Nile Tilapia (*Oreochromis niloticus*) Protein Hydrolysate with Antioxidative and Nitric Oxide Inhibitory Activities

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Functional Food and Nutrition
Prince of Songkla University
2014

Thesis Title	Nile Tilapia (*Oreochromis niloticus*) Protein Hydrolysate with Antioxidative and Nitric Oxide Inhibitory Activities
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ชื่อวิทยานิพนธ์ โปรตีนไฮโดรไลเสทของปลานิล (*Oreochromis niloticus*) ที่มีฤทธิ์ต้านออกซิเดชันและต้านไนตริกออกไซด์

ผู้เขียน สุรีย์พร กังสนันท์

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บทคัดย่อ

โฮโมจิเนทจากโปรตีนกล้ามเนื้อปลานิล (*Oreochromis niloticus*) ถูกย่อยเป็นเวลา 120 นาทีโดยใช้เอนไซม์ 3 ชนิด ได้แก่ เอนไซม์ปาเปน, เอนไซม์ฟลาโวไซม์, หรือเอนไซม์อัลคาเลส ศึกษาผลของการใช้อัลตร้าซาวด์ที่ระดับพลังงาน 10, 40, หรือ 70 วัตต์ ระหว่างการย่อยด้วยเอนไซม์และผลต่อการผลิตโปรตีนไฮโดรไลเสทที่มีฤทธิ์ทางชีวภาพ พบว่า การใช้อัลตร้าซาวด์ในระหว่างการย่อยช่วยเพิ่มระดับการย่อย (degree of hydrolysate; DH) ในไฮโดรไลเสทที่ย่อยด้วยเอนไซม์อัลคาเลส อัลตร้าซาวด์มีผลเพิ่มความหนืดในระหว่างการย่อยของไฮโดรไลเสทในทุกชุดการทดลอง และอัลตร้าซาวด์ยังมีผลต่อโครงสร้างจุลภาคของอนุภาคกล้ามเนื้อปลานิลที่ไม่ผ่านการย่อย ผลของอัลตร้าซาวด์ต่อความสามารถในการต้านอนุมูลอิสระ DPPH และ ABTS ของไฮโดรไลเสทขึ้นอยู่กับชนิดของเอนไซม์ที่ใช้ในการย่อย โดยการใช้อัลตร้าซาวด์ร่วมกับการย่อยด้วยเอนไซม์ปาเปนมีผลให้ความสามารถในการยับยั้งอนุมูลอิสระ DPPH, ABTS, ความสามารถในการรีดิวส์ และความสามารถในการจับโลหะ ของโปรตีนไฮโดรไลเสทดีขึ้น นอกจากนี้ไฮโดรไลเสทยังแสดงความสามารถในการยับยั้งอนุมูลอิสระในระบบเซลล์โดยใช้ระบบจำลองการเกิดอนุมูลอิสระในเซลล์แมคโคเฟจ (RAW 264.7 macrophage cell lines) การย่อยโปรตีนกล้ามเนื้อปลานิลด้วยเอนไซม์ปาเปนร่วมกับการใช้อัลตร้าซาวด์ที่ระดับพลังงาน 40 วัตต์ มีผลในการเพิ่มความสามารถในการต้านอนุมูลอิสระในระบบเซลล์จำลองถ้าการย่อยใช้เวลาในช่วง 60 – 120 นาที จากการทดลอง อัลตร้าซาวด์จึงสามารถใช้ในการเตรียมเปปไทด์ที่มีความสามารถในการต้านอนุมูลอิสระโดยการให้โฮโมจิเนทของโปรตีนกล้ามเนื้อปลานิลสัมผัสกับอัลตร้าซาวด์เป็นเวลามากกว่า 60 นาที ร่วมกับการใช้เอนไซม์เพื่อเพิ่มฤทธิ์ทางชีวภาพของเปปไทด์ที่ได้จากโปรตีนกล้ามเนื้อของปลานิล

จากการศึกษาการใช้อัลตร้าซาวด์ในการปรับสภาพ (Pretreatment) โปรตีนกล้ามเนื้อปลานิลก่อนการย่อยด้วยเอนไซม์และการใช้อัลตร้าซาวด์ในระหว่างการย่อยด้วยเอนไซม์ ผลการศึกษาพบว่าการย่อยโปรตีนกล้ามเนื้อปลานิลในสภาวะที่มีอัลตร้าซาวด์มีผลให้ระดับการย่อยของไฮโดรไลเสทที่ได้ลดลงอยู่ในช่วง 23% ถึง 35% เมื่อเปรียบเทียบกับกระบวนการย่อยโดยการกวน

การใช้อัลตร้าซาวด์ที่ระดับพลังงาน 70 วัตต์ ร่วมกับการย่อยด้วยเอนไซม์ฟราโวไซม์มีผลเพิ่มความสามารถในการยับยั้งอนุมูลอิสระ DPPH และความสามารถในการรีดิวส์ของไฮโดรไลเสทของปลานิลที่ไม่ผ่านการปรับสภาพ ก่อนการย่อยได้เป็น 33% และ 45% ตามลำดับ นอกจากนี้ โปรตีนไฮโดรไลเสทของปลานิลที่เตรียมได้จากทุกชุดการทดลองไม่แสดงความเป็นพิษต่อเซลล์แมคโครฟาจที่ระดับความเข้มข้นสูงสุด 20 มิลลิกรัมโปรตีนต่อมิลลิลิตร โปรตีนไฮโดรไลเสทของปลานิลที่เตรียมได้จากสภาวะ การปรับสภาพโปรตีนกล้ามเนื้อของปลานิลด้วยอัลตร้าซาวด์ที่ระดับพลังงาน 70 วัตต์ เป็นเวลา 30 และ 45 นาที ร่วมกับการย่อยด้วยเอนไซม์ฟราโวไซม์เป็นเวลา 1 ชั่วโมงด้วยกระบวนการย่อยแบบการกวนสามารถผลิตโปรตีนไฮโดรไลเสทของปลานิลที่มีความสามารถในการต้านการหลั่งของไนตริกออกไซด์และต้านการเกิดอนุมูลอิสระในระบบเซลล์เซลล์แมคโครฟาจได้

จากการศึกษาสภาวะที่เหมาะสมในการผลิตโปรตีนโดรไลเสทของปลานิลที่มีความสามารถในการยับยั้งการหลั่งไนตริกออกไซด์และต้านการเกิดอนุมูลอิสระในระบบเซลล์ นำเปปไทด์ที่ได้ไปแยกด้วยวิธีการเจลคอลัมน์โครมาโทกราฟี โดยใช้ Sephadex G-25 และ วิธีการโครมาโทกราฟีของเหลวสมรรถภาพสูงแบบกลับเฟส (Reverse-phase high performance chromatography (RP-HPLC)) ผลการศึกษาพบว่า เปปไทด์ที่มีความสามารถในการต้านการเกิดอนุมูลอิสระในระบบเซลล์มีลำดับกรดอะมิโนจากปลายคาร์บอกซิล (C-terminal) คือ KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (มวลโมเลกุล 6,334.49 ดาลดัน) และเปปไทด์ผ่านการทำบริสุทธิ์ยังแสดงความสามารถในการต้านอนุมูลอิสระในระบบเซลล์ นอกจากนี้ เปปไทด์ที่มีความสามารถในการยับยั้งการหลั่งไนตริกออกไซด์ มีลำดับกรดอะมิโน คือ AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (มวลโมเลกุล 6,309.46 ดาลดัน) โดยเปปไทด์ที่ผ่านการทำบริสุทธิ์แสดงความสามารถในการยับยั้งการหลั่งไนตริกออกไซด์ในระบบเซลล์ จากผลการทดลองแสดงให้เห็นว่า อัลตร้าซาวด์สามารถนำไปประยุกต์ใช้ทั้งในการปรับสภาพตัวอย่างหรือในระหว่างการย่อย ผลการทดลองชี้ให้เห็นว่าเปปไทด์ที่ผ่านการทำบริสุทธิ์มีความสามารถในการต้านการเกิดอนุมูลอิสระและต้านหลั่งไนตริก ออกไซด์ในระบบเซลล์แมคโครเฟจ RAW264.7

Thesis Title Nile Tilapia (*Oreochromis niloticus*) Protein Hydrolysate with Antioxidative and Nitric Oxide Inhibitory Activities
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Major Program Functional Food and Nutrition
Academic Year 2013

ABSTRACT

Tilapia (*Oreochromis niloticus*) muscle homogenate was hydrolyzed for 120 min by using papain, Flavourzyme, or Alcalase. The effects of simultaneous ultrasonication at 10 W, 40 W, or 70 W on proteolytic hydrolysis and bioactivities of the hydrolysates were investigated. The ultrasonic treatments caused an improved degree of hydrolysis in Alcalase hydrolysis. The treatments increased the viscosity of all homogenates during digestion and yielded different microscopy of non-digested fish muscle particle. Effect of ultrasonication on reactivity of hydrolysate against DPPH and ABTS was varied according to the enzymes used. The combination of ultrasonic and papain hydrolysate showed an improvement in DPPH and ABTS scavenging activity, reducing power and metal chelating at least to some extent. The hydrolysates exhibited promising antioxidative activity in RAW 264.6 cell lines. Catalysis with papain and ultrasonication at 40W yielded an increase antioxidative hydrolysate based on macrophage cell line study if the hydrolysis was extending from 60 to 120 min. Ultrasonic treatment could thus be used for preparation of antioxidative peptides if the hydrolysis time was over 60 min and can be incorporated into the enzymatic process to increase the bioactive activities of tilapia peptide.

Ultrasound was applied to tilapia (*Oreochromis niloticus*) muscle protein to facilitate homogenate pretreatment and during enzymatic hydrolysis. Their effects on Flavourzyme hydrolysis and biological activities of the tilapia hydrolysate were examined. The ultrasound-assisted enzymatic hydrolysis caused reduction in degree of hydrolysis ranging from 23% to 35% relative to that of the conventional process. The 70 W ultrasound-assisted enzymatic hydrolysis process increased DPPH radical scavenging activity and reducing power of tilapia hydrolysate prepared from the non pretreatment homogenate by 33% and 45%, respectively. All treatments have non

cytotoxicity on RAW264.7 cell lines at the maximum concentration of 20 mg protein/ml. The 70W ultrasound pretreatment at 30 and 45 min combined with conventional hydrolysis are observed to be the suitable condition for producing tilapia hydrolysate with nitric oxide (NO) inhibitory and antioxidative activities on RAW264.7 cell lines, respectively.

Tilapia (*Oreochromis niloticus*) muscle protein was hydrolyzed using ultrasound pretreatment before Flavourzyme hydrolysis. The hydrolysates pretreated with ultrasound at 70 W for 30 and 45 min before conventional hydrolysis process possessed the NO-inhibitory and antioxidant activities, respectively. The derived peptides were purified using gel column chromatography (Sephadex G-25) and reverse-phase high performance chromatography (RP-HPLC) and antioxidative and NO-inhibitory activities of purified peptide were evaluated. The antioxidative peptide was identified from C-terminal as KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (Mw 6,334.49 Da). The purified antioxidant peptide was shown to scavenge the radical in macrophage cell lines system. Furthermore, NO-inhibitory activity peptide was AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (Mw 6,309.46 Da). The NO-inhibition was also determined using Griess reaction. In this study, the purified peptide could inhibit LPS-induced NO production in RAW264.7 macrophage cell lines. As a result, ultrasound could be applied to enzymatic protein hydrolysis either at pretreatment or during hydrolysis. Our results indicated that the purified peptide showed the potential antioxidative and NO-inhibitory activities in RAW264.7 macrophage cell lines.

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Chapter 1

Introduction and review of literature

1.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is widely cultivated and consumed species among freshwater fishes reared in Thailand. Highly nutritive and functional protein hydrolysates have been successfully produced from tilapia (Cândido and Sgarbieri, 2003; Foh *et al.*, 2011). Moreover, peptide derived from tilapia also exerted biological properties (Charoenphun *et al.*, 2012; Dekkers *et al.*, 2011; Foh *et al.*, 2011; Raghavan and Kristinsson, 2009). For an instant, tilapia protein hydrolysates with antioxidative activity has been reported (Raghavan and Kristinsson, 2008; Raghavan *et al.*, 2008).

Bioactive peptides refer to specific protein fragments that have a positive impact on body function or condition and which ultimately may influence health beyond their basic role as nutrient sources (Hartmann and Meisel, 2007). The bioactive peptide could be prepared by enzymatic hydrolysis, an accepted and safe process providing protein with improved functional, nutritive and bioactive properties (Liceaga-Gesualdo and Li-Chan, 1999). Many documents are claim that the bioactive peptide from fish protein hydrolysate showed the biological activities. For instance, antioxidative activity (Je *et al.*, 2008), anti-hypertension (Yokoyama *et al.*, 1992), anti-inflammatory activity (Sung *et al.*, 2012), and antibacterial (Song *et al.*, 2012).

Several techniques can be applied to alter the structural characteristics of protein in order to increase its accessibility to enzymes and to obtain the most potent bioactive peptides. Recently, several publications described the use of ultrasound treatment for either pretreatment or assisted enzymatic hydrolysis. Jian and co-workers reported that ultrasonic treatment during proteolysis enhanced the enzymatic hydrolysis of leather waste by increasing the digestion yield by fleshing and shaving (Jian *et al.*, 2008). This approach also enhanced the Alcalase hydrolysis of defatted wheat germ protein, whereas ultrasonic pretreatment improved the release of ACE-

inhibitory peptides during the enzymatic hydrolysis as described by the same group (Jia *et al.*, 2010).

Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. ROS are generated during mitochondrial oxidative metabolism as well as part of the cellular response to xenobiotics, cytokines, and bacterial invasion (Ray *et al.*, 2012). Exposure to ROS is strongly linked with chronic diseases such as cancer, coronary heart disease, and Alzheimer's disease (Abdul-Hamid *et al.*, 2002). Therefore, it is important to inhibit the oxidation and formation of free radicals occurring in the living body.

Nitric oxide (NO) is produced from L-arginine by a chemical reaction catalyzed by the enzyme inducible nitric oxide synthase (iNOS) in living systems. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO(Darley-Usmar *et al.*, 1995). Low concentrations of nitric oxide produced by the constitutive and nNOS inhibit adhesion molecule expression, cytokine, and chemokine synthesis as well as leukocyte adhesion and transmigration. Large amounts of NO, generated primary by iNOS can be toxic and pro-inflammatory (Guzik *et al.*, 2003). The interactions between ROS and NO generate potentially cytotoxic agents which may mediate some of the pathology associated with Parkinson's disease, chronic inflammation, atherosclerosis and cancer(Darley-Usmar *et al.*, 1995).

In the present study, ultrasonic pretreatment or assist during enzymatic hydrolysis was performed. Ultrasonic power (10 W, 40 W, and 70 W), duration of pretreatment or hydrolysis process, enzyme types (Alcalase, papain, and Flavourzyme), and hydrolysis processes (conventional and ultrasonic-assisted processes) were optimized in order to improve peptide with antioxidant (*in vitro* and *ex vivo*) and Nitric oxide (NO) inhibitory activities (*ex vivo*). Furthermore, characterization of purified antioxidant and NO inhibition peptides were evaluated.

1.2 Review of literature

1.2.1 Nile tilapia (*Oreochromis niloticus*)

Nile tilapia (*Oreochromis niloticus*) is a well known fresh water fish of the big order Perciformes, family Cichlidae. It originated exclusively from the African continent and from Palestine. Introduction of tilapia outside Africa was begun since 1939. Now tilapia occurs in natural waters throughout the tropics, even in Australia (Srijunngam and Wattanasirmkit, 2001) cited by Philippart and Ruwet (1982). It is fish of economic importance in tropical and subtropical countries. Nile tilapia was first introduced to Thailand in March 1965 by His Imperial Majesty Akihito, the Emperor of Japan. Consequently, they were given to the Department of Fisheries for further development of culturing by His Majesty the King of Thailand (Srijunngam and Wattanasirmkit, 2001) cited by Phumipat (1981). Nowadays, Nile tilapia is an essential food fish, widely cultured in many areas throughout Thailand.

Nile tilapia body (Figure 1) is compressed; caudal peduncle depth equal to length. Scales is cycloid. A knob is like protuberance absent on dorsal surface of snout. Upper jaw length is showing no sexual dimorphism. First gill arch is consisting of 27 to 33 gill rakers. Lateral line interrupts. Spinous and soft ray parts of dorsal fin are continuous. Dorsal fin consists of 16 - 17 spines and 11 to 15 soft rays. Anal fin has 3 spines and 10-11 rays. Caudal fin is truncated. In spawning season, the pectoral, dorsal and caudal fins are become reddish; beside, numerous black bars are observed only on the caudal fin (Pieroni and Gentile, 2009)

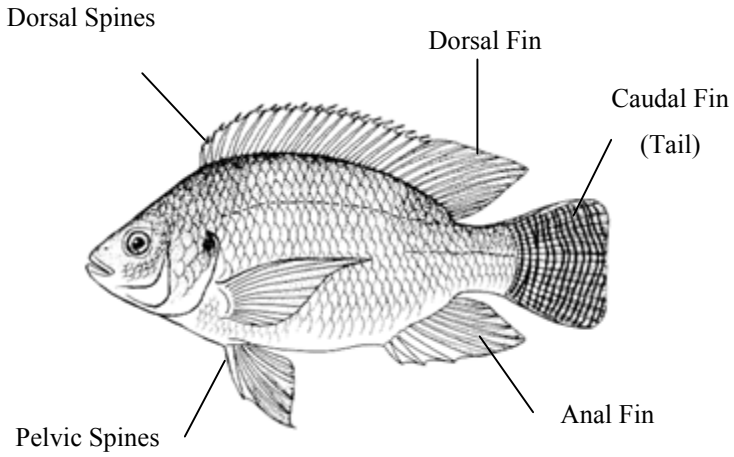


Figure 1 Nile tilapia (*Oreochromis niloticus*)

Source: Rakocy, (2005)

Nile tilapia processing yield (total fish weight minus weight of head, skin and viscera) and fillet yield are 51% and 25.4%, respectively. Nutrient composition of nile tilapia, fat and protein content of nile tilapia are 5.7 % and 20.3%, respectively. Caloric value of fillet is 139 kcal/100 g. Moreover, monounsaturated fatty acid and n-3 highly unsaturated fatty acids account for an average of 54.6 and 2.2 g/100 g of total fatty acids, respectively. A cholesterol content of fillet is 31.3 mg/100 g (Clement and Lovell, 1994).

Nile tilapia is rich of essential amino acids. The essential amino acids of tilapia reached the FAO/WHO requirement for the essential amino acids for adult (FOA, 2007). Moreover, amino acid score, particularly lysine, methionine and threonine were much higher than the recommended FAO/WHO standard (Table 1). In addition, there are some reports available in the literatures that describe bioactive peptides from tilapia (Charoenphun *et al.*, 2012; Foh *et al.*, 2010). To date, no work on ultrasound pretreatment or ultrasound assisted hydrolysis of fish protein particularly tilapia protein has been published. Therefore, tilapia was used as a raw material for protein hydrolysate production.

Table 1 The amino acid profiles of tilapia (*Oreochromis niloticus*)

Amino acid	% protein (dry matter basis)	Amino acid requirements for adult (g/100 g protein)* FAO/WHO	Score (%)
Essential amino acid			
Isoleucine	2.39	3.0	79
Leucine	4.26	5.9	72
Lysine	4.58	4.5	101
Methionine	3.36	1.6	210
Phenylalanine	2.03	3.0	67
Threonine	3.07	2.3	133
Valine	2.45	3.9	62
Histidine	1.41	1.5	94
Tryptophane	0.58	0.6	96
Non-essencetial amino acid			
Arginine	4.60	-	-
Aspartic acid	5.39	-	-
Serine	3.10	-	-
Glutamic acid	9.29	-	-
Proline	4.33	-	-
Glysine	6.38	-	-
Alanine	5.00	-	-
Cystine	1.22	0.6	-
Valine	2.45	-	-
Tyrosine	1.72	-	-

Source: FOA (2007)

1.2.2 Protein hydrolysate

Protein hydrolysate was obtained by breakdown peptide bond of proteins into peptide fragments with different sizes and free amino acids. This can be achieved by a protease enzyme, acid or alkali. Acid and alkali processes are difficult to control and provide product with meager nutrition. Chemical hydrolysis can transform L amino acids to D amino acids and this process can generate toxic substance including lysino-alanine (Clemente, 2000). Enzymatic hydrolysis is performed under a mild condition, pH 6-8 and temperature 40-60 $^{\circ}$C. The overall amino acid composition of enzymatic hydrolysis is similar to that of the starting material. The advantage using of enzymes is a better control of the hydrolysis process and thereby the properties of the resulting product. Biological processes using endogenous or exogenous enzymes are less frequently employed in industrial practices. Enzyme hydrolysis is usually a gentle process that results in products of high functionality, good organoleptic properties, and excellent nutritional value.

To develop commercial protein hydrolysates with bioactive peptide defined physical, chemical and nutritional characteristics, many different factors must be taken into account to achieve a certain. Among them, suitable source of protein, enzymatic hydrolysis conditional, post-hydrolysis processes have special relevance.

1.2.3 Protein source

The nature and quality of the raw material can have a great impact on the hydrolysis process and the quality and functionality of the final product (Kristinsson, 2006). Therefore, there is a growing interest in finding food bioactive derived from natural sources, which may have less potential health hazard compared with synthetic antioxidants.

Recently, bioactive activities derived fish protein hydrolysate were reported relate to many activity as illustrate in Table 2. Therefore, fish protein was played attention as a source for produced protein hydrolysate with biological properties.

Table 2 Bioactivities of fish protein hydrolysate

Source	**Enzyme used for hydrolysis**	**Bioactivities**	**References**
Monkfish (*Lophius litulon*)	Trypsin	Antioxidative activity	Chi *et al.*(2014)
Ribbonfish (*Trichiurus haumela*)	Acid protease	Angiotensin I-converting enzyme inhibitory (ACEI)	Zou *et al.*(2013)
Anchovy (*Engraulis japonicus*)	Alcalase	Antimicrobial	Tang *et al.* (2013)
Salmon	Pepsin	Antioxidative activity and anti-inflammatory	Ahn *et al* (2012).
tilapia (*Oreochromis niloticus*)	Alcalase	Ca-binding	Charoenphun *et al.* (2012)
Alaska pollack (*Theragra chalcogramma*)	Mackerel intestine crude enzyme	Antioxidative activity	Je *et al.* (2005)
	Trypsin	immunomodulatory	Hou *et al.*(2012)
Sweetfish (*Plecoglossus altivelis*)	trypsin and α-chymotrypsin	anti-inflammatory	Sung *et al.*(2012)
Flounder fish (*Paralichthys olivaceus*)	α-chymotrypsin	Antioxidative activity	Ko *et al.*(2013)
Sardinelle (*Sardinella aurita*)	crude enzyme extract from sardine (*Sardina pilchardus*)	Antioxidative activity	Bougatef *et al.*(2010)

1.2.4 Enzymatic hydrolysis process

Enzyme hydrolysis is usually a gentle process that results in products of high functionality, good organoleptic properties, and excellent nutritional value. Typical process for preparation of fish protein hydrolysate is shown as Figure 2. The process of preparation of FPH consists of proteolytic digestion of fish or fish waste at optimum conditions of pH and temperature of enzymes. The enzymes can be from plant (papain, Ficin), animal (Trypsin, Pancreatin), or microbial (Pronase, Alcalase, Flavourzyme) sources (Venugopal, 2009). The hydrolysis condition is controllable; it may be possible to produce hydrolysates with desirable activities. When producing hydrolyzed proteins, it is important to determine the degree of hydrolysis (DH). The DH, which is expressed as the percentage of α-amino nitrogen in the soluble fraction, is important in optimizing the process parameters (Venugopal, 2009). Increasing in DH increased the amount of low molecular weight peptides (Raghavan and Kristinsson, 2009).

