Functional and Biological Properties of Enzymatic Hydrolysate from Defatted Rice Bran by Using Partial Purified Nile Tilapia (*Oreochromis niloticus*) Viscera Extract

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ชื่อวิทยานิพนธ์ สมบัติเชิงหน้าที่และทางชีวภาพของไฮโดรไลเสทจากกากรำข้าวหลังสกัดน้ำมันโดยใช้สารสกัดเอนไซม์จากเครื่องในปลานิล (*Oreochromis niloticus*) ที่ผ่านการทำบริสุทธิ์บางส่วน

ชื่อผู้เขียน นางสาวธัชชพร ไชยเจริญ
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บทคัดย่อ

การสกัดเอนไซม์จากเครื่องในปลานิล (*Oreochromis niloticus*) โดยใช้น้ำ, สารละลายโซเดียมคลอไรด์ และอะซิโตน พบว่า การใช้สารละลายโซเดียมคลอไรด์ที่ความเข้มข้น 5% w/v จะได้สารสกัดที่มีกิจกรรมของเอนไซม์โปรติเอสและอะไมเลสสูงสุด คือ 1,300 และ 1,600 ยูนิตต่อมิลลิกรัมโปรตีน ตามลำดับ โดยเอนไซม์ทั้งสองชนิดนี้มีกิจกรรมสูงสุดที่สภาวะเดียวกัน คือ ที่ pH 8.0 และอุณหภูมิ 60°C และเมื่อบ่มสารสกัดที่สภาวะดังกล่าว พบว่า กิจกรรมของเอนไซม์โปรติเอส ลดลง 50% ของกิจกรรมเริ่มต้นเมื่อบ่มไว้ 30 นาที ในขณะที่กิจกรรมของเอนไซม์อะไมเลสเพิ่มขึ้น 5 เท่าในช่วงแรกก่อนที่จะลดลงประมาณ 3 เท่าของกิจกรรมเริ่มต้นเมื่อบ่ม 90 นาที ซึ่งการแยกเอนไซม์โปรติเอสและอะไมเลสออกจากกันด้วยเมมเบรน ชนิดอัลตราฟิวเทรชัน ที่มีรูพรุนขนาด 30 กิโลดาลตัน พบว่า ไม่สามารถแยกเอนไซม์ทั้งสองชนิดนี้ออกจากกันได้อย่างสมบูรณ์ ส่วนการตกตะกอนโปรตีนอื่นที่ไม่ใช่เอนไซม์ด้วยแอมโมเนียมซัลเฟต พบว่า ที่ความเข้มข้นของแอมโมเนียมซัลเฟต 30 % และ 35% มีผลให้กิจกรรมของเอนไซม์โปรติเอสและอะไมเลสในสารละลายเพิ่มขึ้น เมื่อวิเคราะห์ด้วย SDS-PAGE พบว่า โปรตีนในสารสกัดจากเครื่องในปลานิลมีน้ำหนักโมเลกุล 26.5, 37.3 และ 56.4 กิโลดาลตัน และพบกิจกรรมของเอนไซม์โปรติเอส ไลคีเนส และอะไมเลส ในแต่แถบโปรตีนดังกล่าวตามลำดับ แสดงให้เห็นว่าสารสกัดเอนไซม์ที่มีเอนไซม์ 3 ชนิด สามารถเตรียมได้จากเครื่องในปลานิล

ไฮโดรไลเสทรำข้าวที่เตรียมจากกากรำข้าวหลังการสกัดน้ำมันซึ่งเป็นเศษเหลือจากกระบวนการผลิตน้ำมันรำข้าว โดยใช้สารสกัดจากเครื่องในปลานิลที่ความเข้มข้น 1, 2 และ 3% w/v และใช้เวลาย่อย 30, 60 และ 120 นาที พบว่า เมื่อย่อยโดยใช้สารสกัดจากเครื่องในปลานิล 1% เป็นเวลา 30 นาที จะได้ไฮโดรไลเสทที่มีปริมาณเบต้ากลูแคนสูงสุดในขณะที่ %DH ต่ำสุด และสภาวะนี้

ยังช่วยเพิ่มคุณสมบัติในการจับกับน้ำของไฮโดรไลเสท นอกจากนี้การเพิ่มเวลาย่อยมีผลให้สีของไฮโดรไลเสทเข้มขึ้น

ไฮโดรไลเสทรำข้าวที่ผลิตโดยใช้สารสกัดจากเครื่องในปลานิล 1% เป็นเวลา 30 นาที แสดงกิจกรรมการเป็นสารต้านการเกิดออกซิเดชั่นในระบบหลอดทดลองสูงสุดในทุกการทดสอบ ได้แก่ การต้านอนุมูลอิสระ ABTS, อนุมูลอิสระ DPPH, อนุมูลอิสระไฮดรอกซิล, อนุมูลอิสระซุปเปอร์ออกไซด์, อนุมูลอิสระรีดิวซิ่งพาวเวอร์ และการจับโลหะ นอกจากนั้นไฮโดรไลเสทที่ได้จากทุกสภาวะไม่เป็นพิษต่อเซลล์แมคโครฟาจน์ RAW 264.7 เมื่อใช้ที่ความเข้มข้นสูงสุดที่ 500 ไมโครกรัมต่อมิลลิลิตร ซึ่งไฮโดรไลเสทที่ได้จากการย่อยด้วยสารสกัดเครื่องในปลานิลเข้มข้น 1% และ 2% เป็นเวลา 30 นาที มีฤทธิ์ยับยั้งการสร้างไนตริกออกไซด์ดีที่สุด โดยมีค่า IC_{50} เท่ากับ48 และ 49 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ซึ่งไฮโดรไลเสทที่ย่อยด้วยสารสกัดเครื่องในปลานิล 2% เป็นเวลา 30 นาที มีฤทธิ์ลดการหลั่งไซโตไคน์ TNF-α, IL-1β และ IL-6 ดังนั้นกากรำข้าวหลังการสกัดน้ำมันจึงสามารถใช้ผลิตไฮโดรไลเสทที่มีฤทธิ์ต้านการเกิดออกซิเดชั่นและต้านการอักเสบได้

Thesis Title Functional and biological properties of enzymatic hydrolysate from defatted rice bran by using partial purified Nile tilapia (*Oreochromis niloticus*) viscera extract

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Major Program Functional Food and Nutrition

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*) viscera extracts were prepared using water, NaCl solution and acetone homogenization. The saline solution (5% w/v) yielded the extract with the highest protease and amylase with the specific activities of 1,300 and 1,800 unit/mg protein, respectively. These two enzymes were active at the identical optimum pH (8.0) and temperature (60˚C). At this condition, the protease activity was gradually lost and remained at 50% of its initial value after 30 min. In contrast, the amylase activity had risen 5 times before declining to about 3 times of its initial activity after 90 min. The protease and amylase could not be successfully separated by 30 kDa molecular cut-off ultrafiltration. Precipitation of non-enzyme protein by using 30 or 35% ammonium sulphate effectively improved specific activity of both enzymes of the extract. This partial purified fraction and the crude exhibited three protein bands by using SDS-PAGE assigned as 26.5, 37.3 and 56.4 kDa. And these protein bands showed the activity of protease, lichenase and amylase, respectively. Therefore, the results revealed that crude enzymes extract with three enzyme activities could be prepared from tilapia viscera.

Defatted rice bran, a leftover rice bran oil production, and crude tilapia viscera extract were used for preparation of rice bran hydrolysate (RBH). Effects of concentration of the viscera extract (1, 2 and 3% w/v) and hydrolysis duration (30, 60 and 120 min) on catalytic process and hydrolysate characteristics were investigated. The mildest hydrolysis using 1% viscera extract for 30 min yielded the hydrolysate with

the highest β-glucan content and the lowest degree of hydrolysis (DH) value. This condition also improved hydration properties of the RBH (water holding, water binding and swelling capacity). Extension of hydrolysis time caused the darkening of RBH.

The hydrolysis using 1% viscera extract for 30 min yielded the hydrolysate with the highest *in vitro* anti-oxidative activities. All obtained RBHs exhibited no cytotoxicity on RAW 264.7 cell lines at the maximum concentration of 500 µg/ml. The RBHs obtained by using 1% and 2% viscera extract with 30 min hydrolysis time showed the best nitric oxide inhibitory in the RAW 264.7 cell with an IC_{50} of 48 and 49 µg/ml, respectively. The RBH produced by 2% viscera extract with 30 min hydrolysis time exhibited the strongest reduction of TNF-α, IL-1β and IL-6 cytokines. Thus, the defatted rice bran could be used for preparation of RBH with strong antioxidative and anti-inflammatory activities.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is a cichlid fish native of Africa that prefers to live in shallow water. Tilapia is one of the most popular fish for culture and have been introduced into many countries around the world (FAO, 2001). In recent years, attention has been focused on developing tilapia culture. The production of farmed tilapia in the world is rising, and global tilapia production is expected to almost double from 4.3 million tons per year in 2010 to 7.3 million tons a year in 2030 (FAO globefish, 2014). In Thailand, tilapia is a freshwater fish that is widely consumed and cultivated. Tilapia exports of Thailand to the European Union's market is worth several million dollars annually and likely to increase over time. The volume of exports was 9,515.9 tons in 2012 (Nurit, 2012). The tilapia industry in Thailand has grown significantly over the past decade. The most exported products of tilapia are frozen whole fish amount of 6,867.8 tons, followed by chilled fish fillets 2,120.1 tons and frozen fish fillets 386.2 tons (Nurit, 2012). The mass industrial processing of the tilapia, fish fillets as a priority, generates large amount of by-products especially viscera (Freitas-Júnior *et al*., 2012).

Fish viscera have been reported to be a good source of digestive enzymes (Simpson *et al*., 1991; Bezerra *et al*., 2005; Khantaphant and Benjakul, 2010; Klomklao *et al*., 2008; Souza *et al.*, 2007) and their properties are highly valued in a wide range of industrial applications and processes (Espisito *et al.*, 2009; Klomklao *et al*., 2005). Proteases represent an important class of industrial enzymes, accounting about 50% of the total sale of the enzymes in the world (Souza *et al*., 2007). Digestive protease have been studied in several species of fish (De Vecchi and Coppes, 1996) and are the primary enzymes which have been isolated and characterized from various parts of tilapia digestive tract (Tengjaroenkul *et al*., 2000; Hinsui *et al*., 2006). Enzyme from tilapia viscera is however likely to contain more than one type of enzyme. Because of

tilapia is a herbivorous fish and its digestive system also displays greater activity of amylase than protease and a lesser lipase activity (Tengjaroenkul *et al*., 2000).

Rice bran is a by-product of the rice milling process (the conversion of brown rice to white rice). Rice bran contains a non-significant amount of protein (12-20%), with fairly high nutritional quality (Saunders, 1990). Rice bran protein is also very digestible (more than 90%) (Wang *et al*., 1999) and maybe hypoallergenic (Helm and Burks, 1996). Rice bran protein holds great promise as an alternative protein source such as a food ingredient (Yeom *et al.,* 2010). Rice bran has been utilized to produce protein isolates and respective protein hydrolysates for potential application in various food products (Bandyopadhyay *et al.,* 2008). Rice bran protein hydrolysates have various pharmacological activities such as anti-diabetic effects (Boonloh *et al*., 2015) or antioxidant activity (Chanput *et al*., 2009; Daou and Zang, 2011). However, the large portions of rice bran protein cannot be solubilized by regular solvents such as salt, alcohol and acid due to extensive disulfide bonding and aggregation (Silpradit *et al*., 2010). The most common method of isolating proteins from rice bran is alkali extraction. Though this treatment with high temperature and concentration of alkaline solution solubilizes most of the rice bran proteins, it also cause the occurrence of denaturation and hydrolysis of proteins, increased maillard reaction (which causes dark-colored products) and increased extraction of non-protein components which co-precipitate with protein and lower the isolate quality (Wang *et al*., 1999). Enzymatic method has been used to enhance solubilization of rice bran protein and achieve a wide range of protein hydrolysates (Silpradit *et al*., 2010). Therefore, the present study produced rice bran hydrolysate by using tilapia viscera enzyme extract and investigated it’s properties.

1.2 Review of literature

1.2.1 Nile Tilapia (*Oreochromis niloticus*)

Nile tilapia is the ordinary name for nearly a hundred species of freshwater and some brackish water fishes. The fish labeling regulations designate that all species of the genera *Oreochromis*. It is also commercially known as Mango fish or Nilotica. Tilapias are very important in world fisheries, and are the second most important group of food fishes in the world (FAO, 2011). While capture production is relatively small, several species of tilapia are cultured commercially on a significant scale. The most important and abundant species (capture and aquaculture) is Nile tilapia (*Oreochromis niloticus*) which is cultivated in large scale production units and often insignificant volumes by small local producers (Nurit, 2012). Many species of tilapia have been cultured in developing countries, where animal protein is lacking such as Africa, China, Indonesia, Mexico, Honduras, Colombia and Brazil and account for 75% of worldwide production (Azevedo-Santos *et al*., 2011). Tilapia fish is nutritious and forms a healthy part of a balanced diet to human being that is high in protein as much of required protein as in meat (Foh *et al.*, 2011). The tilapias are considered suitable for culture, because of their rapid growth and palatability, high tolerance to adverse environmental conditions, resistance to disease, excellent quality of its firmly texture flesh and finely appetizing fish to consumers (Jhingram, 1987; Mohamed *et al.*, 2011).

1.2.2 Fish Enzyme

Fish processing generates large amount of waste. Fish processing waste, especially viscera, head, bones and frames, stick-water and effluent from processing account for more than 70% of the total weight of some aquatic animals. Fish viscera are non-edible parts and seem to be a rich source of digestive enzyme, especially proteases (KlomKlao *et al*., 2007) that are highly valued in a wide range of industrial applications and processes (Simpson *et al*., 1991).

1.2.2.1 Protease

Proteases may be classified base on their similarities to well characterized proteases, as trypsin like, chymotrypsin-like, etc., their pH activity profiles as acid, neutral or alkaline proteases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994). Proteases are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors.

Proteases are classified according to their source (animal, plant and microbial), their catalytic action (endopeptidase or exopeptidase) and the nature of the catalytic site. Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, but exopeptidases hydrolyze one amino acid from N terminus (amino peptidase) or C terminus (carboxypeptidases).

a. Endopeptidases

Endopeptidases or endoproteinases are proteolytic peptidases that break peptide bonds of nonterminal amino acids (i.e. within the molecule). Endopeptidases cannot break down peptides into monomers. A particular case of endopeptidase is the oligopeptidase, whose substrates are oligopeptides instead of proteins. They are usually very specific for certain amino acids. Examples of endopeptidases include trypsin, chymotrypsin, elastase, thermolysin and pepsin.

b. Exopeptidases

Exopeptidases are proteolytic peptidases enzyme that catalyze the cleavage of the terminal (last) or next-to-last peptide bond from a polypeptide or protein, releasing a single amino acid or dipeptide. Examples of exopeptidases include carboxypeptidase, metallo carboxypeptidase and cysteine carboxypeptidase.

The International Union of Applied Biochemists classified that proteases from fish and aquatic invertebrates may be classified into four major groups (Simpson, 2000) include acid proteases, serine proteases, cysteine proteases and metalloproteases.

1.2.2.1.1 Acid proteases

The acid or aspartyl protease have been described as a group of endopeptidase characterized by high activity and stability at acid pH. They are referred to as “aspartyl” proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994). Three common acid protease that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin, and gastricsin (Simpson, 2000).

Among these acid proteases, pepsin is the major enzyme for this group found in fish viscera. Pepsin is secreted as a zymogen (pepsinogen) activated by acid in stomach (Clarks *et al.*, 1985). Pepsin prefers specifically the aromatic amino acids phenylalanine, tyrosine and tryptophan. Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35 kDa (Simpson, 2000). However, the pepsins from marine animals were reported to have molecular weights ranging from 27 to 42 kDa (Klomklao, 2008). Pepsin activity is very dependent on pH values, temperatures and type of substrate. Pepsin from polar cod stomach showed a maximal activity against hemoglobin at pH 2.0 and 37°C (Arunchalam and Haard, 1985). Gildberg *et al.* (1990) reported that the optimal pH of Atlantic cod pepsin for hemoglobin hydrolysis was 3.0. Pepsin is quite stable from pH 2 to 6 but it rapidly loses activity at pH above 6 due to the denaturation (Simpson, 2000). Pepsin from sardine stomach was stable between pH 2 and 6 and showed harsh loss of activity at pH 7.0 (Noda and Murakami, 1981). Castillo-Yanez *et al.* (2004) found that Monterey sardine acidic enzymes were stable at pH ranging from 3.0 to 6.0.

1.2.2.1.2 Serine proteases

The serine proteases have been explained as a group of endopeptidase with a serine residue together with the imidazole group and an aspartyl carboxyl group in their catalytic site (Simpson, 2000). Serine protease exhibits high activity under alkaline rather than neutral pH and sensitivity to serine protease inhibitors (Simpson, 2000). The common serine proteases have been recovered from digestive glands of marine animals are trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.11) (Klomklao, 2008).

Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine, while chymotrypsin has a much broader specificity for amino acids with bulky side chains and nonpolar amino acid such as tyrosine, phenylalanine, tryptophan and leucine. Trypsins from marine animals tend to be more stable at alkaline pH, but are unstable at acidic pH. On the other hand, mammalian trypsins are most stable at acidic pH (Simpson, 2000; Klomklao *et al.*, 2006). Trypsins from marine animals are similar to mammalian trypsins with respect to their molecular size (22-30 kDa), amino acid composition and sensitivity to inhibitors. Their pH optima for the hydrolysis of various substrates were from 7.5 to 10.0, while their temperature optima for hydrolysis of those substrates ranged from 35 to 65°C (De Vecchi and Coppes, 1996). Trypsin from tongol tuna spleen showed the high stability in the pH range of 6-11, but the inactivation was more pronounced at pH values below 6 (Klomklao *et al.*, 2006). The stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez *et al.*, 2005). Trypsin might undergo the denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly (Klomklao *et al.*, 2006).

Chymotrypsins have been isolated and characterized from marine species such as anchovy (Heu *et al.*, 1995) and Monterey sardine (Castillo-Yanez *et al.*, 2006). In general, these enzymes are single-polypeptide molecules with molecular weights between 25 and 28 kDa. They are most active in the pH range of 7.5 to 8.5 and are most stable at pH 9.0 (Simpson, 2000). Chymotrypsin has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine (Simpson, 2000).

1.2.2.1.3 Cysteine proteases

Cysteine or thiol protease are a group of endopeptidase that have cysteine and histidine residues as the essential groups in their catalytic sites. The sample of cysteine protease from the digestive glands of marine animals is cathepsin B (EC 3.4.22.1) (Simpson, 2000).

1.2.2.1.4 Metalloproteases

The metalloproteases are hydrolytic enzymes whose activity rely on the presence of bound divalent cations. The metalloproteases have been studied from marine animals such as rockfish, carp, and squid mantle but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000).

1.2.2.2 Amylase

Amylases are starch degrading enzymes that catalyzes starch into sugars. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4-glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Aiyer, 2005). They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved.

Amylases are divided into endo-amylase (α-amylase, EC 3.2.1.1) and exo-amylase (β-amylase, EC 3.2.1.2 and γ-amylase, EC 3.2.1.3) and are widely distributed in animals, plants and microbes. They can hydrolyze starch on non-reducing ends into mono-, di-, tri- and oligo- dextroses, and are usually used to produce syrup, wine and fermented foods.

1.2.3 Enzyme from fish viscera

Recently, there has been increasing demand for proteolytic enzymes in the pharmaceutical and food biotechnology industries. Proteases are mainly derived from plants, animals, and microbial sources. However, their marine and aquatic counterparts have not been used extensively. One of the most important by-products in fishery processing is the viscera, and it has wide biotechnological potential as a source of digestive enzymes (Rawdkuen *et al.*,2012).

Fish viscera are known to be a rich source of digestive enzymes. The recovery of proteolytic enzymes from fish viscera represents an interesting alternative when the aim is to minimize the economic losses and ecological hazards caused by this

waste (Bougatef *et al.*, 2007). Fish viscera were reported to be used as a source of enzymes particularly proteolytic digestive enzymes or serine protease (Heu *et al*., 1995). Among industrial enzymes, proteases are most widely used and account for 60% of the industrial enzymes quantity (Haard, 1992). Commercial protease has been used in seafood processing plant for production of fish extract in Southern Thailand.

Hidalgo *et al.* (1999) compared the activities of proteolytic and amylase enzyme in six species of fish with different nutritional habits: rainbow trout, gilthead seabream, European eel, common carp, goldfish and tench showed in Table 1.

Table 1 Total proteolytic and amylase activities in liver and digestive tract of the different species of fish, determined at 37°C.

Species	Liver		Digestive tract	
	Proteolytic activity (U/mg protein)	Amylase activity (U/mg protein)	Proteolytic activity (U/mg protein)	Amylase activity (U/mg protein)
Carp	1.045 ± 0.436	107.96 ± 7.32	2.50 ± 0.62	72.53 ± 8.46
Goldfish	0.072 ± 0.011	23.80 ± 4.19	2.01 ± 0.11	75.47 ± 15.76
Tench	0.788 ± 0.108	13.13 ± 1.32	1.70 ± 0.29	19.37 ± 2.67
Seabream	0.082 ± 0.012	2.66 ± 0.38	0.81 ± 0.24	1.75 ± 0.28
Trout	0.124 ± 0.006	0.00 ± 0.00	3.44 ± 0.45	1.30 ± 0.07
Eel	0.017 ± 0.008	0.76 ± 0.08	0.46 ± 0.05	1.40 ± 0.07

Total proteolytic activity was obtained as the sum of those determined at pH 1.5, 3, 4, 7, 8.5, 9 and 10. Amylase activity was determined at pH 7.5. Values are mean ± SEM of five observations.

Source: Hidalgo *et al.* (1999)

1.2.4 Isolation of enzyme

1.2.4.1 Isolation of fish digestive protease

Fish viscera or internal organs are a relatively large portion of the animal round weight; approximately 5% (Gildberg, 1992). Fish digestive organs are the important source of enzymes, especially proteases. Digestive proteases from marine animals are produced by digestive glands of marine animals. Digestive proteases from marine animals are hydrolytic in their action and catalyze the cleavage of peptide bonds with the participation of water molecule as reactants (Klomklao, 2008). Digestive proteases have been studied in several species of fishes. Proteases found in the viscera

of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase (Haard, 1994). Pepsin and trypsin are two main groups of protease found in fish viscera. Acidic proteases from fish stomachs display high activity between pH 2.0 and 4.0, while alkaline digestive proteases are most active between pH 8.0 and 10.0 (Hayet *et al.*, , 2011). There are summarized in Table 2.

Beltagy *et al.* (2004) studied the extraction of acidic protease from the digestive tracts (viscera) of bolti fish (*Tilapia nilotica*) defatted with acetone. The acetone dried powder of viscera was extracted with distilled water (1:20, w/v) for 1 h and adjusted the supernatant to pH 2.5, precipitated from the resulting extract by ammonium sulfate followed by dialysis. The crude enzyme was purified using gel filtration; the enzyme showed the highest activity and purification-fold when precipitated at 40–60% ammonium sulfate and showed a molecular weight of 31.0 kDa. The optimal pH and temperature were 2.5 and 35°C. The enzyme showed pH stability between 2 and 6. It retained more than 50% of its activity after heating between 50 and 60°C for 30 min, and 40.2 and 74.9% after heating between the same temperatures for 120 min. While the extraction of alkaline protease was studied by Bezerra *et al.* (2005) from the intestine of Nile tilapia, *Oreochromis niloticus*. Forty mg of tissue/ml (w/v) in 0.9% (w/v) NaCl was homogenized by using a tissue homogenizer. The resulting preparation was centrifuged at 10,000 xg for 10 min at 10°C to remove cell debris and nuclei. The supernatant (crude extract) presented a yield of 30% and showed a single band by SDS-PAGE (23.5 kDa). The optimum temperature was found at 50°C and it was stable for 30 min at 50°C. The optimum pH of 8.0 was noticeable.

Table 2 Thermal stability, pH stability and kinetic properties of digestive proteases from fish[a].

Enzyme	Identified species	Optimum Temperature (°C)	Optimum pH	k_m value[b] (Mm)	Arrhenius Activation Energy (kcal/mol)	Reference
Protease I	Sardine	55	4.00	-	-	Noda and Murakami (1981)
Protease II	Sardine	40	2.00	-	-	Noda and Murakami (1981)
Pepsin I	Arctic capelin	38	3.70	-	-	Gildberg and Raa (1983)
Pepsin II	Arctic capelin	43	2.50	-	-	Gildberg and Raa (1983)
Pepsinogen	Rainbow trout	37	3.00	-	9.13	Twining *et al.* (1983)
Pepsinogen A	Polar cod	37	3.75	0.060	3.20	Arunchalam and Haard (1985)
Pepsinogen B	Polar cod	37	4.75	1.330	2.90	Arunchalam and Haard (1985)
Protease I	Orange roughy	37	2.50	0.124	-	Xu *et al.* (1996)
Protease II	Orange roughy	37	3.50	0.517	-	Xu *et al.* (1996)
Pepsin	Palometa	37	3.50	-	-	Pavlisko *et al.* (1997)
Pepsin	Greenland cod	30	3.00-3.50	1.140	4.70	Haard *et al.* (1982)
Pepsin	Greenland cod	-	3.50	0.860	-	Squires *et al.* (1986)
Pepsin	Arctic cod	32	3.00-3.50	0.400	4.10	Haard *et al.* (1982)
Pepsin I	Atlantic cod	40	3.50	0.175	-	Gildberg *et al.* (1997)
Pepsin IIa	Atlantic cod	40	3.00	0.033	-	Martinez and Olsen (1989)

[a] Experiment conditions of measurements used by different authors are not the same; therefore, attention should be made when making comparison.
[b] K_m = Michaelis constant.

Source: Shahidi and Kamil (2001)

Bougatef *et al.* (2007) isolated of trypsin from the viscera of *Sardina pilchardus* by homogenized with 500 ml of 10 mM Tris– HCl pH 8.0 and 10 mM $CaCl_2$ for 30 s, and centrifuged at 10,000 g for 15 min at 4°C. The supernatant was collected and purified by fractionation with ammonium sulphate, heat treatment and Sephadex G-100 gel filtration was estimated to be 25,000 Da on SDS–PAGE. This enzyme showed esterase specific activity on Na-benzoyl- L-arginine ethyl ester. The optimum pH and temperature for the enzyme activity were pH 8.0 and 60°C, respectively. And the enzyme showed pH stability between 6.0 and 9.0. And a novel aspartic protease was extracted from the defatted viscera of sardinelle (*Sardinella aurita*) with two volumes of cold acetone for 30 s in a tissue homogeniser (Hayet *et al.*, 2011). The acetone dried powder was then suspended in 50 mM phosphate buffer, pH 7.0, at a ratio of 1:10 (w/v) and stirred continuously for 3 h at 4°C. The mixture was centrifuged at 13,000 g for 20 min at 4°C to remove the tissue debris. The supernatant was adjusted to pH 3.0 by adding 0.1 M HCl. The acidified extract was incubated for 45 min at room temperature and then centrifuged at 13,000 g for 20 min to remove precipitated debris. The

supernatant obtained was referred to as crude acidic protease with 23.3% recovery had 17 kDa of the molecular weight. The optimum pH and temperature for protease activity were around 3.0 and 40°C, respectively. The enzyme showed pH stability between 2.0 and 5.0 and retained more than 50% of its activity after heating for 30 min at 50°C. Unajak *et al.* (2012) reported that trypsin from intestinal extracts of Nile tilapia (*Oreochromis niloticus L.*) with three-step purification by ammonium sulphate precipitation, Sephadex G-100, and Q Sepharose – was applied to isolate trypsin, and resulted in 3.77% recovery with a 5.34-fold increase in specific activity. Only one major trypsin isozyme was isolated with high purity appearing as a single band of approximately 22.39 kDa protein. The purified trypsin was stable, with activity over a wide pH range of 6.0–11.0 and an optimal temperature of approximately 55–60°C.

1.2.4.2 Isolation of fish digestive amylase

Herbivorous fishes often exhibit higher carbohydrase activities, apparently to digest the storage carbohydrates of macroalgae, which can contain up to 50% carbohydrate, whereas carnivorous fishes frequently show higher proteolytic enzyme activities, to digest their high-protein animal diets (German *et al.* 1986). Tongsiri *et al.* (2010) studied the activity of amylase in stomach and intestine of the Mekong giant catfish (*Pangasianodon gigas*), put in dry ice immediately and added into a centrifuge tube containing phosphate buffer pH 7 for homogenization and centrifuged at 10,000 x g at 4ºC for 10 min and the supernatant was collected. The amylase enzyme from the stomach and intestine of the Mekong Giant Catfish were studied at pH 2-12 and at temperatures between 25-80ºC. Amylase activities of the stomach were alkaline amylase and the optimal temperatures to be 25 and 50ºC. Amylase activities of the intestine were neutral amylase as well as alkaline amylase and the optimal temperature range was at 25-30ºC.

Nile tilapia, which were previously known to be able to be fed with a high carbohydrate content diet, are certainly able to digest dietary starch by their own. Only three tissues liver, mesenteric tissue and intestine were found to contain any significant amount of amylase. No activity was recovered from the stomach (Moreau *et al*., 2001). α-Amylases from the intestinal cavity of two tilapia species, *Oreochromis niloticus* and *Sarotherodon melanotheron*, are active similar to those of human and

porcine pancreatic α-amylase (Al Kazaz *et al*., 1996). Moreau *et al.* (2001) extracted α-amylases from the intestinal cavity of these two tilapia species and purified using ammonium sulfate precipitation. The purification was approximately 100-fold. The molecular mass of the enzyme differs slightly, 56.6 and 55.5 kDa of *O. niloticus* and *S. melanotheron*, respectively. Only three tissues (liver, mesenteric tissue and intestine) were found to contain any significant amount of amylase. No activity was recovered from the stomach. Although large inter-individual differences in the total activity (15-48 μmol min^{-1} g^{-1}) were observed, the activity was found to be distributed in roughly equal amounts among all the tissues from the eight fishes (*S. melanotheron*) studied (Figure 1).

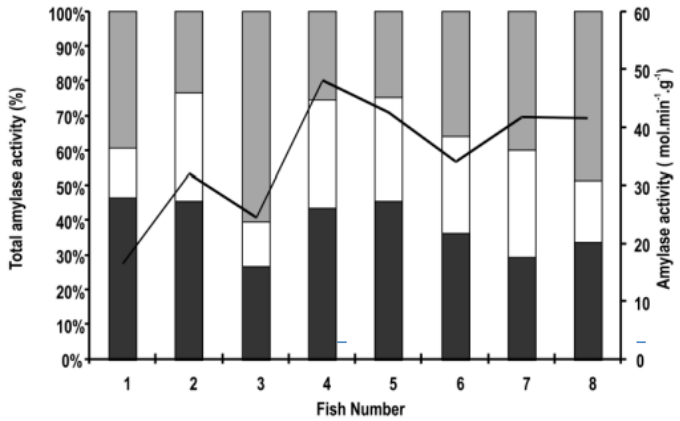


Figure 1 Distribution histogram of amylase activity between liver, intestine and mesenteric tissue from 8 tilapias (*Sarotherodon melanotheron*). (▪) Liver, (▫) intestine, (grey box) mesenteric tissue. The solid line indicates the total activity in each fish. Activities are given per gram of fresh tissue

Source: Moreau *et al.* (2001)

1.2.5 Stability of enzyme

1.2.5.1 Effect of pH

Enzymes are affected by changes in pH. The most favorable pH value - the point where the enzyme is most active is known as the optimum pH. This is graphically illustrated in Figure 2**.** Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of

enzymes. As with activity, for each enzyme there is also a region of pH optimal stability.

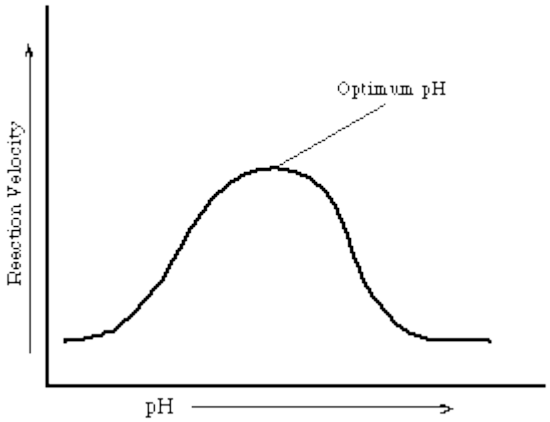


Figure 2 Effect of pH on reaction rate.

Source: Martinek (1969)

1.2.5.2 Effect of temperature

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. As shown in Figure 3, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most animal enzymes rapidly become denatured at temperatures above 40°C, most enzyme determinations are carried out somewhat below that temperature. Bezerra *et al*.,(2005) studied thermal stability of Nile tilapia *(Oreochromis niloticus)* intestine trypsin by assaying its activity after pre-incubation for 30 min at the indicated temperatures. The values (mean S.D.) were expressed as percentage of the highest one (Figure 4).

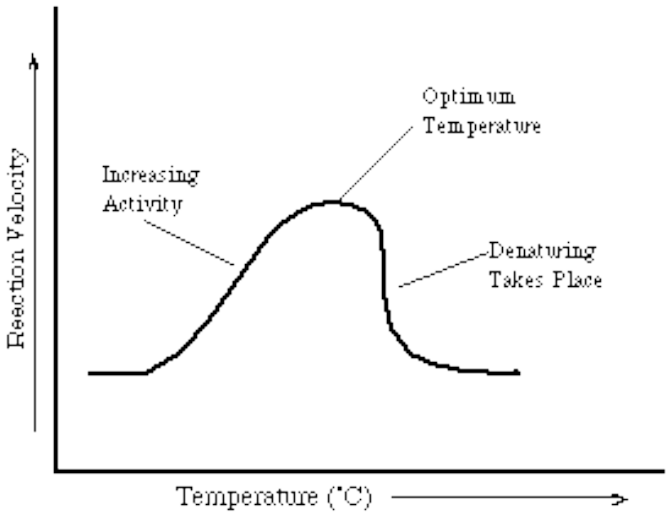


Figure 3 Effect of temperature on reaction rate.

