg)

Figure 19. Electron microscopic image of surimi gel added with different biogenic amines (5 mmol/kg) in the absence and presence of MTGase (0.4 units/g) (Magnification: 10,000x).

5.5 Conclusion

Putrescine, histamine and tyramine were able to serve as the acyl acceptors in MTGase catalysed reaction. Biogenic amines had no profound effect on gelling properties of surimi in the absence of MTGase. However, biogenic amines, especially at high concentration, acted as the competitive substrates for MTGase, thereby lowering the cross-linking between muscle proteins. This led to the slight decrease in gel strength of resulting surimi gel.

5.6 References

Ahhmed, A. M., Kuroda, R., Kawahara, S., Ohta, K., Nakade, K., Aoki, T., and Muguruma, M. 2009. Dependence of microbial transglutaminase on meat type in myofibrillar proteins cross-linking. Food Chem. 112: 354-361.

APHA-AWWA/WPCF 1998. Standard methods for the examination of water and waste water. American Public Health Association. Washington, DC.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Chin, K. B., Go, M. Y., and Xiong, Y. L. 2009. Konjac flour improved textural and water retention properties of transglutaminase-mediated, heat-induced porcine myofibrillar protein gel: Effect of salt level and transglutaminase incubation. Meat Sci. 81: 565-572.

DeJong, G. A. H., and Koppelman, S. J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

Department of Fisheries. 2010. Fisheries Statistics of Thailand. Technical paper No.12/2010. Information Technology Center, Department of Fisheries, Ministry of Agriculture and Cooperatives. Bangkok.

Duangmal, K., and Taluengphol, A. 2010. Effect of protein additives, sodium ascorbate, and microbial transglutaminase on the texture and colour of red tilapia surimi gel. Int. J Food Sci. Technol. 45: 48-55.

Folk, J., and Finlayson, J. 1977. The ε-(γ-glutamyl) lysine crosslink and the catalytic role of transglutaminases. Adv. Prot. Chem. 31: 1-133.

Gaspar, A. L. C., and de Góes-Favoni, S. P. 2015. Action of microbial transglutaminase (MTGase) in the modification of food proteins: a review. Food Chem. 171: 315-322.

Han, M., Zhang, Y., Fei, Y., Xu, X., and Zhou, G. 2009. Effect of microbial transglutaminase on NMR relaxometry and microstructure of pork myofibrillar protein gel. Eur. Food Res. Technol. 228: 665-670.

Herrero, A. M., Cambero, M., Ordóñez, J., De la Hoz, L., and Carmona, P. 2008. Raman spectroscopy study of the structural effect of microbial transglutaminase on meat systems and its relationship with textural characteristics. Food Chem. 109: 25-32.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000. Combination effects of microbial transglutaminase, reducing agent, and protease inhibitor on the quality of hairtail surimi. J. Food Sci. 65: 241-245.

Kaewudom, P., Benjakul, S., and Kijroongrojana, K. 2012. Effect of bovine and fish gelatin in combination with microbial transglutaminase on gel properties of threadfin bream surimi. Int. Aquat. Res. 4: 1-13.

Kulawik, P., Özoğul, F., and Glew, R. H. 2013. Quality properties, fatty acids, and biogenic amines profile of fresh tilapia stored in ice. J. Food Sci. 78: 1063-1068.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lai, T. S., Tucker, T., Burke, J., Strittmatter, W., and Greenberg, C. 2004. Effect of tissue transglutaminase on the solubility of proteins containing expanded polyglutamine repeats. J. Neurochem. 88: 1253-1260.

Min, B., and Green, B. 2008. Use of microbial transglutaminase and nonmeat proteins to improve functional properties of low NaCl, phosphate-free patties made from channel catfish (*Ictalurus punctatus*) belly flap meat. J. Food Sci. 73: 218-226.

Motoki, M., and Kumazawa, Y. 2000. Recent research trends in transglutaminase technology for food processing. Food Sci. Technol. Res. 6: 151-160.

Motoki, M., and Seguro, K. 1998. Transglutaminase and its use for food processing. Trends Food Sci. Technol. 9: 204-210.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

NFI 1991. A manual of standard methods for measuring and specifying the properties of surimi. National Fisheries Institute. Washington, DC.

Niwa, E. 1992. Chemistry of surimi gelation. *In* Surimi technology (Lanier, T. C., and Lee, C. eds.). p. 389-427. Marcel Dekker, New York.

Özogul, F., and Özogul, Y. 2006. Biogenic amine content and biogenic amine quality indices of sardines (*Sardina pilchardus*) stored in modified atmosphere packaging and vacuum packaging. Food Chem. 99: 574-578.

Punakivi, K., Smolander, M., Niku-Paavola, M.-L., Mattinen, J., and Buchert, J. 2006. Enzymatic determination of biogenic amines with transglutaminase. Talanta. 68: 1040-1045.

Ramirez-Suarez, J. C., and Xiong, Y. L. 2002. Transglutaminase cross-linking of whey/myofibrillar proteins and the effect on protein gelation. J. Food Sci. 67: 2885-2891.

Rawdkuen, S., Sai-Ut, S., Khamsorn, S., Chaijan, M., and Benjakul, S. 2009. Biochemical and gelling properties of tilapia surimi and protein recovered using an acid-alkaline process. Food Chem. 112: 112-119.

Ruiz-Capillas, C., and Jiménez-Colmenero, F. 2005. Biogenic amines in meat and meat products. Crit. Rev. Food Sci. 44: 489-599.

Schrode, J., and Folk, J. 1978. Transglutaminase-catalyzed cross-linking through diamines and polyamines. J. Biol. Chem. 253: 4837-4840.

Sil, S., Joseph, J., and Kumar, K. A. 2008. Changes in biogenic amines during iced and ambient temperature storage of tilapia. J. Sci. Food Agric. 88: 2208-2212.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

USFDA 2001. Scombrotoxin (histamine) formation. *In* Fish and fishery products hazards and controls guide. p. 73-93. Department of Health and Human Services, Washington, DC.

Visciano, P., Schirone, M., Tofalo, R., and Suzzi, G. 2012. Biogenic amines in raw and processed seafood. Front. Microbiol. 3: 1-10.

Zarei, M., Najafzadeh, H., Enayati, A., and Pashmforoush, M. 2011. Biogenic amines content of canned tuna fish marketed in Iran. Am. Eura. J. Toxicol. Sci. 3: 190-193.

Zhai, H., Yang, X., Li, L., Xia, G., Cen, J., Huang, H., and Hao, S. 2012. Biogenic amines in commercial fish and fish products sold in southern China. Food Control. 25: 303-308.

CHAPTER 6

PHYSICOCHEMICAL CHANGES OF MYOSIN AND GELLING PROPERTIES OF WASHED NILE TILAPIA MINCE AS INFLUENCED BY OXIDATIVE STRESS AND MICROBIAL TRANSGLUTAMINASE

6.1 Abstract

Physicochemical properties of myosin from tilapia subjected to oxidation via Fenton's reaction using H_2O_2 (0, 0.05, 0.1, 1 and 5 mM) were determined. With increasing H_2O_2 concentrations and times (from 0 to 12 h), sulfhydryl group content and Ca^{2+}-ATPase activity decreased, while carbonyl content and surface hydrophobicity increased to a higher extent. After being subjected to oxidation, cross-linking via disulfide bond along with increased storage modulus (G´) was observed. Microbial transglutaminase (MTGase) induced polymerisation of myosin in both nonoxidised and oxidised forms and increased gel G´. Gel properties of washed mince and oxidised washed mince were determined in the presence and absence of MTGase. A stronger gel was observed when 0.3 units/g was added, regardless of oxidation process. Nevertheless, the gel strengthening effect of MTGase was hampered when mince was subjected to severe oxidation. Excessive protein aggregation of oxidised samples prior to gelation resulted in the reduction of gel strength and water-holding capacity. Negative effect of protein oxidation on gelation could therefore be alleviated to some degree by MTGase addition.

6.2 Introduction

Oxidation processes generally affect the quality of foods via inducing a number of changes in proteins such as modification of amino acid side chains, protein fragmentation, polymerisation, and structural alteration. Several chemicals have been known to induce the oxidation. Fenton's reaction is one of the mechanisms to generate hydroxyl radicals ($OH^{\cdot}$) from H_2O_2 in the presence of Fe^{2+} Hydroxyl radicals are reactive species, which can undergo side reaction with amino acid residues, react with

another carbon-centered radical to form a protein-protein crosslinks, etc. Those reactions lead to the changes in composition and configuration (Liu and Xiong, 2000a; Berlett and Stadtman, 1997).

Myofibrillar proteins, especially myosin, are well known to be primarily responsible for gelation therefore textural properties of comminuted muscle foods (Sun and Holley, 2011). The heat induced gelation of myosin results in the formation of three-dimensional networks that hold water in a less mobile state. However, myosin is susceptible to oxidation, causing cross-linking between individual protein molecules. Crosslinked heterogeneous myosin can be formed in oxidised myofibrillar proteins (Liu and Xiong, 2000a). The increase in carbonyl contents (Srinivasan and Hultin, 1997; Rowe *et al.*, 2004) was associated with the reductions in thermal stability of myosin (Liu and Xiong, 2000b). Gelation of comminuted muscle foods is generally influenced by several physicochemical processes (Ooizumi and Xiong, 2006). Oxidised proteins have varying functional properties, particularly gelation. Decker *et al.* (1993) reported that oxidation of turkey white muscle myofibrillar proteins by iron or copper and ascorbate caused a decreased gel strength. Srinivasan and Hultin (1997) also reported that frozen cod surimi treated with free-radical generating system had a poorer gel quality.

Transglutaminase (EC 2.3.2.13) is a transferase capable of catalysing cross-linking, resulting in the formation of ε-(γ - glutamyl) lysine cross-link in target proteins via acyl transfer between the ε-amino groups of a lysine residue and γ-amide group of a glutamine residue (DeJong and Koppelman, 2002). Microbial transglutaminase (MTGase) has been widely used in processed fish and meat in order to strengthen protein-based gels. MTGase has been reported to increase gel strength of surimi from different fish such as lizardfish (Benjakul *et al.*, 2008), threadfin bream (Benjakul *et al.*, 2004; Jiang *et al.*, 2000) , and sardine (Kudre and Benjakul, 2013; Karayannakidis *et al.*, 2008). Efficiency of MTGase in improving gel properties of proteins depends on many factors, e.g., the amount of MTGase as well as protein substrates (Asagami *et al.*, 1995; DeJong and Koppelman, 2002).

Structural unfolding and aggregation are two common changes induced by oxidants, which result in, respectively, the exposure and masking of amino side chain groups recognised by enzymes. These changes could either favour or suppress the MTGase induced protein gelation (Li *et al.*, 2012). However, there is no published report on the impact of MTGase on gelling properties of oxidised myosin in minced fish products despite the critical importance of gel formation and the susceptibility to oxidation (Decker *et al.*, 1993; Srinivasan and Hultin, 1997; Ooizumi and Xiong, 2006). Thus, the objectives of this study were to determine the reactivity of MTGase toward both oxidised and nonoxidised myosin from tilapia, a major aquacultural species, and to study the effect of MTGase on gelling properties of washed mince as influenced by oxidation process.

6.3 Materials and Methods

6.3.1 Chemicals

All chemicals were of analytical grade. Ascobic acid, sodium dodecyl sulphate (SDS), β-mercaptoethanol (βME), Trolox, propyl gallate and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide, hydrochloric acid, *N, N, N′, N′*-tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fisher Sciencetific (Fair Lawn, NJ, USA). Microbial transglutaminase (MTGase; Activa-TI with the activity of 100 units/g powder) from *Streptoverticilium mobaraense* containing 1%pure enzyme blended with 99%maltodextrin was donated by Ajinomoto Food Ingredients (Chicago, IL, USA).

6.3.2 Fish samples

Live Nile tilapia (*Oreochromis niloticus*) with the average size of 1-1.2 kg were obtained from a local market in Kentucky, USA. Fish were transported to the Department of Animal and Food Science, University of Kentucky within 1 h. Fish was then humanely killed, washed and filleted manually. The fillets were subjected to mincing using a mincer with the hole diameter of 5 mm and kept on ice during preparation.

6.3.3 Extraction of myosin

Myosin was isolated from fillet as described by Wang and Smith (1994) with a slight modification. All steps were performed below 10 °C to minimise proteolysis and protein denaturation. Fish mince was homogenised with 3 volumes of Guba-Straub solution (0.3 M KCl containing 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA, and 4 mM sodium pyrophosphate) for 1 min using a Polytron model PT 10/35 (Brinkmann Instruments, Westbury, NY, USA) at a speed of 11,000 rpm. Thereafter, 3 volumes of distilled water were added to the mixture. The homogenate was filtered through 2 layers of cheesecloth and diluted with 6.5 volumes of 1 mM EDTA with rapid stirring. The mixture was kept at 4 °C overnight before centrifugation at 1,000xg for 1 h using a refrigerated centrifuge (Sorvell RC-5B, Newtown, CT, USA). The pellet was collected and resuspended with 2 volumes of 25 mM PIPES buffer, pH 7 containing 3 M KCl. The mixture was stirred for 30 min on ice and subsequently diluted with 5 volumes of distilled water. Magnesium chloride and sodium pyrophosphate were added to obtain the final concentrations of 5 mM and 3 mM, respectively. The mixture was centrifuged at 40,000 xg for 2 h at 4 °C. Myosin in the supernatant was isolated by fractional precipitation with ammonium sulfate (35–48 % saturation). The precipitate was collected and redissolved in 20 mM Tris-HCl (pH 6.8) containing 0.6M KCl. After 24 h of dialysis against 100 volumes of the same buffer with two changes. The dialysed solution was collected and measured for protein concentration according to the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as standard. Densitometric data showed that the myosin sample had approximately 90 % purity.

6.3.4 Physicochemical changes in tilapia myosin as affected by oxidation

6.3.4.1 Oxidation of myosin

Myosin was firstly diluted to a final protein concentration of 20 mg/ml in 20 mM Tris buffer containing 0.6 M NaCl (pH 6.8). This solution was then subjected to oxidation by hydroxyl radical generated by the Fenton's reaction. Hydroxyl radicals were produced by mixing 10 μM $FeCl_3$/100 μM ascorbic acid with

H_2O_2 at different concentrations (0.05, 0.1, 1 and 5 mM) for various times (2, 6, and 12 h) at 4 °C. The oxidation reaction was terminated by propyl gallate/ Trolox C/EDTA to obtain the final concentration of 1 mM each. The non-oxidised myosin solution containing propyl gallate/Trolox C/EDTA was used as the control. All samples were subjected to analyses:

6.3.4.2 Total sulfhydryl group content

Total sulfhydryl group content was determined using 5,5′ -dithiobis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) with a slight modification. To 1 ml of myosin solution (4 mg/ml), 9ml of 0.2MTris–HCl buffer, pH 8, containing 8Murea, 2% SDS and 10 mM EDTA, were added. To 3 ml of the mixture, 0.3 ml of 0.1 % DTNB, dissolved in 0.2MTris–HCl (pH 8.0) was added and the mixture was incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a double beam spectrophotometer (model UV-1800, Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 M KCl. Sulfhydryl group content was calculated, using the extinction coefficient of 13,600 M^{-1} cm^{-1} and was expressed as mol/10^5 g protein.