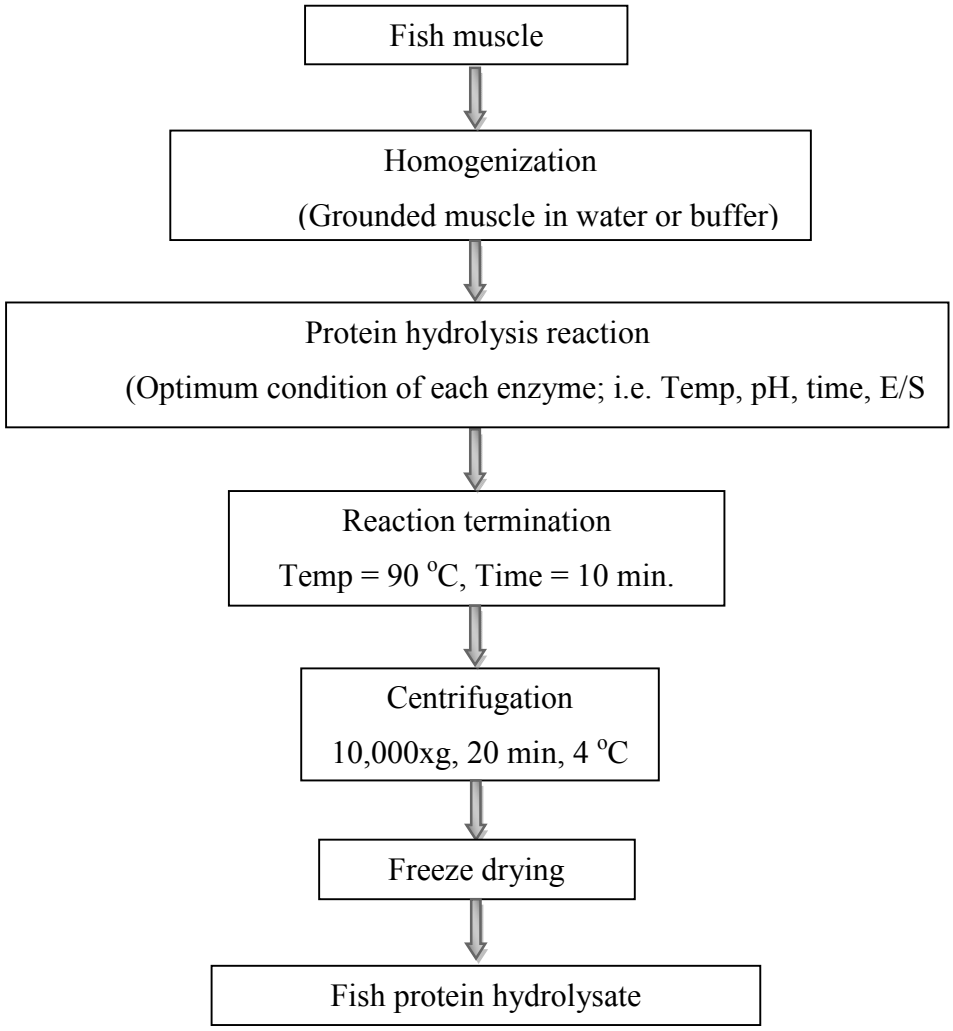


Figure 2 Processing of fish protein hydrolysate

Source: Raghavan and Kristinsson (2009)

Currently, the numerous technologies were applied in hydrolysis process in order to improved functional properties and bioactivity of protein. The technologies used must be safe, inexpensive, simple, non-toxic, and non side effect. In recent years, various techniques have been applied before or during enzymatic hydrolysis process, including microwave and ultrasound. Uluko *et al.* (2013) reported that pretreatments of milk protein concentrates with either microwave or ultrasound significantly improve the bioactivity and functional characteristics of the resulting hydrolysates. Moreover, ultrasound incorporated during enzymatic hydrolysis could be produce antioxidant protein hydrolysate from peanut (Tang *et al.*, 2012; Yu *et al.*, 2012), wheat gluten (Zhu *et al.*, 2011), or soy sauce lees (Yang *et al.*, 2011). Furthermore, incorporation of ultrasound could enhance angiotensin-converting enzyme inhibitory activity of protein hydrolysate (Huang *et al.*, 2013; Ren *et al.*, 2013; Uluko *et al.*, 2013).

Since ultrasound is a safety technology that can use to produce protein hydrolysate with biological properties as mentions above. However, there is lack of information related to ultrasound that used for tilapia protein hydrolysate preparation which the objective to increase the efficiency of bioactive peptide derived tilapia hydrolysate. Therefore, in this study, ultrasound technology was considered as an assisted technique for production bioactive peptide from tilapia protein.

1.2.5 Ultrasound technology

Ultrasound technology is based on mechanical waves at a frequency above the threshold of human hearing (>16 kHz) (Soria and Villamiel, 2010). When ultrasound passes through a liquid medium, the interaction between the ultrasonic waves, liquid and dissolved gas leads to an exciting phenomenon known as acoustic cavitations (Chandrapala *et al.*, 2012). The acoustic cavity formations are two known mechanism (Figure 3). First, pre-existing bubbles in the liquid which are stabilized against dissolution because the surface is coated with contaminants such as a skin of organic impurity. Second, the mechanism relies on the existence of solid particles in liquid with gas trapped in this particle, where nucleation takes place. There can also be tiny crevices in the walls of the vessel or container where gas is trapped. The pressure

inside a gas crevice is lower than the outside liquid pressure. Consequently, gas diffuses into the gas pocket, causing it to grow. A bubble is then created as the gas pocket departs from the crevice under the influence of a radiation force (Leong *et al.*, 2011).

However, the cavitations' bubbles can behave in two ways. One is called stable cavitation, bubbles formed at fairly low ultrasonic intensities (1–3Wcm^{-2}) oscillate about some equilibrium size for many acoustic cycles. While another is transient cavitation, bubbles are formed using sound intensities in excess of 10Wcm^{-2}. Transient bubbles expand through a few acoustic cycles to a radius of at least twice their initial size, before collapsing violently on compression. Transient bubble collapsing is considered to be the main source of the chemical and mechanical effects of ultrasonic energy (Santos *et al.*, 2009).

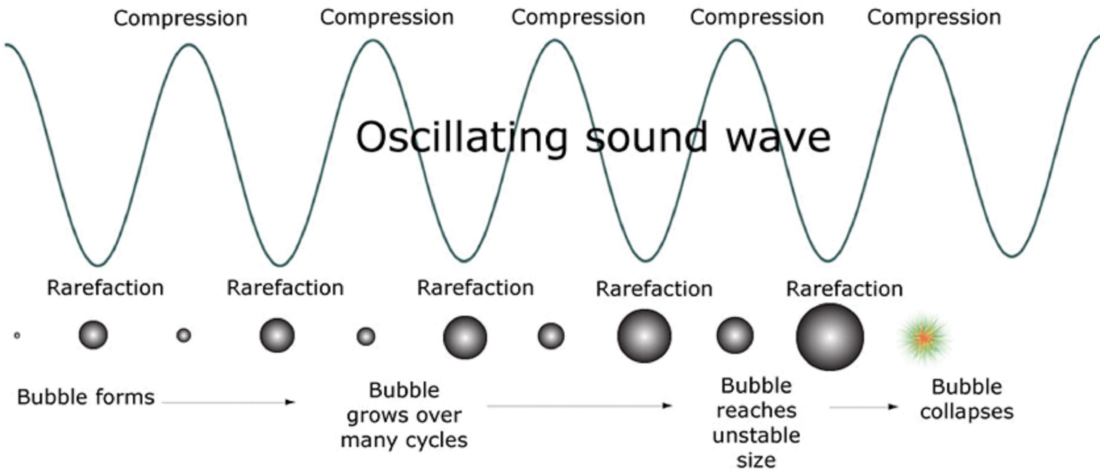


Figure 3 Graphical summary of cavitation bubble formation.

Source: Leong *et al.* (2011)

Ultrasound in liquids generates a number of physical forces including vibration, heating and physical agitation that can be generated in the absence of acoustic cavitation. In addition to these physical forces, acoustic cavitation generates microjets, shear, shockwaves and acoustic streaming (Chandrapala *et al.*, 2012).

In recent years, the physical and chemical effects of ultrasound in liquid and solid media have been extensively used in food processing application. The utilization of ultrasound not only expected for improve process yields but also expectation for produced or enhanced biological properties of raw material. Nevertheless, recently research articles discussed about biological properties and factors effecting the used of ultrasound in protein especially enhance biological properties of food protein hydrolysate. Most of the reported related to factors affecting the biological properties of protein hydrolysate can be summarized as followed:

Ultrasound frequency: The positive effect of ultrasonic frequency was found the study of Wheat gluten. The ultrasound at 25 kHz, 47 kHz, and 69 kHz was improved the antioxidant capacity (ferrous ion-chelating activity, reducing power, inhibition of linoleic acid emulsion peroxidation and 2, 2′-azinobis (3-ethylbenzothaizoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging activity) of wheat gluten hydrolysate (Zhu *et al.*, 2011). In addition, the highest ACE inhibitory activity of corn gluten meal protein hydrolysate was obtained when applied ultrasound at 20 kHz and at the power 1000 W (Zhou *et al.*, 2013). ACE-inhibitory activity for the hydrolysates of unpretreated and ultrasonic-pretreated (900–1800 W) defatted wheat germ protein hydrolysed by Alcalase was studied by Jia *et al.*(2010). The IC_{50} value of the hydrolysate of defatted wheat germ protein with Alcalase was 0.42–0.26 mg/ml after ultrasonic pretreatment at 900–1800W of ultrasonic power. The ACE-inhibitory activity increased with the increase of ultrasonic power from 900W to 1500 W, while the ACE-inhibitory activity declined slightly when ultrasonic power was extended to 1800 W. Nevertheless, negative effect of ultrasonic was foiund in the study of effect of ultrasonic frequency on DPPH free radical scavenging activity of peanut hydrolysate. When ultrasonic frequency was increased from 28 kHz to 100 kHz, DPPH free radical scavenging rate decreased first rapidly, and then slowly (Yu *et al.*, 2012).

Type of ultrasound: The ultrasonic horn and bath reactors were compared based on production of angiotensin-convertingenzyme (ACE) inhibitory peptides from defatted wheat germ proteins (DWGP). The highest ACE inhibitory activity of DWGP hydrolysate was obtained at power intensity of 191.1 W/cm^2 for 10 min in the ultrasonic horn reactor (Zhou *et al.*, 2013).

Time: Incorporation of ultrasound at the 60 kHz in thermolysin hydrolysis of ovotransferrin can enhance the angiotensin-converting enzyme (ACE) inhibitory activity as the prolonged ultrasonic time (Lei *et al.*, 2011). Uluko *et al.*(2013) reported about that the optimum conditions for producing milk protein concentrate hydrolysates with the highest ACE inhibition were ultrasound pretreatment time, hydrolysis time and enzyme-to-substrate ratio of 4.11 min, 2.32 h and 2.33%, respectively.

However, as above mention, it can be seen that most of using ultrasound with the purpose of produce bioactive peptide with antioxidative activities have been lack of information. In addition, peptide with nitric oxide inhibitory activity derived from ultrasound assisted during or pretreatment enzymatic hydrolysis has not reported at the present. Therefore, in this study, ultrasound was interested in the application to prepare protein hydrolysates with antioxidant and nitric oxide inhibitory activities.

1.2.6 Biologically active peptides

Bioactive peptides refers to different peptides of their origin protein that may have regulatory functions in the human system beyond normal and adequate nutrition (Hartmann and Meisel, 2007). Generally, bioactive peptides range in size from 2 to 20 amino acid residues and are encrypted within the sequence of parent protein and may be released during gastrointestinal digestion and/or during food processing (Harnedy and FitzGerald, 2012). Their activity is based on size, amino acid composition, and sequence (Korhonen and Pihlanto, 2006).

Factors affecting the bioactive properties of peptide such as hydrolysis process and processing conditions are important for resulting peptides. These effects can cause the ability of peptide to cross the intestinal epithelium and enter the blood

circulation, or to bind directly to specific epithelial cell surface receptor sites (Samaranayaka *et al.*, 2010; Udenigwe and Aluko, 2012).

1.2.7 Antioxidant

1.2.7.1 Oxidation in biological system

Oxidation and reduction (Redox) reactions are characterized the transfer of electrons from an electron donor (reducing agent) to an electron acceptor (oxidizing agent). The cellular redox environment is influenced by the production and removal of reactive oxygen species (ROS) (Sarsour *et al.*, 2009).

ROS are oxygen-containing molecules that are highly reactive in redox reactions. It is a collective term and includes oxygen free radicals and several non-radical agents (Table 3). ROS generation procedures in biological system are illustrated in Figure 4. The partial reduction of molecular oxygen results in the production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). $O_2^{\bullet-}$ and H_2O_2 react with transition metal ions (e.g., cuprous and ferrous ions) through Fenton and Haber–Weiss chemistry, generating the highly reactive hydroxyl radical ($HO^{\bullet}$). ROS levels also are dependent on oxygen concentrations. ROS were traditionally thought of as toxic byproducts of living in an aerobic environment because they are known to damage cellular macromolecules, which could subsequently lead to cell death (Sarsour *et al.*, 2009). Thus they play an important role in many disease conditions such as cancer, cardiovascular disease or diabetes. In a normal aerobic cell, ROS usually exist in balance with biological antioxidant. However, disruption of this critical balance could result in oxidative stress, leading to many diseases (Raghavan *et al.*, 2008).

Table 3 Reactive oxygen species (ROS).

Radical	**Non-radicals**
Superoxide radical, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxy radical, $HO^{\bullet}$	Hypochloric acid, HOCl
Peroxyl radical, $RO_2^{\bullet}$	Singlet oxygen, O_2^1
Alkoxyl radical, $RO^{\bullet}$	Organic peroxides, ROOH

Source: Abrahamsen (2009)

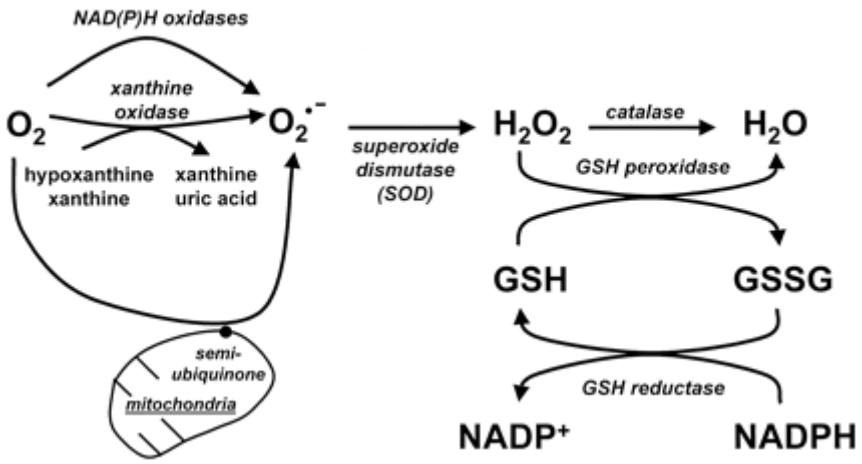


Figure 4 Generation of ROS. The major forms of ROS and their metabolism in biological system.

Source: Droge (2002)

1.2.7.2 Antioxidant

Antioxidant may define as any substance which is capable of delaying, retarding or preventing oxidation reaction.

Antioxidant can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a primary antioxidant, or by a mechanism that does not involve direct scavenging of free radical, in which this compound is a secondary antioxidant (Gordon, 2001).

Primary antioxidant includes phenolic compounds such as vitamin E (α-tocopherol), butylated hydroxyanisole (BHA), flavanoids, gallates, etc. BHA, butylhydroxyl toluene (BHT), tertiary butyl hydroxyquinone (TBHQ), and esters of gallic are the major synthetic primary antioxidants. These compounds are consumed during the induction period.

Secondary antioxidants operate by a variety of mechanisms including biding of metal ions, scavenging oxygen, converting hydropeoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. The secondary antioxidant such as citric acid which are effective only in the present of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants (Gordon, 2001), peroxide decomposers such as thioethers, methionine, metal chelaters, glutathione peroxidase, and catechins (Venugopal, 2009).

1.2.7.3 Antioxidative activity of fish protein hydrolysate

The numerous recently researches are published on antioxidative properties of fish derived protein hydrolysate. The operational conditions employed in the processing of protein isolates are type of protease and the degree of hydrolysis. Degree of hydrolysate (DH) is used as a tool to control hydrolysis condition to obtain hydrolysate with desired oxidative activity.

Some of these studies further fractionated the hydrolysates in order to isolate and identify individual antioxidative peptides. The antioxidant activity of proteins and peptides may be the result of scavenging of specific radicals formed during

peroxidation, scavenging of compounds containing oxygen, or the chelating capacity of metals (Kristinsson and Rasco, 2000). Nevertheless, antioxidative peptide is depending on their amino acid composition, sequence, and concentration.

1.2.7.3.1 Factors effecting the antioxidant activity of protein hydrolysate

Properties of bioactive peptide derived protein hydrolysate are mainly depends on degree of hydrolysis, the specificity of the enzyme and the composition and sequence of amino acid obtained.

Degree of hydrolysis: In protein hydrolysate, the key parameter for monitoring the reaction is the degree of hydrolysis (DH). DH is defined as the proportion of cleaved peptide bonds in a protein hydrolysate (Nielsen *et al.*, 2001). As showed in the Table 4, DH showed a correlation with the ability of fish protein hydrolysate to scavenge radical.

However, many documents are report that the antioxidative activity of protein hydrolysate associated with its DH in the different ways (Batista *et al.*, 2010; Samaranayaka and Li-Chan, 2008; Sathivel *et al.*, 2003). (i) Antioxidative activity of protein hydrolysate increased with increasing DH. (ii) Antioxidative activities increased with increasing DH up to the optimum DH and then decreased with increasing DH. (iii) In contrast, the excellence antioxidative activity was found in low DH. However, the suitable DH for produced

Enzyme: Commercial proteases were used to hydrolyze hydrolysis of fish protein. They included those from plant sources such as papain, bromilane or from organ of animal origin like pepsin pancreatin. Proteolytic enzymes of microbial origin like Alcalase, Neutrase, Protamex and Protease N have been also applied to the hydrolysis of fish protein (See *et al.*, 2011). However, Alcalase has been reported to be one of the highly efficient bacterial protease used to prepare functional fish and other protein hydrolysates (Kristinsson and Rasco, 2000). As illustrated in Table 4, alcalalse is seemed a famous used for produce protein hydrolysate with antioxidative activity. Furthermore, enzyme per substrate ratio in the range of 0.2-4% possesses the ability to produce protein hydrolysate with antioxidative activities.

Hydrolysis time:

As showed in the Table 4, antioxidative peptide derived from different hydrolysis time. The optimum hydrolysis time may be due to the limitation of the enzyme activity to produce the antioxidant peptide at the desired DH. The optimum hydrolysis duration is in the range of 0.5 – 6 h.

Table 4 Factors affecting the antioxidative activities of fish protein hydrolysate

Source	Enzyme	E/S ratio (%)	Hydrolysis time (h)	DH (%)	Antioxidative activities assay (% inhibition)					
					DPPH	ABTS	Metal chelating	Reducing power	Hydroxyl radical	Inhibition of linoleic acid
Silver carp (*Hypophthalmichthys molitrix*)	Alcalase	0.5	1.5	~16	-	-	-		-	~68
			2	~18	-	-	-		~60	-
			6	~22	-	-	92.97		-	-
	Flavouzyme	0.5	4	~6	-	-	-		-	~50
Dong *et al.* (2008)			5	~7	-	-	-		55	-
			6	~8	-	-	~65		-	-
Loach (*Misgurnus anguillicaudatus*)	papain	0.28	4	23	95.5	2.80** mM	-	1.46	56.1	-
	Protamex	0.24	4	23	92.2	2.81** mM	-	-	-	
You *et al* .(2009)		0.28	6	28	-	-	-	-	55.0	
		0.33	20	33	-	-	-	1.17	-	

** Trolox equivalent antioxidant capacity (TEAC)

Table 4 (Continued)

Source	Enzyme	E/S ratio (%)	Hydrolysis time (h)	DH (%)	IC_{50} (*) or % scavenging value					
					DPPH	ABTS	Metal chelating	Reducing power	Hydroxyl radical	Inhibition of linoleic acid
Black scabbard fish (*Aphanopus carbo*) Batista *et al.*(2010)	protamex™	0.5	2	25	30	-	-	0.7	50	-
		1	2	40	20	-	-	~0.9	~52	-
		4	2	56.8	12	-	-	~0.95	~40	-
Blue mussel (*Mytilus edulis*)	papain	3	2	-	13	-	-	-	-	-
			3	-	23	-	-	-	-	-
			4	-	20	-	-	-	-	-
	Neutrase	3	2	-	17	-	-	-	-	-
			3	-	28	-	-	-	-	-
			4	-	23	-	-	-	-	-
	Pepsin	3	2	-	20	-	-	-	-	-
			3	-	25	-	-	-	-	-
			4	-	24	-	-	-	-	-

Table 4 (Continued)

Source	Enzyme	E/S ratio (%)	Hydrolysis time (h)	DH (%)	IC_{50} (*) or % scavenging value					
					DPPH	ABTS	Metal chelating	Reducing power	Hydroxyl radical	Inhibition of linoleic acid
Blue mussel (*Mytilus edulis*) Wang *et al.* (2013)	Alcalase	3	2	-	23	-	-	-	-	-
		1	3	-	21	-	-	-	-	-
		4	4	-	14	-	-	-	-	-
Monkfish (*Lophius litulon*) Chi *et al.* (2014)	Trypsin	2	4	19.8	-	-	-	-	58.1	-
Tuna cooking juice Hsu *et al.* (2009)	Orientase	0.5	1	20	80	-	-	-	-	-
Fresh water carp (*Catla Catla*) Elavarasan *et al.* (2013)	Alcalase	1	0.5	6.7	64.6	-	-	0.4	-	54
	Bromelain	0.5	1	10.6	77.9	-	-	0.9	-	50
	Flavouzyme	1	1.15	7.8	70.4	-	-	0.6	-	49
	Protamex	2	1	12.6	67.9	-	-	0.5	-	36

Table 4 (Continued)

Source	Enzyme	E/S ratio (%)	Hydrolysis time (h)	DH (%)	IC_{50} (*) or % scavenging value					
					DPPH	ABTS	Metal chelating	Reducing power	Hydroxyl radical	Inhibition of linoleic acid
Small yellow crocker (*Pseudosciaena polyactis*) Ji *et al.*(2013)	Alcalase	0.2	4	29.3	1.78*	-	-	-	-	-
	Protamex	0.2	4	28.6	1.72*	-	-	-	-	-
	papain	0.2	4	30.4	1.56*	-	-	-	-	-
Patin (*Pangasius sutchi*) Najafian *et al*. (2013)	Alcalalse	1	1	65.83	71.1	-	-	0.31	-	-

1.2.7.3.2 Structure of antioxidative peptide

Since inhibitory effect of a peptide is effected by the type of amino acid present in its N- or C-terminal sequence which totally depend on the original primary structure (Ketnawa and Rawdkuen, 2013). In addition, Forghani and coworkers suggest that the total amounts of amino acid are not more critical than position of amino acid in the peptide sequence (Forghani *et al.*, 2012). Currently, many documents reported about amino acid sequence of potential antioxidant peptide derived from fish protein hydrolysate (Table 5).

Even if amino acid sequence of antioxidant peptide was reported in different amino acid residue, the function of each amino acid depends on their special structure characteristics. The antioxidant activity of histidine-containing peptides has been reported and attributed to the chelating and lipid radical-trapping ability of the imidazole ring (Park *et al.*, 2001). Cys and Met containing nucleophilic sulfur-containing side chains, and Trp, Tyr, and Phe having aromatic side chain are easily donate hydrogen atoms (Samaranayaka, 2010). In addition, it is also reported that the peptides containing Pro, Leu, Ala, and Tyr played an important role in the radical-scavenging effects (Je *et al.*, 2008).