Source: Martinek (1969)

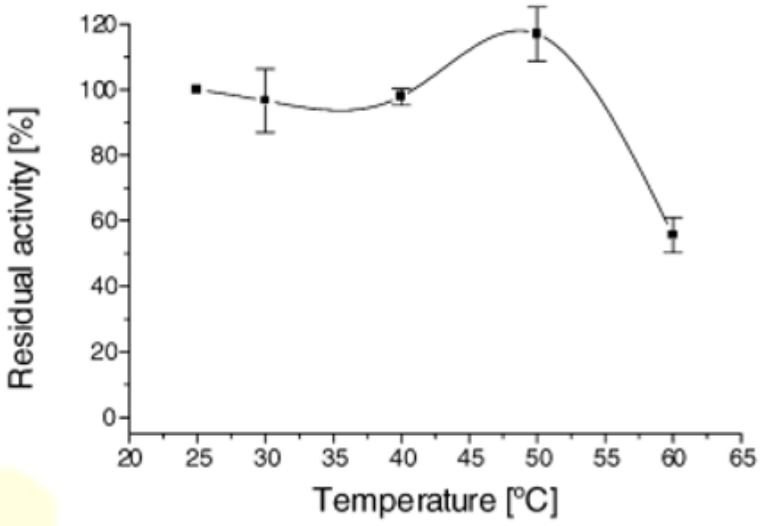


Figure 4 Thermal stability of *O. niloticus* intestine trypsin.

Source: Bezerra *et al*. (2005)

1.2.6 Enzyme Purification

There have been many reports about the purification of proteolytic enzymes from fish viscera by various separation techniques. Typically, reports of extraction and purification of proteases describe salt and organic precipitation, chromatography, or phase separation by an aqueous two-phase system. Most of these operations are time consuming, difficult to scale up, involve expensive reagents, and require technical skill.

Enzymes, groups of proteins, are commonly isolated from tissue samples and purified for experimental use. The experimental methods can enable the understanding of cell function and metabolic pathways. Chromatography and other methods along with adjusted conditions of pH, salt concentration, etc., are chosen based on the types of enzymes being purified. The first method of purification is using the precipitation technique based on salt concentration. Ammonium sulfate is a common solution used in this method. Usually the protein or enzyme solution is brought to a 50 percent saturation with a saturated ammonium sulfate solution. Due to the salt balance, the proteins will coagulate and precipitate to the bottom when centrifuged at a high speed for approximately 15 minutes.

Chromatography methods include ion exchange, bio-affinity and hydrophobicity. Ion exchange uses molecular charge, and bio-affinity uses biomolecular interaction. Initial preparation for such methods includes the lysing of cells and centrifugation for a pure supernatant. Supernatants contain the appropriate isolated enzymes and can be further purified by one of the above chromatography methods. Gel filtration is also a method of chromatography based on the molecular weight of the desired enzyme sample. The method uses beads that come in various pore sizes. The pores of these beads hold the appropriate proteins that are intended to be filtered. Once all of a sample is run through, the beads are extracted for the purified enzymes.

1.2.6.1 Ammonium sulphate precipitation of protein

The most common type of precipitation for proteins is salt induced precipitation. Different types of salts such as ammonium sulphate and sodium sulphate are widely used to precipitate out proteins. Ammonium sulphate is the most widely used salt for the precipitation of proteins as it is highly soluble, inexpensive, available in highest purity level, does not change the protein solution to extreme pH and in most of case it does not denature proteins. Ammonium sulphate can be used for precipitation of total proteins at ~90% saturation or for differential precipitation level of proteins using different saturation of salts. Up to 20% saturation, ammonium sulphate precipitate particulate materials, and preaggregated and very high molecular weight proteins and at 90% saturation it precipitates almost all proteins (Asenjo, 1990).

Proteins have polar amino acids such as glycine, serine etc. Usually in native proteins hydrophilic amino acids are on the surface of proteins whereas hydrophobic amino acids are buried. Attractive interactions between the nearby oppositely charged groups are ion pairs or salt bridges. Analysis has revealed that in folded proteins, 4 attractive ion pairs and 1 repulsive ion pair are present per100 amino acids. Water as powerful solvent, interacts with these surface amino acids and keep them in solution (Janson and Lars, 1989). Protein solubility depends on several factors. It is observed that at low concentration of the salt, solubility of the proteins usually increases slightly. This is termed “Salting in”. But at high concentrations of salt, the solubility of the proteins drops sharply. This is termed “Salting out” and the proteins precipitate out. During ammonium sulphate precipitation the salt has to be added in small amount under constant stirring to avoid accumulation of high concentration of salts. When large amount of salt is added to an aqueous solution of proteins the salt requires more amount of water for its dissolution. This leads to competition for water molecule on the proteins. Completely ionized salts have more affinity for water molecules then protein hence addition of salts takes up water molecule from the protein. Therefore the ionic interactions between water molecules and protein are reduced and as result hydrophobic interactions dominate. The hydrophobic amino acid patches present in all the proteins attract each other and forms aggregates. These aggregates are nothing but the proteins in the form of precipitates (Asenjo, 1990; Janson and Lars,

1989). In salt precipitation, the anions appear to be more significant. Temperature, pH and the most important the protein concentration affect ammonium sulphate precipitation of proteins to large extent. Higher ammonium sulphate is required for precipitating highly soluble proteins (Janson and Lars, 1989).

There have been reported about the purification of fish viscera enzyme by many researcher. The crude acidic protease from viscera of Sardine was purified by 0–80% saturation of ammonium sulphate precipitation and dialysed for 24 h at 4°C. The precipitate was then successively subjected to Sephadex G-100 gel filtration, Mono-S cation-exchange chromatography, ultrafiltration and Sephadex G-75 gel filtration. After the final purification step, the aspartic protease was purified 9.47-fold, with a recovery of 23.3% and a specific activity of 28.41 U/mg, using haemoglobin as a substrate (Table 3) (Hayet *et al.,* 2011).

Table 3 Summary of the purification of the acidic protease from *S. aurita* viscera.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extract	214	70	3	100	1
Acidification	200	55	3.63	93.45	1.21
$(NH_4)_2SO_4$ (0-8%)	162	34	4.76	75.7	1.58
Sephadex G-100	117	14	8.35	54.6	2.78
Mono-S Sepharose	80	5	16	37.38	5.33
Ultrafiltration	64	3	21.33	29.9	7.11
Sephadex G-75	50	1.76	28.41	23.3	9.47

All operations were carried out at 4°C. Protease activity was assayed at pH 3.0 and 40°C for 15 min using haemoglobin as a substrate.

Source: Hayet *et al.* (2011)

1.2.6.2 Diafiltration

In order to obtain a product with the desirable purity, a specialized operation involving membrane filtration, called diafiltration has been applied. Diafiltration is term used reflecting tangential flow filtration (TFF) method of "washing" or removing permeable molecule (impurities, salts, solvents, small proteins, etc) from a solution in an accelerated method when compared to dialysis tubing (Beaton and Klinkowski, 1983). In diafiltration the feed volume is kept constant by adding fresh

solvent (such as water or buffer). Feed is diluted with added solvent to reduce the concentration of permeable components and to remove them by passing this through a membrane. Therefore, the purity of the retained components should be further increased (Li *et al.*, 2009). The process of diafiltration generally includes three steps. These are a pre-concentration step, a diafiltration step and a post-concentration step. The selection of an appropriate membrane, the membrane pores must be large enough to allow the permeable species to pass through (when fractionating) and small enough to retain the larger species. A large variety of membranes are available in the ultrafiltration and microfiltration range for this purpose (Beaton and Klinkowski, 1983). Li *et al.* (2009) collected and used as feed for ultrafiltration, a polysulphone hollow fiber membrane with a molecular weight cutoff of 30 kDa, a fiber diameter of 1 mm, a flow length of 30 cm and an effective area of 0.01 m^2, TMP of 1.6 bar, at ambient temperature (27°C) for pretreated tuna spleen extract in purification of protease.

1.2.7 Rice bran

Rice bran is the hard outer part of the grain that consists of aleurone, a form of protein found in the protein granules of maturing seeds, as well as pericarp, the outer and edible layer of the rice kernel (Figure 5). Apart from these two, it also contains germs and endosperm of the rice kernel. Rice bran is obtained as a by-product during the rice milling process and the outer layers are removed at the time of whitening or polishing (the conversion of brown rice to white rice) of the husked rice.

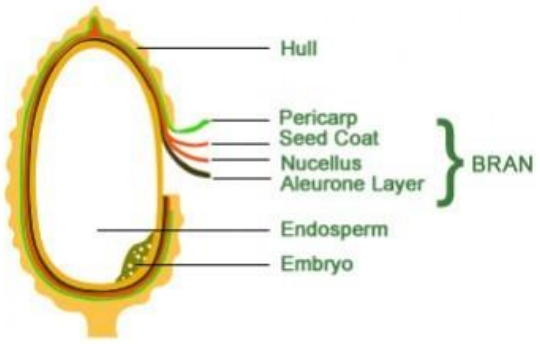


Figure 5 Rice seed composition.

However, since rice bran oil was promoted for its health benefits (Seetharamaiah and Chandrasekhara, 1989; Ausman *et al*., 2005; Zigoneanu *et al*., 2008), the demand for milled rice bran is increasing in the worldwide market and the latent demand estimated in the US in 2011 is 99.18 million dollars (Parker, 2005). Defatted rice bran is a by-product of rice bran oil extraction from whole rice bran. As a by-product, defatted rice bran is actually an excellent source of protein, minerals, dietary fiber and other components (Abdul-Hamid and Luan, 2000).

Rice bran composes of many nutritious substances like protein, fat, fiber, various antioxidants, etc. that have a beneficial effect on human health. Because of its multi-nutritional properties, rice bran is being consumed by humans for thousands of year. A major rice bran fraction contains 12%-13% oil and highly unsaponifiable components (44.3%). This fraction also contains tocotrienols (a form of vitamin E), gamma-oryzanol, and beta-sitosterol; all these constituents may contribute to the lowering of the plasma levels contained in the lipid profile. Rice bran also contains a high level of dietary fibers like beta-glucan, pectin, and gum. The oil present in the rice bran is a rich source of vitamin E, vitamin B, minerals and other essential acids. The composition of rice bran is showed in Table 4.

Table 4 Percent composition of whole barley, β-glucan-enriched barley fraction (GEB), rice bran, and oat bran.

Cereal	Total dietary fiber	Soluble dietary fiber	β-glucan	Fat	Nitrogen	Dry matter
Dehulled whole Barley	17.2	6.0	5.7	3.1	2.2	91
GEB	43.8	19.8	18.9	3.7	2.1	90.2
Rice bran	22.9	1.4	1.8	20.6	2.4	92.2
Oat bran	18.6	8.0	8.3	7.7	3.6	89.7

Source : Kahlon *et al*. (1993)

Rice bran contains many important nutrients that are essential for the body. The various health benefits associated with the use of rice bran are

- It helps in reducing the level of cholesterol, thereby reducing the risks of heart attacks in human beings (Othman *et al*., 2011).
- The presence of dietary fibers and whole grains helps in preventing the occurrence of Type II diabetes (Liu, 2003).
- Rice bran also helps in reducing high blood pressure as well as intestinal cancer (Most *et al*., 2005).

1.2.8 Beta glucan

Beta-glucan (β-Glucan) is a polysaccharides that contain only glucose as structural components, and is linked with β-glycosidic bonds (Mason, 2001). β-glucans are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria. Some forms of β-glucan are useful in human nutrition as texturing agents and as soluble fiber supplements, but can be problematic in the process of brewing. For decades scientists have known β-glucan as a food constituent, and they knew it was abundant in the foods. It is extremely difficult to extract and purify. However, oat bran contains about 7% β-glucan, and is inexpensive, but only good as a food.

1.2.8.1 Structure of β-glucan

β-glucan have 2 forms structure that are (1→3),(1→4)-β-D-glucan and (1→3),(1→6)-β-D-glucan. The name of (1→3),(1→4)-β-D-glucan is mixed-linkage, belongs to a family of polymers which are heterogeneous with respect to the molecular size and fine structure, varying with tissue, stage of maturity and source (Bacic *et al*., 1988). Mixed-linkage β -glucans are exclusively found in cereal grains (Ford and Percival, 1965). On 21 January 1997, the U. S. federal authority Food and Drug Administration (FDA) published health claim on food-product packages stating that “A diet high in soluble fibre from whole oats and low in saturated fat and cholesterol may reduce the risk of heart disease” (FDA, 1996; FDA, 1997). The beneficial effect of oat products is primary attributed to the soluble dietary fibre compound that is mixed-linkage β -glucans (FDA, 1997).

Mixed-linkage β -glucans is a linear molecule of partially water-soluble polysaccharide consisting of glucose linked composed of (1→4) linked glucopyranosyl (Glcp) residues (approx. 70%) substituted at position 3 or 4 and (1→3)-linked Glcp residues (approx. 30%) substituted at position 4 (Aspinall, 1984) (Figure 6).

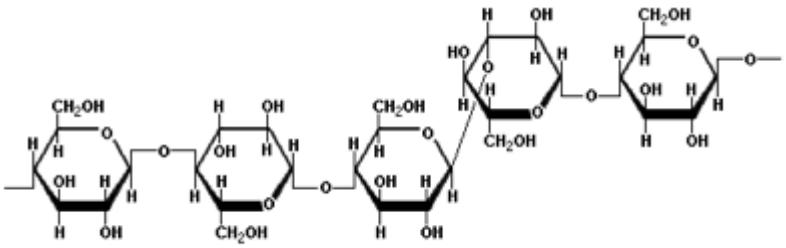


Figure 6 Mixed-linkage β –glucans structure.

Source: Roubroeks *et al*. (2001).

1.2.8.2 Health effect of β –glucans

Mixed-linkage β-glucan is known to reduce blood cholesterol levels. The Food and Drug Administration of the USA has accepted a health claim in which it is stated that a daily intake of 3 g of soluble oat β-glucan can lower the risk of coronary heart disease (FDA, 1997). Oats also reduce the glucose and insuline responses (Johansson, 2006). Ripsin *et al*. (1992) showed in a meta-analysis that oat products in a diet cause a modest reduction in blood cholesterol level. They concluded that a daily intake of 3 g of oat soluble fiber was needed to reduce cholesterol 0.13-0.16 mmol/l. Kalra and Jood (2000) showed that barley β-glucan lowered the levels of total cholesterol, LDL-cholesterol and triglycerides in rats. Cereal β-glucan showed positive physiological effects on the cardiovascular system but also their antibacterial, antitumoral, immunomodulant, and radioprotective properties are mentioned (Havrlentová *et al*., 2011).

1.2.9 Hydrolysate

Protein hydrolysate was obtained by breakdown peptide bond of proteins into peptide fragments with different sizes and free amino acids. It can be achieved by acid, alkaline or enzyme. Acid and alkaline processes can transform L amino acids to D amino acids and it can generate toxic substance include lysinoalanine (Clement, 2000). Enzymatic hydrolysis is performed under a mild condition, pH 6-8 and temperature 40-60°C, that more gently than acids and do not require high temperature and usually target specific peptide bonds (Taherzadeh and Karimi, 2007). There are several advantages and disadvantages of acid and enzymatic hydrolysis, which are lists in Table 5. Using of enzymes is a better control of the hydrolysis process and results in high functionality products, good organoleptic properties and excellent nutritional value. The material that results from a proteolytic digestion is a mixture of amino acids and polypeptides of varying lengths

Table 5 Comparison between acid and enzymatic hydrolyses.

Comparing variable	Acid hydrolysis	Enzymatic hydrolysis
Mild hydrolysis condition	No	Yes
High yield of hydrolysis	No	Yes
Product inhibition during hydrolysis	No	Yes
Formation of inhibitory by-products	Yes	No
Low cost of catalyst	Yes	No
Short time of hydrolysis	Yes	No

Source: Taherzadeh and Karimi (2007)

1.2.10 Antioxidant

Antioxidant is defined as any substance which is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due

to oxidation (Gordon, 2001). In general, antioxidants act by reducing the rate of initiation reaction in the free radical chain reactions (Schafer *et al*., 2002).

1.2.10.1 Classification of antioxidant

1.2.10.1.1 Primary antioxidant

Primary antioxidant terminate the free radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also interact with the lipid radicals, forming lipid antioxidant complexes (Gordon, 2001).

1.2.10.1.2 Secondary antioxidant

Secondary or preventing antioxidant function by decomposing the lipid peroxides into stable end product (Rajalakshmi and Narasimhan, 1996). Synergistic antioxidant can be broadly classified as oxygen scavengers and chelators. Transition metals, such as iron, copper, cobalt etc in foods affect both the rate of autoxidation and the breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one electron donors to form alkoxyl radical (Godon, 2001). Peptides in hydrolysate could chelate the pro-oxidant, leading to decreased lipid oxidation. So, chelation of transition metal ions by antioxidant or antioxidative peptides retarded the oxidationreaction (Sherwin, 1990).

1.2.10.2 Oxidation in biological system

An oxidation-reduction (redox) reaction is a type of chemical reaction that involves a 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 chemically reactive molecules containing oxygen that are highly reactive in redox reaction such as oxygen ions and peroxides. This is a collective term and includes oxygen free radicals and several non-radical agent (Table 6).

Table 6 Reactive oxygen species.