6.3.4.3 Carbonyl content

Protein carbonyl content, an index of protein oxidation, was measured according to the method of Levine *et al.* (1990) with slight modifications. A 100-µl aliquot of protein solution (20 mg protein/ml) was reacted with 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl for 1 h at room temperature; another 100 µl of sample was added with 1 ml 2 N HCl (control). After incubation, 1 ml of 20 % trichloroacetic acid was added to precipitate the protein. The mixture was centrifuged at 10,000 xg for 10 min using a microcentrifuge (Eppendof-5415D, Eppendof, Hamburg, Germany). The precipitate was washed twice with 1.5 ml of an ethanol:ethyl acetate (1:1; v/v) mixture to remove unreacted DNPH, blow-dried, and dissolved in 1 ml of 20 mM potassium phosphate (pH 2.3) containing 6 M guanidine hydrochloride. The absorbance was read at 370 nm for carbonyl content and 280 nm

for protein content. The carbonyl concentration was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

6.3.4.4 Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of myosin with and without oxidation was determined according to the method of Benjakul *et al.* (1997). Diluted myosin solution (1 ml) was mixed with 0.6 ml of 0.5 M Tris-maleate (pH 7.0) and 1 ml of 0.1 M $CaCl_2$. Deionised water was added to make up a total volume of 9.5 ml. To the mixture, 0.5 ml of 20 mM adenosine 5-triphosphate (ATP) solution was added to initiate the reaction. The reaction was conducted for 8 min at 25 °C and terminated by adding 5 ml of chilled 15 % (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3,500 ×g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). The Ca^{2+}-ATPase activity was expressed as micromoles inorganic phosphate released/mg protein/min. A blank was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

6.3.4.5 Surface hydrophobicity

Surface hydrophobicity was determined by assessing fluorescence intensity using 8-anilino-1-naphthalene sulfonate (ANS), as a fluorescence probe. A series of protein solutions (0.2−3 mg/ml, 4 ml) were thoroughly mixed with 20 µl of 8.0 mM ANS, and the fluorescence intensity was measured after 20 min using a FluoroMax-3 spectrofluorometer (Horiba JobinYvon Inc., Edison, NJ, USA) 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”.

6.3.4.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of

5% (w/v) SDS solution (85 °C) were added to the sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 × g for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

6.3.5 MTGase cross-linking of oxidatively stressed myosin

Oxidised myosin was prepared using 10 µM $FeCl_3$/100 µM ascorbic acid in the presence of H_2O_2 at different concentrations (0.05, 0.1, 1 and 5 mM) for various times (2, 6, and 12 h) at 4 °C. Myosin (non-oxidised) and oxidised myosin samples in 20 mM Tris-HCl containing 0.6 M NaCl (pH 6.8) were incubated with MTGase at 25 units/g protein at 4 °C for 2 h. All samples were subjected to analyses as below:

6.3.5.1 SDS-polyacrylamide gel electrophoresis(SDS-PAGE)

Protein patterns were analysed by SDS-PAGE according to the method of Laemmli (1970) as mentioned above.

6.3.5.2 Rheological test

For oscillatory shear analysis, a Bohlin VOR rheometer (Bohlin Instruments, Inc., Cranbury, NJ, USA) was used to examine the dynamic formation of a protein network during thermal process. Samples were heated from 20 to 80 °C at a 1°C/min between two parallel plates (1 mm gap) in an oscillatory mode at a fixed

frequency of 0.1 Hz with a maximum strain of 0.02. Changes in the storage modulus, G′ (i.e., rigidity due to elastic response), were recorded.

6.3.6 MTGase effect on gel properties of washed mince as affected by oxidation process

6.3.6.1 Preparation of washed mince with and without oxidation

Tilapia fillets were subjected to mincing using a mincer with the hole diameter of 5 mm. Mince obtained were placed in polyethylene bag and imbedded in ice until use.

To prepare washed mince, the conventional washing process was implemented. Mince was washed with cold water (4 °C) using a water/mince ratio of 3:1 (v/w). The mixture was stirred gently for 10 min in a cold room (4 °C) and the washed mince was filtered with a layer of nylon screen. Washing was performed three times. Finally, the washed mince was centrifuged at 700×*g* for 15 min using a basket centrifuge (Model CE 21K, Grandiumpiant, Belluno, Italy). To prepare the oxidised mince, the washed mince was mixed with 10 µM $FeCl_3$, 100 µM ascorbic acid and 0.1 mM H_2O_2 using a mince/solution ratio of 1:3 (w/v). The mixture was stirred gently for 10 min in a cold room (4 °C), followed by filtration using a layer of nylon screen. The sample was then washed with cold water again before centrifugation at 700×*g* for 15 min using a basket centrifuge. The obtained mince samples were used for gel preparation.

6.3.6.2 Gel preparation

To prepare the gels, mince samples were ground for 2 min using a MoulinexMasterchef 350 mixer (Paris, France). Moisture content was adjusted to 82% and NaCl was added to the samples to obtain the concentration of 2.5% (w/w). After grinding for 2 min, the paste was added with various amounts of MTGase (0, 0.3 and 0.6 units/g sample). The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step

heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

6.3.6.2.1 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

6.3.6.2.2 Determination of expressible moisture content.

Expressible moisture content (%) was determined according to the method of Benjakul *et al*. (2003a). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between two pieces of Whatman no. 1 filter paper (Whatman Ltd., Maidstone, UK) at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (*Y)*. Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = [(X - Y)/X] \times 100$$

6.3.6.2.3 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (NFI, 1991).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

6.3.6.2.4 Microstructure

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100%. Samples were subjected to critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

6.3.7 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests (Steel and Torrie, 1980). Analysis was performed using the Statistical Package for Social Science package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and Discussion

6.4.1 Physicochemical properties of myosin as affected by oxidation process

6.4.1.1 Total sulfhydryl group content

Total sulfhydryl (SH) group contents of myosins oxidised with H_2O_2 (0–5 mM) in the presence of $FeCl_3$ and ascorbic acid for 2–12 h are shown in Table 13. SH group content of oxidised myosin decreased as the concentration of H_2O_2 and incubation time increased ($P < 0.05$), suggesting the increasing formation of disulfide bonds. When H_2O_2 at a concentration of 5 mM was used, the decrease in SH group by

50.2–54.8 % was obtained, compared with that observed in the control. SH groups, which are abundant in myofibrillar protein, undergo inter- and intramolecular disulfide cross-linking, which occurs during radical-mediated oxidation of proteins (Dean *et al.*, 1997). Hydroxyl radical formed by the Fenton's reaction caused the reduction of SH group by 7–55 %, compared to that found in the control myosin. Cysteine residues are particularly sensitive to oxidation and converted to disulfides by all forms of reactive oxygen species even under mild condition (Berlett and Stadtman, 1997). Frederiksen *et al.* (2008) found that SH groups of myosin are the main target for oxidative modification induced by hypervalent myoglobin species. Liu *et al.* (2000) also reported the reduction in SH group content of myofibrillar protein from pectolaris muscle after the protein was oxidised for 1 h and continued to decrease after 24 h with the concomitant increase in disulfide bonds. Thus, the oxidation process resulted in the formation of disulfide bond in myosin.

6.4.1.2 Carbonyl content

Carbonyl content is one of the most reliable measures of protein oxidation (Levine *et al.*, 1990). Carbonyl contents of myosin under different oxidation condition are shown in Table 13. Carbonyl contents of oxidised myosin increased as the higher levels of H_2O_2 and longer incubation time were used ($P < 0.05$). Myosin oxidised using 5 mM H_2O_2 for 12 h showed approximately 5-fold increase in carbonyl contents, compared to the control (non-oxidised). Li *et al.* (2012) reported that the exposures of myofibrillar protein extracted from longissimus muscle to 5 mM H_2O_2 in the presence of $FeCl_3$ for 24 h significantly increased carbonyl content (2.76 μmol/g protein) of resulting protein. The increase in protein carbonyls is one of the key biochemical changes that occur during protein oxidation. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidised (Estévez, 2011). Protein-bound carbonyls can also be derived from direct oxidative attack on amino acid side chains, fragmentation of the peptide backbone via α-amidation pathway, or cleavage associated with the oxidation of glutamyl residues (Estévez, 2011). The cleavage of peptide backbones and covalent attachments of secondary lipid oxidation products, such as malondialdehyde and 4-

hexyl-2 nonenal, also contributed to carbonyl formation in myofibrillar proteins (Estévez, 2011; Xiong *et al.*, 2000). In addition, Estévez *et al.* (2009) reported that specific carbonyl compounds in oxidised myofibrillar protein namely, α-aminoadipic and γ-glutamic semialdehydes, were formed from oxidised lysine, proline and/or arginine in the presence of Fe^{3+} and H_2O_2. These semialdehydes contributed to 70 % carbonyls found in oxidised proteins. Therefore, tilapia myosin was susceptible to oxidation when exposed to hydroxyl radicals generated by Fenton's reaction.

6.4.1.3 Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of non-oxidised and oxidised myosin as a function of H_2O_2 levels and incubation time is shown in Table 13. Ca^{2+}-ATPase of oxidised myosin decreased with time and H_2O_2 concentrations increased. The result suggested that denaturation of myosin was pronounced by the oxidation. With increasing level of H_2O_2, hydroxyl radicals were generated to a higher extent. As a consequence, those radicals could induce the oxidation of protein, leading to the loss of ATPase activity found at the head of myosin heavy chain. Ca^{2+}-ATPase activity is considered to be a good indicator of integrity of myosin molecule (Roura and Crupkin, 1995). The decrease in Ca^{2+}-ATPase activity was in agreement with the lowered SH group content and increased carbonyl group content (Table 13). Enhanced protein oxidation and reduced SH group content were suggested to be related with the inactivation of Ca^{2+}-ATPase (Klebl *et al.*, 1998). Reactive SH groups located at myosin head are involved in ATPase activity (Wells *et al.*, 1979). The oxidation of sulfhydryl groups, especially in the head region caused the decrease in Ca^{2+}-ATPase activity (Sompongse *et al.*, 1996; Benjakul *et al.*, 2003b). Moreover, the reduced Ca^{2+}-ATPase activity might be governed by structural changes of myosin heavy chain, especially at head domain induced by hydroxyl radicals (Ishibashi *et al.*, 1996; Xu *et al.*, 1997). The result suggested that hydroxyl radicals more likely induced the denaturation of myosin and the rate of denaturation was determined by H_2O_2 concentration in Fenton's reaction as well as reaction time.

6.4.1.4 Surface hydrophobicity

Changes in surface hydrophobicity of tilapia myosin were observed upon the oxidation by Fenton's reaction as influenced by H_2O_2 concentrations and incubation time (Table 13). Surface hydrophobicity of myosin increased as the concentration of $H_2O_2$and time increased ($P < 0.05$). After incubation for 12 h with 5 mM H_2O_2 in the presence of $FeCl_3$ and ascorbic acid, surface hydrophobicity increased by 80.2 %, compared with the control (non-oxidised). During the oxidation, the proteins underwent the conformational changes, in which the hydrophobic portions which buried inside the protein molecules were exposed. As a consequence, conformational changes of the peptide chain occurred and reformed in a manner different from those in the native structure (Morawetz, 1972). Chao *et al.* (1997) reported that the exposure of liver proteins to a metal-catalysed oxidation system or peroxyl radical generating system led to the increases in surface hydrophobicity. Li *et al*. (2012) also found that hydrophobicity of myofibrillar protein under mild oxidative condition increased. Oxidation process therefore induced the configuration changes of myosin, reflecting the denaturation of myosin.

6.4.1.5 Protein pattern

Protein patterns of non-oxidised myosin and those oxidised by H_2O_2 at different levels in the presence of $FeCl_3$ and ascorbic acid as the function of time are depicted in Figure 20A. In the absence of βME, the oxidised myosin had the decrease in band intensity. of myosin. The decrease was more pronounced when H_2O_2 concentration and incubation time increased. At the high concentration of H_2O_2 (5 mM), the myosin band intensity completely disappeared, especially for samples incubated for 6 h and 12 h. Compared to control (non-oxidised), the changes in band intensity of sample oxidised with H_2O_2 up to 0.1 mM H_2O_2 was negligible, when incubation times of 2 and 6 h were used. The disappearance of myosin band was more likely due to the formation of large aggregates localised on the stacking gel. Under the reducing condition, myosin band was almost recovered, indicating that the cross-linking of myosin took place mainly via disulfide bonds. The result was in accordance with the loss of SH group, particularly when the higher concentration of H_2O_2 was

Table 13 Physicochemical changes of myosin from tilapia as affected by oxidation via Fenton's reaction using H_2O_2 at various concentrations for different times

H_2O_2 (mM)	Time (h)	Total sulfhydryl content (mol/10^5 g protein)	Carbonyl content (µmol/ g protein)	Ca^{2+}-ATPase activity (µmol Pi/mg protein/min)	Surface hydrophobicity
0	0	2.21 ± 0.12^{c}	0.41 ± 0.07^{a}	0.168 ± 0.005^{j}	85.42 ± 1.88^{a}
0.05	2	2.05 ± 0.20^{c}	0.60 ± 0.05^{ab}	0.154 ± 0.002^{ij}	89.46 ± 3.60^{b}
	6	2.02 ± 0.03^{bc}	0.75 ± 0.26^{abc}	0.150 ± 0.003^{hi}	93.93 ± 2.43^{c}
	12	1.94 ± 0.04^{bc}	0.93 ± 0.10^{abcd}	0.148 ± 0.005^{gh}	102.05 ± 0.03^{e}
0.1	2	2.06 ± 0.19^{c}	0.88 ± 0.08^{abcd}	0.150 ± 0.003^{hi}	98.72 ± 0.06^{d}
	6	1.94 ± 0.02^{bc}	0.94 ± 0.06^{abcd}	0.148 ± 0.003^{gh}	101.19 ± 1.02^{e}
	12	1.95 ± 0.01^{bc}	1.25 ± 0.34^{cde}	0.145 ± 0.003^{fg}	109.44 ± 0.29^{f}
1	2	1.71 ± 0.03^{bc}	1.12 ± 0.34^{bcde}	0.141 ± 0.002^{f}	108.28 ± 0.07^{f}
	6	1.69 ± 0.02^{bc}	1.24 ± 0.18^{cde}	0.129 ± 0.001^{e}	119.83 ± 0.21^{g}
	12	1.61 ± 0.18^{bc}	1.45 ± 0.15^{efg}	0.111 ± 0.002^{d}	136.29 ± 0.17^{i}
5	2	1.10 ± 0.17^{a}	1.42 ± 0.43^{efg}	0.106 ± 0.002^{c}	133.58 ± 0.52^{h}
	6	1.03 ± 0.02^{a}	1.61 ± 0.12^{fg}	0.078 ± 0.001^{b}	144.71 ± 0.20^{j}
	12	1.00 ± 0.01^{a}	1.95 ± 0.23^{g}	0.042 ± 0.002^{a}	153.98 ± 0.52^{k}

Values are given as mean ± SD (n=3)
* Different lowercase superscripts in the same column indicate the significant differences ($P < 0.05$).

used (Table 13). A slight decrease in band intensity of myosin was found as the high concentration of H_2O_2 and longer time were used. Liu and Xiong (2000a) reported that the $FeCl_3/H_2O_2$/ascorbate-induced oxidation caused fragmentation and polymerisation of myosin from chicken breast and the cross-linking was mediated mainly by disulfide bonds. As indicated by Stadtman and Berlett (1997), oxidation may induce formation of protein aggregates through Schiff base adducts or through formation of carbon-carbon covalent bonds by the interaction of carbon-centered radicals in protein molecules or due to the formation of other covalent bonds, such as Tyr−Tyr and active carbonyl−NH_2 interactions (Baron *et al.*, 2007; Li *et al.*, 2012; Xiong *et al.*, 2010). Thus, the non-disulfide covalent cross-links could be formed in myosin, when hydroxyl radicals were generated at higher extent.