Table 5 Amino acid sequence and molecular weight of antioxidative peptide derived from fish protein hydrolysate

Source	Amino acid sequence	Mw (Da)	IC_{50}(*) or % scavenging value	References
Flounder fish (*Paralichthys olivaceus*)	Val-Cys-Ser-Val	406.1	DPPH (111.32 μM*)	Ko *et al.* (2013)
	Cys-Ala-Ala-Pro	360.1	DPPH (26.89 μM*)	
Croaker (*Otolithes ruber*)	Lys-Thr-Phe-Cys-Gly-Arg-His	861.6	DPPH (85.5%)	Nazeer *et al.* (2012)
Tilapia frame (*Oreochromis niloticus*)	Asp-Cys-Gly-Tyr	456.12	Hydroxyl radical (27.6 μg/ml*)	Fan *et al.* (2012)
	Asn-Tyr-Asp-Glu-Tyr	702.26	Hydroxyl radical (38.4 μg/ml*)	
Sardinelle (*Sardinella aurita*)	Leu-His-Tyr	467.5	DPPH (63 ± 1.57%; at 150 μg/ml)	Bougatef *et al.*(2010)
Loach (*Misgurnus anguillicaudatus*)	Pro-Ser-Tyr-Val	464.2	Hydroxyl radical (1.86 ± 0.19 mg/ml*)	You *et al.* (2010)
Tuna dark muscle	Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr	978	DPPH (79.6 ± 2.86%)	Hsu, (2010)
	Pro–Met–Asp–Tyr–Met–Val–Thr	756	DPPH (85.2 ± 3.38%)	

Table 5 (Continues)

Source	Amino acid sequence	Mw (Da)	IC_{50}(*) or % scavenging value	References
Bigeye Tuna (*Thunnus obesus*)	Leu-Asn-Leu-Pro-Thr-Ala-Val-Tyr-Met-Val-Thr	1,222	-	Je *et al*.(2008)
Tuna backbone	VKAGFAWTANQQLS	1519	DPPH(~65%: at 1.2 mg/ml)	Je *et al.* (2007)
Hoki (*Johnius belengerii*)	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	1,801	DPPH (41.37 μM*) hydroxyl (17.77 μM*) peroxyl (18.99 μM*)	Kim *et al.*(2007)
Conger eel (*Conger myriaster*)	LGLNGDDVN	928	Hydrolxyl radical (74.1μM*)	Ranathunga *et al.* (2006)
Yellowfin sole (*Limanda aspera*)	RPDFDLEPPY	1410.4	-	Jun *et al.* (2004)

1.2.8 Nitric Oxide (NO)

1.2.8.1 Nitric oxide

Nitric oxide (NO) is a member of the labile radical entities knows as reactive oxygen species (ROS) and contains 1 nitrogen atom covalently bonded to an oxygen atom with one unpaired electron. It is particularly reactive with oxygen and heme-iron containing groups which reduce NO to more stable nitrate compounds (Ignarro, 1989). It is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the human body. These include circulation and blood pressure, platelet function, host defense, and neurotransmission in central nervous system and in peripheral nerves (Korhonen and Pihlanto, 2006). On the other hand, NO is a free oxygen radical ($NO^{\cdot}$) and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Alderton *et al.*, 2001).

1.2.8.2 Biosynthesis of NO

Nitric oxide is a pancreatine mediator that is released by endothelial cells and by certain neurons (Sharma *et al.*, 2007). The formation of NO is synthesized form L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzyme. Active NOS is a tetramer formed by two NOS proteins and two calmodulin molecules. Conversion of L-arginine to NO and L-citrulline requires also NADPH (nicotinamide adenine dinucleotide phosphate with extra hydrogen) and Oxygen as necessary co-factors. FAD (flavin adenine di-nucleotide), (6R)-tetrahydrobiopterin (H_4B), FMN (flavin mono-nucleotide), and iron protoporphyrin IX (heam) are also co-factors (Korhonen and Pihlanto, 2006). General mechanism of NO formation by NOS was illustrated in Figure 5.

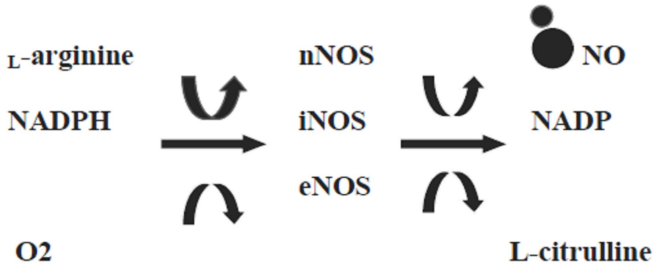


Figure 5 General mechanism of NO formation by NOS

Source Sharma *et al* (2007)

In animal tissue, NO is generated enzymatically by systhases (NOS). Three different isoform of NOS have been characterized (Guzik *et al.*, 2003).

1. NOS I or nNOS is the neuron form. nNOS is bound to plasma membranes and known to be strongly activated by the entry of calcium through membrane-bound receptors (Novo and Parola, 2008).

2. NOS II or iNOS is inducible nitric oxide synthase. Inducible NO synthase (iNOS), which was first identified in macrophages and then in other cells, including hepatocytes, is known to be up-regulated by pro-inflammatory cytokines and/or lipopolysaccharide (LPS), and is able to generate low levels of NO compared with the other NOS isoforms (Novo and Parola, 2008).

3. NOS III or eNOS is a constitutive enzyme primarily discover in endothelium.

All three isoforms have a similar molecular structure and require multiple cofactors.

1.2.8.3 Mechanism of action of NO

NO is a reaction molecule that has a variety of effect depending on the relative concentrations of NO and the surrounding environment in which NO is produced. There are both direct effects of NO that are mediated by the NO molecules itself, and direct effect of NO that are mediated by reactive oxygen species produced by the interaction of NO with superoxide anion or with oxygen (Korhonen and Pihlanto, 2006).

The molecular mechanism that mediate the biological activities of NO can be divide into three categories (Korhonen and Pihlanto, 2006).

1. Nitric oxide reacts readily with transitions metals, such as iron, copper and zinc. These metals are abundantly present in prosthetic groups of enzymes and other proteins, and by that mechanism, NO regulates the activity of various enzymes.

2. NO is able to induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation. Nitrosylation has been shown to modify the activity of several protein involved in cellular regulatory mechanism.

3. NO reacts very quickly with superoxide anion (O_2^-), resulting in the formation of peroxynitrite ($ONOO^-$). Peroxynitrite is a nitrating agent and a powerful oxidant that is able to modify protein, lipids and nucleic acids.

1.2.8.4 Nitric oxide and Inflammation

Inflammation is a defense mechanism initiated by invasion of pathogens or by tissue injury caused by biological, chemical, or physical damage. The activation of machophage is an important part of initiating defensive reactions, and macrophage-release inflammatory mediators such as nitric oxide, and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interluekine-6, -1β are released to enhance defense capacity (Ahn *et al.*, 2012)

Nitric oxide gives an anti-inflammatory effect under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations

(Sharma *et al.*, 2007). Overproduction of NO and proinflammatory cytokines is associated with many diseases, including rheumatoid arthritis, asthma, atherosclerosis, and endotoxin-induced multiple organ injury (Ahn *et al.*, 2012).

1.2.8.5 Nitric oxide inhibitory peptide

Several studies have reported that protein and its peptide from many source conferred anti-nitric oxide inhibitory activity. Most of nitric oxide inhibitory activity documents focus on the active compound plant only a few studies are considered peptide with nitric oxide inhibitory activity from animal protein. Among these documents, peptide from fish, milk, and clam were reported.

Ahn *et al.* (2012) studied the NO inhibitory activity of salmon by product protein hydrolysate (SPH) in RAW 264.7 macrophage cell line. Treatment RAW 264.7 macrophage cell line with LPS and pepsin derived SPH at various concentrations resulted in concentration-dependent inhibition of LPS-induced NO production. The maximum concentration is 400 μg/ml. The SPH contained 1000-2000 Da molecular weight peptides and amino acid such as Tyr, Phe, Pro, Ala, His, and Leu. In addition, Sung and co-workers examined the NO inhibitory activity effect of sweetfish-derived protein and its hydrolysate on LPS induced RAW 264.7 macrophage cell. The results showed that cell treated with trypsin hydrolysate or α-chymotrypsin hydrolysate could reduce NO production as compared to the cell treated with total protein or pepsin hydrolysate (Sung *et al.*, 2012). The hydrolysate from short-necked clam, *Ruditapes philippinarum* is also tested for NO inhibitory activity by Lee and his colleagues. The hydrolysate (Alcalase derived hydrolysate) exhibited the highest reduction of NO production at the concentration 1.0 mg/ml. The sequence of NO-inhibitory peptide obtained was composed of 10 amino acid residues, Gln-Cys-Gln-Gln-Ala-Val-Gln-Ser-Ala-Val at N-terminal position (Lee *et al.*, 2012). Another peptide from animal that exhibited the NO inhibitory activity is yak milk casein. The yak casein hydrolyzed by Alcalase possessed concentration-dependent inhibition of LPS-induced NO production at the maximum 2mg/ml (Mao *et al.*, 2011).

The NO inhibitory derived animal peptide is depending on their amino acid composition, sequence, concentration. However, the peptide contained hydrophobic amino acid residues such as Tyr, Phe, Ala, Leu, Ser, and Val possessed the NO inhibitory activity. Therefore, it could be concluded that hydrophobic amino acids attributed to nitric oxide inhibitory activity. Since hydrophobic hydrophobic amino acids could penetrate through cell membrane and consequently inhibit the inducible nitric oxide synthase (iNOS) expression. Additionally, no documents reported that animal derived protein hydrolysate have cytotoxic on cell lines.

Objective

- To identify an optimum condition for ultrasonic application in material pretreatment and incorporation during enzymatic hydrolysis.
- To investigate bioactive peptides with antioxidative activity *in vitro* and in RAW264.7 macrophage cell lines as well as cytotoxicity.
- To investigate bioactive peptides with anti-inflammatory activity in RAW264.7 macrophage cell lines.
- To identify and characterize bioactive peptides with antioxidative activity and NO inhibitory activity.

Chapter 2

Effect of enzymatic hydrolysis combined with ultrasonic treatment on antioxidative activity of tilapia (*Oreochromis niloticus*) protein hydrolysate

2.1 Abstract

Tilapia muscle homogenate was hydrolyzed for 120 min by using papain, Flavourzyme, or Alcalase. The effects of simultaneous ultrasound at 10, 40, or 70w on proteolytic hydrolysis and bioactivities of the hydrolysates were investigated. The ultrasonic treatments caused an improved degree of hydrolysis by Alcalase. The treatments increased the viscosity of all homogenates during digestion and yielded different microscopy of non-digested fish muscle particle. Effect of ultrasound on reactivity of hydrolysate against DPPH and ABTS was varied according to the enzymes used. The combination of ultrasonic and papain hydrolysate showed an improvement in DPPH and ABTS scavenging activity, reducing power and metal chelating at least to some extent. The hydrolysates exhibited promising antioxidative activity according to the *in vivo* study also displayed antioxidative activity in Raw 264.7 macrophage cell lines. Catalysis of papain with ultrasonic at 40W yielded with an increase antioxidative hydrolysate based on RAW 264.7 cell line study if the hydrolysis was extending from 60 to 120 min. Ultrasonic treatment could thus be used for preparation of antioxidant peptides if the hydrolysis time was over 60 min and can be incorporated into the enzymatic process to increase the bioactive activities of tilapia peptide.

2.2 Introduction

The preparation of protein hydrolysate by enzymatic hydrolysis is an accepted and safe process providing protein with improved functional, nutritive and bioactive properties (Liceaga-Gesualdo and Li-Chan, 1999). Several techniques can be applied to alter the structural characteristics of protein in order to increase its accessibility to enzymes and to obtain the most potent bioactive peptides. Recently, several publications (Chen *et al.*, 2011; Gülseren *et al.*, 2007; Hou *et al.*, 2011; Zhu *et al.*, 2011) described the use of ultrasound treatment for either pretreatment or assisted enzymatic hydrolysis. The beneficial use of ultrasound is due to the chemical, mechanical, and physical effects of acoustic cavitations (Kuldiloke, 2002) . This treatment can accelerate chemical reactions in heterogeneous systems as results of an increased contact area between the phases. In addition, ultrasound treatment during enzymatic hydrolysis might promote process efficiency via the modification of the protein structure (Jambrak *et al.*, 2008). The ultrasonic treatment during proteolysis enhanced the enzymatic hydrolysis of leather waste by increasing the digestion yield by fleshing and shaving (Jian *et al.*, 2008). This approach also enhanced the Alcalase hydrolysis of defatted wheat germ protein, whereas ultrasonic pretreatment improved the release of ACE-inhibitory peptides during the enzymatic hydrolysis (Jia *et al.*, 2010). In contrast, ultrasonic-assisted treatment inhibited Alcalase to some extent during the hydrolysis of soy sauce lees slurry (Yang *et al.*, 2011). In contrast to these results, ultrasound at 20 and 60 kHz applied at various intervals showed no significant effect on the digestibility of ovotransferrin by thermolysin (Lei *et al.*, 2011). These discrepancies point out the need of research to identify the effects of key factors and their interaction with the enzymatic process.

Highly nutritive and functional protein hydrolysates have been successfully produced from tilapia (Abdul-Hamid *et al.*, 2002). Moreover, the antioxidative activity of these tilapia protein hydrolysates has been reported (Dekkers *et al.*, 2011; Raghavan and Kristinsson, 2008; Raghavan *et al.*, 2008). To the best of our knowledge, no study on the application of ultrasonic treatment for enhancing the hydrolysis of tilapia protein for the production of bioactive tilapia hydrolysates has been carried out to date.

The overall goal of this study was to determine the effects of continuous ultrasonic treatment on enzymatic hydrolysis of tilapia muscle protein by papain, Flavourzyme, and Alcalase. The influence of ultrasonic energy on the hydrolytic process and the antioxidative activity of the hydrolysates were also investigated.

2.3 Materials and methods

2.3.1 Material and chemicals

Nile tilapia (*Oreochromis niloticus*) (700-900 g/fish) was purchased from a local market in Hat-Yai, Songkhla province, Thailand and transported to the Faculty of Agro-Industry on ice.

Alcalase (protease from *Bacillus licheniformis*), Flavourzyme (protease from *Aspergillus oryzae*), and papain (from papaya (*Carica papaya*) latex) were purchased from Sigma Chemical Co.(St. Louis, MO, USA).

Testing chemicals, including 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTs, $FeCl_2$, 3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4, 4'-disulfonic acid sodium salt (Ferrozine), the fluorescence probe 2′, 7′-dichlofluorescin diacetate (DCFH-DA), MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromind), Lipopolysaccharide (LPS) were purchased from Sigma Chemical Co.(St. Louis, MO, USA).

Mouse macrophage, RAW 264.7, cell lines were a gift from Dr.Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences Prince of Songkla University, Songkhla, Thailand. Roswell Park Memorial Institute medium (RPMI), penicillin/streptomytocin, and the other materials required for culturing of cell were purchased from Gibco BRL, Lift Technology (Thailand).

2.3.2 Sample preparation

Tilapia muscle was obtained by manual filleting. Fine fish muscle particles were obtained by using a grinder. Tilapia muscle was mixed with distilled water at a

ratio of 1:3 and homogenized at 11,000 rpm for 1 min using Polytron, PT2100 (Switzerland). The homogenate, for each enzyme applied, was adjusted to a suitable pH with NaOH and pre-heated to an appropriate temperature (pH 8, 55 °C for Alcalase and pH 7, 55 °C for papain and Flavourzyme). The enzyme ratios of 0.2 unit/g proteins for Alcalase and 20 unit/g protein for papain and Flavourzyme were used for enzymatic hydrolysis.

2.3.3 Ultrasound treatment

After the addition of enzyme the mixture of fish homogenate and enzyme was immediately exposed to ultrasound at different power outputs (10W, 40W, and 70W) for 120 min (pulse duration of on-time 10 s and off-time 20 s). This was performed by the immersion of the tip of the stainless steel probe of an ultrasonic processor (Sonic, VCX 750, USA) into the centre position of a 1000 ml beaker. The temperature of the hydrolysate was risen and maintained at about 55±1 °C by the ultrasonic protocol. The hydrolysate was withdrawn after 0, 30, 60, 90, and 120 min of sonication. The enzyme activity was terminated by heating to 90 °C for 10 min. The insoluble residues were separated by centrifugation at 15,000xg for 20 min at 4 °C (Beckman Coulter, Avanti J-E, California, USA). The supernatant was used for analysis. The control process was carried out using identical conditions except the sonication which was replaced by continuous stirring at 200 rpm with an overhead stirrer.

2.3.4 Degree of hydrolysis

The degree of protein hydrolysis (DH) was based on the availability of the free amino group upon hydrolysis according to modifications of the methods described by (Dambmann *et al.*, 1999; Wanasundara *et al.*, 2002). The *o*-phthaldialdehyde (OPA) reagent was prepared by dissolving 6 mM OPA and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate containing SDS 2%. Then 0.4 ml of the hydrolysate (at a suitable concentration) was added to 3 ml of OPA solution. The reaction mixture was incubated at room temperature for 20 min and its absorption at λ 340 nm was recorded by a spectrophotometer. The blank value was obtained by adding only the buffer. The OPA test was validated using a serine standard solution

(10% w/v). The amounts of free amino groups were calculated on basis of the serine standard.

The acid hydrolysis of tilapia protein was carried out in 6 M HCl for 24 h at 110 °C for the determination of total free amino groups. The degree of hydrolysis (DH%) was calculated as the ratio of enzymatically released free amino groups related to total free amino groups released by complete acid hydrolysis:

$$\% DH = [(NH_2)T_X - (NH_2)T_0 \ / \ (NH_2)_{Total} - (NH_2)T_0] \times 100$$

Where: $(NH_2)T_0$ = Number of free $-NH_2$ group at 0 min of hydrolysis

$(NH_2)T_X$ = Number of free $-NH_2$ group at x min of hydrolysis

$(NH_2)_{Total}$ = Number of free $-NH_2$ group (completed hydrolysis of protein is assumed)

2.3.5 Viscosity of tilapia protein hydrolysate

The viscosity of hydrolysate was measured using a low viscosity viscometer, Model DV-II+ (Brookfield, Stoughton, MA, USA). Samples were placed in a 600 ml beaker and viscosity was measured using spindle number RV/HA/HB-1 (diameter 56.26 mm) to take torque measurements at 120 rpm. One measurement was done per sample. The apparent viscosity (Centipoise; cP) was recorded.

2.3.6 Scanning electron microscopy (SEM)

The hydrolysate samples were centrifuged at 15,000xg for 20 min at 4^{o}C. The sediments were collected and lyophilized (FTS Systems DuraDry). The flasks containing the thin layer of dried hydrolysate were mounted on holders with silver cement and coated twice with AuPd. The SEM was conducted using a SEM JOEL JSM-5800 with an accelerating voltage of 15 kV and a working distance of 15 mm. The micrographs were taken at a magnification of x 5,000.

2.3.7 Antioxidative activities of tilapia protein hydrolysate

2.3.7.1 DPPH radical scavenging activity

One milliliter of the hydrolysates or trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was mixed with 1 ml of 0.2 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in a test tube. The reaction mixtures were incubated for 30 min in the dark room before the measurement of the absorption at 517 nm. Trolox (10-150 μM) was used as the standard for calibration, and the DPPH radical scavenging activity was expressed as μM trolox equivalents per milligram of protein (Orhan *et al.*, 2007).

2.3.7.2 ABTS radical scavenging activity

ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate)) was dissolved in distilled water to a concentration of 4 mM according to Re *et al*. (1999). The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting the ABTS stock solution with 2.5 mM potassium persulfate (at a ratio of 1:1) and allowing the mixture to stand in the dark at room temperature for 12-16 h prior to use. For the study of the hydrolysate, the $ABTS^{\bullet+}$ solution was diluted with deionized water (DI) to obtain an absorption of 0.8 ± 0.01 at 734 nm. The hydrolysates (150 μl) were added to $ABTS^{\bullet+}$ solution (2.85 ml). The reaction mixture was incubated in the dark for 2 h prior to absorbance measurement at 734 nm. The ABTS radical-scavenging activity was expressed as μM trolox equivalents per milligram of protein.

2.3.7.3 Metal chelating activity

One ml of sample solution (at a suitable concentration) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 10 min at room temperature. The control was prepared in the same manner except that distilled water was used instead of the sample. The absorption was measured at 562 nm and used to determine the Fe^{2+} chelating activity using EDTA as the reference

standard (Decker and Welch, 1990). The activity was expressed as µM EDTA per milligram of protein.

2.3.7.4 Reducing power assay

One milliliter of the hydrolysate was mixed with one milliliter of phosphate buffer pH 6.6 and one milliliter of potassium ferricyanide (1% w/v). The mixtures were incubated at 50 ^{o}C for 20 min followed by the addition of 10% trichloroacetic acid. Finally, 100 µl of the mixtures were mixed with a 100 µl of deionized water and 20 µl of 0.1% (w/v) ferric chloride. After 10 min of reaction, the absorbance of the resulting solutions was measured at 700 nm using a spectrophotometer. The absorbance values were compared with those of the standard reducing agent, ascorbic acid. High absorption represents high reducing power (Yıldırım *et al.*, 2001). The reducing power was expressed as milligrams of ascorbic acid per milligram of protein.

2.3.8 Cell culture and viability determination

Mouse macrophage (RAW 264.7) cells were cultured and maintained in RPMI medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum (FBS) and thermostated at 37 °C in a humidified atmosphere with 5% CO_2.

The cytotoxicity of the hydrolysates was measured using MTT methods (Hansen *et al.*, 1989). The cells were cultured in 96-well plates at a density of $1.5x10^5$ cell/well and allowed to grow for 24h at 37^{o}C under a humidified atmosphere with 5% CO_2. After growth the cells were washed with PBS (Phosphate Buffered Saline) and treated with different concentrations of the protein hydrolysate (100 µl). After 24 h of incubation, the cells were rewashed and 20 µl of MTT (5mg/ml) were added. After 4 h of incubation, the mixture was removed and 100 µl of isopropanol containing 0.04 M HCl was added for dissolving the formazan crystal. The amount of formazan salts was determined by measuring the absorption at 540 nm using a microplate reader (BioTek, Power XS, USA).

2.3.9 Determination of intracellular oxidation using DCFH-DA labeling

The formation of reactive oxygen species (ROS) was evaluated using the oxidation-sensitive dye DCFH (Engelmann *et al.*, 2005). The cell line RAW 264.7 was cultured in a 96-well black plate with $1.5x10^5$ cells/well and allowed to grow for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The cultured cells were treated with 20 µM DCFH-DA in PBS (100 µl) and incubated in the dark for 20 min. The cells were then treated with the protein hydrolysate at various concentrations and incubated for 1 h. After washing the cells with PBS, 100 µl of H_2O_2 in PBS (500µM) were added. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of various ROS was measured at 0 and 30 min using the fluorescence (F) at λex = 485 nm and λ_{em} = 535 nm (SpectraMax M5, Multi-detection Reader, USA). The antioxidative activity was calculated as follows as a function of the emitted fluorescence (% increase in fluorescence; % IF), shown in the following equation:

$$\%IF = [(F_{30min} - F_{0min})/ F_{0min}] \times 100$$

2.4 Results and discussion

2.4.1 Degree of hydrolysis (DH)

Figure 1 shows the results of the hydrolysis experiments obtained under experimental conditions, resulting in a gradual increase in DH as a function of reaction time regardless of the enzyme types and application of ultrasound. The curves show that the highest DH was obtained by using Flavourzyme followed by Alcalase and papain. It is worth noting that the concentration of each enzyme was different with the aim to obtain similar ranges of DH ranges. Therefore, a direct comparison on catalytic activity of each enzyme against tilapia muscle protein cannot be done by this experiment. However, a better proteolytic activity of Alcalase toward tilapia proteins was reported by Foh *et al.*(2010). In addition, our preliminary experiment with tilapia homogenate revealed the highest activity of Alcalase relative to the other two enzymes.

When ultrasound was applied, a slight increase in the DH was noticed only in Alcalase-hydrolysed tilapia especially during the initial digestion phase. These observations differed from the hypothesis that the application of ultrasound would yield a higher DH, due to a better accessible peptide bond of the treated protein. These unexpected results might originate from the low ultrasound power intensity compared to those applied in earlier experiments for the hydrolysis of plant proteins (150-600 W) which showed a significant increase in the DH at increasing ultrasound power (Chen *et al.*, 2011; Jin *et al.*, 2012). It should be pointed out that the detrimental effect of sonication on a DH reduction was seen in the last phase of the Flavourzyme digestion. This was probably associated with the fragility of the enzyme against the continuous sonication. A gradual reduction in enzyme activity throughout the sonication occurred only in the case of Flavourzyme which showed a 20% reduction after a treatment time of 120 min (data not showed).

According to Hamada (2000), Alcalase is composed mainly of the serine endopeptidase subtilisin Carlsberg, while Flavourzyme is a mixture of proteases of both exo- and endopeptidase activities. In addition, papain is an endoprotease (Vernet *et al.*, 1991). Thus the observation made in the Flavourzyme-hydrolyzed tilapia protein suggests the possibility that applied ultrasound reduces the availability of susceptible bonds especially for the combination of exo- and endopeptidase activities of Flavourzyme.