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	$O_2^{\cdot\cdot}$
Superoxide ion	$O_2^{-\cdot}$
Hydroxyl	$OH^{\cdot}$
Peroxyl	$ROO^{\cdot}$
Alkoxyl	$RO^{\cdot}$
Nitric oxide	$NO^{\cdot}$
Nonradical oxygen derivatives	
Hydrogen peroxide	H_2O_2
(Organic peroxide)	ROOH
Hypochlorous acid	HOCL
Ozone	O_3
Aldehydes	HCOR
Singlet oxygen	$^1O_2$
peroxynitrite	ONOOH

Source: Kohen and Nyska (2002)

1.2.11 Inflammation

1.2.11.1 Inflammation

Inflammation is the body's attempt at self-protection; the aim being to remove harmful stimuli, including damaged cells, irritants, or pathogens and begin the healing process**.** When something harmful or irritating affects a part of our body, there is a biological response to try to remove it, the signs and symptoms of inflammation, specifically acute inflammation, show that the body is trying to heal itself. Inflammation does not mean infection, even when an infection causes inflammation. Infection is caused by a bacterium, virus or fungus, while inflammation is the body's response to it (Gould, 2002; Gupta *et al*., 2003). Inflammation is characterized by several familiar signs, redness, swelling, heat, fever, pain and loss of function.

Therefore, the main purpose of inflammation is to identify and eliminate injurious agents and to repair the surrounding tissue. The inflammation response involves several stages include dilation of capillaries to increased blood flow, microvascular structural change and escape of plasma proteins from the blood stream, leukocyte-adhesion cascade, elimination of possible pathogens and resolution of inflammation.

Common acknowledge, inflammation plays important roles in the initiation and progress of many diseases including cancer in multiple organ sites (Schetter *et al*., 2010). Inflammation, classified either acute or chronic, has been described as a basis of many human. Acute inflammation occurs from minutes to hours and days following tissue damage caused by physical force or an immune response. Chronic inflammation occurs over a longer period of time and is cause by pro-inflammation mediators. Chronic inflammation in linked to rheumatoid arthritis, diabetes, atherosclerosis, and cancer (Guo *et al*., 2008). Therefore, inhibition of the production of pro-inflammatory mediators is an important point in the treatment of various inflammatory diseases.

1.2.11.2 Nitric oxide (NO) and nitric oxide synthase (NOS)

Nitric oxide (NO), a colorless gas, has been considered as an important biological regulator which is a fundamental component in the fields of neuroscience, physiology and immunology (Derosa *et al*., 2008; Jiang *et al*., 2012). Nitric oxide (NO) or nitrogen oxide also known as nitrogen monoxide, is a free radical and is a member of the labile radical entities knows as reactive oxygen species (ROS). It is a gaseous signaling molecule that regulates various physiological and pathophysiological response in the human body (Alderton *et al*., 2001). These include circulation and blood pressure, platelet function, host defense and neurotransmission in central nervous system and in peripheral nerves (Korhonen and Pihlanto, 2006). However, NO can act as a cytotoxic reagent in pathological processes, particularly in inflammatory disorder (Alderton *et al*., 2001).

NO is produced by various group of enzyme termed as nitric oxide syntheses (NOS) which are present in body (Brero *et al*., 2010). The synthesis of NO takes place by the conversion of L-arginine to L-citrulline, the reaction being catalyzed

by nitric oxide synthases (NOS) (Boucher *et al*., 1999) (Figure 7). The two cofactors have been found to be involved in this process which includes oxygen and NADPH. Moreover, three isoforms of NOS are present whose names are termed on the basis of their activities, which include following neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Bath *et al*., 2000).

Furthermore, scientists have represented their names by numbers like NOS 1 for nNOS, NOS 2 for iNOS and NOS 3 for eNOS. The isoforms of NOS can be broadly categorized as constitutive (cNOS) and inducible NOS. the cNOS is calcium-dependent and continuously present whereas iNOS is Ca^{2+} independent and has been found to be expressed only after cytokine exposure (Bath *et al*., 2002). Based on this category nNOS and eNOS are constitutively expressed and require elevated levels of Ca^{2+} along with activation of calmodulin in order to produce NO for short period of time. In addition, it has been shown that nNOS and eNOS synthesize NO in response to intracellular Ca^{2+} levels, whereas the reactivity of NOS isoform depend upon their binding with calmodulin (Bath *et al*., 2002). When intracellular Ca^{2+}level is leads to increased production of calmodulin ultimetly leading to augmented binding of calmodulin to eNOS and nNOS which further leads to enhanced production of NO by their enzymes (Sánchez *et al*., 2012).

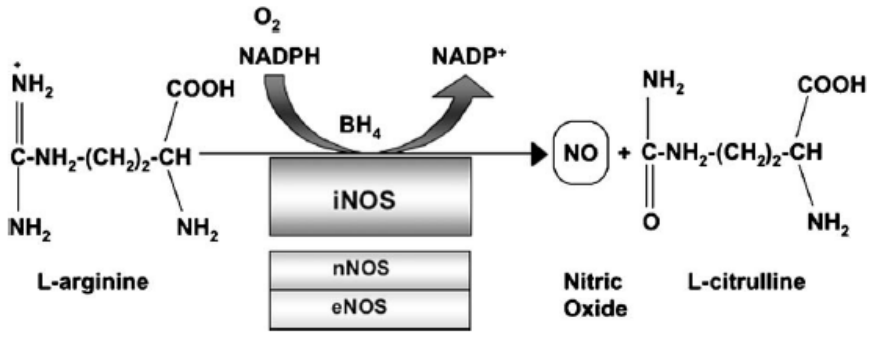


Figure7 Synthesis of nitric oxide by NOS.

Source: Karpuzoglu and Ahmed (2006)

The endothelial nitric oxide synthase (eNOS) is present in endothelium, which is known to play an important role in the dynamic control of vascular tone. The

neural nitric oxide synthase (nNOS) is mainly present in neural tissue and serves as a neurotransmitter. eNOS and nNOS are constitutive isoforms of NOS and are also known as cNOS. iNOS-derived nitric oxide is principally generated from cells of the macrophage–monocyte lineage such as monocytes, macrophages (Assreuy *et al*., 1994), peritoneal macrophages (Matsuura *et al*., 2003), microglia, and Kupffer cells (Karpuzoglu and Ahmed, 2006). iNOS derived nitric oxide has also been reported in certain T (Taylor-Robinson, 1997) and B cell lines (Koide *et al*., 2003), optic nerve astrocytes (Neufeld and Liu, 2003), hepatocytes, neutrophils, vascular smooth muscle cells, and endothelial cells (Bogdan, 2000).

Infected or activated macrophages by such as bacterial lipopolysaccharide (LPS) produce high levels of iNOS-derived nitric oxide. iNOS is induced upon activation (of mostly macrophages or monocytes) in response to inflammatory cytokines such as IFN-γ, IL-1β, and TNF-α (Bogdan, 2001).

1.2.12 Cytokine

Cytokines are small secreted proteins released by cells have a specific effect on the interactions and communications between cells. Cytokine is a general name, other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (Zang *et al*., 2007).

Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Cytokines may be produced in and by peripheral nerve tissue during physiological and pathological processes by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells. Following a peripheral nerve injury, macrophages and Schwann cells that gather around the injured site of the nerve secrete cytokines and specific growth factors required for nerve regeneration. Localized inflammatory irritation of the dorsal root ganglion (DRG) not only increases pro-inflammatory cytokines but also decreases anti-inflammatory cytokines (Xie *et al*., 2006). Cytokines can also be synthesized and released from the

herniated nucleus pulposus, synthesized inside the spinal cord (DeLeo *et al*., 1996) the DRG soma (Schafers *et al*., 2003) or the inflamed skin (Heijmans *et al*., 2006). Furthermore, cytokines may be transported in a retrograde fashion from the periphery, via axonal or non-axonal mechanisms, to the DRG and dorsal horn, where they can have profound effects on neuronal activity (Ozaktay *et al*., 2006) and therefore contribute to the etiology of various pathological pain states.

1.2.12.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological pain (Zang *et al*., 2007).

a. TNF-α

Tumor necrosis factor alpha (TNF-α) also known as cachectin is a multifunctional cytokine that can regulate many cellular and biological processes for instance immune function, inflammation, cell differentiation, proliferation, apoptosis and energy metabolism (Bazzoni and Beutler, 1996; Cawthorn and Sethi, 2008). TNF-α is a potent pro-inflammatory cytokine released primarily from stimulated macrophages. TNF-α was originally identified and isolated for two known characteristic activities, the ability to induce hemorrhagic necrosis of certain tumors and the ability to induce cachexia during states of chronic infection (Crisafulli *et al.*, 2009). TNF-α now represents a key mediator of inflammatory responses. Many aspects of tissue damage following acute or chronic inflammatory reactions can be directly attributed to the concomitant induction of TNF biosynthesis and release, and provide the therapeutic rationale for developing TNF antagonists (Crisafulli *et al.*, 2009).

b. IL-1β

Interleukin-1 beta (IL-1β) also known as catabolin, is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation,

differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 (Dinarello, 1994).

IL-1β has been described in endothelial cells (Warner *et al*., 1987) and in keratinocytes (Nylander and Egelrud, 1997). Low-dose administration of this cytokine induces local inflammatory responses followed by activation of protective immunity, whereas high-dose administration causes broad inflammation accompanied by tissue damage and tumor invasiveness (Apte and Voronov, 2002)

c. IL-6

Interleukin-6 (IL-6) is a multifunctional cytokine that plays key roles not only in the immune system but also in a variety of biological processes. IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine (Akira *et al*., 1990). IL-6 is a primary regulator of both acute and chronic inflammations. It has a dual effect; at some levels it acts as a defense mechanism but in chronic inflammation it is rather pro-inflammatory (Gabay, 2006).

IL-6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. IL-6 also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae* (Van der Poll *et al*., 1997). IL-6 is a pro-inflammatory cytokine that signals via binding to a soluble or membrane bound receptor, while nitric oxide (NO), an oxidative stress molecule, diffuses through the cell membrane without a receptor. Both mediators signal through different mechanisms, yet they are dependent on nuclear factor kappa B (NFκB) (Maalouf *et al*., 2010).

IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier (Banks *et al*., 1994) and initiating synthesis of PGE_2 in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilization that leads to increased body temperature. IL-6 can be secreted by macrophages in response to

specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs).

1.3 Objective

1. To optimize the extraction of crude enzyme from Nile tilapia viscera.
2. To prepare the enzyme extract from tilapia viscera with active protease and amylase activities.
3. To elucidate the characteristic and property of crude enzyme extract from tilapia viscera
4. To prepare rice bran hydrolysate from defatted rice bran by using crude tilapia enzyme extract.
5. To investigate anti-inflammatory activity of rice bran hydrolysate in RAW 264.7 macrophage cell lines.

CHAPTER 2

EXTRACTION OF CRUDE ENZYME FROM NILE TILAPIA (*Oreochromis niloticus*) VISCERA

2.1 Abstract

Nile tilapia (*Oreochromis niloticus*) viscera extracts were prepared using water, NaCl solution and acetone homogenization. The saline solution (5% w/v) yielded the extract with the highest protease and amylase specific activities of 1,300 and 1,800 unit/mg protein, respectively. These two enzymes were active at the same optimum pH (8.0) and temperature (60˚C). At this condition, protease activity was gradually lost and remained at 50% of its initial value after 30 min. In contrast, amylase activity had risen 5 times before declining to about 3 times of its initial activity after 90 min. Protease and amylase could not be successfully separated by 30 kDa molecular cut-off ultrafiltration. Precipitation of non-enzyme protein by using 30 or 35% ammonium sulphate effectively improved specific activity of both enzymes of the extract. This partial purified fraction and the crude exhibited three protein bands by using SDS-PAGE assigned as 26.5, 37.3 and 56.4 kDa. And these protein bands showed the activity of protease (570.28 unit/ml), lichenase (702.04 unit/ml) and amylase (3,079.17 unit/ml), respectively. Therefore, the results revealed that crude enzymes extract could be prepared from tilapia viscera.

2.2 Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most important fish species in Thailand's aquaculture (Nurit, 2012). The mass industrial processing of Nile tilapia, frozen fillets as a priority, generates large amount of waste especially viscera (Nurit, 2012; Freitas-Júnior *et al*., 2012). Fish viscera have been reported to be a good source of digestive enzymes (Simpson *et al*., 1991; Bezerra *et al*., 2005; Khantaphant and Benjakul, 2010; Klomklao *et al*., 2008) and their properties are highly valued in a wide range of industrial applications and processes since some proteases are stable and active under harsh conditions (high temperature and pH) and in the presence of oxidizing agents or surfactants (Klomklao *et al*., 2005). Proteases are primary enzymes which have been isolated and characterized from various parts of Nile tilapia digestive tract (Tengjaroenkul *et al*., 2000, Hinsui *et al*., 2006). Enzyme from Nile tilapia viscera is however likely to contain more than one type of enzyme. Nile tilapia is a herbivorous fish. Its digestive system displays greater activity of amylase than protease and a lesser lipase activity (Tengjaroenkul *et al*., 2000).

Fish digestive proteases are hydrolytic in their action. Proteases found in the viscera of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase (Haard, 1994). Amylases from the intestinal cavity of tilapia species, *Sarotherodon melanotheron*, are active similar to those of human and porcine pancreatic α-amylase (Al Kazaz *et al*., 1996). Only three tissues (liver, mesenteric tissue and intestine) were found to contain any significant amount of amylase. No activity was recovered from the stomach (Moreau *et al.*, 2001).

Extraction of enzymes from whole digestive tract is more practicable for this soft and perishable by-product. Optimization of extraction and separation in order to obtain those active enzymes are thus concerned. Until now, no information has been reported on preparation of crude visceral extract containing protease and amylase especially from tilapia viscera. Hence, it was the primary objective of this study to optimize an extraction of crude enzyme from Nile tilapia viscera and to evaluate properties of this mixed enzyme.

2.3 Materials and Methods

2.3.1 Materials and chemicals

The viscera of Nile tilapia (*Oreochromis niloticus*) was obtained from a local market and a frozen tilapia manufacturer in Songkhla province, Thailand. They were transported to the laboratory at Prince of Songkla University on ice within 2 hrs. The visible fat and gallbladder were removed manually before being washed with water and stored at -20ºC until used for extraction.

Testing chemicals, including chemicals for tilapia viscera extraction, enzyme activity assay, ammonium sulfate precipitation and polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3.2 Crude tilapia viscera enzyme preparation

To produce a homogeneous sample, the tilapia viscera were minced using a blender for 2 min at 4ºC with different media; distilled water, acetone, and 0.3% (w/v) NaCl solution at the ratio of 1:3 (w/v). The mixtures were centrifuged at 10,000 × g for 15 min at 4ºC. The supernatants were then collected and used as "crude enzyme extract" for further studies includes:

2.3.2.1 Analysis of protein concentration by lowry method (Lowry *et al.*, 1951).

The protein concentration was determined by the Lowry method according to the procedure described by Gerhardt *et al.* (1994). In this procedure, the proteins were first pretreated with copper ions in an alkali solution. The aromatic amino acids in the treated sample reduced the phosphomolybdic-phosphotungstic acid present in the Folin reagent. Since the endpoint of the reaction has a blue color, the amount of protein in the sample could be estimated by reading the absorbance using a spectrophotometer (Biochrom, Libra S4 Visible spectrophotometer, Holliston, Massachusetts, USA) at 750 nm.

Solution A was prepared by mixing 2.8598 g NaOH and 14.3084 g Na_2CO_3 in 500 ml distilled water. Solution B was made by adding 1.4232 g $CuSO_4.5H_2O$ to 100 ml distilled water. Solution C was prepared by adding 2.85299 g

sodium tartarate to 100 ml water. Lowry solution was prepared fresh daily by combining solutions A + B + C together in the ratio 100:1:1. Folin Reagent was prepared fresh by adding 5 ml of 2 N Folin Ciocalteu's Phenol Reagent to 6 ml distilled water. Bovine serum albumin (BSA) was used as the standard protein solution.

2.3.2.2 Estimation of protease activity by the modified Hagihara method using casein as a substrate (Banik and Prakash, 2006).