6.4.2 MTGase cross-linking of myosin as affected by oxidation process

6.4.2.1 Protein pattern

When MTGase was incorporated in myosin and those subjected to oxidation under various conditions, it was noted that band intensity of myosin in all samples were decreased (Figure 20B), compared with that found in the corresponding sample without MTGase (Figure 20A). It was postulated that MTGase could induce the cross-linking in both non- and oxidised myosin. It was noted that similar protein patterns were found between those without and with MTGase addition (Figure 20A and 20B). Under reducing condition, most of MHC band was regained. However, the band intensity was still lower than those found in sample without MTGase addition. The result indicated that MTGase could induce cross-linking of myosin via nondisulfide covalent bond to some degree. Those bonds were not destroyed by βME used. Li *et al.* (2012) found that more loss of MHC band intensity from oxidised myofibrillar protein from pork was noticed, compared to non-oxidised one when MTGase was incorporated. MTGase was therefore capable of cross-linking of myosin, regardless of oxidation.

6.4.2.2 Dynamic Rheology

Non-oxidised and oxidised myosin under different oxidising conditions showed varying rheological property. Storage modulus (G′) of different myosins during heating was monitored as shown in Figure 21. Generally, G′ value is a measure of deformation energy stored in the sample during shear process, representing the elastic behavior of a sample (Tabilo-Munizaga and Barbosa-Cánovas, 2005). The increase in G′ of all myosins was observed at 40 °C, indicating the onset of gelation or the formation of an elastic protein network. In control sample (without oxidation), the G′ reached the maximum of the first peak at around 42-43 °C, followed by a slight decrease. The initial increase of G′ could be related to interactions that occurred between protein molecules at low temperatures.

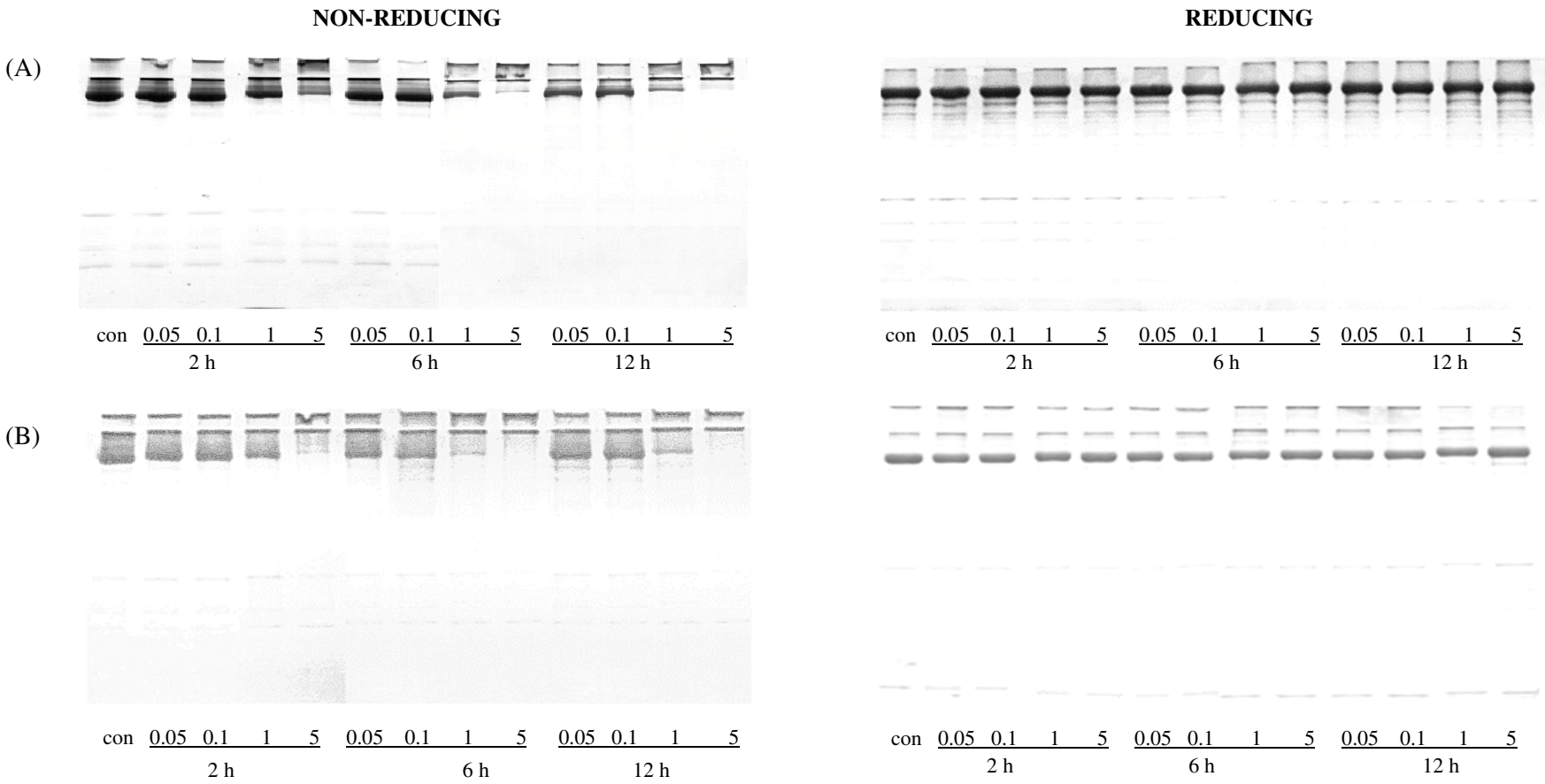


Figure 20. Protein patterns of myosin and those subjected to oxidation via Fenton′s reaction using H_2O_2 at various concentrations for different times in the absence (A) and presence (B) of MTGase at 25 units/g protein. SDS-PAGE was conducted under non-reducing and reducing conditions. Numbers designate the H_2O_2 concentration (mM) and incubation time (h)

The subsequent decrease was possibly due to disentanglement and the increased molibility of myosin molecules as a result of breaking of protein–protein bonds (Chen *et al.*, 1999; Lanier *et al.*, 2004). Denaturation (unfolding) of heavy meromyosin and cross-linking of myosin filaments were responsible for the initial G′ increase at < 50 °C (Egelandsdal *et al.*, 1986). Denaturation of light meromyosin and subfragment-1 (S-1), which leads to increased filamental "fluidity", caused G′ to temporarily decrease (Brenner *et al.*, 2009; Choi and Kim, 2009; Liu *et al.*, 2014). Subsequently, G′ of myosin solution increased. This second increase in G′ was more likely related to the formation of more permanent, irreversible myosin filaments or complexes. The phase angle, a ratio of G″/G′, decreased when the samples were heated from 20 to 37 °C. It increased and reached the maximum at 40 °C. Thereafter, it decreased gradually and remained constant during heating at 50–80 °C (data not shown). Changes in the phase angle reflected a transition of the viscous myosin sol to elastic myosin gel, which was in accordance with the increase of G′. Yongsawatdigul and Park (2003) reported that the unfolding of actomyosin helical structure, hydrophobic interaction and disulfide formation took place and became greater at high temperature (>50 °C).

When myosin was oxidised, a higher G′ with the lower phase angle was found, compared with that of the control (data not shown). This indicated that the larger aggregation induced by oxidation condition resulted in enhanced entanglement, associated with increased viscosity. At final temperature, oxidised myosin using 1 mM H_2O_2 for 12 h exhibited the highest G′, while the control sample showed the lowest G′. Further oxidation using higher H_2O_2 concentration slightly lowered the G′. Thus, degree of protein cross-linking directly affected the network formation as evidenced by different G′.

After oxidation, most myosin underwent cross-linking, mainly via disulfide bond as evidenced by the loss of SH group and reduction of myosin band in SDS-PAGE (Figure 20). Therefore, less viscosity with more solid-like was found due to strong protein aggregation. When oxidation took place under the strong condition (5 mM H_2O_2), the excessive cross-linking of myosin before heating could cause premature aggregation, thereby limiting ordered interactions of reactive functional

groups. This resulted in the inhibition of fine gel network formation (Liu *et al.*, 2000). Xiong *et al.* (2010) investigated the oxidation in pectoralis myofibrillar protein from chicken. Myosin tail (light meromyosin or rod) was likely susceptible to hydroxyl radicals attack and the subsequent aggregation of myosin monomers via tail–tail interaction occurred. However, aggregation of non-oxidised myosin occurred by head–head interaction (Ooizumi and Xiong, 2006).

When MTGase (25 units/g) was added, both non-oxidised and oxidised samples had the higher final G′ at 80 °C than those without MTGase addition. MTGase catalyses the cross-linking of polypeptides through the formation of isopeptides between lysine and glutamine residues (Folk 1970). For the oxidised samples, the final G′ decreased when oxidation took place under strong condition (using 5 mM H_2O_2). Due to high reactivity of carbonyls with free amines, particularly ε-NH_2 of lysine, oxidation-induced carbonyl production was probably an important cause for the reduced MTGase cross-linking in myosin exposed to higher concentrations of H_2O_2. Both the production and the consumption of carbonyls appeared to be at the expense of lysine and affected MTGase catalysis (Li *et al.*, 2012). Moreover, disulfide bond formed during oxidation might lower the accessibility of reactive Glu and Lys or yielded the steric hindrance for MTGase cross-linking. Therefore, the degree of protein oxidation affected the rheological property of tilapia myosin.

6.4.3 Effect of MTGase on gelling properties of washed mince as affected by oxidation process

6.4.3.1 Breaking force and deformation

Breaking force and deformation of gels from washed mince (control) and oxidised washed mince added without and with MTGase at different levels (0-0.6 units/g) are depicted in Figure 22. Generally, breaking force of the gel was positively correlated with gel strength, while the deformation represented the elasticity of the gels.

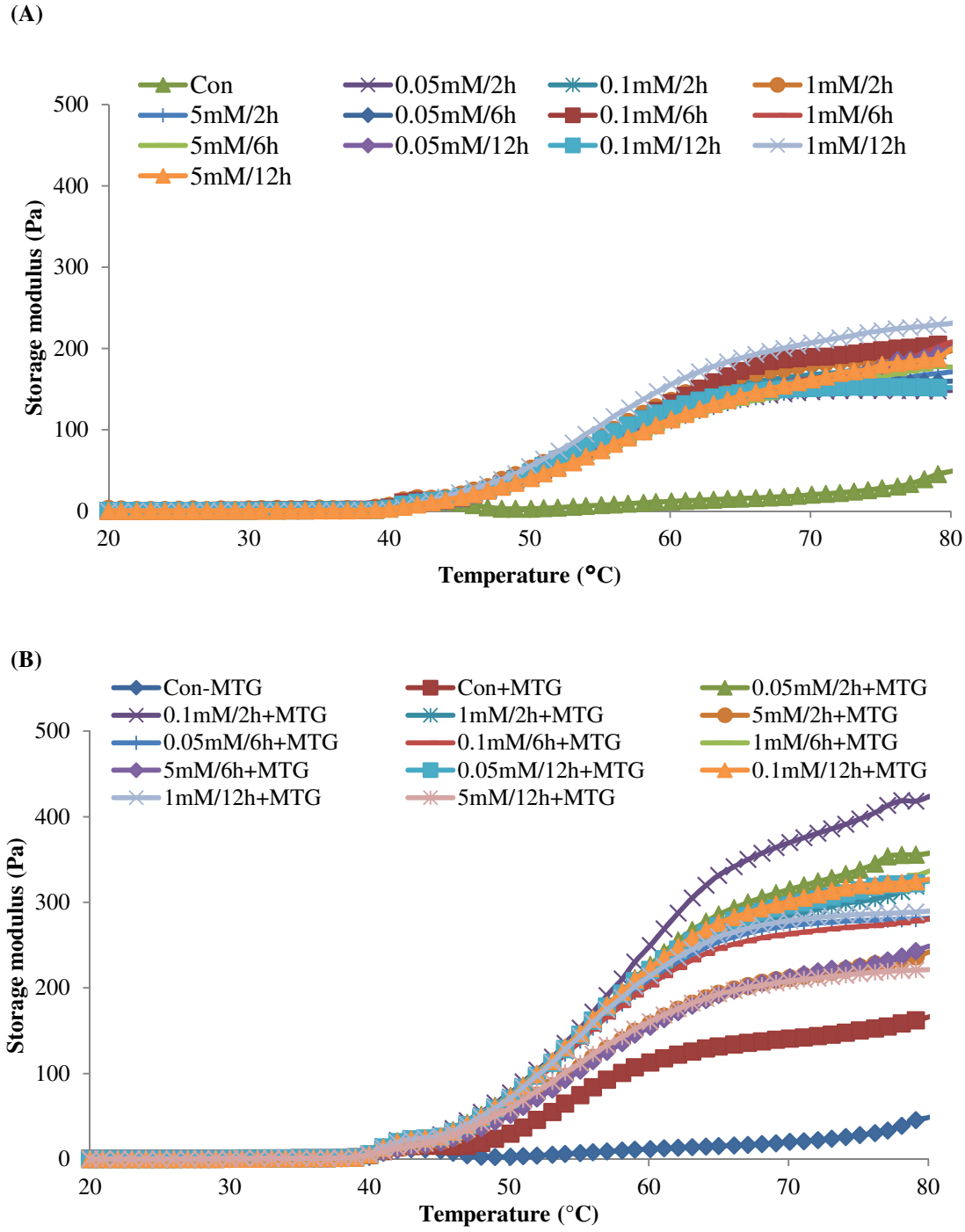


Figure 21 Rheogram of myosin and myosin subjected to oxidation via Fenton's reaction using H_2O_2 at various concentrations for different times without (A) or with MTGase at 25 units/g protein (B)

Without MTGase addition, the control gels had higher breaking force and deformation, compared to those from oxidised washed mince ($P < 0.05$). Breaking force and deformation of gel from oxidised washed mince decreased by 24% and 32%, respectively, compared with those of control gel. Oxidation was associated with lowered solubility, caused by cross-linking. Protein conformation and cross-linking were induced by the oxidation as evidenced by the increases in surface hydrophobicity and carbonyl content, with the loss of SH group content and Ca^{2+}-ATPase activity (Table 13). Li *et al.* (2012) also reported that changes in protein induced by radicals were manifested by carbonylation of amino acid side chains, cleavage of peptide backbones and formation of disulfide cross-linking. Those changes might be associated with poorer gelation. Xiong *et al.* (1993) reported that the inhibition of oxidation by using propyl gallate, ascorbate and tripolyphosphate during washing resulted in a strong gel of restructured meat. When protein was oxidised, cross-linking via disulfide bonds or protein-protein interaction via Schiff-based occurred, leading to lower solubility of protein. During setting at 40 °C, the formation of isopeptide catalysed by endogenous transglutaminase has been reported to play a crucial role in gel strengthening of processed fish product (Kamath *et al.*, 1992). Oxidation mediated by hydroxyl radicals could induce the loss of activity of endogenous transglutaminase. As a result, the setting phenomenon could be impeded. Thus, oxidation taken place before gel setting resulted in the lower breaking force and deformation of gels. In addition, not only protein was oxidised but some lipids which were still retained after washing process could also be oxidised. The compounds generated from lipid oxidation can modify proteins by inducing cross-linking, resulting in modifications of amino acids and a decrease in protein functionality including gelation (Eymard *et al.*, 2009).