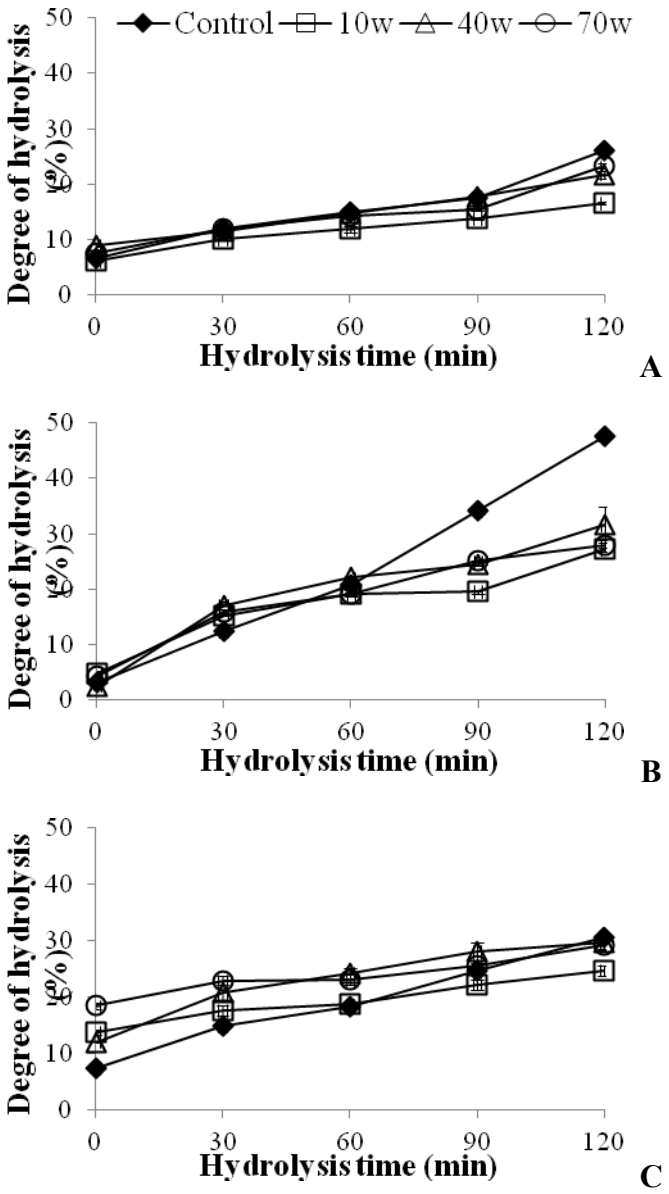


Figure 1 Degree of hydrolysis (DH) of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication. Value represent the mean ± standard deviation of n=3 duplicate assays. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

2.4.2 Viscosity of tilapia protein hydrolysate

Viscosity was used to demonstrate the physical change of the hydrolysate due to ultrasonic irradiation and the apparent viscosity of hydrolysates as a function of hydrolysis times, enzyme types, and ultrasound powers are shown in Figure 2. Steady and low viscosity value is observed in all hydrolysates prepared by a conventional stirring process. In general, hydrolysate with low viscosity is expected to be obtained by the prolonged hydrolysis due to the enzymatic break down of the fish muscle particles and reduction in size of soluble peptides. The observation made in the control samples suggested that these changes may not be critical enough to produce a noticeable change in the viscosity of the hydrolysates.

A considerable increase in viscosity was observed by the combining the sonication with the hydrolysis process. The highest viscosity was obtained in the process using Flavourzyme. This effect was not so pronounced when papain or Alcalase were tested. They showed a common trend of increasing viscosity during an initial hydrolysis phase where the increase of the magnitude of viscosity and the decrease of time for reaching the highest viscosity were correlated with an increased ultrasound power. Their profiles additionally revealed a viscosity reduction phase during extended hydrolysis. The observed profiles probably reflect the modifications that were induced in the fish muscle particles by the ultrasonic treatment. It is also suggested that the cavitation effect induced by the ultrasound waves, which involves the formation, growth, and collapse of microscopic bubbles (Soria and Villamiel, 2010) taking place in the very fine fish tissues.

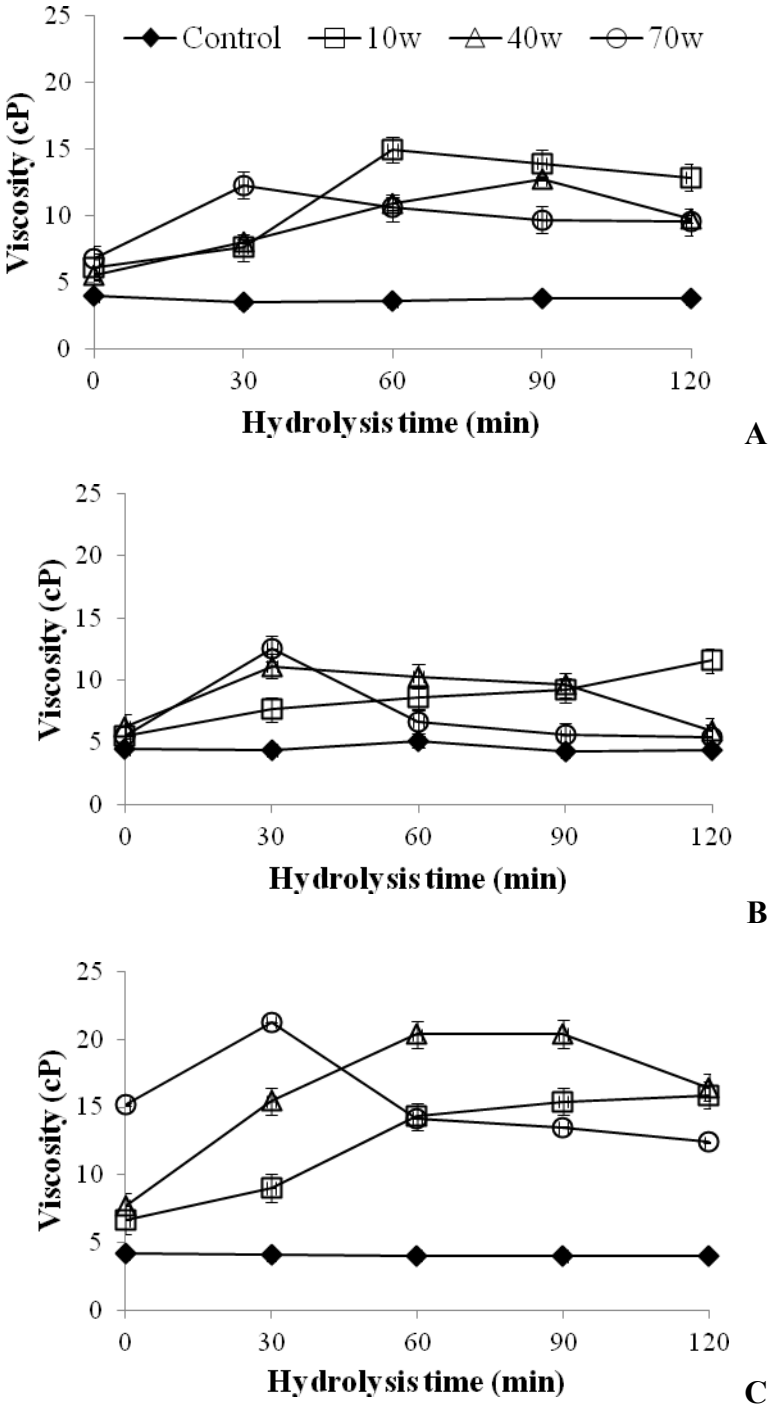


Figure 2 Viscosity of tilapia hydrolysate during enzymatic hydrolysis combined with simultaneous sonication. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

2.4.3 Morphological characteristics of tilapia muscle particles

The SEM photomicrographs of fish particles after a 60 min hydrolysis are shown in Figure 3. It can be seen in the micrographs that papain-digested fish particle residues after the control process had a smoother surface and exhibited a big hole. In addition, the Flavourzyme-digested fish particle residues showed a coarse irregular surface with some hollow openings while the surface of the Alcalase-digested fish particle residue appeared to be smooth and neat.

The process with simultaneous sonication further altered the microstructure of partly digested fish particles, forming holes which are distributed over the whole particle surface. There was distinguishable morphology between papain or Flavourzyme-digested and Alcalase-digested fish particle residues. The particle residues of the first two treatments showed big holes when the ultrasound intensity was set to 10 W. The non digested residues became less porous with a smaller size of the hole if the ultrasound intensity was increased up to 70 W. These observations can be explained by a considerable pressure built up inside the material during the sonication process (Carcel *et al.*, 2011). In addition, a large cavitation bubble would be generated at low frequency ultrasound (Patist and Bates, 2008). The high pressure drastically changes the physical properties and destroys the cell structure thus improving the capillary-porous structure of the tissues (Mason *et al.*, 1996). In contrast, the Alcalase-digested fish particle residues exhibited a bubble-like structure on the surface at low ultrasound power and showed a mixture of big and small size holes on their surface at 70 W ultrasound power. The bubble-like structure may originate from fat globules formed by pressure generated inside the fish particle. The large hole is probably left behind after the fat globule is expelled from the particle surface.

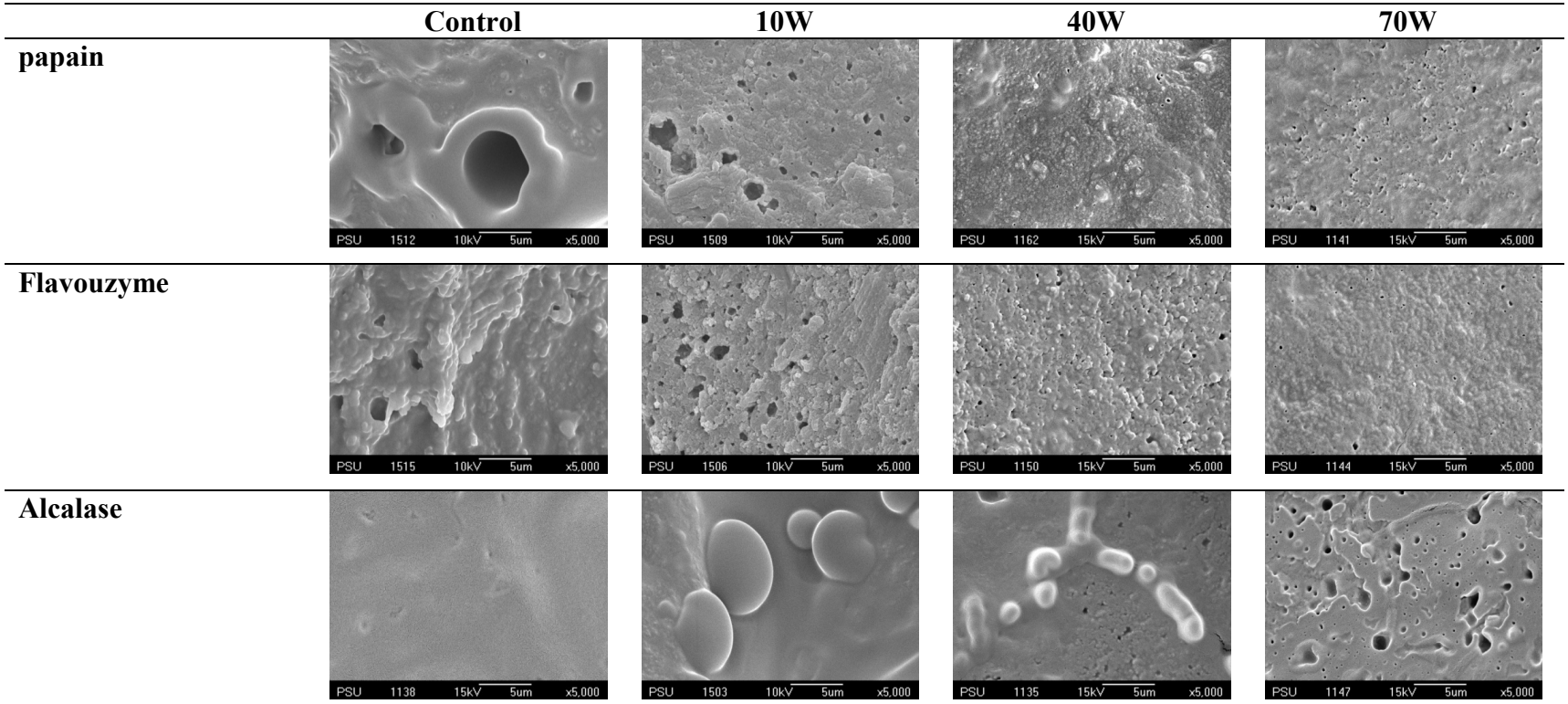


Figure 3 Scanning electron micrographs of non digested fish particle residues after 60 min of papain (A), Flavourzyme (B), and Alcalase (C) hydrolysis combined with simultaneous sonication. The micrographs were taken at magnification of x5,000, accelerating voltage of 15 kV and a working distance of 15 mm.

2.4.4 Antioxidative activity of tilapia protein hydrolysate

2.4.4.1 DPPH radical scavenging activity

The antioxidant activity of the hydrolysates as measured by DPPH scavenging activity is shown in Figure 4. By using the control process (stirring without ultrasonic treatment), a reduction of the activity with the extension of hydrolysis time was observed in papain, Flavourzyme and Alcalase hydrolysates. The results suggest that most of the released peptides are less active than their parent proteins. As the peptide size decreases, more hydrophilic amino acid are likely to be exposed reducing the reactivity with DPPH. Peptide structure included the followed amino acid were claimed to react with DPPH radical such as Ala–Cys–Phe–Leu (Sampath Kumar *et al.*, 2011), peptide structures from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins were identified as Leu-His-Tyr, Leu-Ala-Arg-Leu, Gly-Gly-Glu, Gly-Ala-His, Gly-Ala-Trp-Ala, Pro-His-Tyr-Leu and Gly-Ala-Leu-Ala-Ala-His, respectively (Bougatef *et al.*, 2010).

Raghavan and co-workers (2008) however reported an increased $DPPH^{\bullet}$ inhibition of Flavourzyme treated tilapia hydrolysate with an increased DH or hydrolysis time. This difference may originate from their reported procedure. Their percentage of $DPPH^{\bullet}$ inhibition did not take into account the soluble protein content which increased with hydrolysis time.

Incorporation of sonication into the enzymatic hydrolysis obviously improved DPPH scavenging activity with the highest being the Flavourzyme hydrolysate. The impact of ultrasound clearly depended on enzyme type, hydrolysis time and power. For instance, a significant effect of sonication on enhancing the free radical scavenging activity was only evident in the papain and Flavourzyme hydrolysates. Moreover, papain and Flavourzyme needed a different ultrasound intensity to obtain the optimal antioxidant activity. This may due to sonication at a certain intensity facilitating the exposure of hydrophobic side chains. This would make it more accessible to the reaction with the lipid-soluble DPPH radicals. In addition, the hydrogen-donating capability of peptides in Flavourzyme and papain hydrolysates

could be increased by exposing certain amino acids.

2.4.4.2 ABTS radical scavenging activity

The scavenging activity of tilapia hydrolysates against the aqueous $ABTS^{\bullet+}$ is shown in Figure 5. By using the control process without sonication the highest reactivity was seen in the Flavourzyme hydrolysate although the activity was gradually reduced by prolonging the hydrolysis time. This suggests that most released peptides were low electron-donating peptides. For papain and Alcalase hydrolysates the hydrolysis time did not influence the antioxidant activity significantly. The use of sonication showed both positive and negative effects on the reactivity of the hydrolysates against the free radical. An increase in the activity was seen in the Alcalase hydrolysate where no marked effect of different ultrasound power intensities was noted. In contrast, a critical reduction of the activity was exhibited in Flavourzyme hydrolysate if 40 W and 70 W ultrasound were applied. The incorporation of sonication into the papain hydrolysis showed a promising effect only if the 40W ultrasound was used.

The ABTS and DPPH -scavenging assay rely on a single electron and hydrogen transfer to inactivate each of the radicals (Antolovich *et al.*, 2002). The marked influence on the scavenging activity of the sonicated hydrolysates toward ABTS (Figure 5) and DPPH (Figure 4) thus points to a contrary ultrasonic effect on the modification of the peptides if each active peptide in the hydrolysates is responsible for the two activities. For example, the improved H contribution of Flavourzyme hydrolysate by simultaneous sonication may lead to an increase in the steric hindrance of the electron donation of the hydrolysate.

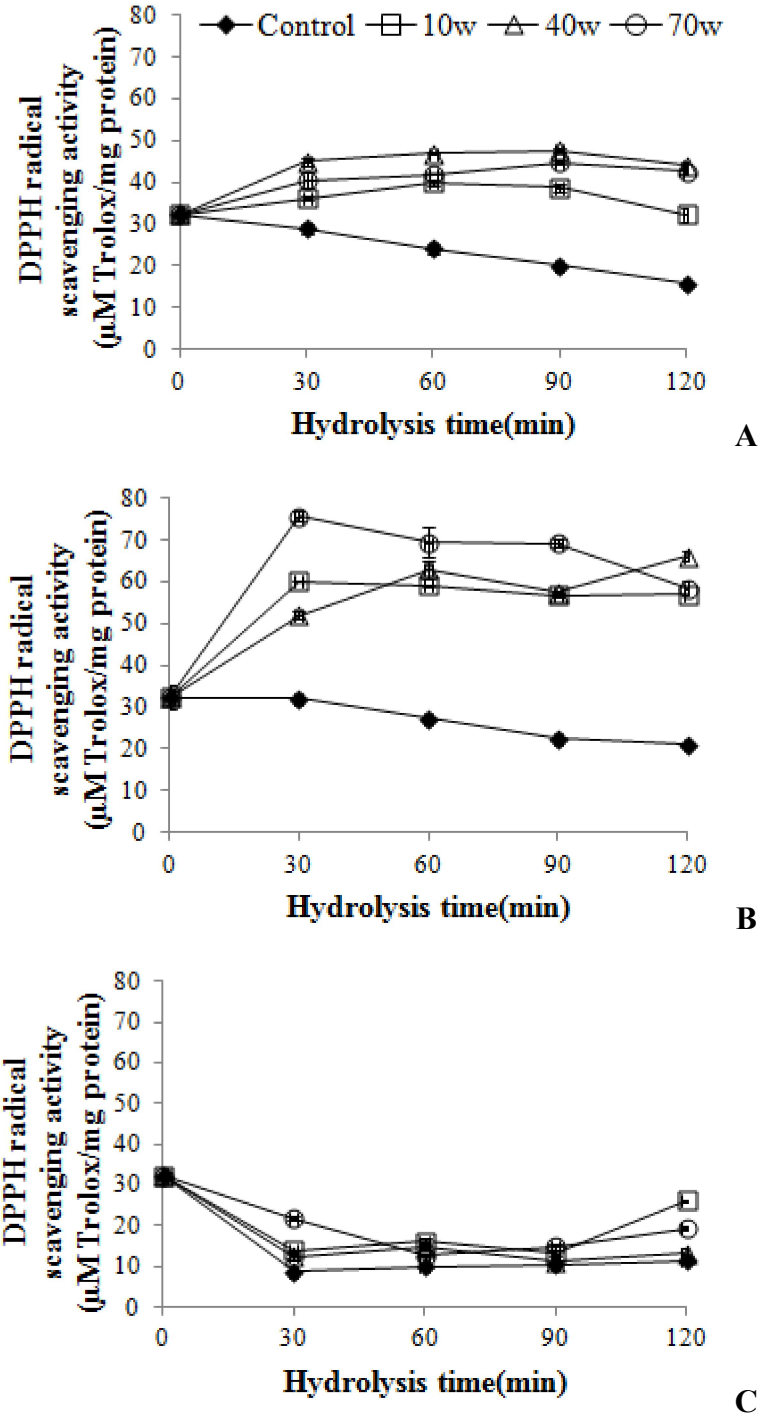


Figure 4 DPPH scavenging activity of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication. Values represented are the mean of triplicate analysis. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

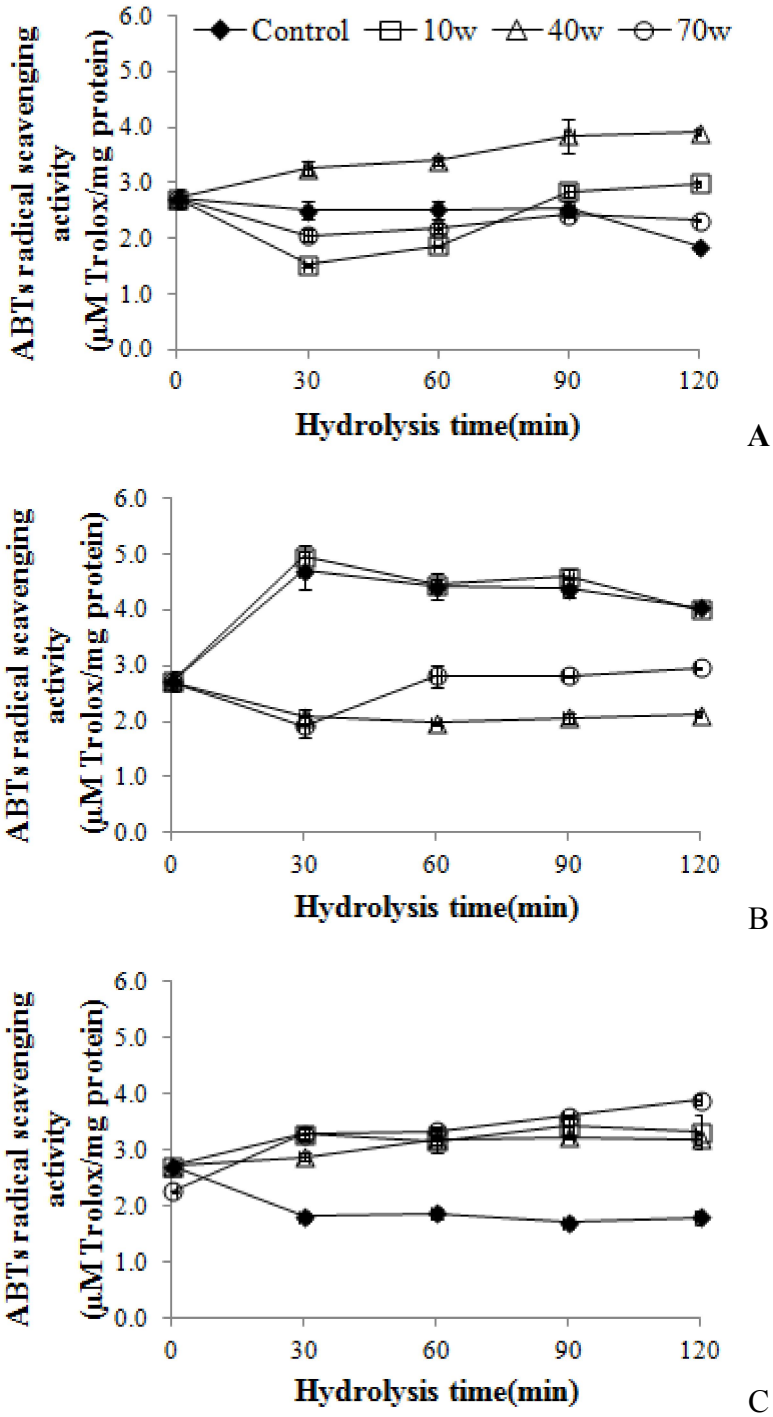


Figure 5 ABTS radical scavenging activity of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication. Values represented are the mean of triplicate analysis. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

2.4.4.3 Reducing power

The effects of enzymes and ultrasound intensities on reducing power are shown in Figure 6 a marked reduction in the reducing power of Flavourzyme and Alcalase hydrolysates upon hydrolysis in the control process was noted. Incorporation of sonication, regardless of intensity and times, caused a steady reduction of the activity of the Alcalase hydrolysate. In the Flavourzyme hydrolysate when sonication, at either 10 or 70 W was introduced, the hydrolysates had an improved reducing power although its efficacy varied according to the hydrolysis time. In contrast, an enhancement of the reducing power of the papain hydrolysate was observed only if 10 W-ultrasound was used. The results therefore showed a limited range of sonication for an effective gain of the activity. It was generally found that the reducing profiles correspond well with those of $ABTS^{\bullet+}$ scavenging activity (Figure 5) with the exception of Alcalase hydrolysate. The reducing power assay is often used to evaluate the electron-donating activity of an antioxidant. Thus it is possible that ultrasound treatment under specific conditions could reduce the steric hindrance for certain electron donating groups.