The protease activity of the crude extract was determined as described by Banik and Prakash (2006) using casein as a substrate. To initiate the reaction, 1,000 µl of 100-fold diluted viscera crude extract with Tris-HCl buffer pH 7.0 was added into 1,000 µl of 1% casein and incubated at 37ºC 15 min. The enzymatic reaction was terminated by adding 2,000 µl of stop buffer (0.1M TCA: 0.22 M sodium acetate: 0.33 M acetic acid, 1:1:1). Unhydrolyzed protein substrate was allowed to precipitate for 30 min, followed by centrifuged at 4,390 x g for 10 min. The supernatant was measured using spectrophotometer at 275 nm. The absorbance value were determined by comparing with tyrosine standard curve to obtain the tyrosine concentration. The protease activity was calculated by using the following equation:

$$\text{Protease activity (unit/ml)} = \frac{\text{mg of tyrosine x 1,000 x fold of dilution}}{\text{Mw of tyrosine x incubation time (min) x enzyme solution volume (ml)}}$$

$$\text{Specific activity (unit/mg protein)} = \frac{\text{protease activity (unit/ml)}}{\text{protein concentration (mg/ml)}}$$

2.3.2.3 Determination of amylase activity by the starch hydrolysis method (Pongsawadi and Yagisawa, 1988).

Amylase activity of the crude extract was determined by the starch hydrolysis method, described by Pongsawadi and Yagisawa (1988). To initiate the reaction, 1,000 µl of 100-fold diluted viscera crude extract with Tris-HCl buffer pH 7.0 was added into 3,000 µl of 1% starch solution and incubated at 37ºC 30 min. Then 200 µl of the mixed solution was transferred into 5,000 µl of iodine solution. The

absorbance of the solution at 540 nm was measured using a spectrophotometer. The amylase activity was calculated according to the following equation:

$$\text{Amylase activity (unit/ml)} = D\,[(Ro - R) / Ro] \times 100$$

when D = dilution factor of enzyme
Ro = absorbance of blank
R = absorbance of enzyme

$$\text{Specific activity (unit/mg protein)} = \frac{\text{amylase activity (unit/ml)}}{\text{protein concentration (mg/ml)}}$$

2.3.3 Effect of NaCl on crude tilapia viscera enzyme

Effect of NaCl concentration on enzyme extraction and activity was determined by homogenization of the minced tilapia viscera with different concentration of NaCl (0.1%, 0.5%, 1%, 5%, 10%, 15%, 20% w/v) at the ratio of 1:3. The suspended particle was removed by centrifugation at 10,000 × g for 15 min at 4 ºC. The supernatant (crude extract) was then collected and determined protease and amylase activities at 37ºC pH 7.0. The optimum NaCl concentration with the highest activity of enzyme was selected.

2.3.4 pH and temperature profile of crude enzyme extract

2.3.4.1 pH profile

pH profile of crude enzyme extract was determined at 37ºC, over the pH range of 2.0-11.0 (0.1 M sodium citrate buffer for pH 2.0-5.0, 10 mM Tris-HCl buffer for pH 6.0-9.0, 0.05 M carbonate-bicarbonate buffer for pH 10.0-11.0). Effects of the pH values on protein content including protease and amylase activities were evaluated and selected the optimum pH for crude enzyme extract.

2.3.4.2 Temperature profile

Temperature profile for crude enzyme extract was determined at optimum pH over the thermal range of 30-80°C. Evaluation of protein content, protease and amylase activities and select the optimum temperature for crude enzyme extract.

2.3.5 Stability of crude enzyme extract

The stability of crude enzyme was examined by pre-incubating the crude enzyme in optimum pH and temperature for up to 6 hrs. Samples were removed at intervals of 30 min, protein content and the residual activities of protease and amylase were examined.

2.3.6 Separation of protease and amylase from tilapia viscera extract

Membrane used for ultrafiltration was a polysulphone hollow fiber membrane (UFP-30-E-3MA, Amersham Biosciences, UK) with molecular weight cut-off of 30 kDa, fiber diameter of 1 mm, flow length of 30 cm and effective area of $0.01m^2$. By using the membrane, it was possible that retentate and permeate riched of amylase and protease, respectively, would be obtained. Both retentate and permeate were then investigated for their protease and amylase activities.

2.3.7 Partial removal of non-enzyme protein

2.3.7.1 Ammonium sulphate precipitation

The crude enzyme extract was first submitted to ammonium sulphate precipitation at 10%, 20%, 30%, 40%, 50% and 60%. The supernatant obtained after centrifugation at 10,000 × g and 4ºC for 15 min was analyzed for protein content and enzyme activities. The supernatant from the optimum precipitation was later diafiltration by using a hollow-fiber membrane (molecular weight cut-off 30 kDa) at ambient temperature (28-30ºC), pressure 1.3 bars and flow rate 17 ml/sec. The operational modes included a pre-diafiltration and a post-concentration processes. In all runs, the retentate was recycled to the feed tank while any permeate was collected and removed from the system. After

that, the protein content, protease and amylase activities were monitored. Protein profile of desalted extract was investigated by using SDS-PAGE.

2.3.7.2 SDS-PAGE

SDS–PAGE was performed according to the method of Laemmli (1970). Sample solutions were mixed at 1:1 (v/v) ratio with the SDS–PAGE sample buffer (0.06 M Tris–HCl, pH 6.8; 2% SDS; 25% glycerol; 5% β-mercaptoethanol; 0.01% bromophenol blue) and boiled for 4 min. The samples were loaded onto the gel made of 4% stacking and 12% separating gels. After that the gels were stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 7% acetic acid and destained with 7% acetic acid in 40% methanol. Each protein band of partial purified extract were confirmed by determined enzyme activity via native-PAGE.

2.3.7.3 Native-PAGE

Native–PAGE was carried out according to the method of Laemmli (1970). The sample solution was mixed at 1:1 (v/w) ratio with sample buffer (240 mM tris-HCl, pH 6.8; 30% glycerol; 0.5% bromophenol blue). The sample solution was loaded onto the gel made of 4% stacking and 10% separating gels. Then, the gel was stained with 0.3% coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destained with 10% acetic acid and 20% methanol. After that, cut out the desired protein band with a scalpel and crushed the gel in a small amount of phosphate buffer solution, pH 8.0 (Pollard, 1998). And each band were used for determination the activity of protease, amylase and lichenase (McCleary and Nurthen, 1986).

Activity of protease and amylase of the band were measured by using the methods previously described in section 2.3.2.2 and 2.3.2.3, respectively. The lichenase activity was assayed using Azo-barley glucan as the substrate according to the manufacturer's instruction of Megazyme. Briefly, 125 µl of pre-warmed Azo-barley glucan substrate solution and 125 µl of pre-incubated lichenase in assay buffer were mixed vigorously and incubated at 50ºC for 10 min. To terminate the enzyme reaction, 750 µl of precipitant solution A, 4% (w/v) sodium acetate, 0.4% (w/v) zinc acetate, was added. After centrifugation, the absorbance of the supernatant at 590 nm was measured.

One unit of enzyme activity was defined as the amount of enzyme that release 1 µmol of glucose reducing sugar equivalent per minute under the specified assay condition.

2.3.8 Statistical analysis

All experiments were run in triplicate. Results were expressed as the mean value ± standard deviation from three replicates. A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980).

2.4 Results and Discussion

2.4.1 The optimum condition for extraction of crude enzyme from Nile tilapia viscera

Effects of different extract media on protein content and specific activity of protease and amylase of tilapia viscera extract are shown in Table 7. It is obvious to be found that crude extract with the highest protein concentration and specific activity of protease and amylase were derived by using saline solution as an extract medium. The finding suggested that digestive enzyme accounted for principal protein concentration of the extract. Although an enzyme is known as a water soluble protein, it is likely that water used as an extract medium decreased the ionic strength of endogenous solution of the visceral system. Salt soluble protein may be caused to precipitate due to the diluted medium resulting co-precipitation of water soluble protein including enzyme. On the other hand extraction with 50 mM salt solution may cause insignificant change on ionic strength of the visceral liquid fraction to affect solubility of salt soluble protein.

Pre-extraction of viscera with organic solvent to remove fat and lipid is used as a material pretreatment by some research groups (Kishimura and Hayashi, 2002; Beltagy, 2004; Hayet *et al*., 2011). Thus, acetone was used to investigate whether this fat removal medium affect enzyme extraction. The result revealed that significant amount of protease was co-removed with lipid by adoption of this treatment. The loss may be facilitated by existence of emulsion. Moreover, organic solvents are known to

cause protein denaturation and loss of its functional properties (Fukushima, 1969). Park *et al*. (2008) reported that digestive enzyme from mackerel viscera denatured by solvent extraction with protease activity decreased more than 50% while amylase activity decreased up to 80%.

Table 7 Effect of extractants on protein concentration and specific activity of protease and amylase in the crude extracts of Nile tilapia viscera.

Extractant	Protein concentration (mg/ml)*	Specific activity of protease (unit/mg protein)*	Specific activity of amylase (unit/mg protein)*
acetone	4.82 ± 0.03	471.88 ± 2.70	1,360.40 ± 4.27
water	4.88 ± 0.06	410.04 ± 2.98	1,414.69 ± 3.78
NaCl (0.3% w/v)	5.47 ± 0.06	551.55 ± 3.78	1,453.82 ± 2.68

* The values are expressed as mean ± standard deviation from triplicate determinations. There are significant differences among means within the same column at $p \leq 0.05$.

2.4.2 Effect of NaCl on protease and amylase activities

Effects of NaCl on protein concentration and protease and amylase activities of the viscera extracts are shown in Figure 8. Protein concentration of the extract was increased gradually with increasing of salt concentration reaching the highest value of 7.31 mg/ml at 10% NaCl. Thereafter a plateau of protein content was found with further increasing of salt concentration. Effect of salt concentration on protein content of the extract however differed from its effect on specific activity of both protease and amylase. The enzyme activity with relative to that of the water extract was increased only if 5% NaCl was used. Decrease of the specific enzyme activities was noted if concentration of salt solution was higher than 5%. It is possible that both protease and amylase were precipitated more with an increase of salt concentration due to the effect of salting out. The difference between these two responses (protein concentration and specific enzyme activity) against salt concentration suggested at least that extraction of other proteins is enhanced when salt concentration higher than 5%.

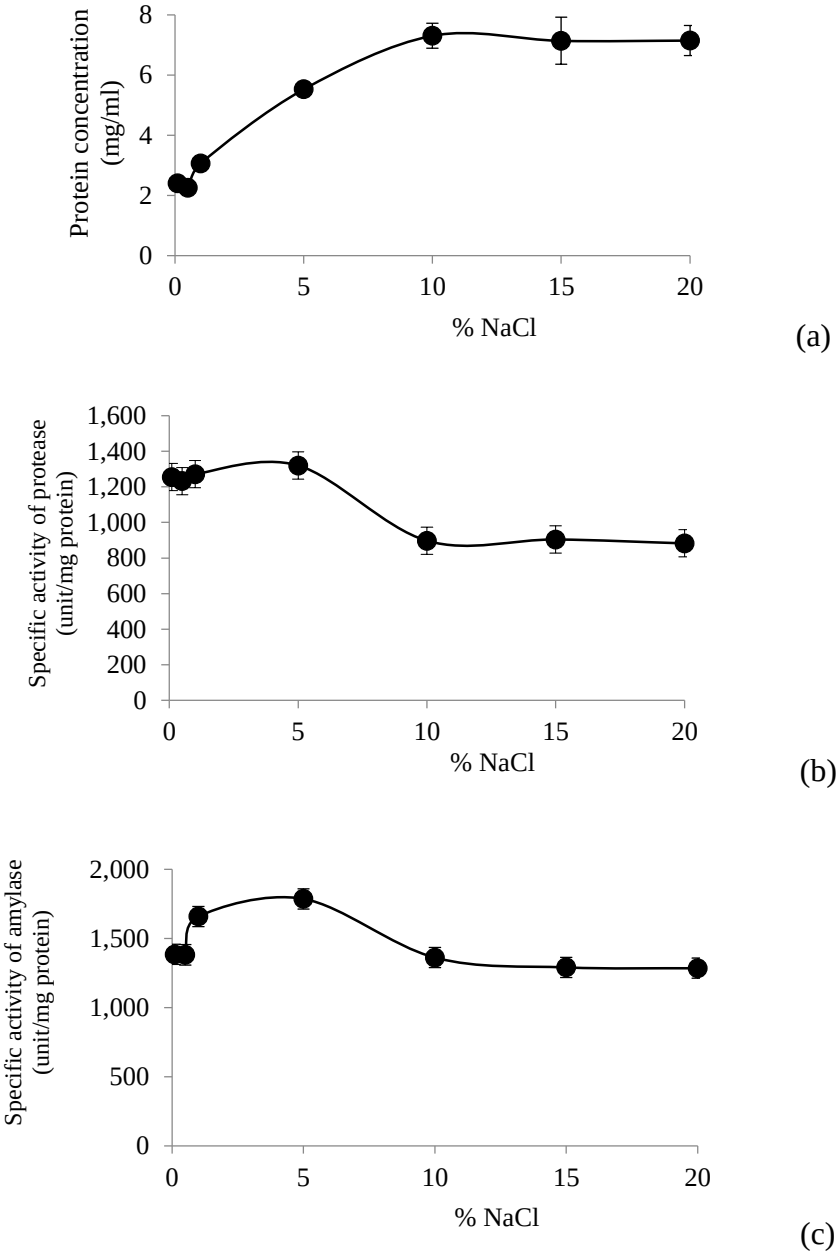


Figure 8 Effect of NaCl concentration on protein concentration (a) and specific activities of protease (b) and amylase (c). Bars represent standard deviation from 3 determinations.

2.4.3 pH and temperature profiles of tilapia viscera extracts

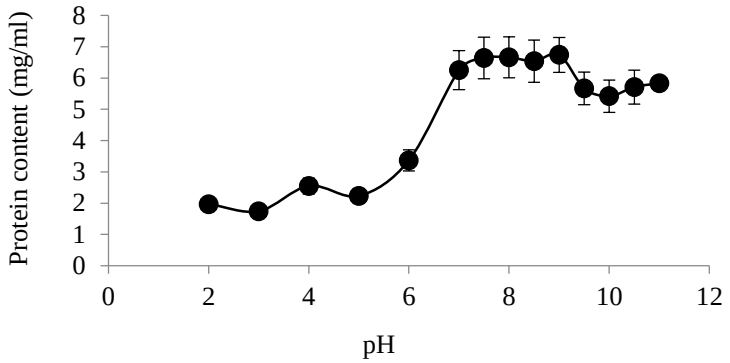
Effect of pH on specific activity of crude enzyme extract derived from the tilapia viscera was determined over the pH range of 2.0-11.0 as shown in Figure 9. The protease and amylase of the extract were highly active within pH range of 7.5-8.5 and 7.0-9.0, respectively. The increase in protein concentration was observed with increasing pH from 2.0-7.0 and stable at pH 7.0-9.0 then slightly decreased when pH more than 9.0. This protein behavior at different pH may be relevant to the net charge of the protein at that pH.

Crude enzyme extract from tilapia viscera exhibited the maximum activity of protease and amylase at pH 8.0 (Figure 9b and Figure 9c). The activity of both enzymes decreased above the optimum pH values. The optimum pH values of protease was in accordance with Bezerra *et al*., (2005) who reported that the optimal pH of proteolytic enzyme from the intestine of Nile tilapia was 8.0. The intestinal extract from catfish viscera isolated by Villalba *et al.* (2011) showed the optimum pH value of 9.0. Klahan *et al*., (2008) reported that the optimal pH of amylase from liver, stomach and intestine of tilapia were 7.0, 8.0 and 7.0, respectively.

Effect of temperature on specific activity of crude enzyme extract at pH 8.0 was determined at different temperatures from 30-80°C as shown in Figure 10. The protease and amylase of the tilapia crude extract were highly active at temperature ranges of 40-60°C and 50-65°C, respectively. Their optimal temperature was identical at 60°C. This protease has optimum temperature slightly higher than those commonly reported such as those of the extracts from the intestine of Nile tilapia and hybrid *Tilapia nilotica/aurea* which were 50°C and 40°C, respectively (Bezerra *et al.*, 2005). In addition, the optimal temperature for β-amylase from tilapia intestine was 40°C (Tsao *et al.*, 2004). The optimum temperature may associate with the fact that Nile tilapia live in warm water (Bezerra *et al.*, 2005).

The protein contents decreased with temperature above 40°C that might be due to the thermal precipitation of other thermal labile protein in the extract. Coagulation of this protein fraction may partly account for an increase of the specific activity of both enzymes. Moreover, it is also possible that both enzymes found in the

crude extract are thermal stimulated as their activity risen up to 60°C. The appreciable decrease in specific activity of both enzymes was observed at temperatures above 60°C, presumably as a result of thermal inactivation and coagulation. Enzymes were inactivated at high temperature, possibly due to the partial unfolding of the enzyme molecule (Klomklao *et al.*, 2006) and the subsequent loss of activity (Villalba *et al*., 2011).



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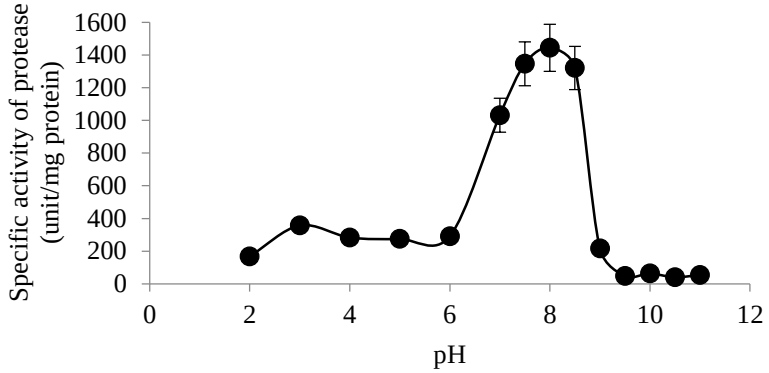

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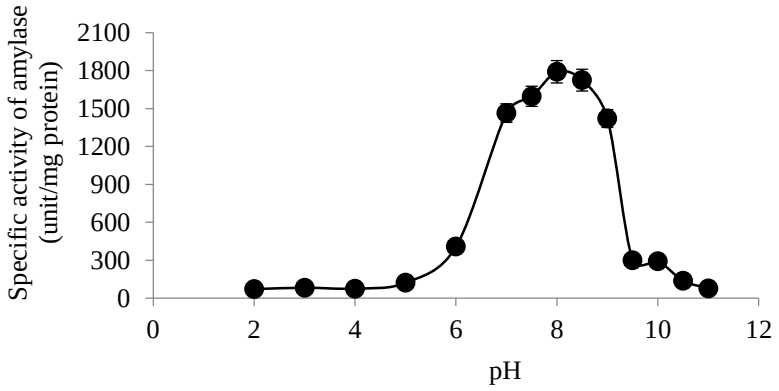

(c)

Figure 9 pH profiles of protein concentration (a) and specific activities of protease (b) and amylase (c) at 37°C. Bars represent standard deviation from 3 determinations.