Breaking force of control gels increased as MTGase at the higher levels was incorporated. Higher amount of MTGase might induce the formation of non-disulphide covalent bond to a greater extent. As a result, the strength of gel matrix was enhanced. MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins. For oxidised samples, MTGase at 0.3 units/g increased breaking force ($P < 0.05$). Nevertheless, the addition of MTGase at 0.6 units/g did not

increase breaking force of the gel ($P > 0.05$). There was no difference in breaking force and deformation between oxidised and non-oxidised sample when MTGase 0.3 units/g was added. However, with the addition of 0.6 units/g MTGase, gel from the oxidised washed mince had the decreases in breaking force and deformation by 19.7% and 9.8%, respectively, compared to those of non-oxidised samples. Disulfide bond regulated by hydroxyl radicals might cause the cross-linking, in the way which lowered the accessibility of glutamine and lysine for MTGase reaction. Oxidation induced carbonyl production was probably one of the important causes for reduced MTGase cross-linking (Li *et al*., 2012). The addition of MTGase at 0.3 units/g was able to induce protein cross-linking and improve property of gel from oxidised mince to be equivalent to the control gel. Visessanguan *et al*. (2003) also reported that the iron-catalysed oxidation decreased the gel-forming ability of bigeye snapper (*Priacranthus tayenus*) and the addition of MTGase could partially recovered the gel strength and setting response to some degree. Therefore, oxidation of muscle protein mainly reduced breaking force and deformation of gel. With the excessive amount of MTGase, the cross-linking of previously oxidised proteins with the large aggregates might occur, in which gel with coagulated network was formed. This led to poor gel network. However, the addition of MTGase at an appropriate level was able to improve gel strength of oxidised mince to some extent.

6.4.3.2 Expressible moisture

Expressible moisture content of gels from washed mince (control) and oxidised washed mince added with MTGase at different levels (0-0.6 units/g) is shown in Table 14. In the absence of MTGase, oxidised samples showed the non-significantly higher expressible moisture content, compared to control gel. The lower expressible moisture content of gels suggested more water retained in the gel network (Niwa, 1992). When MTGase was added, the decrease in expressible moisture content was observed. For the control gel, the expressible moisture content decreased as MTGase level increased.

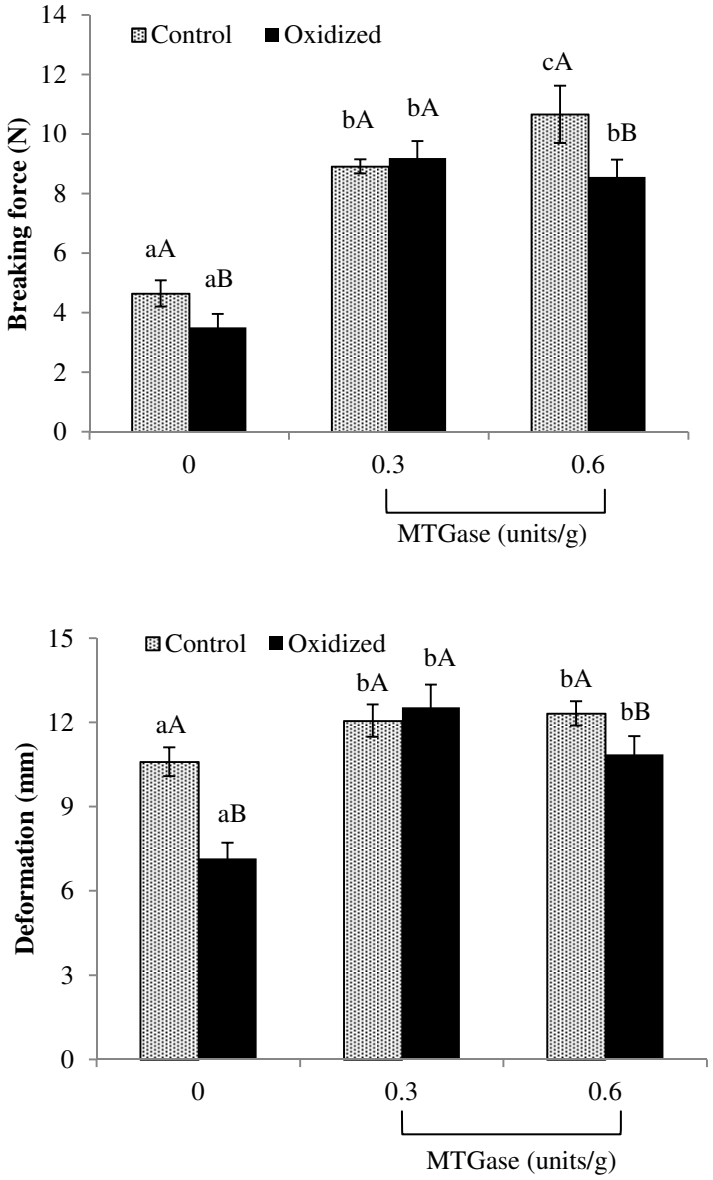


Figure 22. Breaking force and deformation of gels from washed mince and oxidised washed mince as affected by addition of MTGase at different levels. Bars represent the standard deviation (n=3). Different letters within same oxidative condition indicate significant differences ($P < 0.05$). Different capital letters within the same level of MTGase indicate significant differences ($P < 0.05$). Numbers designate the level of MTGase added (units/g).

However, no significantly differences in expressible moisture content were obtained between gels from oxidised washed mince added with MTGase at 0.3 and 0.6 units/g ($P > 0.05$). Bertram *et al.* (2007) reported that reduced WHC of purified myofibrils upon oxidation with Hb and H_2O_2 was found together with an increase in formation of the crosslinked oxidation product. When MTGase at 0.6 units/g was added, expressible moisture content of control and oxidised sample was reduced by 18.3% and 8.2%, respectively compared to those without MTGase addition. It was noted that the addition of MTGase could increase the ability of gel in water holding as evidenced by the lowered expressible moisture content ($P > 0.05$). MTGase induced protein cross-linking via covalent cross-linking, causing the water to be bound or retained in the gel network with more inter-connection. Monero *et al.* (2008) reported that the addition of 1% MTGase increased water holding capacity of restructured fish muscle. The result suggested that water holding capacity of gel network was determined by protein substrates and level of MTGase incorporated.

6.4.3.3 Whiteness

Whiteness of gels made from washed mince and oxidised washed mince added with MTGase at different levels is shown in Table 14. No differences in whiteness were found between gels from washed mince and oxidised washed mince. At the same level of MTGase, no differences in whiteness were found between the control and oxidised sample ($P > 0.05$). However, the addition of MTGase slightly lowered the whiteness of gel. The higher gel strength induced by MTGase may cause gel network of samples to become denser. This might be associated with the higher light absorption. Kang *et al.* (2007) reported that gels from post-rigor pork had small pockets with denser myofibrillar gel matrix. This might cause more light to be absorbed in the gel matrix, resulting in the darker color of gel.

6.4.3.4 Microstructure

Microstructures of gel from washed mince and oxidised washed mince added with different levels of MTGase (0-0.6 units/g sample) are illustrated in Figure 23. Gel of oxidised washed mince displayed a coarse gel matrix with a slightly larger

Table 14 Expressible moisture content and whiteness of gels from washed mince and oxidised washed mince added with MTGase at different levels.

MTGase levels	samples	Expressible moisture (%)	Whiteness
0	Control	3.88 ± 0.41^{bA}	86.09 ± 0.27^{bA}
	Oxidised	4.03 ± 0.26^{bA}	86.62 ± 0.38^{bA}
0.3	Control	3.72 ± 0.18^{abA}	85.32 ± 0.51^{bA}
	Oxidised	3.76 ± 0.19^{aA}	85.30 ± 0.59^{aA}
0.6	Control	3.17 ± 0.25^{aB}	85.04 ± 0.55^{aA}
	Oxidised	3.70 ± 0.15^{aA}	85.24 ± 0.47^{aA}

Values are given as mean ± SD (n=3)
* Different lowercase superscripts in the same column indicate the significant differences ($P < 0.05$).
** Different uppercase superscripts in the same column under the same MTGase levels indicate the significant differences ($P < 0.05$).

void, while the control gels (without oxidation) had a finer three-dimensional filamentous protein network with smaller void. The finer and more ordered structure of gel was in accordance with higher breaking force (Figure 22) along with higher water holding capacity (Table 14). Less continuous network with slightly larger strands were observed in gels prepared from oxidised washed mince. This might be caused by the large aggregate of proteins induced by oxidation process. Those large bundles as indicated by the increased G' (Figure 21) could not form the fine network. Therefore, those large aggregates induced by oxidation could not be completely dissociated prior to thermal aggregation. This led to the coarser network with poor water holding capacity.

When MTGase was incorporated, protein could undergo the cross-linking more effectively. In control samples, gel structure became more compact and denser with smaller voids as higher amount of MTGase was added. Gel network became more rigid. The result was in agreement with Kudre and Benjakul (2013) who reported that MTGase addition was able to improve the gel matrix of sardine surimi, which became more compact and filamentous. For oxidised samples, gel became denser with the addition of MTGase up to 0.3 units/g. However, an excessive amount of MTGase (0.6 units/g) resulted in the discontinuous network, leading to the lower

gel strength in comparison with the gel of control sample added with the same MTGase level (Figure 22). Thus, oxidation process and MTGase addition affected gel network formation of tilapia washed mince.

6.5 Conclusion

The oxidation induced the physicochemical and conformation changes of myosin from tilapia. Those changes determined the susceptibility of protein to MTGase cross-linking, in which protein cross-linking induced by MTGase was impeded when the severe oxidation took place. The oxidation also lowered the gel-forming ability of washed mince but MTGase at appropriate level could strengthen the gel. The gel formability and setting response of oxidised samples were partially recovered by the addition of MTGase. Therefore, MTGase could be an effective means to improve gel properties from mince when oxidation occurred at low degree.

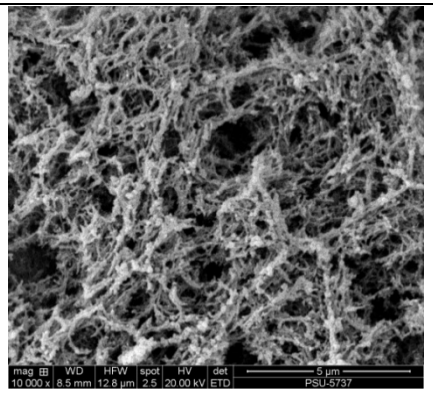
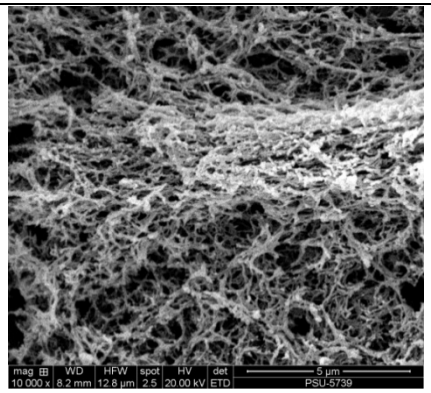
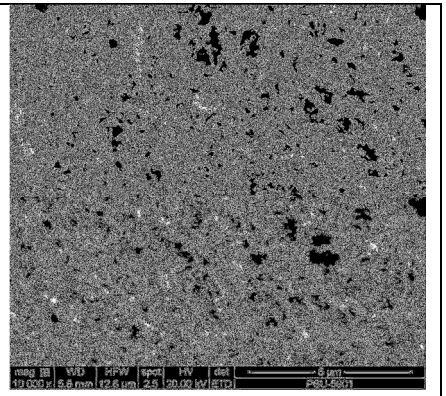
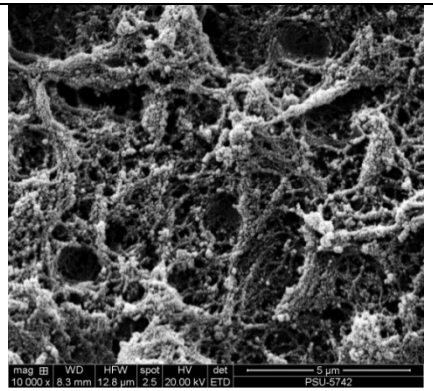
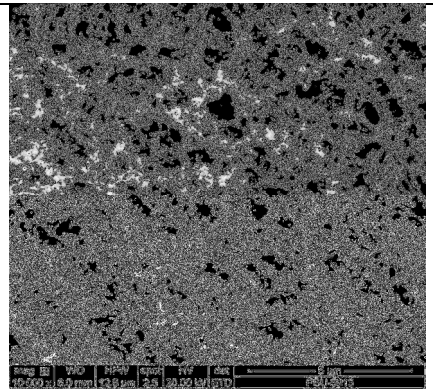
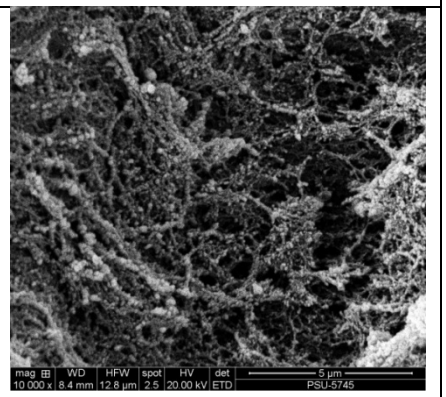
Control			
Oxidised			
MTGase (units/g)	**0**	**0.3**	**0.6**

Figure 23 Electron microscopic image of gels from washed mince and oxidised washed mince as affected by addition of MTGase at different levels (Magnification: 10,000x).

6.6 References

Asagami, T., Ogiwara, M., Wakameda, A., and Noguchi, S. F. 1995. Effect of microbial transglutaminase on the quality of frozen surimi made from various kinds of fish species. Fish. Sci. 61: 267-272.

Baron, C. P., KjÆrsgård, I. V., Jessen, F., and Jacobsen, C. 2007. Protein and lipid oxidation during frozen storage of rainbow trout (*Oncorhynchus mykiss*). J. Agric. Food Chem. 55: 8118-8125.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003a. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Benjakul, S., Visessanguan, W., and Pecharat, S. 2004. Suwari gel properties as affected by transglutaminase activator and inhibitors. Food Chem. 85: 91-99.

Benjakul, S., Visessanguan, W., Thongkaew, C., and Tanaka, M. 2003b. Comparative study on physicochemical changes of muscle proteins from some tropical fish during frozen storage. Food Res. Int. 36: 787-795.

Berlett, B. S., and Stadtman, E. R. 1997. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. 272: 20313-20316.

Bertram, H. C., Kristensen, M., Østdal, H., Baron, C. P., Young, J. F., and Andersen, H. J. 2007. Does oxidation affect the water functionality of myofibrillar proteins? . J. Agric. Food Chem. 55: 2342-2348.

Brenner, T., Johannsson, R., and Nicolai, T. 2009. Characterisation and thermo-reversible gelation of cod muscle protein isolates. Food Chem. 115: 26-31.

Chao, C. C., Ma, Y. S., and Stadtman, E. R. 1997. Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. P. Natl. Acad. Sci. USA. 94: 2969-2974.

Chen, J., Dickinson, E., and Edwards, M. 1999. Rheology of acid-induced sodium caseinate stabilized emulsion gels. J. Texture Stud. 30: 377-396.

Choi, Y., and Kim, B. 2009. Muscle fiber characteristics, myofibrillar protein isoforms, and meat quality. Livest. Sci. 122: 105-118.

Dean, R., Fu, S., Stocker, R., and Davies, M. 1997. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 324: 1-18.

Decker, E. A., Xiong, Y. L., Calvert, J. T., Crum, A. D., and Blanchard, S. P. 1993. Chemical, physical, and functional properties of oxidized turkey white muscle myofibrillar proteins. J. Agric. Food Chem. 41: 186-189.

DeJong, G. A. H., and Koppelman, S. J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

Egelandsdal, B., Fretheim, K., and Samejima, K. 1986. Dynamic rheological measurements on heat-induced myosin gels: Effect of ionic strength, protein concentration and addition of adenosine triphosphate or pyrophosphate. J. Sci. Food Agric. 37: 915-926.