2.4.4.4 Metal chelating activity

As shown in Figure 7 the conventional enzymatic hydrolysis process decreased the ferrous ion chelating activity of the hydrolysates with a considerable drop noted in the Flavourzyme hydrolysate. The reduction is probably due to the release of active amino acid residues or peptide fragments from its active parent protein by the hydrolysis thus resulting in low active peptides. However by replacing the stirrer with sonication, an improved chelating activity of the hydrolysates was noticed even though its efficacy varied according to the ultrasound intensity and hydrolysis time. The greatest effect of the sonication was recorded in the Flavourzyme hydrolysate where ultrasound power at 10 W yielded the highest chelating activity, especially during the final digestion phase. Ultrasound treatment may weaken steric hindrance on the peptides released. This leads to increased concentration of the metal chelating sites, the carboxylic and amino groups, on the

surface of the molecules. This change may take place as a negative effect of sonication by decreasing the electron donating activity of the obtained hydrolysates as noted in the previous section.

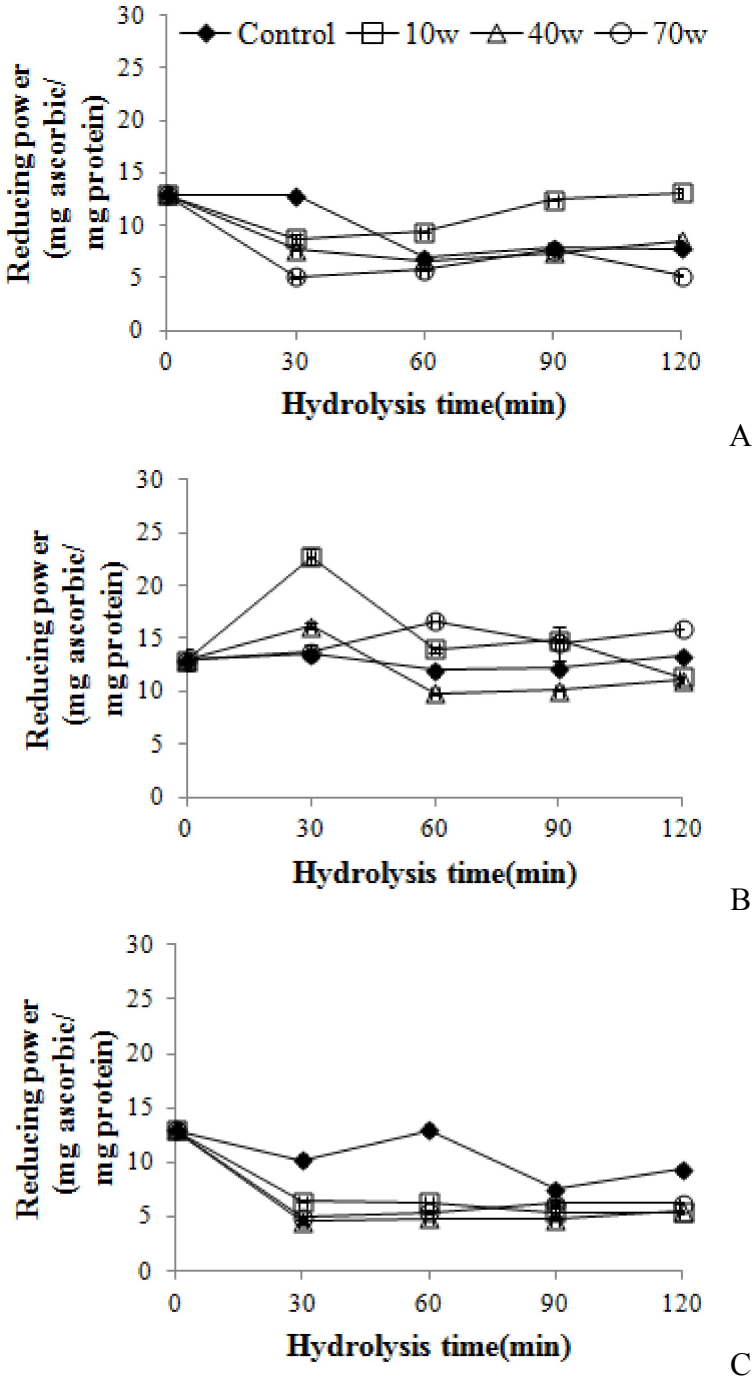


Figure 6 Reducing power activity of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication. Values represented are the mean of triplicate analysis. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

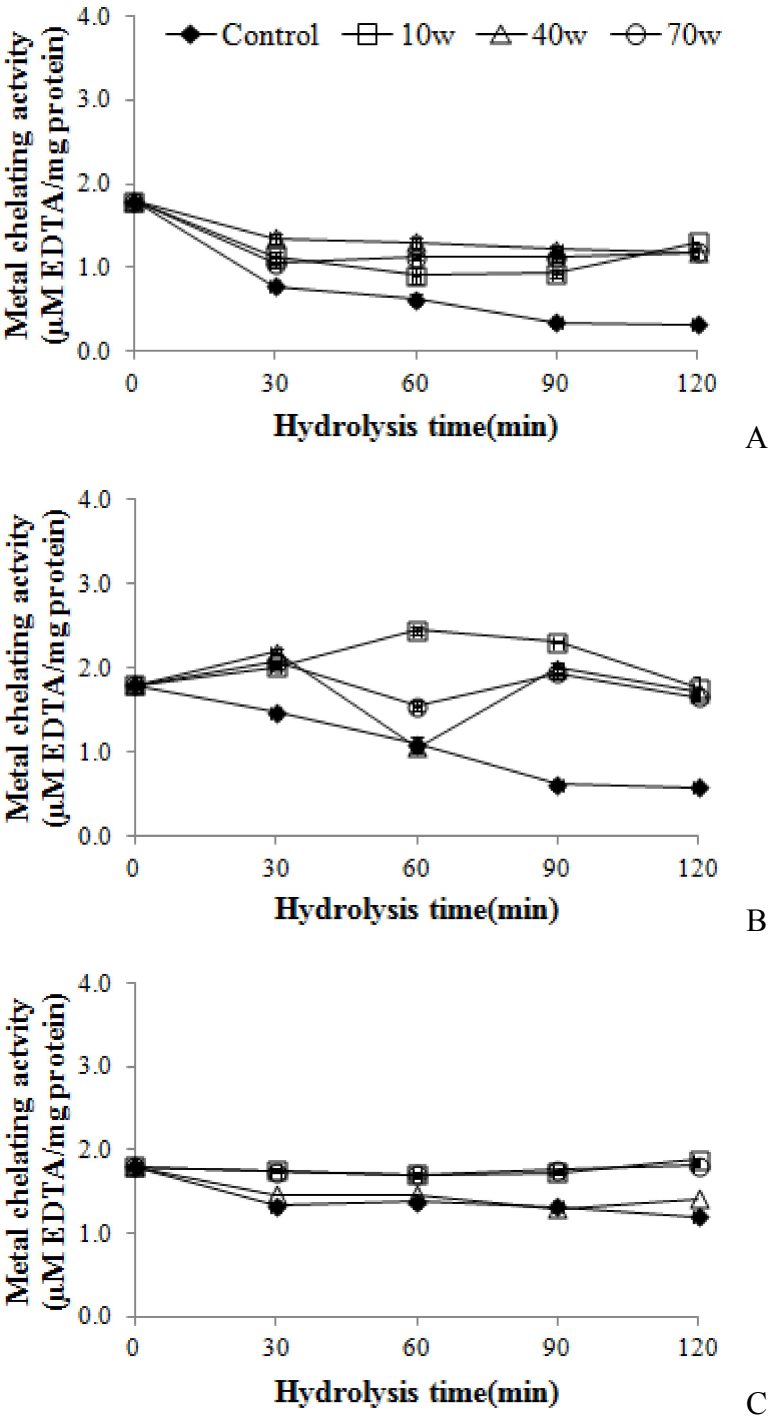


Figure 7 Metal chelating activity of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication. Values represented are the mean of triplicate analysis. A: papain hydrolysate, B: Flavouzyme hydrolysate, C: Alcalase hydrolysate.

2.4.5 Effect of tilapia hydrolysate on cell viability and ROS generation in the cell line RAW 264.7

The results from the *in vitro* analyses pointed to the strong antioxidative activity of hydrolysates obtained by using papain with sonication at 40W and Flavourzyme with sonication at 10W. Only these two hydrolysates were thus used for investigation of their inhibitory effect on the generation of ROS in the macrophage cell line RAW 264.7. It was found that both hydrolysates exerted no cytotoxic effect as measured by the MTT assay at a concentration range of 0.5-20 mg protein/ml. In these experiments the cell viability was higher than 80% (Figure 8). In the following experiments, testing for the intracellular oxidative inhibition the concentration of the hydrolysates was 20 mg protein/ml.

It was, however, an unexpected result to see that a significant reduction of the generation of ROS was noticed only in the cell system to which the hydrolysate obtained with papain was added (Figure 8). And more research is needed to explain in detail the differences observed in the *in vitro* and *ex vivo* cell line studies. Its inhibitory activity increased significantly if the hydrolysis process was extended from 60 min to 120 min. Ultrasound especially at higher energy correlated with an increased radical scavenging activity of the hydrolysate. At higher ultrasound power the accessibility of some peptide bonds may be increased for endo-peptidases resulting in the release of more active peptide.

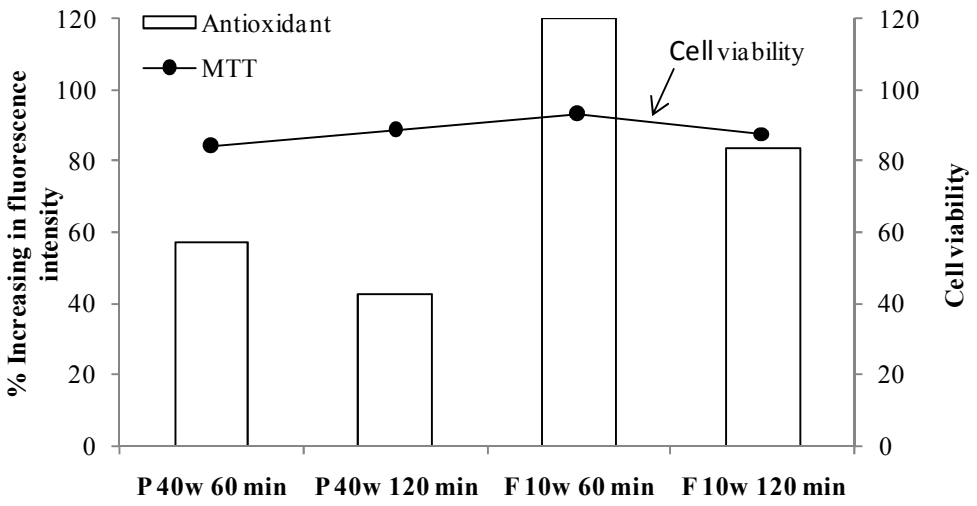


Figure 8 Antioxidative activity and cell viability in RAW 264.7 of tilapia hydrolysates after enzymatic hydrolysis combined with simultaneous sonication.

2.5 Conclusions

The results from the investigation of the hydrolysate viscosity profiles and microscopy of fish particle residues suggested a modification of fish particles during the enzymatic process with simultaneous ultrasonic treatments. Consequent effects of the ultrasonic treatment on the efficiency of the enzymatic hydrolysis of the three enzymes as measured by the degree of hydrolysis cannot be predicted. The enzyme type, ultrasonic intensity, and process time have an influence on the antioxidative activities of the hydrolysates obtained. The explicit effect of sonication was highlighted either from its intensification or reduction of the hydrolysates reactivity against DPPH and ABTS. In general all the antioxidative activities of papain hydrolysates were improved, at least to some extent, by combination with the sonication processes. Hydrolysates from tilapia muscle protein having a potent intracellular radical scavenging activity hydrolysate could be prepared using papain at an ultrasound treatment at 40 W for 120 min. Application of continuous ultrasound treatment with the papain hydrolysis is appropriate if the longer process time is needed.

Chapter 3

Antioxidant and nitric oxide inhibition activities of tilapia (*Oreochromis niloticus*) protein hydrolysate: effect of ultrasonic pretreatment and ultrasonic-assisted enzymatic hydrolysis

3.1 Abstract

Ultrasound was incorporated to processing of fish protein hydrolysate to facilitate homogenate pretreatmentand enzymatic hydrolysis of tilapia (*Oreochromis niloticus*) muscle protein. Their effects on Flavourzyme hydrolysis and biological activities of the tilapia hydrolysate were examined. The ultrasound-assisted hydrolysis caused reduction in degree of hydrolysis ranging from 23% to 35% relative to that of the conventional process. The 70 W ultrasound-assisted hydrolysis process increased DPPH radical-scavenging activity and reducing power of tilapia hydrolysate prepared from the non-pretreatment homogenate by 33% and 45%, respectively. All hydrolysates have no cytotoxicity on RAW264.7 cell lines at the maximum concentration of 20 mg protein mL^{-1}. The 70 W ultrasound pretreatment at 30 and 45 min combined with conventional hydrolysis is the suitable condition for producing tilapia hydrolysate with nitric oxide inhibitory and antioxidative activities on RAW264.7 cell lines, respectively. As a result, ultrasound could be applied to enzymatic protein hydrolysis either as pretreatment or during the hydrolysis.

3.2 Introduction

Reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. ROS are normally generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion (Ray *et al.*, 2012). Its occurrence is strongly linked with chronic diseases such as cancer, coronary heart disease, and Alzheimer's disease (Ahn *et al.*, 2012).

Nitric oxide (NO) is produced from L-arginine by a chemical reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Cirino *et al.*, 2006). The endothelial NOS (eNOS), neurons NOS (nNOS), and inducible NOS (iNOS) are three different NOS isoforms. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amounts of NO (Darley-Usmar *et al.*, 1995). Low concentrations of nitric oxide produced by the constitutive and nNOS inhibit adhesion molecule expression, cytokine, and chemokine synthesis as well as leukocyte adhesion and transmigration. Large amounts of NO, generated primary by iNOS can be toxic and pro-inflammatory. The interactions between ROS and NO generate potentially cytotoxic agents which may mediate some of the pathology associated with Parkinson's disease, chronic inflammation, atherosclerosis and cancer (Darley-Usmar *et al.*, 1995).

Bioactive peptides refer to specific protein fragments that have a positive impact on body function or condition. They ultimately may influence health beyond their basic role as nutrient sources. The bioactive peptide could be prepared by enzymatic hydrolysis, an accepted and safe process providing a product with improved functional, nutritive, and bioactive properties. Many documents claim that the peptides from fish protein hydrolysate have biological activities. For instance, antioxidative activity (Je *et al.*, 2008) and anti-inflammatory activity (Sung *et al.*, 2012).

Ultrasound technology is based on mechanical waves at a frequency above the threshold of human hearing (>16 kHz) (Soria and Villamiel, 2010). Ultrasound has been used for a variety of purposes in food technology such as improved functional properties of protein (Jambrak *et al.*, 2008), enhanced the biological properties of protein (Yu *et al.*, 2012) and improved yield extraction (Hromádková *et al.*, 2008). Furthermore, ultrasound treatment during enzymatic hydrolysis might promote process efficiency via the modification of the protein structure (Jambrak *et al.*, 2008). The beneficial use is due to the chemical, mechanical, and physical effects of acoustic cavitations.

Nile tilapia (*Oreochromis niloticus*) is a widely cultivated and consumed species among freshwater fish reared in Thailand. Its flesh has high protein content (17-20 %) and contains other valuable compounds which could be beneficial for human consumption. There are some reports available in the literatures that describe bioactive peptides from tilapia (Charoenphun *et al.*, 2012; Foh *et al.*, 2010). To date, no work on ultrasound pretreatment or ultrasound assisted hydrolysis of fish protein particularly tilapia protein has been published.

The objective of this study was to investigate the effect of ultrasonic pretreatment and ultrasonic-assisted hydrolysis on an enzymatic hydrolysis process of tilapia protein and antioxidative activity and anti-inflammatory activity of the hydrolysate. These bioactive activities would be tested, both *in vitro* and in RAW264.7 cell lines.

3.3 Materials and methods

3.3.1 Materials and chemicals

Nile tilapia (*Oreochromis niloticus*) (700-900 g per fish) was purchased from a local market in Hat-Yai, Songkhla province, Thailand.

Flavourzyme (protease from *Aspergillus oryzae*) and testing chemicals including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTs, $FeCl_2$, 3-(2-pyridyl)-5, 6-diphenyl-1,2,4-triazine-4,40-disulfonic acid sodium salt (ferrozine), the fluorescence probe

20,70-dichlorofluorescein diacetate (DCFH-DA), MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) and LPS were purchased from Sigma Chemical Co.(St. Louis, MO, USA).

The mouse macrophage cell lines, RAW 264.7, were obtained from Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences Prince of Songkla University, Songkhla, Thailand. Roswell Park Memorial Institute (RPMI) medium, penicillin/streptomycin and the other materials required for culturing of the cells were purchased from Gibco BRL, Lift Technology (Thailand).

3.3.2 Sample preparation and ultrasound pretreatment

Tilapia flesh obtained by manual filleting was ground into fine particles using a grinder. The fish mince was further homogenised by mixing with three volumes of distilled water at 11,000 r.p.m. for 1 min using Polytron, PT2100 (Lucerne, Switzerland). The ultrasonic pretreatment of the homogenate was performed using an ultrasonic processor (Sonic, VCX 750, USA) at power outputs of 10 W or 70 W. The duration of the ultrasonic pretreatment was 15, 30 or 45 min with pulse duration of on-time 10 s and off-time 20 s. For a control, sample preparation involving only homogenization without ultrasonic pretreatment was performed. During this pretreatment protocol, the homogenate temperature was increased to 55 ± 1 °C.

3.3.3 Enzymatic hydrolysis

All tilapia homogenates with and without ultrasonic pretreatment were used as raw material for enzymatic hydrolysis. The pH of the homogenates was adjusted to 7.0 and heated to 55 °C. The enzymatic hydrolysis was initiated by addition of a Flavourzyme solution to obtain a ratio of 20 unit g^{-1} protein. For the conventional hydrolysis – without sonication – an overhead stirrer was used at 200 r.p.m. In the ultrasonic-assisted process, ultrasound was applied continuously either at a power output of 10 or at 70 W. The ultrasound pulse duration was on-time 10 s and off-time 20 s. During this protocol, the homogenate temperature was controlled at 55 ± 1 °C.

After 60 min, the hydrolysis was terminated by increasing the temperature of

the reaction mixture to 90 °C for 10 min. The insoluble residue was separated by centrifugation at 15,000g for 20 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Avanti J-E, CA, USA). The supernatant was used for further analyses. For the control experiment, conventional hydrolysis was carried out.

3.3.4 Degree of hydrolysis

The degree of protein hydrolysis (DH) was calculated on the basis of the availability of the free amino groups that appear during hydrolysis with a modified method described earlier (Dambmann *et al.*, 1999; Wanasundara *et al.*, 2002). The o-phthaldialdehyde (OPA) reagent was prepared by dissolving 6 mM OPA and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate containing 2% (w/v) SDS. Then, 0.4 mL of hydrolysate (at a suitable concentration) was added to 3 mL of the OPA solution. The reaction mixture was measured after incubation at room temperature for 20 min at 340 nm using a spectrophotometer. The amounts of free amino groups were calculated on the basis of L-serine-NH_2 group which was used as a reference. The acid hydrolysis of tilapia hydrolysate in 6 M HCl for 24 h at 110 °C was carried out for the determination of total free amino groups.

3.3.5 Antioxidative activities of tilapia protein hydrolysate

3.3.5.1 DPPH radical scavenging activity

One milliliter of the hydrolysates or trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was mixed with 1 ml of 0.2 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in a test tube. The reaction mixtures were incubated for 30 min in a dark room before the measurement of absorbance at 517 nm. Trolox (10-150 μM) was used as the standard for the calibration curve, and DPPH radical scavenging activity was expressed as μM trolox equivalents per milligram of protein (Orhan *et al.*, 2007)

3.3.5.2 ABTS radical scavenging activity

ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate)) was dissolved in distilled water to make a 4 mM concentration according to Re *et al.*(1999b). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.5 mM potassium persulfate (at a ratio 1:1) and allowing the mixture to stand in the dark at room temperature for 12–16 h prior to use. For the study of the hydrolysate, the $ABTS^{\bullet+}$ solution was diluted with deionized water to obtain absorbance of 0.8 ± 0.01 at λ734 nm. One hundred and fifty microliter of hydrolysate was added into 2.85 ml of $ABTS^{\bullet+}$ solution. The reaction mixture was incubated in a dark room for 2 h prior to absorbance measurement at 734 nm. The ABTS radical-scavenging activity was expressed as μM trolox equivalents per milligram of protein.

3.3.5.3 Metal chelating activity

One milliliter of sample solution (at suitable concentration) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 10 min at room temperature. The control was prepared in the same manner except that distilled water was used instead of the sample. The absorbance at 562 nm was measured and used to determine Fe^{2+} chelating activity using EDTA as the reference standard (Decker and Welch, 1990). The activity was expressed as μM EDTA per milligram of protein.

3.3.5.4 Reducing power

One milliliter of the hydrolysate was mixed with one milliliter of phosphate buffer pH 6.6 and one milliliter of potassium ferricyanide (1% w/v). The mixtures were incubated at 50 ^{o}C for 20 min followed by the addition of 10% trichloroacetic acid. Finally, a 100 μl of the mixtures were mixed with a 100 μl of deionized water and 20 μl of 0.1% (w/v) ferric chloride. After 10 min of reaction, the absorbance of the resulting solutions was measured at 700 nm using a spectrophotometer. The absorbance values were compared with those of the standard reducing agent, ascorbic

acid. High absorbance value represented high reducing power (Yıldırım *et al.*, 2001). The reducing power was expressed as milligrams of ascorbic acid per milligram of protein.

3.3.6 Assay for nitric oxide inhibitory activity

Nitric oxide production was assessed by Griess reaction (Mao *et al.*, 2011). The RAW 264.7 cell lines were cultured in 96-well plates with 1.5×10^5 cells/well and allowed to adhere for 1 h at 37 °C under a humidified atmosphere with 5% CO_2. After that, the cells were replaced with a fresh medium containing 100 µg/ml of LPS (100 µl) together with the hydrolysate at various concentrations (100 µl). NO production was determined after 48 h by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). Briefly, 100 µl supernatant was removed from each well and placed in a separate 96-well microtitre plate. After addition of equal volumes of the Griess reagent to each well and reacting for 20 min, the absorption was measured at 570 nm using a microplate reader (BioTek, Power XS, USA).

The NO concentration in the media of sample treated cells was calculated using a standard curve obtained for sodium nitrite.

3.3.7 Cell culture and viability determination

Mouse macrophage cells (RAW 264.7) were cultured and maintained in RPMI medium supplemented with 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere with 5% CO_2.

The MTT method was used for testing the cytotoxicity of the hydrolysed samples (Hansen *et al.*, 1989).

3.3.8 Assay for nitric oxide inhibitory effect

Nitric oxide production was assessed by Griess reaction (Mao *et al.*, 2011). The RAW 264.7 cell lines were cultured in 96-well plates with 1.5×10^5 cells/well

and allowed to adhere for 1 h at 37 °C under a humidified atmosphere with 5% CO_2. After that, the medium of the cells was replaced with a fresh medium containing 100 μg mL^{-1} of LPS (100 μL) together with the hydrolysate (100 μL) at various concentrations. NO production was determined after 48 h by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The NO concentration in the media of sample of treated cells was calculated using a standard curve obtained for sodium nitrite. Inhibition (%) was calculated using the following equation:

$$\text{Inhibition \%} = \frac{A - B}{A - C} \times 100$$

A - C: sodium nitrite concentration (μM) [A: LPS (+), sample (-); B: LPS (+), sample (+); and C: LPS (-) sample (-)].

3.3.9 Determination of intracellular oxidation using DCFH-DA labeling

Formation of reactive oxygen species (ROS) was evaluated using the oxidation-sensitive dye DCFH-DA assay (Engelmann *et al.*, 2005). For this, the RAW 264.7 cell lines were cultured in black 96-well plates with 1.5×10^5 cells/well and allowed to grow for 24 h at 37 °C under a humidified atmosphere with 5% CO_2. The growing cells were treated with 20 μM DCFH-DA in PBS (100 μl) and incubated in the dark for 20 min. The cells were then treated with the tilapia protein hydrolysate at various concentrations and incubated for 1 h. After washing the cells with PBS, 100 μl of H_2O_2 in PBS (250 μM) were added. The formation of 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of various ROS was determined by measuring the fluorescence at 0 and after 30 min at λ_{ex} = 485 nm and λ_{em} = 535 nm (SpectraMax M5, Multi-detection Reader, USA). The antioxidative activity was calculated as a function of emitted fluorescence and express as a percentage of inhibition as shown in the following equation:

$$\text{Inhibition (\%)} = [1 - (F_{30min} - F_{0min})/ F_{0min}] \times 100$$

3.3.10 Statistical analysis

Results were expressed as the mean value ± standard deviation from three replicates. The significance between means was statistically determined by the Duncan's multiple-range test. Differences were considered to be significant at $p < 0.05$.