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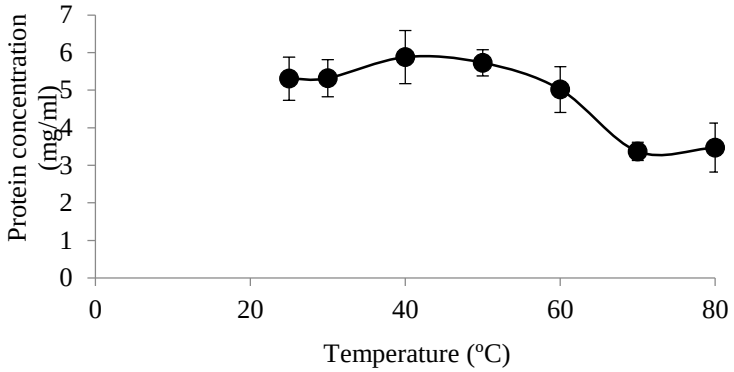

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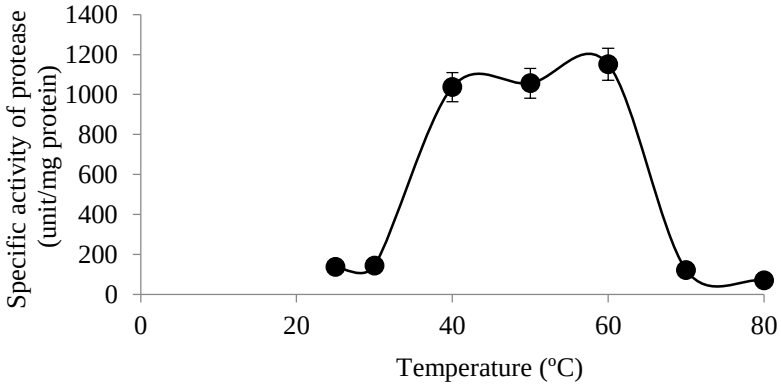

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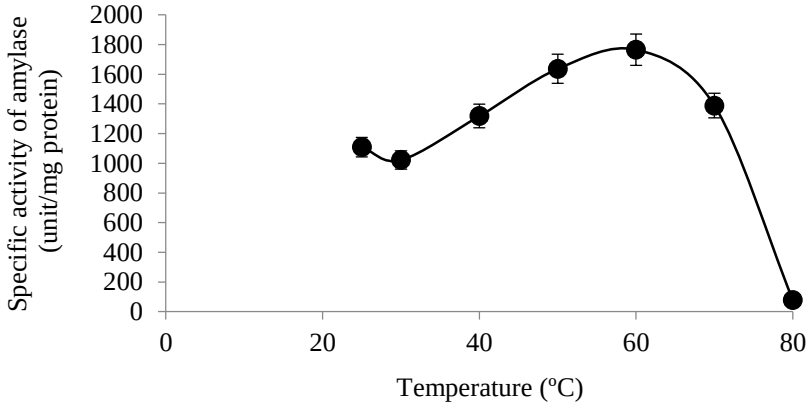

(c)

Figure 10 Temperature profiles of protein concentration (a) and specific activities of protease (b) and amylase (c) at pH 8.0. Bars represent standard deviation from 3 determinations.

2.4.4 Stability of tilapia viscera enzyme extracts

The stability of tilapia viscera enzymes at 60ºC and pH 8.0 is shown in Figure 11. Protease activity of the crude extract decreased significantly after 30 min incubation thereafter exhibited a plateau phase at about 50% of its initial activity with extension of incubation time up to 360 min. In contrast, amylase activity of the extract rose 5 times after incubation for 30 min. It later declined gradually reaching a stationery phase of about 3 times of its initial activity after 90 min incubation. The results thus suggest a difference between these two enzymes. Protease activity obviously has low heat tolerance while amylase activity is not only more heat tolerance but also well heat stimulate. Since only about 15 and 30% drop of protein concentration of the extract was noted due to the 30 and 90 min incubation, respectively, thus thermal coagulation of the enzymes could not primarily account for those changes of the enzyme specific activity. On other hand conformation changes of the enzymes due to thermal inactivation is likely responsible for the observation made. Nasri *et al*. (2011) reported the activity of alkaline protease from goby (*Z. ophiocephalus*), thornback ray (*R. clavata*) and scorpionfish (*S. scrofa*) that decreased immediately after incubation at 60ºC and complete inactivation when incubated for 30, 30 and 45 min respectively. Castillo-Yanez *et al*. (2005) also reported that activity of trypsin from sardine viscera decreased rapidly after incubation for 15 min at 55ºC pH 8. While, Thy *et al*. (2011) incubated amylase enzyme from the intestine of Tra (*PANGASIUS*) catfish at 55ºC found that amylase activity increased at early of incubation time and decreased after 5 min. Dutta *et al*. (2006) reported that amylase from *Heliodiaptomus viduus* decreased after incubated for 10 min and retained 50% activity for 85 min when heated to 50ºC. The rate of thermal inactivation was faster at higher temperatures. At 60 and 70ºC, the enzyme lost activity after heating for 120 and 60 min, respectively.

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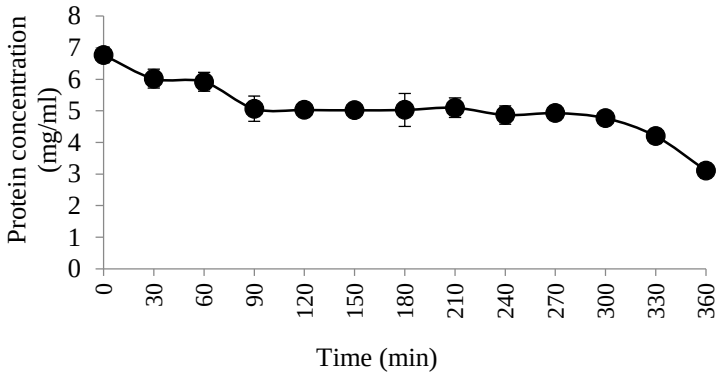
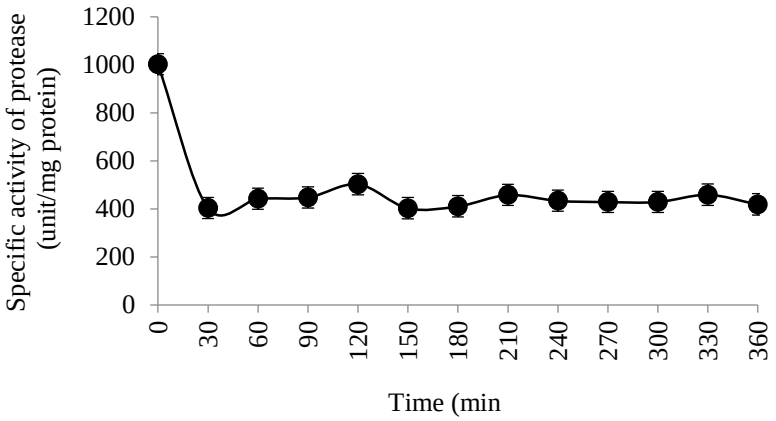


(a)



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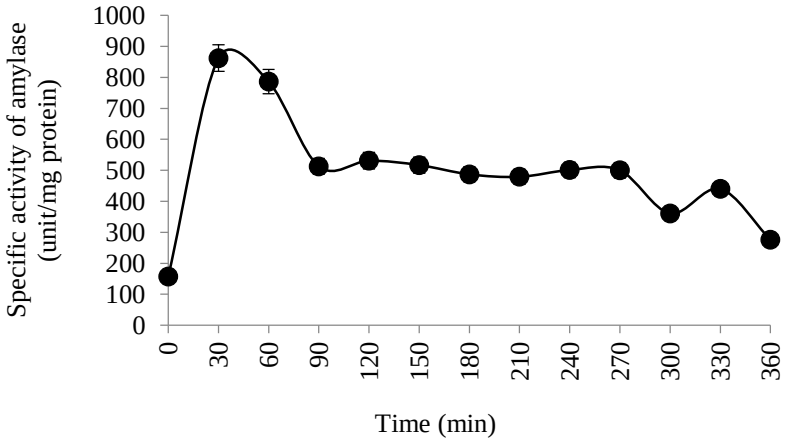


(c)

Figure 11 Stability of protein concentration (a) and specific activities of protease (b) and amylase (c) at 60ºC pH 8.0.

2.4.5 Separation of protease and amylase from tilapia viscera extract

Separation of protease and amylase enzyme in tilapia viscera extract using ultrafiltration membrane with molecular cut-off of 30 kDa showed in Table 8. By using 30 kDa molecular weight cut-off membrane, amylase and protease, about 25 kDa difference in their molecular weight, were expected to be separated effectively. It was, however, found that retentate and permeate displayed both enzyme activities. Size of the component is simply a general guide regarding to its ability to cross the membrane. Hydration ability of protein created its hydration shell may increase virtual size of protein limiting its capability to cross membrane (Smith, 2014). In addition, smaller molecule is also able to bind with larger components which could limit its passage through a membrane despite an apparent small size (Smith, 2014). These may account for significant residual of protease activity found in retentate. Moreover, general characteristic of membrane pores are irregular shape, vary in size (She *et al*., 2008) and low density (<10%), high pressure is thus required in order to increase flux of the operation. This may flavor flow across membrane of high molecular weight molecule (Smith, 2014; Nakao, 1994).

Table 8 Specific activity of protease and amylase in retentate and permeate after ultrafiltration.

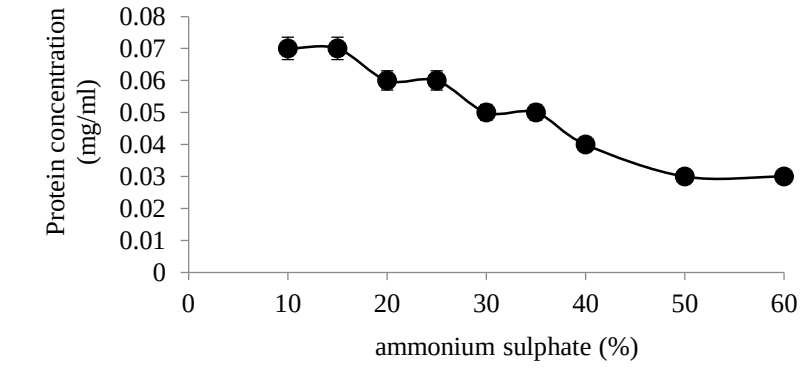
Samples	Specific activity of protease (Unit / mg protein)*	Specific activity of amylase (Unit / mg protein)*
Tilapia viscera extract	401.92 ± 1.74	608.48 ± 2.22
Retentate	351.64 ± 0.93	454.07 ± 1.92
Permeate	418.14 ± 1.21	222.42 ± 0.95

* The values are expressed as mean ± standard deviation from triplicate determinations. There are significant differences among means within the same column at $p \leq 0.05$.

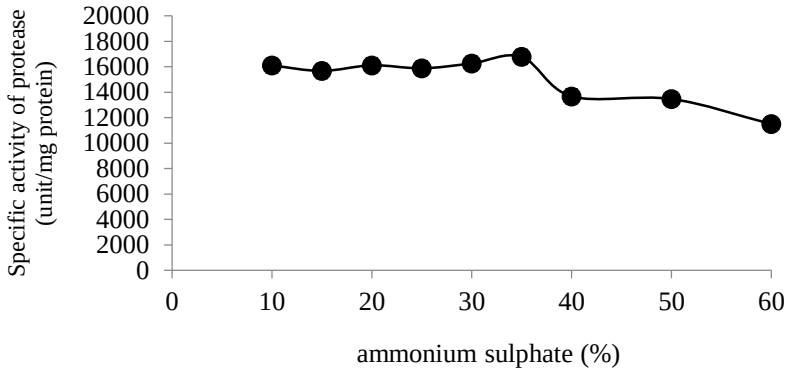
2.4.6 Partial removal of non-enzyme protein

Removal of soluble protein of the crude extract occurred progressively with increasing of ammonium sulphate concentration from 10% to 60% (Figure 12). According to specific enzyme activity the result suggested that the appropriated ammonium sulphate concentration ranges for partial recovery protease and amylase are 10-35% and 30-40%, respectively. Thus, the extract with improved specific activity of both enzymes could be obtained by precipitation of other proteins by using a very narrow range (30-35%) of ammonium sulphate concentration. At ammonium sulphate concentration higher than 35%, a decrease on specific activity of the protease in the residue extract was noted which reflected a better precipitation of the enzyme with relative to other non-enzyme proteins. Therefore, the partial purified of tilapia viscera enzyme extract with 35% ammonium sulphate concentration was selected to use in further study. Rawdkuen and Benjakul. (2012) found that the sediment with the highest proteases activity of farmed giant catfish (*Pangasianodon gigas*) could be obtained by partitioning with 50% ammonium sulphate. In contrast, Temiz *et al*. (2013) reported that 20 to 40% ammonium sulphate precipitation of anchovy digestive tract extract yielded the pellet rich of alkaline protease. The result also revealed that partial separation of amylase from protease and other proteins could be possible by using 40% w/v ammonium sulphate whereas partial separation of protease from amylase and other proteins could be performed at ammonium sulphate concentration lower than 30% w/v.

Moreau *et al.* (2001) isolated α-amylases form Nile tilapia viscera by subjected to ammonium sulfate fractionation. Non-enzyme protein contaminant was precipitated by using 30% ammonium sulfate thereafter amylase was precipitated from the supernatant by using 60% ammonium sulfate.



(a)



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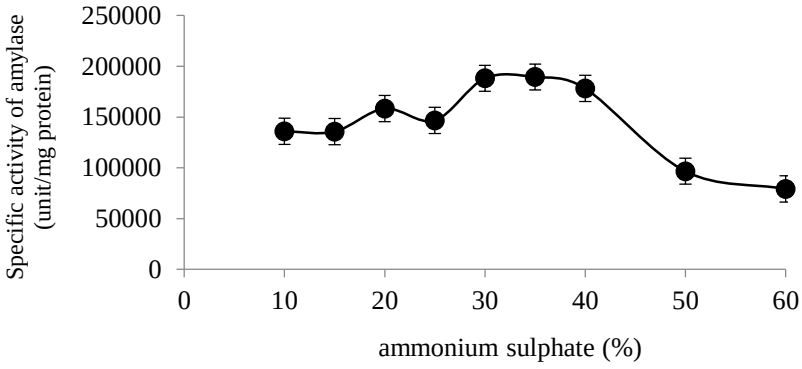


(c)

Figure 12 Effect of ammonium sulphate precipitation on protein concentration (a) and specific activities of protease (b) and amylase (c).

Protein patterns of the tilapia viscera, the crude extract and the partial purified extracts obtained by using 35% ammonium sulphate under SDS-PAGE are shown in Figure 13. The major protein bands with the molecular weight of 26.5, 37.3 and 56.4 kDa were found in both crude extracts and partial purified extract. The latter

band may be represent of protease since its molecular weight is within a range of 23-28 kDa (Freitas-Júnior *et al*., 2012). Molecular weight of protease derived from several fish visceral. The band with molecular weight of 37.3 kDa might be the band of lichenase enzyme that was found in intestine of the herbivorous fish (Linton *et al*., 2014; German *et al*., 2004). Johansson *et al*. (2004) reported the molecular weight of lichenase was in the range of 27-64.5 kDa. And, Moreau *et al*. (2001) reported that the molecular weight of α-amylases derived from the intestinal cavity of two tilapia species, *Oreochromis niloticus* and *Sarotherodon melanotheron*, were 56.6 and 55.5 kDa, respectively. Therefore, the band with the molecular weight of 56.4 might be the band of amylase enzyme. The smear band existed in tilapia viscera (Figure 13 lane A) suggested that it is non-enzyme protein removed by NaCl and ammonium sulphate precipitation.

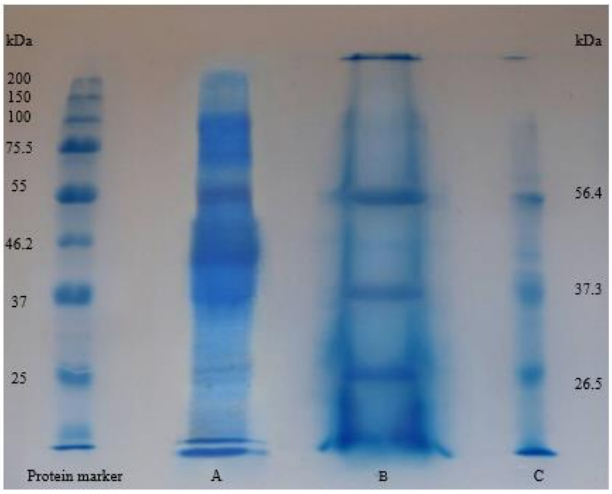


Figure 13 SDS-PAGE patterns of tilapia viscera (A); crude enzyme extract (B) and partial purified extract (C) from tilapia viscera.