Ellman, G. L. 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70-77.

Estévez, M. 2011. Protein carbonyls in meat systems: a review. Meat Sci. 89: 259-279.

Estévez, M., Ollilainen, V., and Heinonen, M. 2009. Analysis of protein oxidation markers α-Aminoadipic and γ-Glutamic semialdehydes in food proteins

using liquid chromatography (LC)−electrospray ionization (ESI)− multistage tandem mass spectrometry (MS). J. Agric. Food Chem. 57: 3901-3910.

Eymard, S., Baron, C. P., and Jacobsen, C. 2009. Oxidation of lipid and protein in horse mackerel (*Trachurus trachurus*) mince and washed minces during processing and storage. Food Chem. 114: 57-65.

Fiske, C. H., and Subbarow, Y. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Frederiksen, A. M., Lund, M. N., Andersen, M. L., and Skibsted, L. H. 2008. Oxidation of porcine myosin by hypervalent myoglobin: the role of thiol groups. J. Agric. Food Chem. 56: 3297-3304.

Ishibashi, T., Lee, C. I., and Okabe, E. 1996. Skeletal sarcoplasmic reticulum dysfunction induced by reactive oxygen intermediates derived from photoactivated rose bengal. J. Pharmacol. Exp. Ther. 277: 350-358.

Jiang, S. T., Hsieh, J. F., Ho, M. L., and Chung, Y. C. 2000. Microbial transglutaminase affects gel properties of golden threadfin bream and pollack surimi. J. Food Sci. 65: 694-699.

Kamath, G., Lanier, T., Foegeding, E., and Hamann, D. 1992. Nondisulfide covalent cross-linking of myosin heavy chain in "setting" of Alaska pollock and Atlantic croaker surimi. J. Food Biochem. 16: 151-172.

Kang, G., Yang, H., Jeong, J., Moon, S., Hur, S., Park, G., and Joo, S. 2007. Gel color and texture of surimi-like pork from muscles at different rigor states post-mortem. Asian Austral. J. Anim. 20: 1127-1134.

Karayannakidis, P., Zotos, A., Petridis, D., and Taylor, K. 2008. The effect of washing, microbial transglutaminase, salts and starch addition on the functional properties of sardine (*Sardina pilchardus*) kamaboko gels. Food Sci. Technol. Int. 14: 167-177.

Klebl, B. M., Ayoub, A. T., and Pette, D. 1998. Protein oxidation, tyrosine nitration, and inactivation of sarcoplasmic reticulum Ca^{2+}-ATPase in low-frequency stimulated rabbit muscle. FEBS Lett. 422: 381-384.

Kudre, T. G., and Benjakul, S. 2013. Combining effect of microbial transglutaminase and bambara groundnut protein isolate on gel properties of surimi from sardine (*Sardinella albella*). Food Biophy. 8: 240-249.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lanier, T. C., Carvajal, P., and Yongsawatdigul, J. 2004. Surimi gelation chemistry. *In* Surimi and surimi seafood (Park, J. W. eds.). p. 451-470. Marcel Dekker. New York.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., and Stadtman, E. R. 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186: 464-478.

Li, C., Xiong, Y. L., and Chen, J. 2012. Oxidation-induced unfolding facilitates myosin cross-linking in myofibrillar protein by microbial transglutaminase. J. Agric. Food Chem. 60: 8020-8027.

Liu, G., Xiong, Y., and Butterfield, D. 2000. Chemical, physical, and gel-forming properties of oxidized myofibrils and whey and soy protein isolates. J. Food Sci. 65: 811-818.

Liu, G., and Xiong, Y. L. 2000a. Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidized myosin. J. Agric. Food Chem. 48: 624-630.

Liu, G., and Xiong, Y. L. 2000b. Oxidatively induced chemical changes and interactions of mixed myosin, β-lactoglobulin and soy 7S globulin. J. Sci. Food Agric. 80: 1601-1607.

Liu, Q., Bao, H., Xi, C., and Miao, H. 2014. Rheological characterization of tuna myofibrillar protein in linear and nonlinear viscoelastic regions. J. Food Eng. 21: 58-63.

Morawetz, H. 1972. Rate of conformational transitions in biological macromolecules and their analogs. *In* Advanance in protein chemistry (Anfinsen, C. B. ed.). p. 243-277. Academic Press, Inc. New York.

NFI 1991. A Manual of Standard Methods for Measuring and Specifying the Properties of Surimi. National Fisheries Institute. Washington, DC.

Ooizumi, T., and Xiong, Y. L. 2006. Identification of cross-linking site(s) of myosin heavy chains in oxidatively stressed chicken myofibrils. J. Food Sci. 71: 196-199.

Robinson, H. W., and Hogden, C. G. 1940. The biuret reaction in the determination of serum proteins. 1. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135: 707-725.

Roura, S., and Crupkin, M. 1995. Biochemical and functional properties of myofibrils from pre and post-spawned hake (*Merluccius hubbsi Marini*) stored on ice. J. Food Sci 60: 269-272.

Rowe, L., Maddock, K., Lonergan, S., and Huff-Lonergan, E. 2004. Influence of early postmortem protein oxidation on beef quality. J. Anim. Sci. 82: 785-793.

Sompongse, W., Itoh, Y., and Obatake, A. 1996. Effect of cryoprotectants and a reducing reagent on the stability of actomyosin during ice storage. Fish. Sci. 62: 73-79.

Srinivasan, S., and Hultin, H. O. 1997. Chemical, physical, and functional properties of cod proteins modified by a nonenzymic free-radical-generating system. J. Agric. Food Chem. 45: 310-320.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

Sun, X. D., and Holley, R. A. 2011. Factors influencing gel formation by myofibrillar proteins in muscle foods. Comp. Rev. Food Sci. 10: 33-51.

Tabilo-Munizaga, G., and Barbosa-Cánovas, G. V. 2005. Rheology for the food industry. J. Food Eng. 67: 147-156.

Visessanguan, W., Benjakul, S., and Tanaka, M. 2003. Effect of microbial transglutaminase on rheological properties of oxidised and non-oxidised natural actomyosin from two species of bigeye snapper. J. Sci. Food Agric. 83: 105-112.

Wang, S. F., and Smith, D. M. 1994. Dynamic rheological properties and secondary structure of chicken breast myosin as influenced by isothermal heating. J. Agric. Food Chem. 42: 1434-1439.

Wells, J. A., Werber, M. M., and Yount, R. G. 1979. Inactivation of myosin subfragment one by cobalt (II)/cobalt (III) phenanthroline complexes. 2. cobalt chelation of two critical thiol groups. Biochem. 18: 4800-4805.

Xiong, Y., Decker, E., Robe, G., and Moody, W. 1993. Gelation of crude myofibrillar protein isolated from beef heart under antioxidative conditions. J. Food Sci. 58: 1241-1244.

Xiong, Y. L., Blanchard, S. P., Ooizumi, T., and Ma, Y. 2010. Hydroxyl radical and ferryl generating systems promote gel network formation of myofibrillar protein. J. Food Sci. 75: 215-221.

Xiong, Y. L., Decker, E., Faustman, C., and Lopez-Bote, C. 2000. Protein oxidation and implications for muscle food quality. *In* Antioxidants in muscle foods: nutritional strategies to improve quality (Decker, A. E., Faustman, C., and Lopez-Bote, C. J. eds.). p. 85-111. John Wiley and Sons Inc. New Jersey.

Xu, K. Y., Zweier, J. L., and Becker, L. C. 1997. Hydroxyl radical inhibits sarcoplasmic reticulum Ca^{2+}-ATPase function by direct attack on the ATP binding site. Circ. Res. 80: 76-81.

Yongsawatdigul, J., and Park, J. 2003. Thermal denaturation and aggregation of threadfin bream actomyosin. Food Chem. 83: 409-416.

CHAPTER 7

IMPACT OF MICROBIAL TRANSGLUTAMINASE ON GELLING PROPERTIES OF INDIAN MACKEREL FISH PROTEIN ISOLATES

7.1 Abstract

Impacts of microbial transglutaminase (MTGase) (0–0.6 units/g) on gel properties of Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing were studied. Generally, lower myoglobin and lipid contents were found in protein isolate with and without prewashing, compared to those of unwashed mince and surimi ($P < 0.05$). Protein isolate had the decreased Ca^{2+}-ATPase and protein solubility, indicating protein denaturation. When MTGase was incorporated, breaking force and deformation of all gels markedly increased, especially as MTGase levels increased ($P < 0.05$). At the same MTGase level, gel from protein isolate with prewashing exhibited the highest breaking force and deformation ($P < 0.05$). The addition of MTGase could lower the expressible moisture content of most gels. No change in whiteness of gel was observed with the addition of MTGase ($P > 0.05$), but gel from protein isolate gels had decreased whiteness as MTGase at high level was added. The microstructure of protein isolate gels without prewashing showed a similar network to unwashed mince gels, whilst a similar network was observed between surimi gel and gel from protein isolate with prewashing. Nevertheless, a larger void was noticeable in gels from protein isolates. All gels incorporated with MTGase (0.6 units/g) showed a slightly denser network than those without MTGase. Thus, gel with improved properties could be obtained from protein isolate from Indian mackerel with added MTGase.

7.2 Introduction

Surimi is the concentrated myofibrillar protein obtained from mechanically deboned fish flesh, which is washed with cold water. Theoretically, any fish can be used to produce surimi but the properties of surimi gel vary, depending on fish species. Due to insufficient amount of lean fish as raw material, dark-fleshed

pelagic fish such as mackerel, etc. have gained increasing attention for surimi production. However, it is difficult to obtain high quality surimi from those species due to the high content of dark muscle, which contains a large amount of lipids and myoglobin. Generally, high quality surimi with the improved gel strength and whiteness can be obtained when as much dark muscle as possible is removed prior to the washing process (Balange and Benjakul, 2009).

Conventional surimi production aims to concentrate myofibrillar proteins by removing sarcoplasmic proteins, fat, blood and pigments through continuous washing of the mechanically separated fish mince (Park *et al.*, 1997). However, the conventional washing renders a low yield (Kristinsson *et al.*, 2005). To overcome this problem, a new approach of recovering protein by a pH-shift process, developed by Hultin and Kelleher (2000) can be used. The extraction mechanism of the pH-shift process is to solubilise the muscle protein at low or high pH to separate soluble proteins from bone, skin, connective tissue, cellular membranes, and neutral storage lipids through the centrifugation (Nolsøe and Undeland, 2009). The solubilised proteins are recovered by isoelectric precipitation to give a protein isolate (Kristinsson and Ingadottir, 2006). Alkaline solublisation of proteins is widely used for recovery of proteins from dark muscle fish (Kristinsson and Hultin, 2003). The major advantages of this process, compared with surimi, include economical feasibility, high recovery yield, and improved functionalities of the recovered proteins, (Kristinsson *et al.*, 2005; Undeland *et al.*, 2002). Better gel properties were obtained for protein isolate from fatty fishes containing more than 5% fat content, compared with those of surimi produced by conventional method (Kristinsson and Hultin, 2003; Kristinsson and Ingadottir, 2006; Undeland *et al.*, 2002).

To improve gel properties of surimi or protein isolate, microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins via non-disulphide covalent bonds (Benjakul *et al.*, 2008). MTGase induces the formation of ε-(γ-glutamyl) lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and c-amide group of a glutamine residue (Chanarat *et al.*, 2012). However, efficiency of MTGase in improving gel property of proteins depends on many factors, e.g. amount of MTGase, type of fish, fat content (Asagami *et al.*, 1995; DeJong and Koppelman, 2002; Visessanguan *et al.*,

2003). Protein substrate is another factor determining the amount of MTGase. Protein isolate, obtained from pH-shift process, more likely contains the dissociated protein caused by repulsion at pH far away from their isoelectric points. Those individual monomers may have the higher exposed reactive group for cross-linking induced by MTGase. Although MTGase has been successfully used for improving the gel property of surimi, no information regarding the use of MTGase in protein isolate, especially from dark fleshed fish, has been reported. Therefore, the objective of this study was to investigate the effect of MTGase at different levels on the gel properties of protein isolates from Indian mackerel in comparison with those from mince and surimi obtained from conventional process.

7.3 Materials and Methods

7.3.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, phosphatidylcholine and glutaraldehyde were purchased from Sigma (St. Louis, Mo., USA). Sodium hydroxide, hydrochloric acid, *N, N, N', N'*-tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fluka (Buchs, Switzerland). Microbial transglutaminase (MTGase) from *Streptoverticilium mobaraense* (TG-K) containing 1% pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

7.3.2 Fish samples

Fresh Indian mackerel (*Rastrelliger kanagurta*) with the sizes of 110–130 g/fish were purchased from a dock in Songkhla province, Thailand. The fish, off loaded approximately 24–36 h after capture, were transported on ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 3 h. The fish were immediately washed, gutted, cleaned and filleted. The fillets were kept on ice during preparation and analysis.

7.3.3 Preparation of fish mince, surimi and fish protein isolate

Fillets were subjected to mincing using a mincer with the hole diameter of 5 mm. Mince obtained were place in polyethylene bag and imbedded in ice until use.

To prepare surimi by the conventional washing process, fish mince was washed with cold water (4 °C) using a water/mince ratio of 3:1 (v/w). The mixture was stirred gently for 10 min in a cold room (4 °C) and the washed mince was filtered with a layer of nylon screen. Washing was performed three times. Finally, the washed mince was centrifuged at 700×*g* for 15 min using a basket centrifuge (Model CE 21K, Grandiumpiant, Belluno, Italy).

To prepare the protein isolate, the alkaline solubilisation process was used following the method of Undeland *et al.* (2002). The mince (250 g) was homogenised for 1 min with 2.25 l of cold distilled water (4 °C) using an IKA homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was adjusted to the pH of 11 using 2 N NaOH. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C to remove the insoluble materials. The soluble proteins were then precipitated by adjusting the pH to 5.5 using 2 N HCl. Precipitated proteins were collected and their pH was adjusted to 7.0 using 2 N NaOH. For the prewashing-alkaline solubilisation process, the mince was prewashed with 3 cycles of cold water using a water/mince ratio of 3:1 (v/w) prior to alkaline solubilisation. Solubilised proteins were collected and precipitated, followed by neutralisation as described previously. The unwashed mince was used as a control. A portion of all samples was directly subjected to analyses.

For another portion, all samples were added with 4% sucrose and 4% sorbitol, mixed well and frozen using an airblast freezer (Patkol Co., Ltd, Bangkok, Thailand). The frozen samples were kept at −18 °C until used for gel preparation. The storage time was not more than 1 month.

7.3.4 Study on chemical compositions and properties of unwashed mince, surimi and protein isolates

7.3.4.1 Measurement of myoglobin content

The extractable myoglobin content was determined by direct spectrophotometric method as described by Benjakul and Bauer (2001). A sample (2 g) was weighed into a 50-ml polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenised at 13,500 rpm for 10 s, followed by centrifuging at 3,000×*g* for 30 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was filtered with Whatman No. 1 filter paper (Whatman Ltd., Maidstone, UK). The absorbance of the filtrate was read at 525 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the millimolar extinction coefficient of 7.6 mM^{-1} cm^{-1} and a molecular mass of 16,110 (Gomez-Basauri and Regenstein, 1992). The myoglobin content was expressed as mg/g dry sample.

7.3.4.2 Lipid and phospholipid content

Lipid content was determined by the Soxhlet apparatus according to the method of AOAC (2000) with the analytical No. of 920.39B. Lipid content was expressed on a dry weight basis.