3.4 Results and discussions

3.4.1 Degree of hydrolysate (DH)

The effect of ultrasonic pretreatment and ultrasonicassisted hydrolysis on the Flavourzyme catalytic process of tilapia protein was monitored using the DH as illustrated in Figure 1. The ultrasonic-assisted hydrolysis of control homogenate apparently yielded a lower DH than that of the conventional process; especially about 30% reduction in DH was noted. Whenever tilapia homogenate is pretreated with ultrasound, both consequence process and pretreatment duration also have an effect on DH. For instance, a reduction in the DH of a conventional hydrolysis was prominent if the homogenate was pretreated using 70 W ultrasound. In addition, in the process of ultrasonic pretreatment and ultrasonic-assisted at 70 W, the effect of the pretreatment on the DH was more associated with its duration than that of 10 W ultrasound, namely increment of pretreatment time results in reduced DH in both hydrolysis processes.

These results thus show that 10 W ultrasound has a prominent role on the retardation of the enzyme catalytic activity especially when applied during the hydrolysis. Furthermore, application of 70 W ultrasound as pretreatment caused a similar effect although to a less extent. The prolonged duration of the 70 W pretreatment has an effect as a gradual reduction in the DH from both processes.

The DH is an indication of the proteolytic level, which is related to the protein structure and enzyme activity. We found that a 1-h pre-exposure of the Flavourzyme solution to ultrasound using the above-mentioned power ranges caused only about 6% reduction in the enzyme activity (data not showed). This result shows that the

deactivation of the enzyme due to sonication is unlikely to be the cause of the observed low DH.

Reduction of the DH due to application of ultrasonic pretreatment suggested that modification of fine fish muscle particle and soluble protein in the homogenate is likely. The treated homogenate was more viscous and homogenous with respect to the untreated homogenate. Explosion of cavitation bubbles generated by ultrasonic may responsible for the transformation of the homogenate (Leong *et al.*, 2011). Moreover, more buried hydrophobic groups were exposed to the protein surface after sonication (Gülseren *et al.*, 2007). This alteration of protein conformation may lead to a decline of accessible peptide bonds.

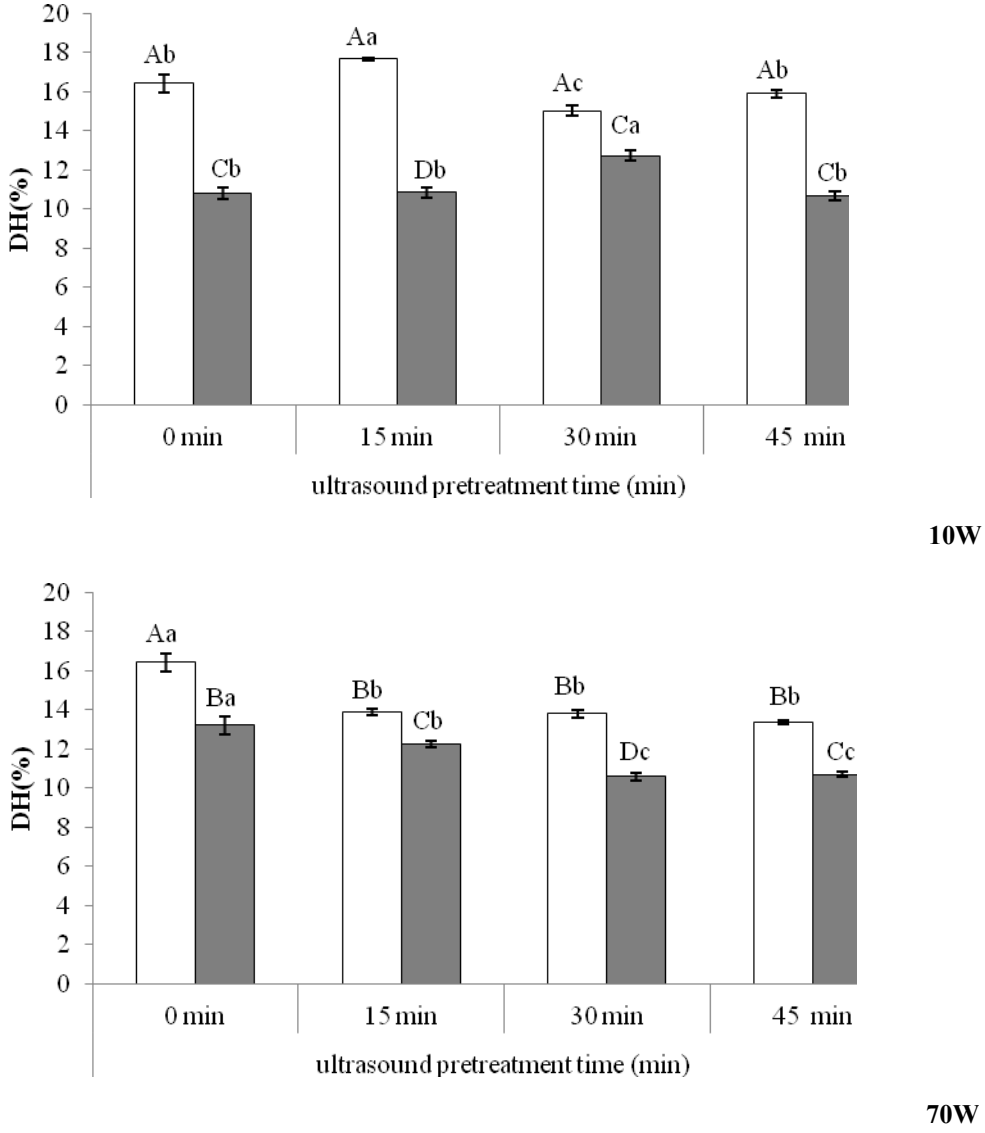


Figure 1 Degree of hydrolysis of tilapia hydrolysed by Flavourzyme for 1 h. The labels indicate the hydrolysate with different ultrasound pretreatment at different times and ultrasound power (□: no ultrasound assisted during hydrolysis, ■: ultrasound assisted during hydrolysis). The data are means ± SD (n = 3). The different lowercase letters and capital letters indicate significant differences as a result of pretreatment time and hydrolysis process, respectively ($p < 0.05$).

3.4.2 Antioxidative activity

3.4.2.1 DPPH radical scavenging activity

As shown in Table 1, the pretreatment time and ultrasound power have an effect on the DPPH radical-scavenging activity of the hydrolysate. Tilapia hydrolysate derived from the control homogenate using the conventional laboratory-scale hydrolysis had the lowest radical-scavenging activity (59.9 ± 1.2 mM Trolox per mg protein). However, this homogenate could be transformed into a protein hydrolysate with an improved radical-scavenging activity if the homogenate was preexposed to ultrasound either at 10 W or at 70 W. The similar benefit was also obtained, to a greater extent, if it was hydrolysed under ultrasound where the highest activity was found at 70 W. However, if the ultrasonic pretreatment was combined with ultrasonic-assisted hydrolysis, a reduction in the activity with pretreatment time was evident especially using 70 W.

Table 1 DPPH radical scavenging activity of tilapia hydrolysate prepare by sonicate pretreatment at various time and during hydrolysis process.

Pretreatment time (min)	**Non-ultrasound-assisted hydrolysis (mM Trolox/mg protein)**		**Ultrasound-assisted hydrolysis (mM Trolox/mg protein)**	
	10 W	70 W	10 W	70 W
0	59.9 ± 1.2^{Cb}	59.9 ± 1.2^{Cc}	72.8 ± 1.2^{Ba}	83.9 ± 0.2^{Aa}
15	65.6 ± 0.3^{Ba}	62.2 ± 1.3^{Bbc}	73.0 ± 1.8^{Aa}	78.2 ± 3.0^{Aa}
30	62.8 ± 0.9^{Cab}	65.4 ± 0.3^{BCa}	73.1 ± 1.7^{Aa}	67.1 ± 1.3^{Bb}
45	62.6 ± 2.1^{Bab}	64.3 ± 1.3^{Bab}	71.2 ± 2.1^{Aa}	63.9 ± 2.9^{Bb}

The data are means ± standard deviations of triplicate values. Column with different lowercase letter and row with different capital letters are indicating statistical differences in pretreatment time and hydrolysis process, respectively ($p < 0.05$).

3.4.2.2 ABTS radical scavenging activity

The ABTS radical-scavenging activity of tilapia hydrolysate using ultrasound for pretreatment and hydrolysis is illustrated in Table 2. The hydrolysate derived from ultrasonic-assisted hydrolysis at 70 W exhibited the highest ABTS radical-scavenging activity ($p < 0.05$) among the non-pretreated hydrolysates (0 min). Increasing the pretreatment time caused the reduction in the ABTS radical-scavenging activity of all treatment conditions. However, hydrolysate obtained by ultrasonic-pretreated and ultrasonic assisted hydrolysis at 70 W possessed a much better ABTS radical-scavenging activity than the other treatments at comparable pretreatment times and ultrasonic intensity.

Table 2 ABTS radical scavenging activity of tilapia hydrolysates after a 1 h hydrolysis with Flavourzyme combined with ultrasound pretreatment or applied during hydrolysis.

Pretreatment time (min)	Non-ultrasound-assisted hydrolysis (mM Trolox/mg protein)		Ultrasound-assisted hydrolysis (mM Trolox/mg protein)	
	10 W	70 W	10 W	70 W
0	1.3 ± 0.1^{Bab}	1.3 ± 0.1^{Ba}	0.7 ± 0.1^{Cb}	1.4 ± 0.0^{Aa}
15	1.4 ± 0.1^{Aa}	1.2 ± 0.2^{Aa}	0.9 ± 0.1^{Ba}	1.2 ± 0.1^{Ab}
30	1.0 ± 0.1^{Bc}	0.9 ± 0.1^{Cb}	0.9 ± 0.0^{Ca}	1.2 ± 0.0^{Aab}
45	1.2 ± 0.0^{Ab}	1.2 ± 0.0^{Aa}	0.7 ± 0.1^{Cb}	1.1 ± 0.0^{Bb}

The data are means ± SD (N = 3). Columns with different lowercase letters and rows with different capital letters indicate significant differences in pretreatment time and hydrolysis process, respectively ($p < 0.05$).

3.4.2.3 Reducing power

The reducing power of tilapia hydrolysates derived using ultrasonic pretreatment and assisted during hydrolysis is illustrated in Table 3. In a comparable non-pretreatment condition, ultrasonic-assisted hydrolysis at 70 W resulted in the highest reducing power of the tilapia hydrolysate with a maximum value of 28.9 ± 0.8 mg ascorbic per mg protein. Pretreatment of the homogenate with high ultrasound intensity (70 W) and further hydrolysis with both hydrolysis processes exhibited the higher reducing ability than that of lower ultrasound intensity (10 W) pretreatment. Increasing in pretreatment time has an effect on the reducing power of tilapia protein hydrolysate especially when pretreated with 70 W. Reducing power decreased with increment of pretreatment time. The other parameters, power intensity and process time might also contribute to this effect.

3.4.2.4 Metal chelating activity

The metal-chelating activity of the tilapia hydrolysates achieved from different ultrasonic pretreatment and hydrolysis conditions is illustrated in Table 4. Among the non-pretreatment conditions, the ultrasonic assisted hydrolysis at 70 W exhibited the highest chelating activity. If the ultrasonic pretreatment was adopted, its effect on the metal-chelating activity was different depending on the processes and the ultrasound intensity. Even a satisfied pattern on the effect of pretreatment could not be drawn in the conventional process, but its effect in the ultrasonic-assisted hydrolysis was very much determined by its intensity. Increasing the duration of the 70 W pretreatment improved the chelating activity. The highest activity was obtained after 45 min. In contrast, a reduction in the activity was noted with an extension of the 10 W ultrasonic pretreatment.

Different combinations of homogenate pretreatment with the hydrolysis process in the context of ultrasonic treatment yielded the tilapia hydrolysates with different in vitro antioxidative activities. The hydrolysate with the highest DPPH and ABTS radical-scavenging activity and reducing power was derived by 70 W ultrasonic-assisted hydrolysis of the control homogenate, whereas that with the

excellent metal-chelating activity was produced by the process composed of the 45-min ultrasonic pretreatment and 70 W ultrasonic-assisted hydrolysis produced.

The antioxidative reactivity of protein hydrolysate associates with its DH (Batista *et al.*, 2010) although there are contradicting reports regarding the relationship between DH and *in vitro* antioxidative activities of fish hydrolysate (Wiriyaphan *et al.*, 2012) where most reports have linked a high DH with strong antioxidative activity (You *et al.*, 2009). The reactive hydrolysates of the present study were however derived by using the hydrolysis processes having a low DH (Figure 1).

However, the previous document also reported that ultrasound assisted during enzymatic hydrolysis could be applied during enzymatic hydrolysis to produce protein hydrolysate with antioxidative activities (Yang *et al.*, 2011). In this study, in vitro antioxidative activities of tilapia protein hydrolysate prepared using ultrasound-assisted Flavourzyme hydrolysis with the DH of 13% possess the excellent activities. Nevertheless, the similarly results were reported by Klompong *et al.* (2008). They found that the Flavourzyme-derived protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) with 15% DH exhibited the strong *in vitro* antioxidative activities.

Theoretically, DPPH radical-scavenging activity represents an antioxidant with hydrogen donor (Kumaran and Joel karunakaran, 2006), while other assays including ABTS radical-scavenging activity and reducing power represent electron donor capability (Sampath Kumar *et al.*, 2011). Thus, an improved antioxidative activity of tilapia hydrolysate suggests that ultrasound intensity at 70 W via an unidentified means could promote both hydrogen and electron donation of the hydrolysate. This may be associated with the modification of liberated peptide. However, hydrophobic amino acid residues including valine or leucine at the N-terminus, alanine or tyrosine at the C-terminus and other amino acids such as proline or histidine in the sequences were identified as antioxidative peptides (Bougatef *et al.*, 2010; Fan *et al.*, 2012; Samaranayaka and Li-Chan, 2008).

The ultrasonic treatment ruptures and mechanically modifies protein structure due to the collapse of cavitation bubbles producing pressure and heat (Leong *et al.*, 2011). A possible interpretation of the information which was not discussed earlier

might be as follows: (i) ultrasound helps to unfold peptide chains and promotes the enzyme to cleave the peptide chains with a subsequent exposure of radical-scavenging amino acid side chains which are not good reaction partners of OPA; (ii) the less effective cleaved peptide was modified by ultrasonic power which enlarges the radical-scavenging activity. By this means, the expression radical-scavenging activity might not correlate with the DH.

Table 3 Reducing power of tilapia protein hydrolysates pretreated with ultrasound with a hydrolysis time of 1 h.

Pretreatment time (min)	Non-ultrasound-assisted hydrolysis (mg Ascorbic acid/mg protein)		Ultrasound-assisted hydrolysis (mg Ascorbic acid/mg protein)	
	10 W	70 W	10 W	70 W
0	19.7 ± 0.2^{Ba}	19.7 ± 0.2^{Bb}	16.4 ± 0.1^{Ca}	28.9 ± 0.8^{Aa}
15	16.0 ± 0.5^{Cc}	20.7 ± 0.3^{Ba}	16.7 ± 0.2^{Ca}	22.4 ± 0.6^{Ac}
30	13.8 ± 0.2^{Cd}	15.1 ± 0.2^{Bc}	15.0 ± 0.2^{Bb}	27.1 ± 0.4^{Ab}
45	18.5 ± 0.3^{Cb}	19.6 ± 0.4^{Bb}	14.0 ± 0.4^{Dc}	20.3 ± 0.1^{Ad}

The data are means ± SD (n = 3). Column with different lowercase letter and row with different capital letters indicate statistical differences in pretreatment time and hydrolysis process, respectively ($p < 0.05$).

Table 4 Metal chelating activity of tilapia hydrolysates after hydrolysis for 1 h with Flavourzyme combined with ultrasound pretreatment or applied during the hydrolysis.

Pretreatment time (min)	Non-ultrasound-assisted hydrolysis (μM EDTA/mg protein)		Ultrasound-assisted hydrolysis (μM EDTA/mg protein)	
	10 W	70 W	10 W	70 W
0	2.7 ± 0.1^{Ba}	2.7 ± 0.1^{Bab}	2.0 ± 0.1^{Ca}	3.2 ± 0.1^{Ac}
15	2.4 ± 0.1^{Bb}	2.6 ± 0.1^{Bb}	2.0 ± 0.1^{Ca}	2.9 ± 0.0^{Ad}
30	1.7 ± 0.1^{Cc}	2.1 ± 0.1^{Bc}	1.7 ± 0.0^{Cb}	3.7 ± 0.1^{Ab}
45	2.6 ± 0.1^{Cab}	2.8 ± 0.1^{Ba}	1.5 ± 0.1^{Db}	4.1 ± 0.1^{Aa}

The data are means ± SD (n = 3). Column with different lowercase letter and row with different capital letters are indicating statistical differences in pretreatment time and hydrolysis process, respectively ($p < 0.05$).

3.4.3 Cell viability

The pretreated tilapia hydrolysate prepared by different ultrasound conditions showed no cytotoxicity on the RAW 264.7 cells. In this study, we used a hydrolysate concentration in the range of 0.1–20 mg protein mL^{-1}, and each concentration resulted in a cell viability of more than 80% (Figure 2). Therefore, the tilapia protein hydrolysate could be used for further analysing the intracellular nitric oxide inhibitory and radical-scavenging activities. Ahn and co-workers reported that a salmon by-product of peptic protein hydrolysate did not show cytotoxicity on RAW 264.7 macrophage cell lines (Ahn *et al.*, 2012). Furthermore, sweetfish-derived protein hydrolysate at the maximum concentration 200 μg mL^{-1} has no effect on cell death (Sung *et al.*, 2012).

3.4.5 Nitric oxide inhibitory activity

Tilapia protein hydrolysates at a concentration of 20 mg protein mL^{-1} were used to verify their NO inhibitory activity of RAW264.7 macrophage cells, and the result is shown in Figure 3. An improved NO inhibitory activity was noticed for tilapia hydrolysate prepared from untreated homogenate using the 10 W ultrasonic-assisted hydrolysis. The 10 W ultrasound pretreatment could also enhance the activity of hydrolysate derived using conventional hydrolysis process to some extent if the pretreatment duration lasted for 30 or 45 min. Nevertheless, the process that included ultrasonic treatment at both steps did not give any additional improvement relative to that of the ultrasonic-assisted hydrolysis. A comparable beneficial effect of 70 W ultrasound with that of 10 W ultrasound was also noted at certain conditions including combination of 30- or 45-min ultrasonic pretreatment followed by conventional or ultrasonic-assisted hydrolysis, respectively. In addition, cell viability after nitric oxide inhibitory activity determination was more than 80% (data not showed).

The hydrolysates with an improved NO inhibitory activity were generally produced by the catalytic process with a low DH. However, it is not straightforward to explain the relation on activity of hydrolysate with DH as there are some process conditions having an improved DH but produced a hydrolysate with low NO

inhibitory activity on the contrary. Apart from a possible alteration of enzyme cleavage, the modification of the liberated peptide by ultrasound power proposed in previous results either in a low or high reactive hydrolysate is likely an account for the observation made. Few studies have reported the NO inhibitory activity of fish protein hydrolysates. Nevertheless, none of them mentioned the mechanism responsible for the reported activity. In a broad sense, inhibition of the inducible nitric oxide synthase (iNOS) expression may be attributed to those reactive hydrolysates (Sung *et al.*, 2012).

3.4.6 Intracellular free radical scavenging activity of hydrolysate

Figure 4 shows the effect of ultrasonic treatments on intracellular radical-scavenging activity of tilapia hydrolysate in RAW264.7 cells. The hydrolysis process with an integration of 10 W sonication caused a drastic loss of the intracellular radical-scavenging activity of the untreated homogenate. Application of ultrasonic pretreatment affected the radical-scavenging activity of the protein hydrolysate differently, depending on its power intensity, duration and the following processes. In this regard, high ultrasound power (70 W) is a promising mean to enhance intracellular radical-scavenging activity. The 10 W ultrasonic pretreatment, however, differs very much from that of the 70 W counterparts as no additional beneficial effect was found regardless of the type of the hydrolytical process used.

The different antioxidative capabilities of the hydrolysates due to different analysis systems, in macrophage cell lines and in vitro studies, have been reported (Wiriyaphan *et al*., 2012), and similar re-marks also hold for the present study. This may associate with a different scavenging reactivity of the hydrolysate against synthetic and biological free radicals (Wiriyaphan *et al*., 2012). Moreover, the hydrolysate exhibited excellent in vitro antioxidative activities which may not be able to pass through cell membranes. Cell permeability and interactions of peptides with the cell membrane limiting or promoting absorption are among critical characteristics of a potent intracellular antioxidant (Carrasco-Castilla *et al*., 2012). However, the principal common observation of those two systems of this study points out that utilisation of 70 W ultrasound could enhance antioxidative activity of tilapia

hydrolysate.

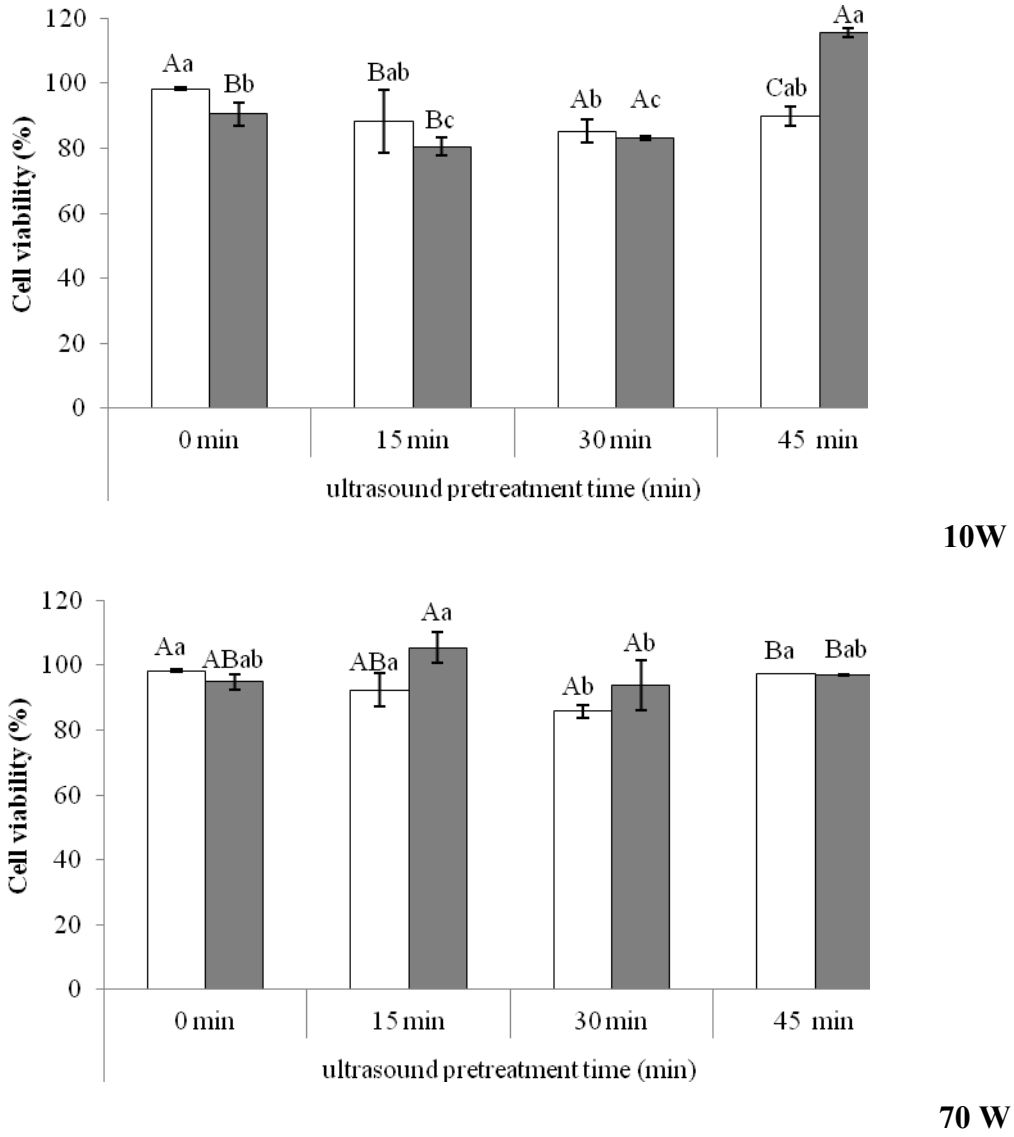


Figure 2 The cytotoxic effect of tilapia hydrolysates (20 mg protein/ml) on the viability of RAW 264.7 cells (□: no ultrasound assisted during hydrolysis, ■: ultrasound assisted during hydrolysis). The data are means ± SD (n = 3). The different lowercase letters and capital letters indicate significant differences as a result of pretreatment time and hydrolysis process, respectively ($p < 0.05$).