Protein pattern of the partial purified extract obtained by using 35% ammonium sulphate precipitation under native–PAGE are shown in Figure 14. The result showed 3 major protein bands of the partial purified extract. The band with molecular weight of 13.1 kDa showed protease activity at 570.28 unit/ml. While, the protein band with molecular weight of 21.4 kDa had lichenase activity at 702.04 unit/ml. And the band of 44.5 kDa showed amylase activity at 3,079.17 unit/ml.

Therefore, it can confirm that the 3 major protein bands of the partial purified extract were protease, lichenase and amylase, respectively.

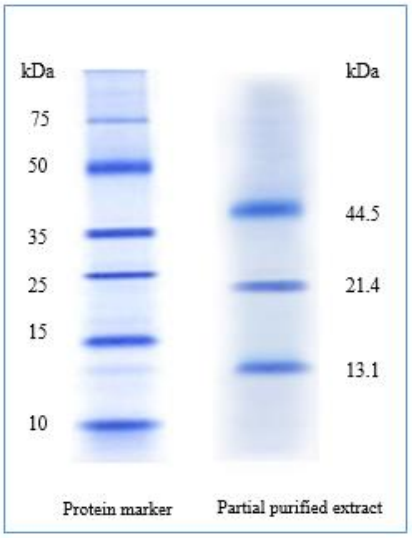


Figure 14 Native–PAGE pattern of the partial purified extract from tilapia viscera.

2.5 Conclusion

The tilapia crude enzyme extract showed high protease and amylase activities in the 5% NaCl solution and exhibited a maximal activity at 60ºC and pH 8.0. The protease activity of the tilapia crude enzyme extract decreased significantly after 30 min incubation while amylase activity increased rapidly by about 5 times within 30 min incubation. The optimum recoveries of protease (68.18%) and amylase (64.30%) were obtained by precipitation using ammonium sulphate at concentration range of 30-35%. The major protein bands with the molecular weight of 56.22, 31.33 and 26.71 kDa were found in both crude enzyme extract and the partial purified extract.

CHAPTER 3

DEFATTED RICE BRAN HYDROLYSATE PRODUCTION BY TILAPIA (*Oreochromis niloticus*) VISCERA ENZYME EXTRACT

3.1 Abstract

Rice bran hydrolysate (RBH) was prepared by using defatted rice bran and crude tilapia viscera extract. Effects of concentration of the viscera extract (1, 2 and 3% w/v) and hydrolysis duration (30, 60 and 120 min) at 60ºC on catalytic process and hydrolysate characteristics were investigated. The hydrolysis of RBH with 1% viscera extract for 30 min yielded the hydrolysate with the highest β-glucan content and the lowest degree of hydrolysis. This condition also improved hydration properties include water holding, water binding and swelling capacity of the RBH. Extension of hydrolysis time yielded the RBH with low L and b value.

3.2 Introduction

Rice bran is an under-utilized milling by-product of rough rice (Jiamyangyuen *et al.*, 2005) containing many bioactive compounds, for instance, 20-27% total dietary fibers, 12-20% protein, a rich source of iron and B vitamin, and small molecules (essential fatty acids, phytosterols, and antioxidants), which give beneficial effects to human health (Borresen and Ryan, 2014). Rice bran has been used as a feedstock for rice bran oil and has the potential to be used as a food ingredient (McCaskill and Zhang, 1999) and it is being used as a low-cost animal feed (Kannan *et al*., 2009). Oil production from rice bran produces defatted rice bran as a waste material. Although it is considered a less valuable product, defatted rice bran still contains useful substances such as fiber and protein (Anderson *et al*., 2009). Therefore, the current interest is to separate the benefit nutrient of rice bran before discarding as waste.

Protein hydrolysis is an effective method for modifying functional properties and improving extraction (David *et al.,* 2009). Strong acids or bases can also be applied to break the peptide bond, which is a simple method, albeit harsh. Furthermore, this either destroys or modifies the essential amino acids, and creates toxic by-products and undesirable side reactions that reduce the quality of the protein (Humiski and Aluko, 2007). However, enzymatic hydrolysis does not affect the nutritional value of the proteins. Additionally, enzymatic hydrolysis can improve the physicochemical, functional, and sensory properties of native proteins (Kristinsson and Rasco, 2000).

Rice bran contains a significant amount of protein (12-20%), with fairly high nutritional quality (Saunders, 1990). The lysine content of rice bran protein is approximately 3-4%, which is higher than that of rice endosperm protein or proteins from other cereal bran or legume. Rice bran protein is also very digestible (more than 90%) (Wang *et al*., 1999) and maybe hypoallergenic (Helm and Burks, 1996). Rice bran protein holds great promise as an alternative protein source such as a food ingredient (Yeom *et al.,* 2010). However, this protein has received only minimal attention and only rarely been employed in the food industry (Yeom *et al.,* 2010). This is due partly to the difficulty inherent to the extraction of a large portion of rice bran

protein, owing to extensive disulfide bond cross-linkages and strong aggregation (Hamada, 1997) and also partly because the protein has exhibited poor functional properties (Yeom *et al.*, 2010).

Rice bran can be also used as a dietary fiber source. One of an active component was a water soluble polysaccharide fraction (Aoe *et al.*, 1993) that ß-glucan is a main composition (Ahmad *et al.*, 2009). Health benefits associated with intake of soluble dietary fiber include low chance of cardiovascular diseases, reduction in problem of obesity (Bourdon *et al*., 1999), lowering of blood cholesterol (Keogh *et al*., 2003), better control on diabetes (Brennan and Tudorica , 2003), hypercholesterolemia (Maki *et al*., 2003), cancer (Sier *et al*., 2004), hypertension (Anderson, 1990) and support in growth of beneficial intestinal micro flora (Tungland, 2003).

The object of this study was to use tilapia viscera crude enzyme extract for preparation of hydrolysate from defatted rice bran.

3.3 Materials and Methods

3.3.1 Materials and Chemicals

Defatted rice bran, a by-product of rice bran oil production was obtained from the CEO Agrifood Co., Ltd, Singburi, Thailand. Information of rice bran oil extraction and other processing step were gathered from the supplier. The defatted rice bran was stored at -20ºC until used.

Testing chemicals, including chemicals for analysis of DH, chemical composition and functional property were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.3.2 Analysis of chemical composition of defatted rice bran

The chemical composition of defatted rice bran was performed include moisture content, protein, ash, fat, starch, crude fiber and total dietary fiber according to the method of AOAC (2000). Total β-glucan content was determined by the method of McCleary and Mugford (1997) using Megazyme (Megazyme International Ltd., Bray, Ireland) mixed linkage β-glucan assay kit. Reducing sugar was determined by 3,5-dinitro salicylic acid (DNS method) (Neureiter *et al*., 2002)

3.3.3 Extraction of rice bran hydrolysate by using tilapia viscera enzyme extract

Enzymatic hydrolysis of defatted rice bran was conducted using tilapia viscera enzyme extract selected from the previously studied in chapter 2. Protease 5,760 units and amylase 8,640 units were obtained from started tilapia viscera 100 g. The specific activities of protease and amylase in this enzyme extract were 16,780 and 189,480 unit/mg protein, respectively. Rice bran was dispersed in a 5-fold volume of distilled water and adjusted to the optimum pH of tilapia viscera enzyme extract at 8.0, and incubated at the optimum temperature (60ºC). Hydrolysis was performed by using the tilapia viscera crude enzyme extract either at 1.0%, 2.0% or 3.0% and hydrolysis time of 0.5, 1 or 2 h. Non-digested residue was removed by using centrifugation at

10,000 ×g for 20 min at 4ºC. The hydrolysate was prepared into a powder form by using a freeze dryer (Figure 15).

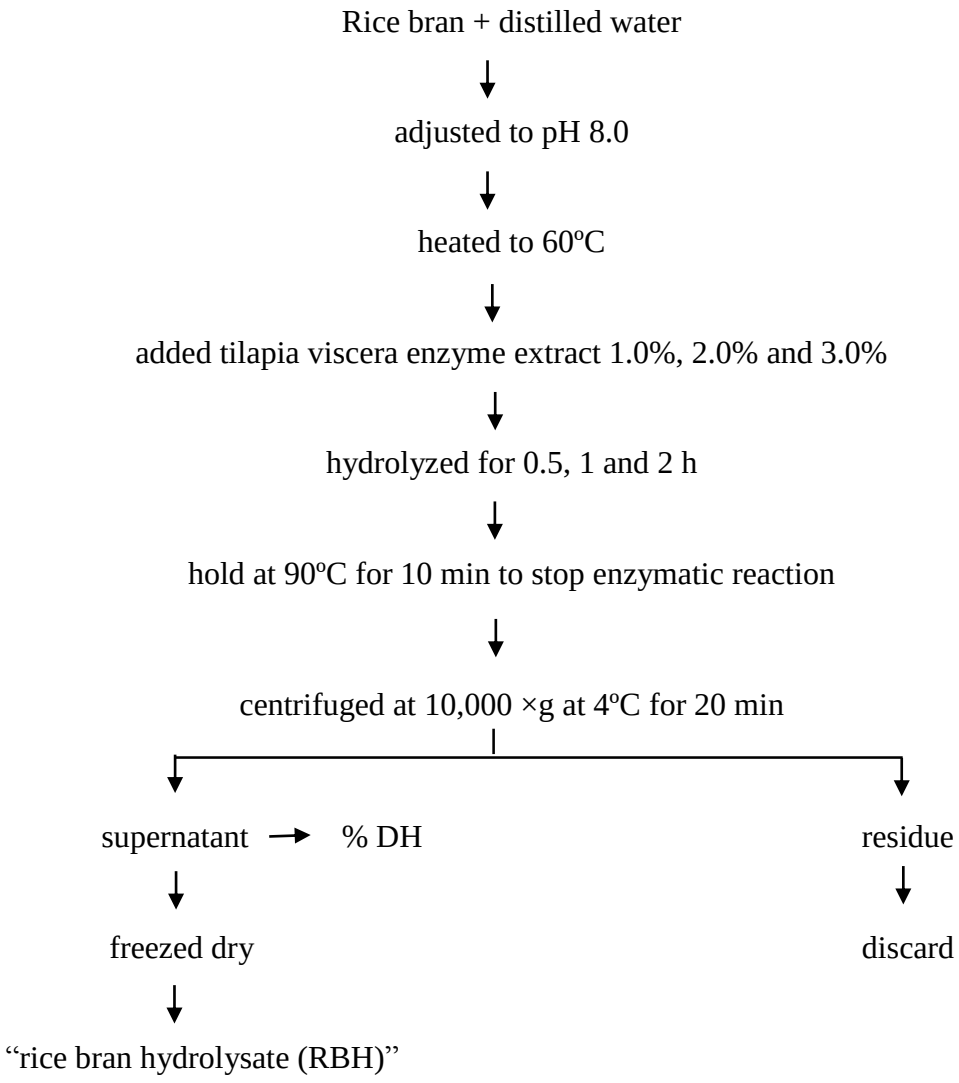


Figure 15 Rice bran hydrolysate production.

3.3.4 Analysis of degree of protein hydrolysis

The degree of protein hydrolysis (DH) in the RBH solution was determined using OPA method with some modifications described by Wanasundara *et al*. (2002), for the reaction of amino nitrogen with ortho-phthalaldehyde (OPA). The 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. A volume of 0.4 ml

of hydrolysate was added to 3 ml of the OPA solution and mixed well. This mixture was allowed to stand for 2 min at ambient temperature and the absorbance was measured at 340 nm using a spectrophotometer. The quantity of free amino groups were calculated as L-serine-NH_2 group which was used as a reference. The acid hydrolysis of RBH in 6 M HCl for 24 h at 110ºC was carried out for determination of total free amino group of defatted rice bran. The DH value was calculated as the ratio of released free amino groups because of enzymatic hydrolysis to total free amino groups released due to complete acid hydrolysis per unit of protein and expressed as a percentage value:

$$\text{DH (\%)} = \frac{\text{free amino groups released due to enzymatic hydrolysis} \quad x \quad 100}{\text{total free amino groups from acid hydrolysis}}$$

3.3.5 Chemical composition of rice bran hydrolysate

The chemical composition of RBH was performed include protein content using the Kjeldahl method and total dietary fiber (AOAC, 2000). Total β-glucan content was determined by the method of McCleary *et al*. (1997) using Megazyme (Megazyme International Ltd., Bray, Ireland) mixed linkage β-glucan assay kit. Reducing sugar was determined by 3,5-dinitro salicylic acid (DNS method) (Neureiter *et al*., 2002)

3.3.6 Color of rice bran hydrolysate

Color of RBH powder was determined by a Hunter-Lab (Miniscan XE Plus, Hunter-Lab, USA). The instrument was calibrated before color measurement with black glass and white calibration tile. Each RBH sample was put in a cuvette and replaced into the sample port, the color parameters (L, a and b) were then read.

3.3.7 Functional properties of rice bran hydrolysate powder

3.3.7.1 Water holding capacity (WHC)

WHC was demonstrated follow the methods of Robertson *et al*. (2000) and Daou and Zhang (2011). Distilled water (30 ml) containing sodium azide (0.02%)

was added into RBH (1 g) and hydrated for 18 h. Then, removed the supernatant by allowing the wet RBH to drain in a sieve. The hydrated RBH was removed, weighed and dried to stable weight (±0.05 mg) in hot-air oven at 110ºC. WHC was expressed as the amount of water retained per gram dry sample (g/g dry weight).

$$\text{WHC (g/g)} = \frac{\text{(Hydrated residue weight - Dry residue weight)}}{\text{Dry residue weight}}$$

3.3.7.2 Water binding capacity (WBC)

WBC was estimated follow the procedure of Robertson *et al.* (2000); Ahmad *et al*. (2010) and Daou and Zhang (2011) with some modifications. Distilled water (30 ml) containing sodium azide (0.02%) was added into the RBH (1 g) and stand at room temperature for 18 h. Then, the RBH was centrifuged (3000×g) for 20 min. The supernatant was removed by passing through a Büchner funnel vacuum suction. The hydrated residue was weighted and RBH was dried for 24 h at 105ºC to get dry weight. WBC was demonstrated as the amount of water retained per gram dry sample.

$$\text{WBC (g/g)} = \frac{\text{(Residue hydrated weight after centrifugation - Residue dry weight)}}{\text{Residue dry weight}}$$

3.3.7.3 Swelling capacity (SC)

SC was investigated follow Robertson *et al.* (2000). Distilled water (10 ml) containing sodium azide (0.02%) was added into dry sample (0.2 g). After that tubes were stand for 18 h at room temperature. The volume was recorded and SC was calculated as ml per gram of dry RBH for 18 h, then the final volume attained was evaluated:

$$\text{SC (ml/g)} = \frac{\text{Volume occupied by sample}}{\text{Original sample weight}}$$

3.3.7.4 Fat binding capacity (FBC)

FBC was examined by the method of Lin *et al*. (1974). The RBH (5 g) was mixed with soybean oil (20 ml) in centrifuge tube. Then, stirred for 30 s in every 5 min and after 30 min centrifuged at 1600×g, 25 min. Free oil was gradually poured and absorbed oil was calculated by difference and illustrated as ml (oil) per gram sample.

$$\text{FBC (ml/g)} = \frac{\text{(Precipitation weight - Dry weight)}}{\text{Dry Weight}}$$

3.3.7.5 Emulsifying capacity (EC)

EC was evaluated according to the method of Yasumatsu *et al*. (1972). Mix 20 ml of aqueous dispersion RBH (by adding RBH 1.5 g to 20 ml of distilled water) with soybean oil (20 ml) and blended for 5 min with high speed blender. Then, centrifuged at 3000×g for 5 min. The total mixture (%) that remained after centrifugation was presented as stability index. The stability index of a good emulsion is greater than 94% while a poor emulsion is lower than 50% (Wang and Kinsella, 1976; Yasumatsu *et al.*, 1972).

3.3.8 Statistical analysis

All experiments were run in triplicate. Results were expressed as the mean value ± standard deviation from three replicates. A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan’s Multiple Range Test (Steel and Torrie, 1980).

3.4 Results and Discussion

3.4.1 Chemical composition of defatted rice bran

The chemical composition of defatted rice bran is shown in Table 9. Defatted rice bran composed of 15.41% protein which Juliano and Villarea (1993) reported that the protein content of *Oryza sativa* bran ranged from 4.5 to 15.9 % depended on rice varieties (Oszvald *et al*., 2008). Ash content was 7.47%, which represented that defatted rice bran was a good source of minerals and trace minerals. The total dietary fiber content of defatted rice bran used in this study was 19.08% that slightly lower than value of 27.04 % reported by Abdul-Hamid and Luan (2000) and 30.98% by Wan (2010). This variation might be due to the different rice milling processing equipment and conditions that may remove rice bran from rice with more or less endosperm (Wan, 2010). In addition, defatted rice bran also contains β-glucan (7.3%).

Table 9 Chemical compositions of defatted rice bran.