Phospholipid content was measured based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids extracted by the Bligh and Dyer method (Bligh and Dyer, 1959) (20 μl) were dissolved in chloroform to obtain a final volume of 2 ml. One millilitre of thiocyanate reagent (a mixture of 0.10 M ferric chloride hexahydrate and 0.40 M ammonium thiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared using phosphatidylcholine (0-50 ppm). The phospholipid content was expressed as mg/100 g dry sample.

7.3.4.3 Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of natural actomyosin (NAM) from unwashed mince, surimi and protein isolate with and without prewashing was determined according to the method of Benjakul *et al.* (1997). NAM prepared as described by Benjakul *et al.* (1997) was diluted to 5 mg/ml with 0.6 M KCl (pH 7.0). Diluted NAM solution (1 ml) was added to 0.6 ml of 0.5 M Tris–maleate (pH 7.0) and 1 ml of 0.1 M $CaCl_2$ was added to the mixture. Deionised water was added to make up a total volume of 9.5 ml. To the mixture, 0.5 ml of 20 mM adenosine 5-triphosphate (ATP) solution was added to initiate the reaction. The reaction was conducted for 8 min at 25 °C and terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3,500×*g* for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). The Ca^{2+}-ATPase activity was expressed as micromoles inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

7.3.4.4 Protein extractability

Protein extractability was performed according to the method described by Yongsawatdigul and Park (2004) with a slight modification. Sample (2 g) was homogenised with 25 mL of 20 mM Tris-HCl containing 50 mM KCl (pH 7.0) at a speed of 11,000 rpm for 1 min. The homogenate was centrifuged at 5,000×*g* for 20 min at 4°C. The supernatant was defined as “water-soluble proteins”. The pellet was then homogenised with 30 mL of 20 mM Tris-HCl containing 0.6 M KCl (pH 7.0) followed by centrifugation at 5,000×*g* for 20 min. The supernatant was defined as “salt-soluble proteins”. Protein concentration in the supernatant was measured by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

7.3.5 Study on the impact of MTGase on properties of gel from unwashed mince, surimi and protein isolates

7.3.5.1 Gel preparation

To prepare the gels, the frozen samples were partially thawed at 4 °C for 6 h. The samples were cut into small pieces. The samples were ground for 2 min using a Moulinex Masterchef 350 mixer (Paris, France). Moisture content was adjusted to 75% and NaCl was added to the samples (2.5%, w/w). After grinding for 2 min, the paste was added with various amounts of MTGase (0, 0.2, 0.4, and 0.6 units/g). The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

7.3.5.2 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

7.3.5.3 Determination of expressible moisture content.

Expressible moisture content (%) was determined according to the method of Benjakul *et al*. (2003). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (*X*) and placed between two pieces of Whatman no. 1 filter paper at the bottom and one piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and

weighed again (*Y*). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content } (\%) = [(X - Y)/X] \times 100$$

7.3.5.4 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, VA., USA). Illuminant C was used as the light source of measurement. CIE L^*, a^*, and b^* values were measured. Whiteness was calculated using the following equation (NFI, 1991).

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

7.3.5.5 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 mL of 5% (w/v) SDS solution (85 °C) were added to the sample (3 g). The mixture was then homogenised using a homogenizer at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500 × g for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

7.3.5.6 Microstructure

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm^3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100%. Samples were subjected to critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

7.3.6 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range tests (Steel and Torrie, 1980). Analysis was performed using the Statistical Package for Social Science package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and Discussion

7.4.1 Chemical compositions and properties of unwashed mince, surimi and protein isolates with and without prewashing

7.4.1.1 Myoglobin content

Myoglobin content of unwashed mince, surimi, and protein isolates with and without prewashing is shown in Table 15. Unwashed mince had myoglobin content of 20.32 mg/g dry sample. Myoglobin content in surimi was decreased by 37.5%, compared with that found in unwashed mince. In general, myoglobin content is governed by fish species, muscle type, storage time, post-mortem handling and the washing process (Thiansilakul *et al.*, 2010). Chaijan *et al* (2010) reported that 61% of

myogloin from short-bodied mackerel was removed via the washing process of mince with cold water. Thus, myoglobin, the major pigment in Indian mackerel muscle, could be removed to some degree by the washing process. Myoglobin in protein isolates without and with prewashing was reduced by 94.2% compared with that of unwashed mince. Similar myoglobin content was obtained between protein isolate without and with prewashing ($P > 0.05$). During alkaline treatment, the pigment underwent a conformation change, which could not be co-precipitated with myofibrillar proteins at pH 5.5. Therefore, they were removed from the pellet after centrifugation (Chaijan *et al.*, 2010; Yamaguchi *et al.*, 1979). It was noted that alkaline solubilisation process could lower myoglobin content effectively. Tongnuanchan *et al.* (2011) reported that fish protein isolate from tilapia showed the lowest haem iron, associated with haemoglobin and myoglobin, whereas unwashed mince had the highest content. Therefore, myoglobin in protein isolate could be eliminated more effectively than that removed by conventional washing.

7.4.1.2. Lipid and phospholipid contents

Lipid content of unwashed mince, surimi and protein isolates with and without prewashing was 9.07%, 4.34%, 1.55% and 1.38% (dry basis), respectively (Table 15). Lipid content of protein isolate without prewashing was decreased by 82.91%, whilst the conventional washing process reduced lipid content by 52.15%, compared with that found in unwashed mince. Lower lipid content was found in protein isolate with prewashing prior to the alkaline solubilisation process than that without prewashing. Washing could therefore remove lipids to some extent. However, lipoproteins associated with other muscle proteins might not be leached out with ease. When the alkaline solublisation process was applied, proteins were more likely dissociated. As a result, lipids could be liberated to a higher extent. After solubilisation, these components were separated on the basis of density and solubility differences. The higher lipid removal by the alkaline solubilisation process might be due to the higher emulsification ability of the proteins at alkaline pH (Kristinsson *et al.*, 2005; Tongnuanchan *et al.*, 2011). Kristinsson *et al.* (2005) reported that the acid- and alkaline-aided processes of channel catfish mince led to higher reduction in lipids than did the surimi process. Lipid reduction in Atlantic croaker mince was more than

4 times higher when using the alkaline-aided process compared with the conventional process (Kristinsson and Ingadottir, 2006). Tongnuanchan *et al.* (2011) reported that lipid content of surimi and alkaline protein isolate from red tilapia was decreased by 14.41% and 98.8%, respectively,in comparison with that found in mince. Rawdkuen *et al*. (2009) reported that the lipid reduction of 85.2 and 86.6% was achieved for protein isolate recovered using acid and alkaline solubilisation processes, respectively. Chaijan *et al*. (2010) reported that the lipid content of the protein isolate prepared by alkaline solubilisation process with and without prewashing was not different but was lower, when compared with washed mince obtained from conventional washing process.

For phospholipid content, it was in accordance with lipid content, in which the lowest phospholipid content was obtained in protein isolates with prewashing (Table 15). In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeletal proteins (Haleva *et al.*, 2004). During the washing process, the polar head of phospholipids associated with cell membrane was more likely leached out to some extent (Khantaphant *et al.*, 2011). Surimi produced using conventional washing process and protein isolate with prewashing had a decrease in phospholipid by 42.7% and 81.5%, respectively, compared with that found in unwashed mince. Nylander (2004) reported that phospholipid is difficult to remove because it is strongly bound to proteins. Moayedi *et al.* (2010) reported that neutral lipids were removed from chicken dark meat protein isolate by 61.5%, whilst phospholipid remained unchanged. Nevertheless, Undeland *et al.* (2002) found that phospholipid to protein ratio from herring light muscle was reduced from 0.13 g lipid/g protein to 0.02 g/g protein by alkaline solubilisation process. During alkaline solubilisation, proteins became dissociated, in which phospholipids could be more exposed to hydrophilic environment. As a result, they were leached out potentially from the proteins. Therefore, the removal of lipid and phospholipid could be achieved by using alkaline solubilisation process in combination with prewashing.

7.4.1.3. Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity of NAM extracted from unwashed mince, surimi and protein isolates with and without prewashing is shown in Table 15. The highest activity of Ca^{2+}-ATPase was found in NAM extracted from unwashed mince. Ca^{2+}-ATPase activity is considered to be a good indicator of integrity of myosin molecule (Roura and Crupkin, 1995). Slightly lower Ca^{2+}-ATPase was observed in NAM extracted from surimi ($P < 0.05$). Although the cold water was used for washing to remove sarcoplasmic proteins, lipids and enzymes (Chaijan *et al.*, 2004), this process might result in the partial denaturation of myosin as indicated by the decrease in Ca^{2+}-ATPase activity. For protein isolates prepared by the alkaline solubilisation process with and without prewashing, a marked decrease in Ca^{2+}-ATPase activity was observed ($P < 0.05$). The results suggested that the denaturation of myosin was pronounced by alkaline solubilisation process. These results were in agreement with Chaijan *et al*. (2006) who reported that myosin of sardine (*Sardinella gibbosa*) and mackerel (*R. kanagurta*) underwent denaturation by alkaline solubilisation. When muscle proteins were adjusted to pH above pI, the protein molecule became negatively charged. As a consequence, the repulsion became dominated, leading to the dissociation of protein as well as unfolding of myosin head with ATPase activity. Moreover, prewashing prior to alkaline solubilisation process resulted in a slightly greater decrease in Ca^{2+}-ATPase activity ($P < 0.05$). It was postulated that soluble components as well as lipids might prevent the myosin from the denaturation caused by the alkali to some degree. Thus, the alkaline solubilisation process directly induced denaturation of myosin, a major protein contributing to gelation (Chaijan *et al.*, 2006).

7.4.1.4. Protein extractability

Amongst all samples, unwashed mince showed the highest protein extractability in water ($P < 0.05$) (Table 15). Sarcoplasmic proteins in unwashed mince generally contributed to high solubility in water. The decrease in water extractability was found in surimi, in comparison with that obtained in unwashed mince. During washing process, sarcoplasmic protein was removed to some extent and myofibrillar proteins became concentrated. Coincidentally, extractability of protein in salt was increased in surimi, compared with unwashed mince. Surimi generally contained higher amount of myofibrillar proteins, which were soluble in

high ionic strength solution. Low ionic-strength buffer (50 mM KCl) was applied to extract sarcoplasmic proteins, whereas myofibrillar protein was solubilised using high ionic-strength buffer (0.6 M KCl) (Yongsawatdigul and Park, 2004). Marked decreases in both protein extractability in water and salt were observed in both protein isolates ($P < 0.05$). The loss of extractability or solubility might be associated with the denaturation of protein during pH-adjustment. This was in accordance with the sharp decrease in Ca^{2+}-ATPase activity. Yongsawatdigul and Park (2004) reported that the isolation of muscle protein after solubilising at either acid or alkaline pH induced denaturation and aggregation of both sarcoplasmic and myofibrillar proteins. Protein denaturation typically causes a decrease in protein solubility due to intermolecular hydrogen or hydrophobic bonds, as well as disulfide bonds and ionic interactions amongst protein molecules (Thawornchinsombut and Park, 2006). Thawornchinsombut and Park (2006) also reported that the solubility of protein isolate from Pacific whiting prepared using alkaline solubilisation process was lower than that of surimi by 7 times. Thus, all processes used for protein isolate preparation had the influence on conformational changes associated with the altered solubility.

7.4.2 Effect of MTGase on gel properties of unwashed mince, surimi and protein isolates with and without prewashing

7.4.2.1 Breaking force and deformation

Breaking force and deformation of gels from Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing added with MTGase at different levels are depicted in Figure 24.Without MTGase addition, gels prepared from protein isolate with prewashing showed the highest breaking force and deformation ($P < 0.05$), followed by those from surimi, protein isolate without prewashing and unwashed mince, respectively. Nevertheless, no difference in deformation of gel from surimi and protein isolate without prewashing was observed ($P > 0.05$). Generally, breaking force of the gel was positively correlated with gel strength, whilst the deformation represented the elasticity of the gels.

Table 15 Chemical compositions and properties of Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing.

Parameters		Unwashed mince	Protein isolate without prewashing	Surimi	Protein isolate with prewashing
Ca^{2+}-ATPase activity (µmoles inorganic phosphate/mg protein/min)		0.133±0.003^{d}	0.020±0.006^{b}	0.126±0.001^{c}	0.010±0.002^{a}
Lipid (% dry basis)		9.07±0.10^{d}	1.55±0.10^{b}	4.34±0.14^{c}	1.38±0.16^{a}
Phospholipid (mg/ 100 g dry sample)		985.91±11.25^{d}	227.08±19.59^{b}	564.63±13.58^{c}	182.99±17.99^{a}
Myoglobin content (mg/100 g dry sample)		7.11±0.23^{c}	0.36±0.07^{a}	4.44±0.23^{b}	0.35±0.05^{a}
Protein extractability (mg protein/g sample)	water	28.30±0.16^{c}	5.41±0.20^{a}	9.58±0.27^{b}	4.50±0.05^{a}
Protein extractability (mg protein/g sample)	salt	44.41±0.78^{c}	5.30±0.82^{a}	63.53±0.17^{d}	8.45±0.69^{b}

Values are given as mean ± SD (n=3)

* Different letters within the same row indicate the significant differences ($P < 0.05$).

The increases in breaking force and deformation of gel from protein isolate with prewashing were plausibly due to the partial denaturation of protein after alkaline treatment, leading to the exposure of reactive groups that subsequently underwent interaction during heat treatment (Chaijan *et al.*, 2010). However, it was noted that protein isolate without prewashing had the lower breaking force than surimi and protein isolate with prewashing ($P < 0.05$). It was postulated that sarcoplasmic protein in fish mince might be co-precipitated with myofibrillar proteins during precipitation at pI. Those sarcoplasmic proteins more likely had a negative impact on gelation (Jafarpour and Gorczyca, 2012; Nakagawa *et al.*, 1989). In general, washing process has been used widely in surimi production to remove sarcoplasmic proteins such as enzymes and haem proteins, pigments, odourous compounds, and other impurities that would reduce surimi quality (Hultin *et al.*, 2005; Martin-Sanchez *et al.*, 2009). Yongsawatdigul and Park (2004) also reported that with the appropriate washing, sarcoplasmic proteins could be removed, resulting in concentrated myofibrillar proteins, and consequently increased breaking force of surimi gel. Moreover, the reduction of lipid also resulted in higher ability of proteins to interact with each other, thereby increasing breaking force of gels. The presence of lipids may interfere with myosin cross-linking during gel matrix formation because they do not form gels and have poor water holding capacity. Therefore, prewashing in combination with alkaline solubilisation process was able to improve the gel forming ability of Indian mackerel effectively. Kristinsson and Ingadottir (2006) reported that washed muscle, and protein isolates had different protein compositions, which contribute to the different gel forming ability.

Breaking force and deformation of all gels increased as MTGase at the higher levels was incorporated ($P < 0.05$). Higher amounts of MTGase added might induce the formation of non-disulphide covalent bonds to a greater extent. As a result, the strength of gel matrix was enhanced. MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins. At the same level of MTGase added, gels from protein isolate with prewashing had the highest breaking force and deformation ($P < 0.05$), followed by those of protein isolate without prewashing. The result indicated that protein isolates were the appropriate substrates for MTGase. A

prerequisite for protein cross-linking induced by MTGase is the availability of those substrates, which undergo either inter- or intra- molecular ε-(γ-glutamyl) lysine cross-linking (DeJong and Koppelman, 2002; Nielsen, 1995). Alkaline solubilisation more likely induced the dissociation of protein complexes, in which protein monomers with the higher exposure of reactive groups, especially lysine and glutamine, could be formed. As a result, the cross-linking of proteins could be enhanced in the presence of MTGase. However, protein isolates with prewashing were shown to be the better substrate for MTGase than that without prewashing. Sarcoplasmic protein or some lipids might impede the crosslinking induced by MTGase. Furthermore, those co-precipitated sarcoplasmic proteins might not serve as the good substrate for MTGase. Pérez-Mateos and Lanier (2007) reported that the addition of 0.2% MTGase into Atlantic menhaden alkaline-processed gels incubated at 40 °C for 30 min prior to cooking showed the increase in penetration forces by 5-fold. Although protein isolate might lose the solubility in salt to some degree (Table 15), those aggregates with the larger size could undergo cross-linking mediated by MTGase. As a result, the interaction between the larger strands could contribute to the strong network as evidenced by the increased breaking force.