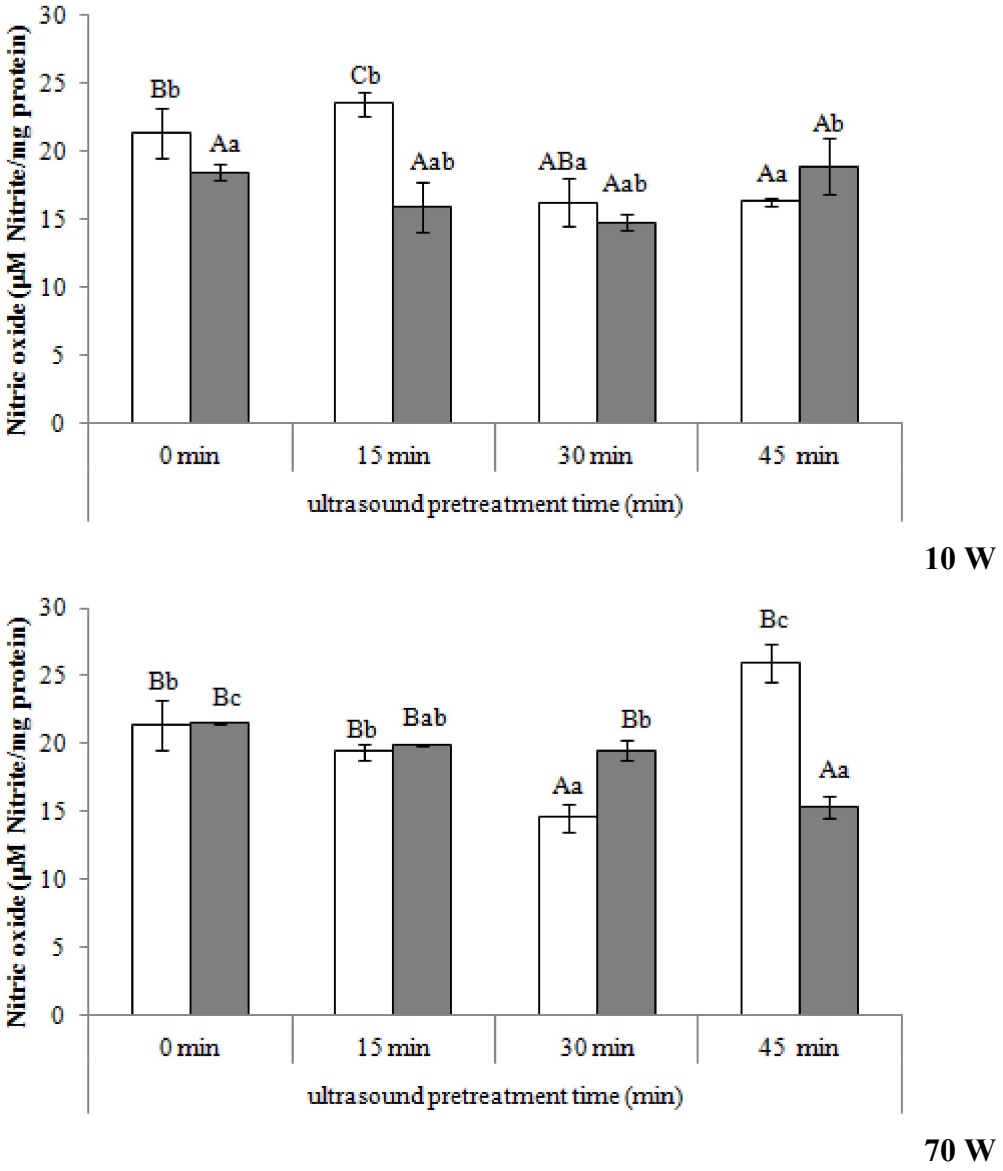


Figure 3 Effect of tilapia hydrolysate (20 mg protein/ml) on LPS-induced in RAW264.7 macrophage cells (□: no ultrasound assisted during hydrolysis, ■: ultrasound assisted during hydrolysis). The data are means ± SD (n = 3). The different lowercase letter and capital letters indicate significant differences in pretreatment time and hydrolysis process, respectively ($p < 0.05$).

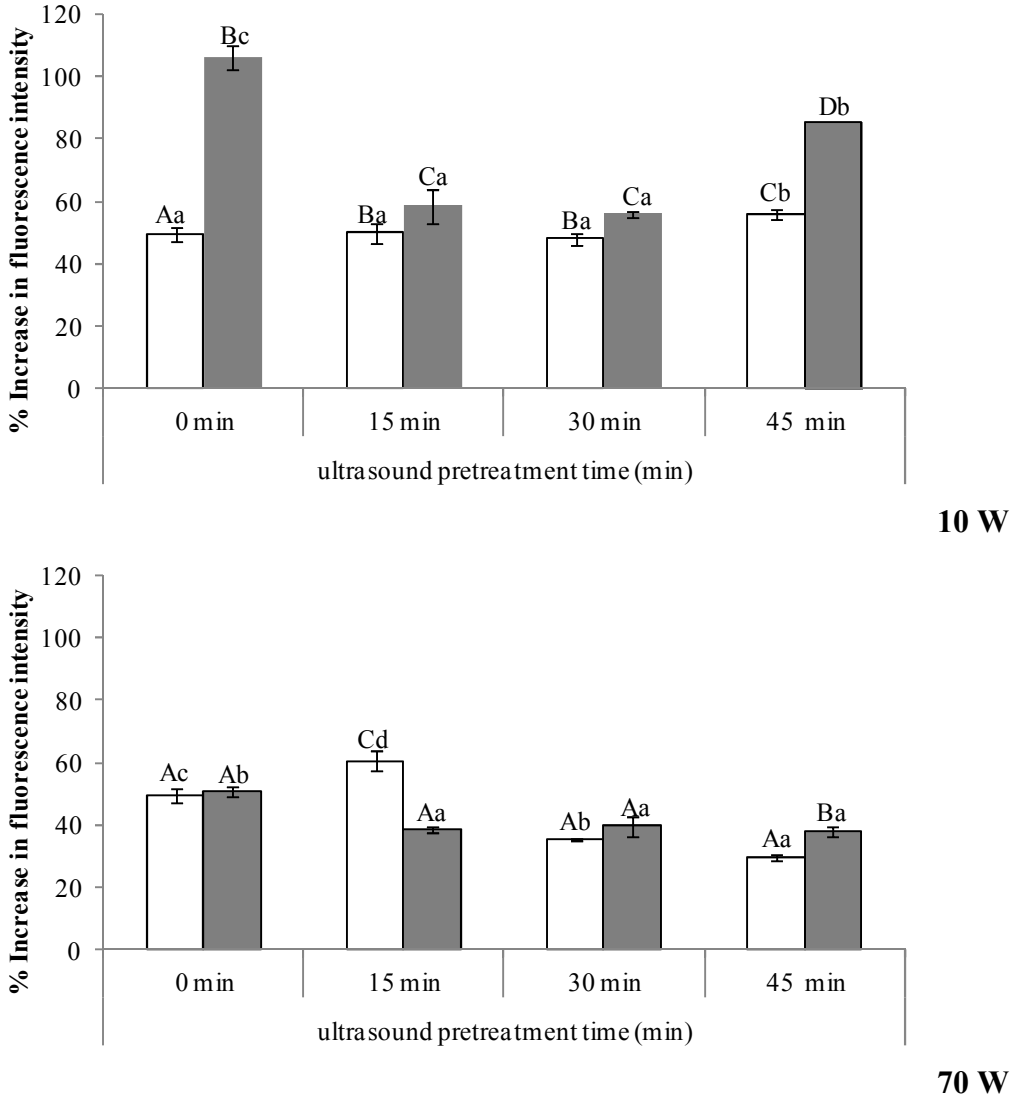


Figure 4 Influence of the tilapia protein hydrolysate on the intracellular antioxidant defense of RAW264.7 cells (□: no ultrasound assisted during hydrolysis, ■: ultrasound assisted during hydrolysis). The data are means ± SD (n = 3). The different lowercase letter and capital letters indicate significant differences as a result of pretreatment time and hydrolysis.

3.5 Conclusion

The application of ultrasound in protein pretreatment and incorporation during Flavourzyme hydrolysis results in bioactive hydrolysates with potential free radical-scavenging and anti-inflammatory properties. The 70 W ultrasonic-assisted hydrolysis of untreated tilapia homogenate yielded the strongest in vitro antioxidative activities. The homogenate pretreated for 30 and 45 min followed by a conventional enzymatic hydrolysis yielded the strongest nitric oxide inhibitory and antioxidative (in macrophage cell lines) activities, respectively. Therefore, ultrasound incorporated in the enzymatic hydrolysis has the potential to be a novel hydrolysis process for preparation of bioactive hydrolysates.

Chapter 4

Purification and characterization of antioxidant and nitric oxide inhibitory peptides from tilapia (*Oreochromis niloticus*) protein hydrolysate

4.1 Abstract

Nitric oxide (NO)-inhibitory and antioxidative activities of tilapia hydrolysates were prepared by using ultrasound pretreatment at 70 W for 30 and 45 min, respectively, followed by Flavourzyme hydrolysis for 1 h. Both hydrolysates were fractionated by using size exclusion chromatography on Sephadex G-25 column and purified by RP-HPLC. The amino acid sequence of the most potent and purified fractions were determined using LC/MS/MS. The antioxidant peptide (KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALV

K ,MW: 6334.49 KDa) and NO inhibitory peptide (AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK, MW: 6309.49Da) produced no cytotoxicity in RAW264.7 macrophage cell lines at concentration 100 mg mL^{-1}. The purified peptides at the concentration 100 μg mL^{-1} possessed the antioxidative and NO inhibitory activities 83.0 ± 1.1% and 40.9 ± 0.2 %, respectively which were about 100 times those of their counterpart crude hydrolysates.

4.2 Introduction

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion (Ray *et al.*, 2012). Exposure to ROS is strongly linked with chronic diseases such as cancer, coronary heart disease, and Alzheimer's disease (Abdul-Hamid *et al.*, 2002). Nitric oxide (NO) is produced from L-arginine by a chemical reaction catalyzed by the enzyme nitric oxide synthase antioxidative activity (Cirino *et al.*, 2006). Overproduction of NO and pro-inflammatory cytokines is associated with many diseases, including rheumatoid arthritis, asthma, atherosclerosis, and endotoxin-induced multiple organ injury (Ahn *et al.*, 2012). The interactions between ROS and NO generate potentially cytotoxic agents which may mediate some of the pathology associated with Parkinson's disease, chronic inflammation, atherosclerosis and cancer (Darley-Usmar *et al.*, 1995).

Bioactive peptides refer to specific protein fragments that have a positive impact on body functions or conditions which ultimately may influence health beyond their basic role as nutrient sources (Hartmann and Meisel, 2007). There are reports claiming that the bioactive peptide from fish protein hydrolysate possess biological activities, including, antioxidative (Je *et al.*, 2008), anti-hypertensive(Yokoyama *et al.*, 1992), anti-inflammatory (Sung *et al.*, 2012), and antibacterial (Song *et al.*, 2012) activities.

Nile tilapia (*O.niloticus*) is a widely cultivated and consumed species among freshwater fishes reared in Thailand. There are some reports describing bioactive peptides from tilapia (Charoenphun *et al.*, 2012; Foh *et al.*, 2010). Recently we found that ultrasonic pretreatment affected reactivity of tilapia protein hydrolysate (Kangsanant *et al.*, 2014). The pretreatment at 70W for 30 and 45 min followed by Flavourzyme hydrolysis yielded the hydrolysates with the most potent NO inhibitory and antioxidative activities on RAW264.7 cell lines, respectively (Kangsanant *et al.*, 2014). Therefore, in this study, we fractionated and purified the hydrolysates in order to establish the amino acid sequence of the potency peptides. Knowledge on characteristic features of the bioactive peptides may lead to utilize tilapia hydrolysate as a functional food ingredient.

4.3 Materials and methods

4.3.1 Materials and chemicals

Nile tilapia (*O.niloticus*) (700-900 g per fish) was purchased from a local market in Hat-Yai, Songkhla province, Thailand. Flavourzyme (protease from Aspergillus oryzae) and testing chemicals including the fluorescence probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide), lipopolysaccharide (LPS), and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Sephadex G-25 was purchased from GE Healthcare (GE Healthcare Bio-Science (Thailand) Ltd). Acetonitrile and water (HPLC grade) were purchased from Merck (New Jersey, USA). All other reagents are analytical grade.

The mouse macrophage cell lines, RAW 264.7, were obtained from Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. Roswell Park Memorial Institute medium (RPMI), penicillin/streptomycin, and the other materials required for culturing of the cells were purchased from Gibco BRL, Lift Technology (Thailand).

4.3.2 Preparation of tilapia protein hydrolysate

Tilapia muscle obtained manual filleting was ground into fine particles by using a grinder. The fish mince was further homogenized by mixing with 3 volumes of distilled water at 11,000 rpm for 1 min using Polytron, PT2100 (Lucerne, Switzerland). The ultrasonic pretreatment of the homogenate was performed by using an ultrasonic processor (Sonic, VCX 750, USA) at power outputs of 70 W with pulse duration of on-time 10 s and off-time 20 s. The pretreatment duration was 30 and 45 min for homogenates to be produced into NO inhibitory and antioxidant hydrolysates, respectively. During these pretreatments the homogenate temperature was risen to

55 ± 1 °C.

The pH of the homogenates was adjusted to 7.0 and heated to 55 °C. The enzymatic hydrolysis was initiated by addition of a Flavourzyme solution to obtain a ratio of 20 unit g^{-1} protein. For the conventional hydrolysis, overhead stirrer was used at 200 rpm for 1h. During this protocol, the homogenate temperature was controlled at 55 ± 1 °C. After 60 min, the hydrolysis was terminated by increasing the temperature of the reaction mixture to 90 °C for 10 min. The insoluble residue was separated by centrifugation at 15,000g for 20 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Avanti J-E, California, USA). The degree of hydrolysis was measured by OPA method (Dambmann *et al.*, 1999; Wanasundara *et al.*, 2002). The supernatant was lyophilized and used for further analyses.

4.3.4 Cell culture and viability determination

Mouse macrophage cells (RAW 264.7) were cultured and maintained in RPMI medium supplemented with 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 10% fetal bovine serum (FBS), and maintained at 37 °C under a humidified atmosphere with 5% CO_2. The MTT method was used for testing the cytotoxicity of the hydrolysed samples (Hansen *et al*., 1989). The cells were cultured in 96-well plates at a density of 1.5 x 10^5 cell/well and allowed to grow for 24 h at 37 ^oC under a humidified atmosphere with 5% CO_2. After growth the cells were washed with PBS (Phosphate Buffered Saline) and treated with different concentrations of the protein hydrolysate (100 µl). After 24 h of incubation, the cells were rewashed and 20 µl of MTT (5mg/ml) were added. After 4 h of incubation, the mixture was removed and 100 µl of isopropanol containing 0.04 M HCl was added for dissolving the formazan crystal. The amount of formazan salts was determined by measuring the absorption at 540 nm using a microplate reader (BioTek, Power XS, USA).

4.3.5 Assay for nitric oxide inhibitory effect

Nitric oxide production was assessed by Griess reaction (Mao *et al*., 2011). The RAW 264.7 cell lines were cultured in 96-well plates with 1.5×10^5 cells/well and allowed to adhere for 1 h at 37 °C under a humidified atmosphere with 5% CO_2. The cells were then replaced with a fresh medium containing 100 µg mL^{-1} of LPS (100 µL) together with the hydrolysate at various concentrations (100 µL). NO production was determined after 48 h by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). Briefly, 100 µl supernatant was removed from each well and placed in a separate 96-well microtitre plate. After the addition of equal volumes of the Griess reagent to each well and reacting for 20 min, the absorption was measured at 570 nm using a microplate reader (BioTek, Power XS, USA). The NO concentration in the media of sample treated cells was calculated using a standard curve obtained for sodium nitrite. Inhibition (%) was calculated using the following equation:

$$\text{Nitric oxide inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

Value represents sodium nitrite concentration (µM) in:

A: LPS (+), sample (-)

B: LPS (+), sample (+)

C: LPS (-), sample (-)

4.3.6 Determination of intracellular oxidation using DCFH-DA labeling

Formation of ROS was evaluated using the oxidation-sensitive dye DCFH-DA

(Engelmann *et al.*, 2005). For this, the RAW 264.7 cell lines were cultured in black 96-well plates with 1.5×10^5 cells/well and allowed to grow for 24 h at 37 °C under a humidified atmosphere with 5% CO_2. The growing cells were treated with 20 µM DCFH-DA in PBS (100 µL) and incubated in the dark for 20 min. The cells were then treated with the tilapia protein hydrolysate at various concentrations and incubated for 1 h. After washing the cells with PBS, 100 µL of H_2O_2 in PBS (250 µM) was added. The formation of 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH (2′,7′-dichlorofluorescein) in the presence of various ROS was determined by measuring the fluorescence at 0 and after 30 min at λ_{ex} = 485 nm and λ_{em} = 535 nm (SpectraMax M5, Multi-detection Reader, USA). The antioxidative activity was calculated as a function of emitted fluorescence and express as a percentage of inhibition as shown in the following equation:

Inhibition (%) = $[1- (F_{30min} - F_{0min})/ F_{0min}] \times 100$

F_0 and F_{30} represented the fluorescence intensity at 0 and 30 min, respectively.

4.3.7 Purification of antioxidant and nitric oxide inhibitory peptide

4.3.7.1 Gel column chromatography

The lyophilized hydrolysates (0.2g mL^{-1}) were dissolved in 50 mM sodium phosphate buffer (pH 7.0) and loaded onto a Sephadex G-25 gel filtration column (∅ 2.5×90 cm) which had previously been equilibrated with the same buffer. Fractions (3 ml each) were collected at a flow rate of 60 ml h^{-1}, and the absorbance was measured at 280 nm to determine the elution profile of the hydrolysate. The fractions with desired peaks were pooled and lyophilized; NO inhibitory and antioxidant activities were also investigated in RAW264.7 macrophage cell lines as well as RP-HPLC determination.

4.3.7.2 Reversed-Phase High-Performance Liquid (RP-HPLC) Chromatography

The lyophilized antioxidative and NO inhibitory activities peaks obtained on the gel column chromatography were dissolved in solvent A at a concentration 30 mg sample mL^{-1} before applied to a reverse-phase high performance liquid chromatography (RP-HPLC) having Kinetex 2.6 u XB-C18 100 A°column (∅ 3.00×150 mm). The solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in water (HPLC grade), and solvent B was 0.1% (v/v) TFA in 80% (v/v) acetonitrile solution (in water). The separation was performed with a linear gradient of eluent B concentrations: 0–30 min, 2-50% and 30-50 min, 50–100% at a flow rate 0.3 ml min^{-1}. The absorbance was measured at 280 nm to determine the elution profile of the sample. Potent peaks were collected, lyophilized and then evaluated for antioxidative and NO-inhibition activities. The fraction that showed the highest biological activities were analyzed for amino acid sequence.

4.3.7.3 Determination of amino acid sequence by LC/MS/MS

Peptide determination was performed according to the method proposed by Kuakarn et al., (2013). Peptide mixtures were analyzed by ultra-performance liquid chromatography (UPLC) (Ultimate 3000, Dionex, united states) coupled to the micrOTOF-Q Ⅱ™ ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an online nanoESI source. The MS and MS/MS spectrometry data were processed using data analysis software (Bruker Daltonics, Germany) and searched against the NCBInr database using the MASCOT search engine. A probability-based Mowse score of more than 43 was considered significant ($p < 0.05$).

4.3.8 Statistic analysis

Results were expressed as the mean value ± standard deviation from three replicates. The significance between means was statistically determined by the

Duncan's multiple-range test. Differences were considered to be significant at $P < 0.05$.

4.4 Results and discussion

4.4.1 Preparation of tilapia protein hydrolysate and their biological activities

The antioxidant and NO inhibitory activities of tilapia hydrolysate were performed in RAW264.7 macrophage cell lines using DCFH-DA assay and Griess reaction, respectively. The tilapia protein hydrolysates have no cytotoxicity on RAW 264.7 cell lines at the concentration range 0.1-20 mg protein mL^{-1} (data not shown). The percentage intracellular radical inhibition was 70.5 ± 1.3 % and the NO inhibitory activity of tilapia hydrolysate was 86.5 + 1.01 % at the concentration 100 mg mL^{-1} (20 mg protein mL^{-1}).

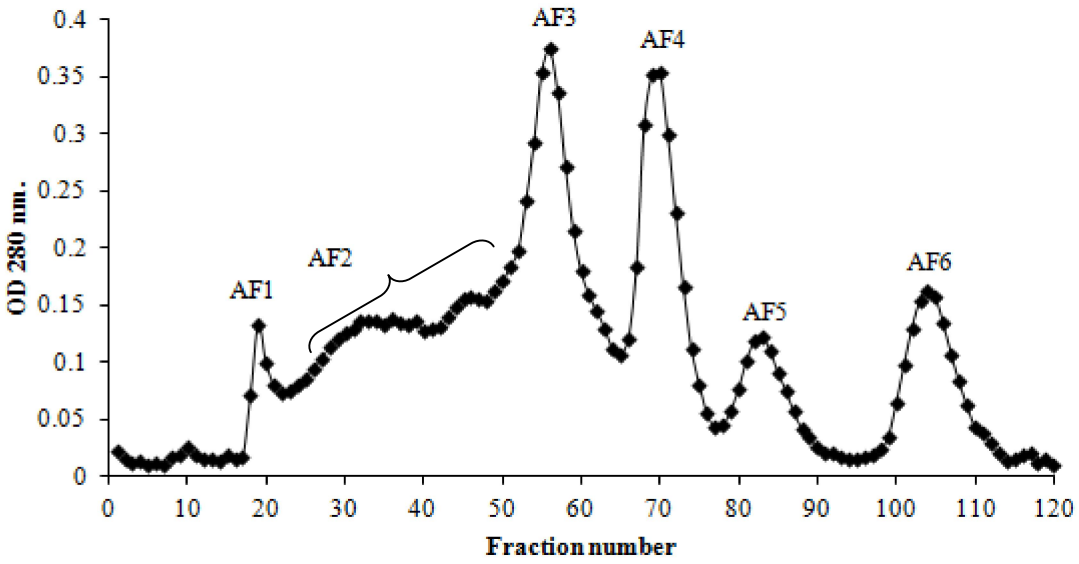
4.4.2 Purification of antioxidant peptide

4.4.2.1. Gel column chromatography and RP-HPLC

The antioxidant tilapia hydrolysate with a DH 13.3 % was subjected to size exclusion chromatography on Sephadex G-25. The elution profile showed six major absorbance peaks, antioxidative fraction (AF1- AF6), at 280 nm (Figure 1A), which were collected and tested for antioxidative activity in RAW 264.7 cell lines using DCFH-DA assay. The fraction AF1 exhibited the strongest antioxidative activity (54.03 + 0.8 %) among all fractions. Its activity was 10 times that of the crude sample (Figure 1B).

The selected fraction, AF1, was collected, lyophilized and further purified by using RP-HPLC. As shown in Figure 2A, the AF1 fraction was separated into three prominent peaks (AF1-1, AF1-2, and AF1-3). At the concentration of 100 μg sample

mL^{-1}, the fraction AF1-1, AF1-2, and AF1-3 exerted strong inhibitory activity with the percentage of inhibition of 76.6 ± 2.76%, 74.0 ± 2.95%, and 83.0 ± 1.12% ($p < 0.05$), respectively (Figure 2B). These purified peptides were no cytotoxicity against the RAW264.7 macrophage cell lines. The antioxidative activity of the most potent peptide (AF1-3) was about 100-fold relative to that of the crude hydrolysate. The result suggests that fraction AF1-3 is strong hydrophobicity since it has the last fraction eluted by using high concentration of acetonitrile (~80%).