7.4.2.2. Expressible moisture content

Expressible moisture content of gels from unwashed mince, surimi and protein isolate with and without prewashing added with different levels of MTGase is shown in Table 16. Different gels showed varying expressible moisture content ($P < 0.05$). Surimi gel had the lowest expressible moisture content, compared with others ($P < 0.05$), suggesting the highest water holding capacity. Gel from protein isolate without prewashing showed the highest expressible moisture content ($P < 0.05$), reflecting the poorest water holding capacity. Precipitated sarcoplasmic proteins along with lipids found in protein isolate without prewashing might lower the amount of water retained in gel network. This led to the decreased capacity of water holding of gel network. Chaijan *et al.* (2006) also reported that higher expressible moisture content was found in the gels of sardine and mackerel muscle prepared by the alkaline solubilisation process, compared with those from the conventional washing method.

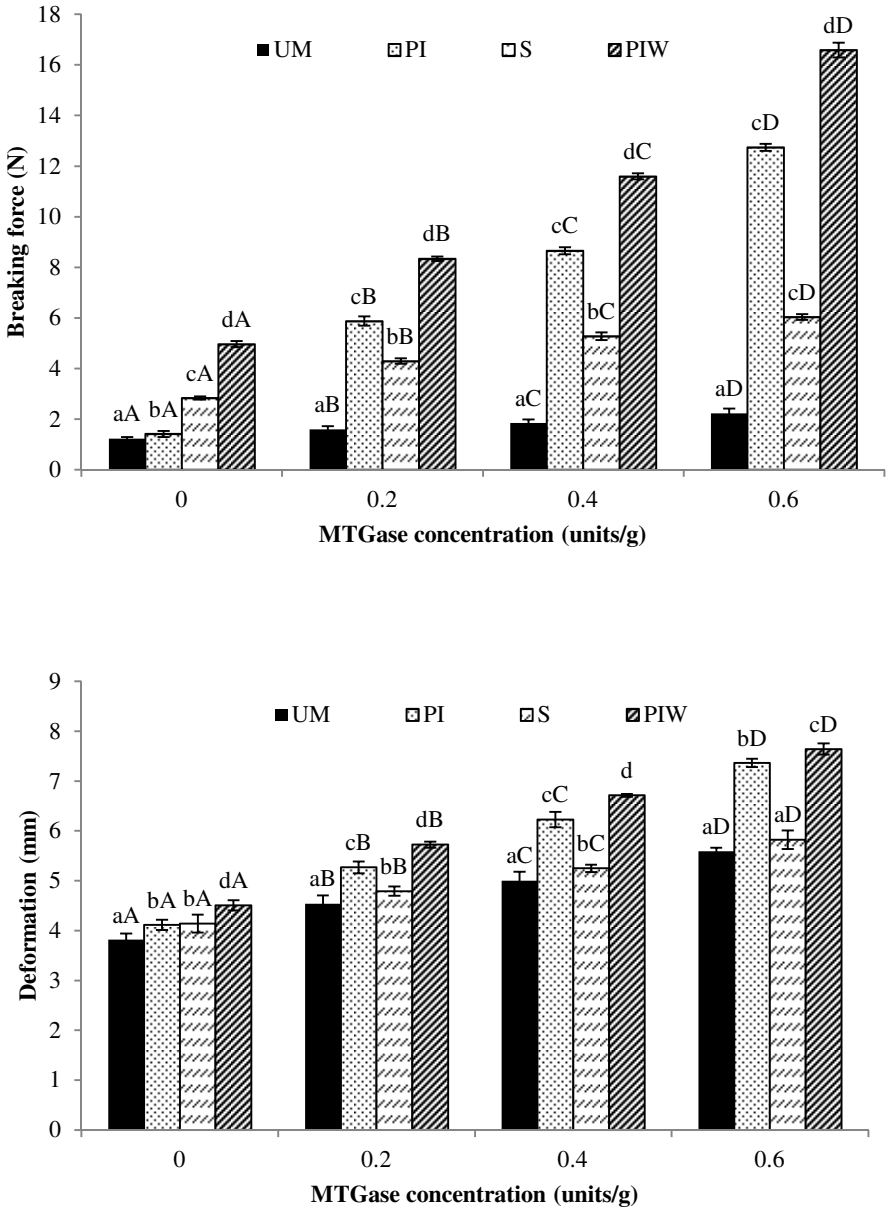


Figure 24. Breaking force and deformation of gels from Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing added with MTGase at different levels (0-0.6 units/g). Bars represent the standard deviation (n=3). Different lowercase letters within the same level of MTGase indicate significant differences ($P < 0.05$). Different uppercase letters within the same processing condition indicate significant differences ($P < 0.05$). UM: unwashed mince; S: surimi; PI: protein isolate without prewashing; PIW: protein isolate with prewashing.

When MTGase at various levels was added, the increase in water holding of gels was noticeable as evidenced by the lowered expressible moisture content ($P < 0.05$). However, with addition of MTGase at 0.6 units/g, gels from protein isolate with prewashing showed the marked increase in expressible moisture content. When protein isolate with prewashing was used as substrate, cross-linking was more pronounced as evidenced by the marked increases in both breaking force and deformation. The greater interaction between protein molecules was associated with the denser network. This might lead to the lower water binding sites of proteins. As a consequence, more free water was released from network. On the other hand, MTGase might induce protein cross-linking in other samples at the appropriate level, where the water was more bound or retained in the gel network with higher interconnection. However, MTGase at level above 0.4 units/g did not cause a reduction in expressible moisture content in gel from unwashed mince and protein isolate without prewashing. For surimi gel, MTGase higher than 0.2 units/g had no effect on expressible moisture content ($P > 0.05$). Moreno *et al.* (2008) reported that the addition of 1% MTGase increased water holding capacity of restructured fish muscle from hake. The result suggested that water holding capacity of gel network was determined by protein substrates and level of MTGase incorporated.

7.4.2.3. Whiteness

Whiteness of gels from unwashed mince, surimi and protein isolates with and without prewashing added with different levels of MTGase is shown in Table 16. In the absence of MTGase, the highest whiteness was found in surimi gel, whilst gel from unwashed mince showed the lowest whiteness ($P < 0.05$). The colour of surimi gels can be affected by the amount of dark muscle, and the presence of blood and pigment. Cortés-Ruiz *et al.* (2001) reported that unwashed sardine mince showed a low whiteness due to the presence of myoglobin in the muscle and possible contaminanation of haemoglobin and skin pigment. Yongsawatdigul and Park (2004) also reported that the highest whiteness was found in conventionally processed surimi from rockfish, followed by alkaline-produced protein isolate and crude mince. No difference in whiteness was found between gels from protein isolates with and without prewashing ($P > 0.05$). The lower whiteness of gels of protein isolates with

and without prewashing, compared to that of surimi, was possibly due to the oxidation of haem proteins in the recovered proteins, which was induced by the alkaline pH. Chaijan *et al.* (2007) reported that alkaline and acid conditions accelerated autoxidation of sardine myoglobin.

When MTGase at different levels was incorporated, no difference in whiteness among gels was observed ($P > 0.05$). However, the whiteness of gel from protein isolate with prewashing became lower as MTGase level increased ($P < 0.05$). Gel network of protein isolate with prewashing was denser as MTGase level increased. This might be associated with the higher light absorption. Kang *et al.* (2007) reported that gels from post rigour muscle of pork had small pockets with denser myofibrillar gel matrix. This might cause more light to be absorbed in the gel matrix, resulting in the darker colour of gel. Light scattering in meat is caused by structural, myofibrillar proteins and size of scattering particle (Kang *et al.*, 2007). Thus, MTGase could affect whiteness of gel, depending on the arrangement of protein in network.

7.4.2.4. Protein pattern

Protein patterns of gels from unwashed mince, surimi and protein isolate with and without prewashing added with MTGase at different levels are depicted in Figure 25. All gels without MTGase addition contained myosin heavy chain (MHC) and actin as the major proteins. Higher band intensity of MHC of gels from both protein isolates was observed, compared with that of surimi and unwashed mince. MHC of unwashed mince might undergo degradation to higher level, whereas the higher cross-linking via non-disulphide bond was presumed in surimi gel. It has been reported that pelagic fish muscle contained high amount of proteases, which could degrade proteins of gel during thermal gelation. For surimi, endogenous transglutaminase might be retained to some extent after washing process, thereby cross-linking via non-disulphide bonds could be formed as indicated by the lower band intensity of MHC. MHC has been known as the substrate for transglutaminase mediated reaction (Visessanguan *et al.*, 2003).

Table 16 Expressible moisture content and whiteness of gels from Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing added with different levels of MTGase (0-0.6 units/g)

Parameter	MTGase levels	Unwashed mince	Protein isolate without prewashing	Surimi	Protein isolate with prewashing
Expressible moisture	0	8.79 ± 0.28^{cC}	14.48 ± 0.47^{cD}	2.83 ± 0.15^{bA}	6.32 ± 0.25^{aB}
	0.2	8.29 ± 0.18^{bC}	9.16 ± 0.42^{bD}	2.44 ± 0.17^{aA}	6.61 ± 0.32^{abB}
	0.4	7.97 ± 0.07^{aC}	8.24 ± 0.13^{aD}	2.32 ± 0.08^{aA}	6.96 ± 0.28^{bB}
	0.6	7.73 ± 0.03^{aB}	8.19 ± 0.29^{aC}	2.27 ± 0.14^{aA}	9.53 ± 0.36^{cD}
Whiteness	0	63.79 ± 0.45^{aA}	64.96 ± 0.75^{aB}	67.13 ± 0.33^{aC}	64.95 ± 0.42^{cB}
	0.2	63.38 ± 0.51^{aA}	64.44 ± 0.20^{aB}	67.28 ± 0.46^{aC}	64.60 ± 0.48^{bcB}
	0.4	63.53 ± 0.66^{aA}	64.61 ± 0.21^{aB}	67.63 ± 0.23^{aC}	63.92 ± 0.65^{bAB}
	0.6	63.67 ± 0.08^{aB}	63.95 ± 0.68^{aB}	67.38 ± 0.39^{aC}	60.81 ± 0.39^{aA}

Values are given as mean ± SD (n=3)

* Different letters within the same column under the same processing condition indicate the significant differences ($P < 0.05$).

** Different capital letters within the same row indicate the significant differences ($P < 0.05$).

It was noted that gel from both protein isolates, especially with prewashing had more MHC band retained. This gel exhibited the highest breaking force and deformation (Figure 24). The result suggested that most cross-linking of this gel was more likely mediated by weak bonds such as H-bond or hydrophobic interaction, which could be destroyed by SDS used for solubilisation and electrophoresis. It was also postulated that endogenous TGase might undergo inactivation to a high degree during alkaline solubilisation process. The dissociated proteins could undergo interaction via exposed reactive groups, which were abundant in protein isolates. Nevertheless, other bonds also contributed to gel formation of all gels. Yongsawatdigul and Park (2004) reported that disulphide linkage plays an important role in gelation of alkaline produced gel. At alkaline pH, muscle protein underwent extensive unfolding, in which sulfhydryl (SH) groups could be exposed. Hence, the oxidation of SH groups to disulphide bonds favourably occurred. It could be presumed that gels from unwashed mince and surimi could form non-disulphide covalent bonds via endogenous transglutaminase. Moreover, alkaline solubilisation and the washing process was able to concentrate myofibrillar protein as evidenced by the higher intensity of MHC bands

in alkaline produced protein isolate surimi than those from unwashed mince and conventional surimi (data not shown).

With the addition of MTGase, the intensity of MHC band decreased in all samples, especially as the levels added increased. The disappearance of MHC indicated the formation of MHC cross-links mainly via the formation the ε-(γ-glutamyl) lysine linkages induced by MTGase. At high concentration of MTGase (0.4–0.6 units/g), tropomyosin band intensity was slightly decreased in all gel samples. Although MHC and troponin band intensity decreased, no changes in actin were observed in all samples. MHC has been known to serve as the preferable substrate for MTGase. Nakahara *et al.* (1999) reported that MTGase preferentially polymerised MHC among individual myofibrillar components, whilst actin could not be crosslinked by MTGase, even though it had active glutamyl residue as acyl donor. It was shown that MTGase preferentially cross-linked connectin, followed by MHC, troponin, respectively (Nakahara *et al.*, 1999). Among all gel samples, MHC was more retained in gel from protein isolate with prewashing. This was probably due to the lower degradation of MHC, caused by the lower protease associated with prewashing and the inactivation of protease to some extent at alkaline pH for solubilisation.

7.4.2.5. Microstructure

Microstructures of gel from unwashed mince, surimi and protein isolates with and without prewashing in the absence and presence of MTGase (0.6 units/g) are illustrated in Figure 26. Gel of unwashed mince displayed a coarse gel matrix with a large void. Compared to unwashed mince gel, surimi gel had a finer three dimensional filamentous protein network with a smaller void. Washing could remove interfering components for gelation, especially sarcoplasmic proteins. The finer and more ordered structure of gel was in accordance with higher breaking force (Figure 24) along with better water holding capacity (Table 16). Similar microstructures of gel from protein isolate without prewashing were noticeable, compared with gels from unwashed mince.

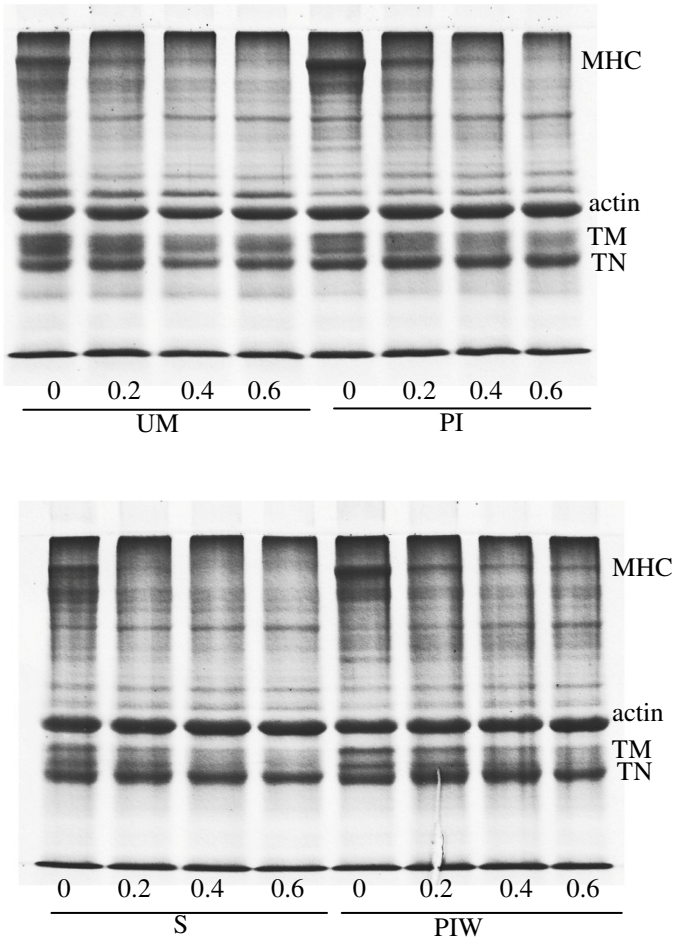


Figure 25. Protein patterns of gels from Indian mackerel unwashed mince, surimi and protein isolates with and without prewashing added with MTGase at different levels (0-0.6 units/g). MHC: myosin heavy chain; TM: tropomyosin; TN: troponin. Numbers designate the level of MTGase added (units/g). UM: unwashed mince; S: surimi; PI: protein isolate without prewashing; PIW: protein isolate with prewashing.