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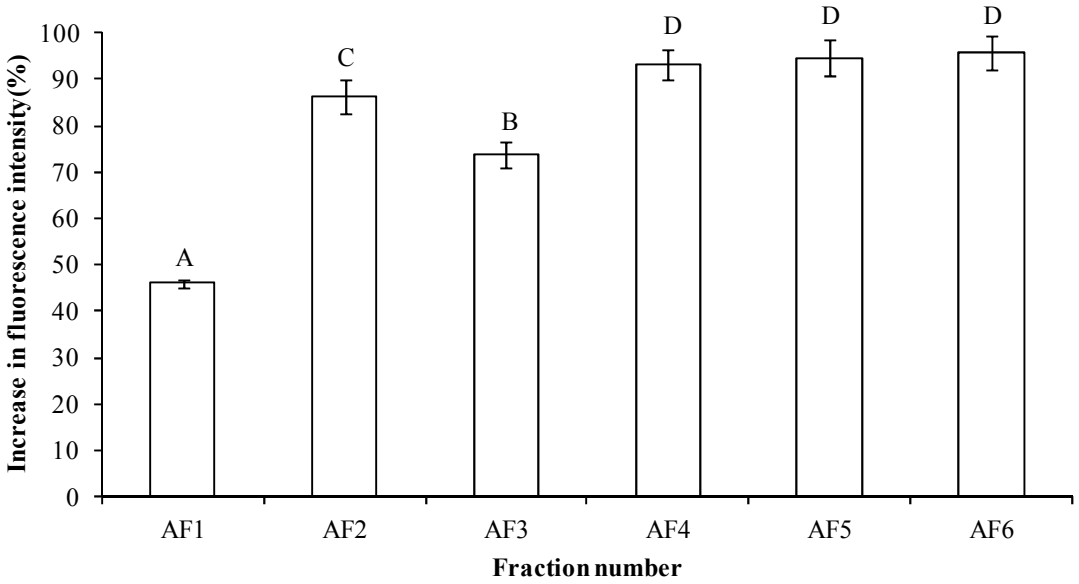
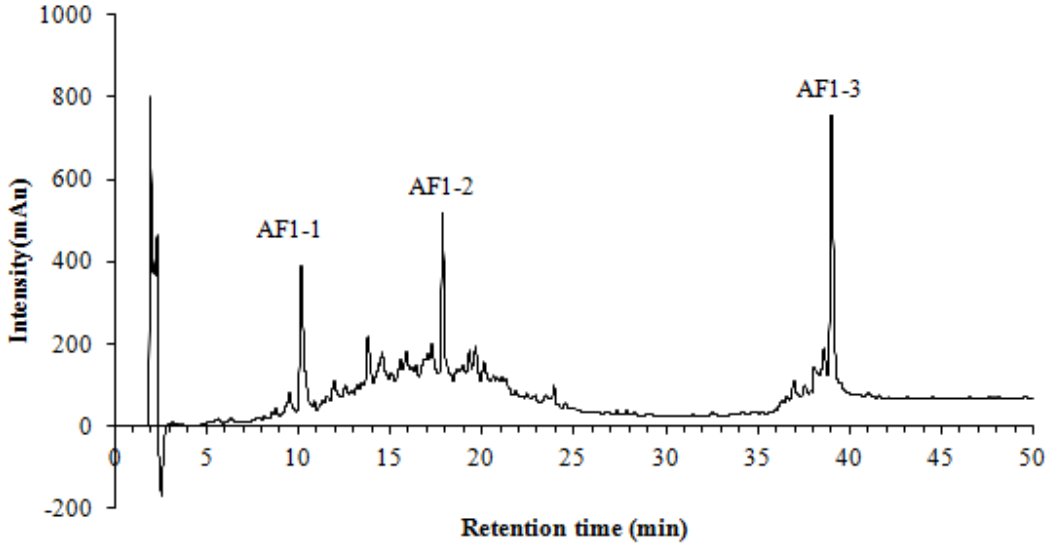


B

Figure 1 Elution profile of tilapia hydrolysate separated by size exclusion chromatography on Sephadex G-25 (A) and antioxidative activity of the separated fractions (B). Elution was carried out with 50mM Phosphate buffer pH 7.0 at a flow rate 1.8 ml/min. Results are mean + SE values of three independent experiments.



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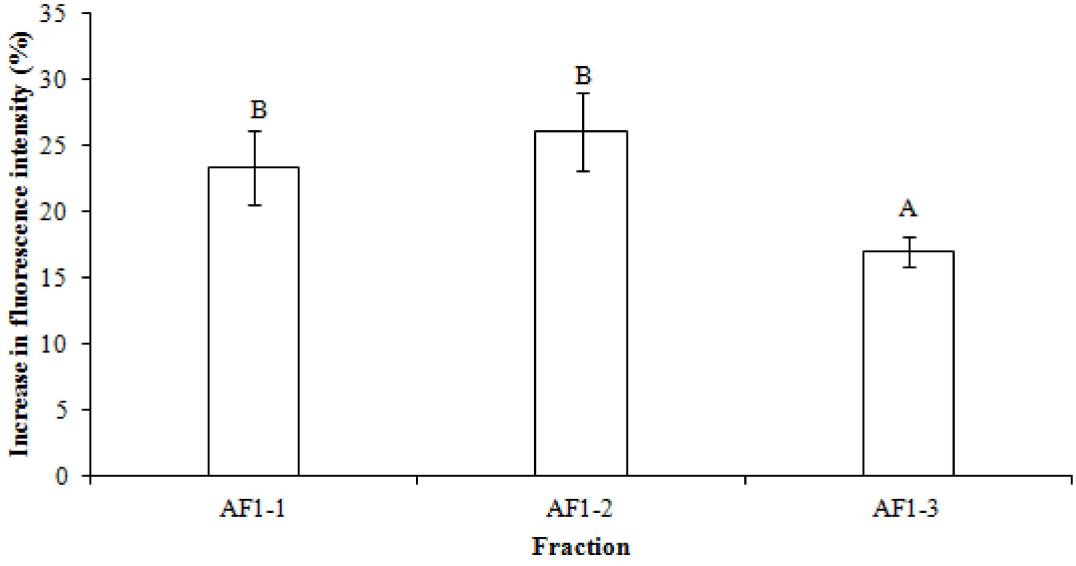


B

Figure 2 Chromatogram of RP-HPLC (Phenomenex C18 Kinetex column) of the AF1 fraction obtained from Sephadex G-25 gel filtration (A). The antioxidative activity of the eluted peaks was measure by DCFH-DA assay. Elution was performed with a linear gradient of acetonitrile (0–80%) containing 0.1% TFA at a flow rate of 0.3 ml/min. Results are mean ± SE values of three independent experiments.

4.4.2.2. Characterization of purified antioxidative peptide

The potent antioxidative and purified peptide was identified by using LC/MS/MS as 51 residues and its amino acid sequence from C-terminal was KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK (Mw 6,334.49 Da). This was in contrast with previous result reporting strong antioxidative peptides are composed with 5–17 amino acid residues(Kang *et al.*, 2013; Nazeer *et al.*, 2012; Wang *et al.*, 2008). This may originate from difference on degree of hydrolysis of the catalytic process used. The process of the current study yielded a DH of 13.38% whereas the reported small-antioxidative peptides were prepared by using the processes yielding DH values in the range of 34 – 80% (Je *et al.*, 2008; Kang *et al.*, 2013; Wang *et al.*, 2008).

According to DCFH-DA method, there are some possible means for the tilapia hydrolysate to express its radical scavenging in cell lines system. Theoretically, the nonpolar and non-ionic DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterase to non-fluorescent 20, 70-dichlorofluorescin (DCFH). In the presence of hydrogen peroxide (H_2O_2), DCFH is oxidized to fluorescent 20, 70-dichlorofluorescein (DCF) (Girard-Lalancette *et al.*, 2009). There is possibility that the antioxidative peptide can exert its activity outside cell membrane. In this case the hydrophobic nature of peptide facilitates scavenging of radicals in cellular system by keeping close contact with the cell membrane and scavenged the radical as described by Mendis *et al*., (2005). This will lead to lower intercellular free radical concentration before approaching to cell membrane.

Absorption of intact protein or polypeptide is possible through the specialized microfold cell (M cell) overlying the lymphoid Peyer's patches of small intestine (Bhutta and Sadiq, 2013; Pichayakorn *et al.*, 2003). Moreover, *in vivo* studies have shown that increased of blood glucose level in rats can be achieved after oral administration of 51 peptides with amino acid residues (insulin) (Roberts *et al.*, 1997). In this case if the peptide could penetrate into murine macrophage cell lines via phagocytosis, the peptide could act as a radical scavenger before it oxidized DCFH to highly fluorescent DCF. The less increase in of fluorescent intensity implied a reduced amount of radical activity in cells.

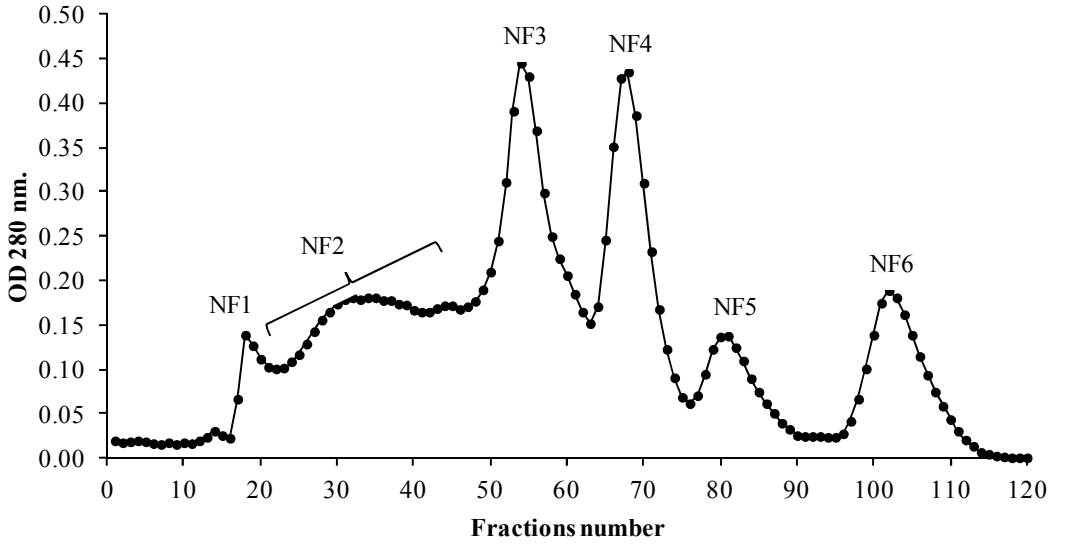
The potential of antioxidant activity of peptides depend on their structure, hydrophobicity, and amino acid composition (Sarmadi and Ismail, 2010). We found that hydrophobic amino acids including Ala, Phe, Ile, Val and Lue accounted for 41% of the purified antioxidant peptide. This is in accordance with hydrophobic amino acid content of antioxidant peptide from hoki (*Johnius belengerii*) frame (44%)(Kim *et al.*, 2007) and skin (50%) (Mendis *et al.*, 2005). The hydrophobicity of peptide is important for its accessibility to hydrophobic targets and affinity and reactivity to cell membrane (Mendis *et al.*, 2005).

Additionally, the major amino acid residues of the antioxidative tilapia peptide were Ala (13.7%), Asp (11.76%), Lys (9.8%), Gly (9.8%), and Phe (9.8%). These amino acids either contain alkyl, amino, or carboxylic group in the side chain which can act as hydrogen donor to facilitate breaking the chain reaction of oxidation by scavenging radicals (Qian *et al.*, 2008).

4.4.3 Purification of nitric oxide inhibitory peptide

4.4.3.1. Gel column chromatography and RP-HPLC

The hydrolysate (DH: 13.8%) was fractionated into NO inhibitory fractions using gel column chromatography. The elution profile exhibited six peptide groups (NF1-NF6) at 280 nm (Figure 3A). At concentration 10 mg mL^{-1}, they exhibited the NO inhibition in the range of 50-87%. The inhibitory activity of the most potent fraction (NF-5; 87.2 ± 0.9 %) was 10 folds of that of crude hydrolysate (Figure 3B). A further purification of this fraction with the RP-HPLC showed three prominent peaks (Figure 4A). At the concentration of 100 μg mL-1, the percentage of NO inhibition of the three fractions; NF5-1, NF5-2, and NF5-3, were 38.1 ± 0.12%, 38.5 ± 0.21, and 40.9 ± 0.24 % ($p < 0.05$), respectively (Figure 4B). The activity of the fraction NF5-3 was about 100-folds relative to that of the crude hydrolysate.



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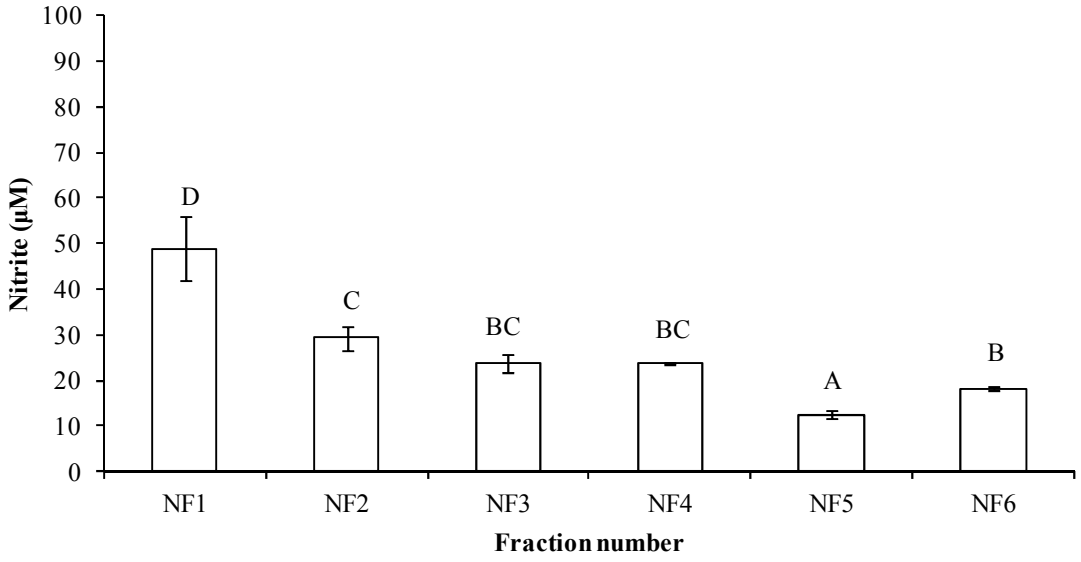
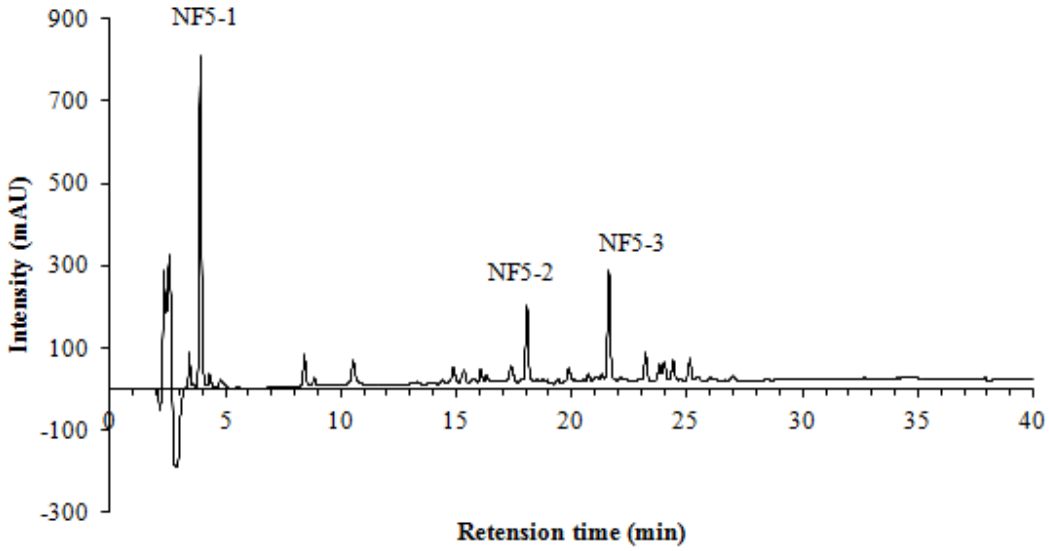


B

Figure 3 Elution profile of tilapia hydrolysate separated by size exclusion chromatography on Sephadex G-25 (A) and nitric oxide inhibitory activity of the separated fractions (B). Elution was carried out with 50mM Phosphate buffer pH 7.0 at a flow rate 1.8 ml/min. Results are mean ± SE values of three independent experiments.



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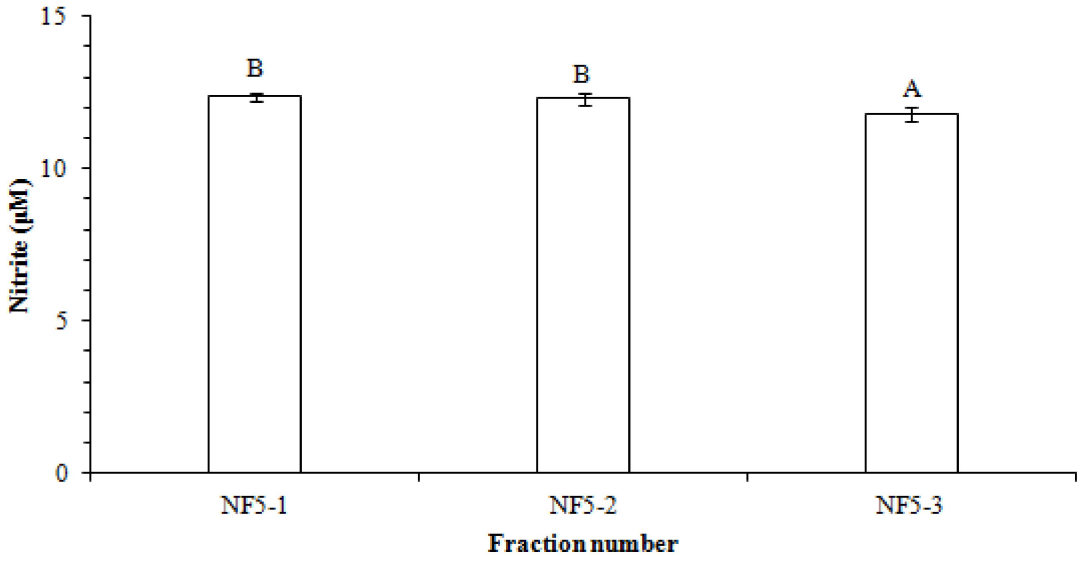

B

Figure 4 Chromatogram of RP-HPLC (Phenomenex C18 Kinetex column) of the NF5 fraction obtained from Sephadex G-25 gel filtration (A). The nitric oxide inhibitory activity of the eluted peaks was measure by DCFH-DA assay. Elution was performed with a linear gradient of acetonitrile (0–80%) containing 0.1% TFA at a flow rate of 0.3 ml/min. Results are mean ± SE values of three independent experiments.

4.4.3.2 Characterization of purified nitric oxide inhibitory peptide

The purified peptide (NF5-3), 6309.46 Da, composed with 51 amino acid residues. It is considerably larger than the previously reported NO inhibitory peptides, which are reported to have 10-14 amino acid residues (Kim *et al.*, 2013; Lee *et al.*, 2012; Ryu *et al.*, 2010). This is associated with the applied catalytic process resulting with 13.80 % DH. The result, however, suggests that the peptide could penetrate into macrophage cell lines and possible absorption route are discussed in the antioxidative section. The inhibitory activity of the peptide might associate with the modulation the expression of inducible nitric oxide systhase (iNOS), which catalyzes the production of NO after LPS stimulation (Sung *et al.*, 2012).

Hydrophobic residue was addressed to play a significant role on bioactive peptide to express their reactivity. The purified peptide contained primarily five hydrophobic amino acids including Ala, Phe, Leu, Val, and Ile which is well corresponded with characteristics of anti-NO inhibitory peptide (Ryu *et al.*, 2010). The hydrophobic amino residue might promote the peptides to access to hydrophobic targets enhancing attraction and reactivity of peptides with the cell membrane (Mendis *et al.*, 2005).

Amino acid sequence of the purified peptide was identified as AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK. There are some documents mentioning on amino acid sequence of NO inhibitory peptide derived from marine animals. The amino acid sequence of the NO-inhibition peptide from hard-shelled mussel (*Mytilus coruscus*) was identified as GVSLLQQFFL at N-terminal (Kim *et al.*, 2013). The anti-inflammatory activity of

purified peptides from *Crossostrea gigas* hydrolysate was QCQCAVEGGL at N-terminal position (Hwang *et al*., 2012). As well as, Lee and his colleagues (2012) found that the NO-inhibition peptide sequence of the short-necked clam (*Ruditapes philippinarum*) obtained was composed of QCQQAVQSAV at the N-terminal positions. Nevertheless, the present amino acids with NO-inhibitory are different in characteristics and are derived from many sources of raw materials.

4.5 Conclusions

Antioxidant and NO-inhibition peptides were purified from tilapia hydrolysates. Amino acid sequence of the purified antioxidant peptide was identified from C-terminal as KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK. And the sequence of NO-inhibitory peptide was AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK.

The results suggest that such bioactive peptides are liberated, depending on their structural, compositional, and sequential properties; they may exhibit various bioactivities. The purified peptides efficiently inhibited radical reaction and NO generation by about 100 times that of the crude hydrolysate while they exhibited non-cytotoxicity on RAW264.7 cell lines. These results suggested that antioxidant and NO-inhibition peptides from tilapia might be advantage for functional food ingredient, diet nutrient and pharmaceutical agent.

Chapter 5

Summary and future works

5.1 Summary

1. The enzyme type, ultrasonic intensity, and process time have an influence on the antioxidative activities of the tilapia hydrolysates obtained.

2. The explicit effect of sonication was highlighted either from its intensification or reduction of the hydrolysates reactivity against radical activities.

3. Hydrolysates from tilapia muscle protein having a potent intracellular radical scavenging activity hydrolysate could be prepared using papain at an ultrasound treatment at 40 W for 120 min.

4. The application of ultrasound in protein pretreatment and incorporation during Flavourzyme hydrolysis results in bioactive hydrolysates with potential free radical-scavenging and anti-inflammatory properties.

5. The homogenate pretreated for 30 and 45 min followed by a conventional enzymatic hydrolysis yielded the strongest nitric oxide inhibitory and antioxidative (in macrophage cell lines) activities, respectively.

6. The purified antioxidant peptide (KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALV K ,MW: 6334.49 KDa) and NO inhibitory peptide (AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK, MW: 6309.49Da) produced no cytotoxicity in RAW264.7 macrophage cell lines at concentration 100 mg mL^{-1}.

5.2 Future works

1. Antioxidative and NO inhibitory activities of tilapia hydrolysate in animal trial should be study.

2. The mechanism of NO inhibitory activity should be determined such as cytokine inhibition.

3. Crude hydrolysate with antioxidative or NO inhibitory activities should be scale up for commercial production.

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Publications

1. Kangsanant, S., Murkovic, M. and Thongraung, C. 2014. Antioxidant and nitric oxide inhibitory activities of tilapia (*Oreochromis niloticus*) protein hydrolysate: effect of ultrasonic pretreatment and ultrasonic-assisted enzymatic hydrolysis. Int J Food Sci Tech. 49. 1932-1938.
2. Sureeporn Kangsanant, Chakree Thongraung, Chaweewan Jansakul, Michael Murkovic, Vatcharee Seechamnanturakit. 2014. Purification and characterization of antioxidant and nitric oxide inhibitory peptides from Tilapia (*Oreochromis niloticus*) protein hydrolysate. Int J Food Sci Tech. Submitted.

Presentations

1.Oral presented at The RGJ-Ph.D Congress VIX, Pattaya, Thailand (5-7 April 2013)

Antioxidant and Nitric Oxide Inhibitory Activities of Tilapia (*Oreochromis niloticus*) Protein Hydrolysate: Effect of Ultrasound Pretreatment and Ultrasound-Assisted Enzymatic Hydrolysis.

2. Oral presented at The International Conference on Food Research 2010 (Sustainable AND Quality Food for All). Kuala Lumpur, Malaysia (22-24 November 2010) Antioxidative Activity and Stability of Tilapia (*Oreochromis niloticus*) Protein Hydrolysate.