Sarcoplasmic proteins in protein isolate (without prewashing) might show the negative effect on gelation as found in unwashed mince. It was noted that a slightly larger strands of network were observed in gel from protein isolates. Aggregated protein induced by alkaline solubilisation more likely provided those strands in the gel network.

In the presence of MTGase, protein could undergo the cross linking more effectively. Gel became slightly more compact, with a denser gel network and smaller voids as MTGase was added. The result was in agreement with Benjakul *et al.* (2008) who reported that MTGase addition was able to improve the gel matrix of lizardfish surimi, which became more compact and filamentous. Thus, alkaline solubilisation process and MTGase addition affected the gelling properties of Indian mackerel mince.

7.5 Conclusion

The alkaline solubilisation process induced the denaturation of the muscle proteins as evidenced by the decrease in the Ca^{2+}-ATPase activity and protein solubility. Moreover, this process either with or without prewashing could lower myoglobin content and lipid from mince, leading to the better properties of gels. The addition of MTGase in protein isolate, especially with prewashing could improve gel properties of Indian mackerel effectively.

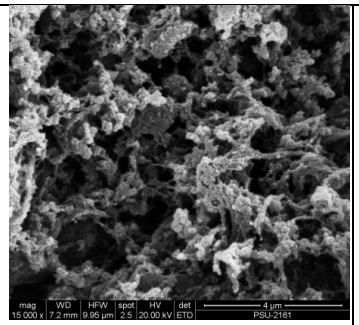
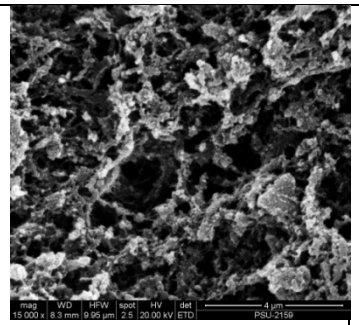
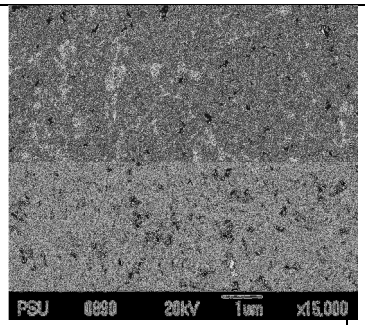
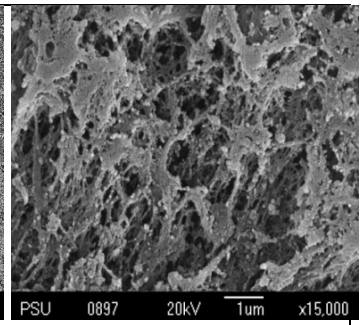
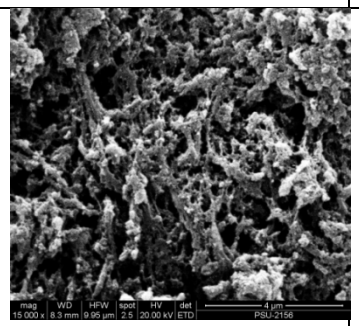
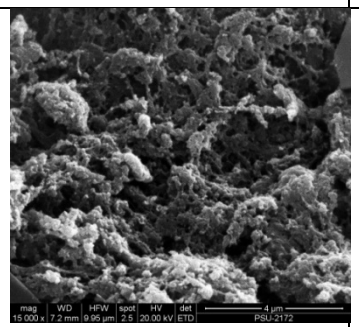
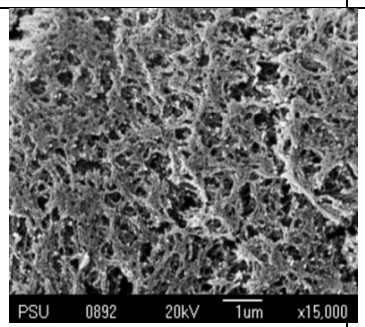
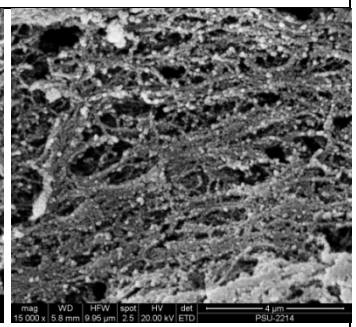
Without MTGase				
With MTGase (0.6 units/g)				
	Unwashed mince	Protein isolate without prewashing	surimi	Protein isolate with prewashing

Figure 26. Electron microscopic image of unwashed mince, protein isolate without prewashing, surimi and protein isolate with prewashing in the absence and presence of MTGase (0.6 units/g) (Magnification: 15,000x)

7.6 References

Asagami, T., Ogiwara, M., Wakameda, A., and Noguchi, S. F. 1995. Effect of microbial transglutaminase on the quality of frozen surimi made from various kinds of fish species. Fish. Sci. 61: 267-272.

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

Benjakul, S., and Bauer, F. 2001. Biochemical and physicochemical changes in catfish (*Silurus glanis Linne*) muscle as influenced by different freeze-thaw cycles. Food Chem. 72: 207-217.

Benjakul, S., Chantarasuwan, C., and Visessanguan, W. 2003. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567-574.

Benjakul, S., Phatcharat, S., Tammatinna, A., Visessanguan, W., and Kishimura, H. 2008. Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. J. Food Sci. 73: 239-246.

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

Bligh, E. G., and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37: 911-917.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2004. Characteristics and gel properties of muscles from sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) caught in Thailand. Food Res. Int. 37: 1021-1030.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2006. Physicochemical properties, gel-forming ability and myoglobin content of

sardine (*Sardinella gibbosa*) and mackerel (*Rastrelliger kanagurta*) surimi produced by conventional method and alkaline solubilisation process. Eur. Food Res. Technol. 222: 58-63.

Chaijan, M., Benjakul, S., Visessanguan, W., and Faustman, C. 2007. Characterisation of myoglobin from sardine (*Sardinella gibbosa*) dark muscle. Food Chem. 100: 156-164.

Chaijan, M., Panpipat, W., and Benjakul, S. 2010. Physicochemical and gelling properties of short-bodied mackerel (*Rastrelliger brachysoma*) protein isolate prepared using alkaline-aided process. Food Bioprod. Process. 88: 174-180.

Chanarat, S., Benjakul, S., and H-Kittikun, A. 2012. Comparative study on protein cross-linking and gel enhancing effect of microbial transglutaminase on surimi from different fish. J. Sci. Food Agric. 92: 844-852.

Cortés-Ruiz, J. A., Pacheco-Aguilar, R., García-Sánchez, G., and Lugo-Sánchez, M. E. 2001. Functional characterization of a protein concentrate from bristly sardine made under acidic conditions. J. Aquat. Food Prod. Technol. 10: 5-23.

DeJong, G. A. H., and Koppelman, S. J. 2002. Transglutaminase catalyzed reactions: impact on food applications. J. Food Sci. 67: 2798-2806.

Gomez-Basauri, J. V., and Regenstein, J. F. 1992. Vacuum packaging, ascorbic acid and frozen storage effects on heme and nonheme iron content of mackerel. J. Food Sci. 57: 1337-1339.

Haleva, E., Ben-Tal, N., and Diamant, H. 2004. Increased concentration of polyvalent phospholipids in the adsorption domain of a charged protein. Biophys. J. 86: 2165-2178.

Hultin, H. O. and Kelleher, S. D. 2000. High efficiency alkaline protein extraction. US patent. 6, 136, 959.

Hultin, H. O., Kristinsson, H. G., Lanier, T. C., and Park, J. W. 2005. Process for recovery of functional proteins by pH shifts. *In* Surimi and surimi seafood. 2nd ed. (Park, J. W. eds.). p. 107-139. CRC Press. Boca Raton, Florida.

Jafarpour, A., and Gorczyca, E. M. 2012. Contribution of sarcoplasmic proteins to myofibrillar proteins gelation. J. Food Sci. 77: 73-81.

Kang, G., Yang, H., Jeong, J., Moon, S., Hur, S., Park, G., and Joo, S. 2007. Gel color and texture of surimi-like pork from muscles at different rigor states post-mortem. Asian Austral. J. Anim. 20: 1127.

Khantaphant, S., Benjakul, S., and Ghomi, M. R. 2011. The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*Lutjanus vitta*). LWT-Food Sci. Technol. 44: 1139-1148.

Kristinsson, H. G., and Hultin, H. O. 2003. Effect of low and high pH treatment on the functional properties of cod muscle proteins. J. Agric. Food Chem. 51: 5103-5110.

Kristinsson, H. G., and Ingadottir, B. 2006. Recovery and properties of muscle proteins extracted from tilapia (*Oreochromis niloticus*) light muscle by pH shift processing. J. Food Sci. 71: 132-141.

Kristinsson, H. G., Theodore, A. E., Demir, N., and Ingadottir, B. 2005. A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. J. Food Sci. 70: 298-306.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Martin-Sanchez, A. M., Navarro, C., Perez-Alvarez, J. A., and Kuri, V. 2009. Alternatives for efficient and sustainable production of surimi: a review. Comp. Rev. Food Sci. 8: 359-374.

Moayedi, V., Omana, D., Chan, J., Xu, Y., and Betti, M. 2010. Alkali-aided protein extraction of chicken dark meat: Composition and stability to lipid oxidation of the recovered proteins. Poultry Sci. 89: 766-775.

Moreno, H. M., Carballo, J., and Borderias, A. J. 2008. Influence of alginate and microbial transglutaminase as binding ingredients on restructured fish muscle processed at low temperature. J. Sci. Food Agric. 88: 1529-1536.

Nakagawa, T., Nagayama, F., Ozaki, H., Watabe, S., and Hashimoto, K. 1989. Effect of glycolytic enzymes on the gel-forming ability of fish muscle. Nippon Suisan Gakkaishi. 55: 1045-1050.

Nakahara, C., Nozawa, H., and Seki, N. 1999. A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. Fish. Sci. 65: 138-144.

NFI 1991. A manual of standard methods for measuring and specifying the properties of surimi. National Fisheries Institute. Washington, DC.

Nielsen, P. 1995. Reactions and potential industrial applications of transglutaminase. Review of literature and patents. Food Biotechnol. 9: 119-156.

Nolsøe, H., and Undeland, I. 2009. The acid and alkaline solubilization process for the isolation of muscle proteins: state of the art. Food Bioprocess. Technol. 2: 1-27.

Nylander, T. 2004. Interactions between proteins and polar lipids. *In* Food emulsions (Friberg, S. E., Larsson, K., and Sjöblom, J. eds.). p. 107-174. Marcel dekker. New York.

Park, J. W., Lin, T. M., and Yongsawatdigul, J. 1997. New developments in manufacturing of surimi and surimi seafood. Food Rev. Int. 13: 577-610.

Pérez-Mateos, M., and Lanier, T. C. 2007. Comparison of Atlantic menhaden gels from surimi processed by acid or alkaline solubilization. Food Chem. 101: 1223-1229.

Rawdkuen, S., Sai-Ut, S., Khamsorn, S., Chaijan, M., and Benjakul, S. 2009. Biochemical and gelling properties of tilapia surimi and protein recovered using an acid-alkaline process. Food Chem. 112: 112-119.

Robinson, H. W. and Hodgen, C. G. 1940. The biuret reaction in the determination of serum protein. I. A study of the conditions necessary for the production of the stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135: 707–725.

Roura, S., and Crupkin, M. 1995. Biochemical and functional properties of myofibrils from pre and post-spawned hake (*Merluccius hubbsi Marini*) stored on ice. J. Food Sci. 60: 269-272.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. McGraw-Hill. New York.

Thawornchinsombut, S., and Park, J. W. 2006. Frozen stability of fish protein isolate under various storage conditions. J. Food Sci. 71: 227-232.

Thiansilakul, Y., Benjakul, S., and Richards, M. P. 2010. Changes in heme proteins and lipids associated with off-odour of seabass (*Lates calcarifer*) and red tilapia (*Oreochromis mossambicus*× *O. niloticus*) during iced storage. Food Chem. 121: 1109-1119.

Tongnuanchan, P., Benjakul, S., Prodpran, T., and Songtipya, P. 2011. Characteristics of film based on protein isolate from red tilapia muscle with negligible yellow discoloration. Int. J. Biol. Macromol. 48: 758-767.

Undeland, I., Kelleher, S. D., and Hultin, H. O. 2002. Recovery of functional proteins from herring (*Clupea harengus*) light muscle by an acid or alkaline solubilization process. J. Agric. Food Chem. 50: 7371-7379.

Visessanguan, W., Benjakul, S., and Tanaka, M. 2003. Effect of microbial transglutaminase on rheological properties of oxidised and non oxidised

natural actomyosin from two species of bigeye snapper. J. Sci. Food Agric. 83: 105-112.

Yamaguchi, K., Takeda, N., Ogawa, K., and Hashimoto, K. 1979. Properties of mackerel and sardine myoglobins. Nippon Suisan Gakkaishi. 45: 1335-1339.

Yongsawatdigul, J., and Park, J. 2004. Effects of alkali and acid solubilization on gelation characteristics of rockfish muscle proteins. J. Food Sci. 69: 499-505.

CHAPTER 8

CONCLUSION AND SUGGESTION

8.1 Conclusion

1. The addition of MTGase enhanced the gel-forming ability of surimi from different fish species, but the strengthening effect varied. The reactivity of muscle proteins toward MTGase was the key factor determining the gel strength of surimi.

2. The presence of urea and formation of ammonia negatively affected protein cross-linking induce by MTGase. However, the washing process, which reduced urea and ammonia contents, was an effective means to lower the inactivation of both endogenous transglutaminase and MTGase caused by ammonia.

3. MTGase was able to increase the gel strength of surimi from lizardfish. However, formaldehyde present in surimi had a negative impact on gel improvement and cross-linking ability induced by MTGase.

4. Biogenic amines including putrescine, histamine and tyramine were able to serve as an acyl acceptor in MTGase catalysed reaction. Biogenic amines, which acted as the competitive substrates, lowered gel strengthening effect mediated by MTGase.

5. The oxidation induced the physicochemical and conformation changes of fish myosin. Protein cross-linking mediated by MTGase was impeded when the severe oxidation took place. The oxidation also decreased the gel-forming ability of washed mince but MTGase at appropriate level could strengthen the gel.

6. Alkaline solubilisation process induced unfolding of the muscle proteins as evidenced by the decrease in the Ca^{2+}-ATPase activity and protein solubility. This process was able to remove myoglobin and lipid from mince, leading

to the better properties of gels. The addition of MTGase in protein isolate, especially with prewashing, could improve properties of gel incorporated with MTGase.

8.2 Suggestion

1. The composition and properties of muscle proteins of varying fish species associated with protein cross-linking mediated by MTGase should be further investigated.

2. The effect of lipid oxidation products on gel-forming ability and protein cross-linking mediated by MTGase should be further studied.

3. Pretreatment of fish muscle to favour the cross-linking reaction mediated by MTGase should be studied.