Compositions	Values (%)
moisture	9.55 ± 0.34
protein	15.41 ± 0.43
ash	7.47 ± 0.37
fat	0.37 ± 0.28
crude fiber	9.11 ± 0.41
total dietary fiber	19.08 ± 0.59
starch	67.20 ± 0.46
total β-glucan	7.30 ± 0.27
reducing sugar	0.015 ± 0.34

The data are mean ± SD (n = 3).

3.4.2 Effect of hydrolysis condition on protein content and degree of protein hydrolysis of RBH

The effects of enzyme content and hydrolysis time on protein and DH of RBH at 60ºC pH 8.0 are shown in Figure 16 and Figure 17. The protein content of RBH was in the range of 0.4-1.49% (Figure 16). The protein content of the hydrolysate depended on the amount of tilapia enzyme as well as the hydrolysis time. The highest protein content of the hydrolysate was obtained by hydrolysis with enzyme concentration of 3% (v/w of rice bran) and hydrolysis time of 60 min. Thereafter gradual reduction of protein content was found with extension of hydrolysis time.

The degree of protein hydrolysis (DH) of RBH derived by Nile tilapia viscera enzyme extract and different hydrolysis time is shown in Figure 17. The result revealed that both enzyme content and hydrolysis time affected DH of the enzymatic process. The DH value was significantly ($p<0.05$) increased when enzyme concentration was increased from 1% to 3%. The significant increase of DH was observed by using 3% (v/w) enzyme suggesting that at concentration at 1% or 2% enzyme content is considerably low relative to available peptide bond. This result is similar to the report of Haslaniza *et al*. (2010) stated that significant improve of DH was occurred after increase enzyme concentration at certain level.

The DH value was increased from 30 min up to 60 min and decreased when the hydrolysis time reaching to 120 min (Figure 17). The result is likely due to enzyme deactivation with long incubation (Amza *et al.*, 2013). The protease activity of the viscera extract decreased about 50% of its initial activity with extension of incubation time up to 120 min.

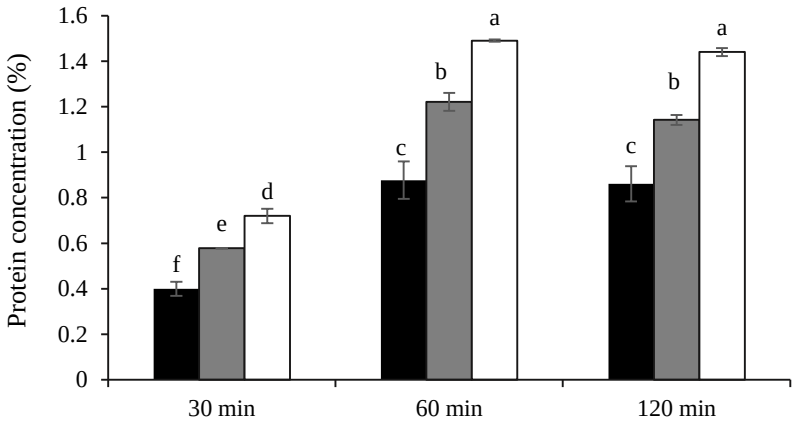


Figure 16 Effect of tilapia viscera extract content and hydrolysis time on protein concentration of RBH (■ : 1% tilapia viscera extract in hydrolysis reaction, ▩ : 2% tilapia viscera extract in hydrolysis reaction, □ 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

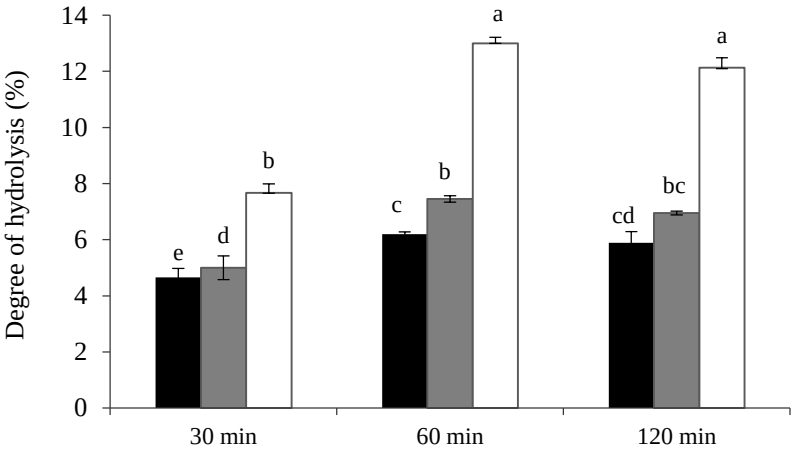
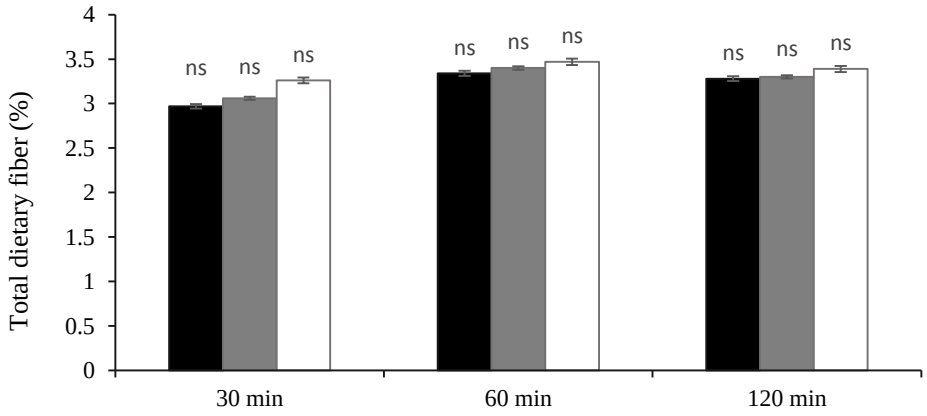


Figure 17 Effect of tilapia viscera extract content and hydrolysis time on degree of hydrolysis (DH) (■ : 1% tilapia viscera extract in hydrolysis reaction, ▩ : 2% tilapia viscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

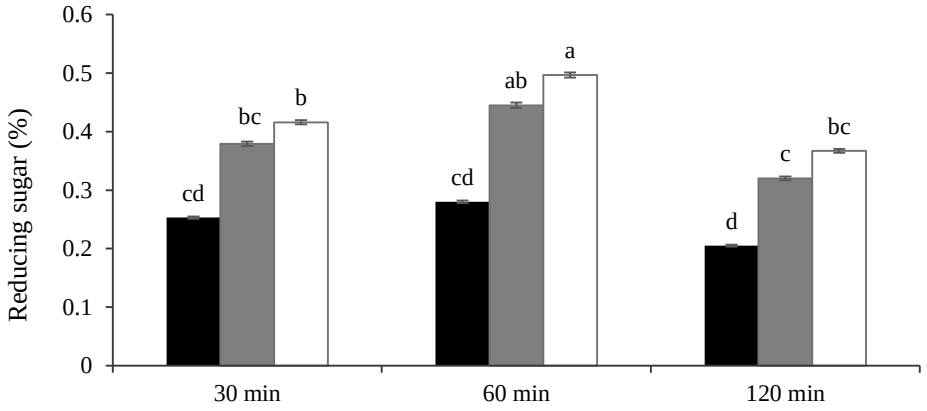
3.4.3 Effect of hydrolysis condition on total dietary fiber and β-glucan content of RBH

The RBH with the highest total dietary fiber was obtained after 60 min hydrolysis with 3% the tilapia viscera extract (Figure 18a). Reducing sugar content associated with hydrolysis level of dietary fiber increased with increasing of hydrolysis duration and enzyme concentration (Figure 18b). These result related with the result of β-glucan content (Figure 18c).

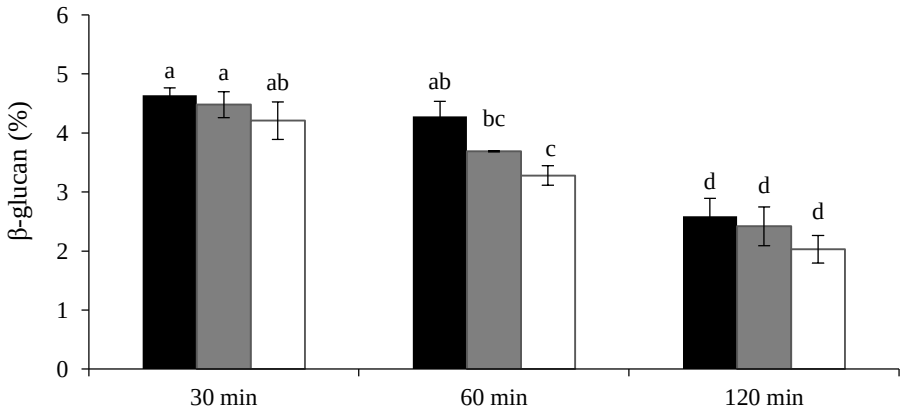
The β-glucan was decreased by increasing amount of tilapia viscera enzyme. The highest content (4.64% w/w) of β-glucan was obtained by using the mildest hydrolysis condition (1% tilapia viscera enzyme for 30 min). The tilapia viscera enzyme has lichenase enzyme (Mw 27-64.5 kDa) (Sasmal and Ray, 2015) that found in digestive tract of herbivorous fish (German *et al*., 2004). The lichenase enzyme is specifically cleaves the (1-4)-β-linkage next to a (1-3)-β-linkage at the reducing end of β-glucan and products are (1-4)-link-oligosaccharide with one (1-3)-linked glucose unit (Johansson *et al.,* 2004). Activity of this enzyme may responsible for reduction of β-glucan after extension of the hydrolysis. This was support by increase of reducing sugar with increasing hydrolysis duration. The report of the hydrolysis of β-glucan from oat bran by Sibakov *et al*. (2013) showed the β-glucan concentration was reduced from 16.4 to 11.6% when the hydrolysis time was prolonged from 60 to 240 min.



(a)



(b)



(c)

Figure 18 Effect of enzyme content and hydrolysis time on total dietary fiber, reducing sugar and β-glucan content (■ : 1% tilapia viscera extract in hydrolysis reaction, ■ : 2% tilapia viscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

3.4.4 Effect of hydrolysis condition on color of RBH

Table 10 shows the color parameters (L, a and b) of the RBH. L value which expresses the brightness was in the range of 22.26-37.36. The values of a (redness) and b (yellowness) were in the range of 4.34-7.17 and 12.93-18.63, respectively. It can be noticed that tilapia viscera extract content have no effect on L, a and b values of the RBH. However, duration of hydrolysis reaction has effect on L, a and b values of the RBH. Increasing of hydrolysis time caused a decrease in L and b value but an increase in a value. It means that the RBH seemed to be darker with longer hydrolysis time.

Table 10 Effect of hydrolysis condition on color of RBH.

% enzyme	Hydrolysis time (min)	L	a	b
1	30	37.36 ± 0.13^{a}	4.70 ± 0.04^{d}	15.22 ± 0.01^{d}
2	30	35.04 ± 0.06^{b}	5.66 ± 0.10^{c}	17.79 ± 0.03^{b}
3	30	33.35 ± 0.05^{d}	6.80 ± 0.03^{a}	17.99 ± 0.05^{b}
1	60	30.19 ± 0.04^{e}	5.63 ± 0.04^{c}	18.63 ± 0.03^{a}
2	60	34.54 ± 0.42^{c}	4.34 ± 0.09^{d}	16.57 ± 0.09^{c}
3	60	28.51 ± 0.46^{g}	5.83 ± 0.07^{bc}	15.30 ± 0.01^{d}
1	120	22.26 ± 0.31^{i}	6.17 ± 0.13^{b}	12.93 ± 0.05^{f}
2	120	29.32 ± 0.09^{f}	6.82 ± 0.04^{a}	16.16 ± 0.14^{c}
3	120	23.37 ± 0.32^{h}	7.17 ± 0.17^{a}	13.72 ± 0.02^{e}

The data are expressed as mean ± SD (n =3). Column with different letters indicate statistical differences ($p \leq 0.05$).

3.4.5 Effect of hydrolysis condition on functional properties of RBH

The functional properties of RBH hydrolyzed by Nile tilapia viscera enzyme are shown in Table 11.

Table 11 Effect of hydrolysis condition on hydration properties of rice bran hydrolysate**.**

% enzyme	Hydrolysis time (min)	Functional properties				
		WHC (g/g)	WBC (g/g)	SC (ml/g)	EC (%)	FBC (ml/g)
1	30	6.70±0.31^{a}	10.03±0.30^{a}	11.30±0.22^{a}	55.02±0.07^{a}	3.28±0.05^{b}
2	30	3.48±0.11^{c}	4.83±0.07^{b}	8.81±0.14^{b}	45.13±0.21^{c}	3.41±0.18^{b}
3	30	2.71±0.26^{d}	2.37±0.07^{c}	4.94±0.06^{d}	50.02±0.16^{b}	3.86±0.66^{b}
1	60	4.80±0.12^{b}	5.10±0.05^{b}	9.30±0.34^{b}	50.48±0.13^{b}	5.26±0.71^{a}
2	60	1.93±0.11^{ef}	4.44±0.16^{b}	6.88±0.15^{c}	50.35±0.04^{b}	5.47±0.14^{a}
3	60	1.40±0.09^{fg}	2.28±0.00^{c}	4.30±0.00^{e}	45.22±0.37^{c}	5.68±0.34^{a}
1	120	2.27±0.06^{de}	2.04±0.12^{c}	8.91±0.21^{b}	50.40±0.08^{b}	5.34±0.03^{a}
2	120	1.48±0.07^{fg}	1.34±0.30^{c}	6.45±0.45^{c}	50.23±0.04^{b}	5.92±0.11^{a}
3	120	1.30±0.21^{g}	0.86±0.00^{d}	4.30±0.39^{e}	50.44±0.34^{b}	5.93±0.07^{a}

Values are expressed as mean ± standard deviation from triplicate determinations. Columns with different letters indicate statistical differences (P≤0.05).

The result revealed that increasing of hydrolysis by extension of hydrolysis duration and increase enzyme concentration caused reduction in water holding capacity (WHC), water binding capacity (WBC) and swelling capacity (SC) of the obtained RBH. Tounkara *et al*. (2013) suggested that water holding ability of protein hydrolysate may be determined by the difference in molecular weight of peptides within protein hydrolysates, type of amino acid, amino acid content and amino acid sequence.

Emulsifying capacity (EC) (Table 11) of the RBH with 4.66% DH showed the highest value ($p < 0.05$). Mutilangi *et al*. (1996) postulated that higher MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. Most

RBH are judged as poor emulsifier based on their stability indices was less than 50%. Prakash and Ramanathan (1995) showed that EC of protein concentrate from rice bran, range from 52 to 57 %, therefore the lower EC of RBH might be due to the lower protein level in the RBH.

FBC of RBH with long hydrolysis time was greater than that of shorter hydrolysis time (Table 11). Generally, the oil absorption is related to the nature of the surface and density or thickness of particles (Amado, 1994). It was reported that lignin-rich samples had higher FBC. The insoluble dietary fiber had higher FBC level than the soluble dietary fiber because it contained high percentage of large particles, and might also contain lignin (Daou and Zhang, 2011).

It is important to note that this observation is correspond with the observation made in the β-glucan content (Figure 18c). β-glucan is the mixed-linkage β-glucan or (1-3)-(1-4) β-D-glucan that is the water soluble dietary fiber (Johansson, *et al.*, 2004). Reduction of β-glucan content associated with an intense hydrolysis may at least partial, impair those properties of the RBH.

3.5 Conclusion

The enzyme content in hydrolysis reaction affected the chemical composition of rice bran hydrolysate, including protein, total dietary fiber, DH and reducing sugar. The protein and total dietary fiber content were in line with DH and reducing sugar, respectively. The β-glucan content decreased with increasing enzyme content and hydrolysis time. The extension of hydrolysis duration and increase tilapia enzyme content caused reduction of hydration properties including WHC, WBC and SC of RBH. On the contrary, the FBC of RBH was increased with enzyme concentration and hydrolysis time.

CHAPTER 4

ANTIOXIDATIVE AND ANTI-INFLAMMATORY PROPERTIES OF RICE BRAN HYDROLYSATE

4.1 Abstract

Hydrolysation of defatted rice bran using 1% viscera extract for 30 min yielded the hydrolysate with the highest *in vitro* anti-oxidative activities. All obtained RBHs exhibited no cytotoxicity on RAW 264.7 cell lines at the maximum concentration of 500 µg/ml. The RBHs obtained by using 1% and 2% viscera extract and 30 min hydrolysis were the best nitric oxide inhibitory in the RAW 264.7 cell with an IC_{50} of 48 and 49 µg/ml, respectively. The latter RBH exhibited the strongest reduction of TNF-α, IL-1β and IL-6 cytokines. Thus, the defatted rice bran could be used for preparation of RBH with strong antioxidant and anti-inflammatory.

4.2 Introduction

Reactive oxygen species (ROS) are highly reactive ions and free radicals (chemicals containing atoms with an unpaired electron in its outer orbit) involving oxygen molecules, such as the superoxide anion, the hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$). ROS consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. ROS are regularly 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 coronary heart disease, cancer and Alzheimer's disease (Ahn *et al.*, 2012).

It has been reported that defatted rice bran has the antioxidant potential but it still remains a relatively unexplored source material, that demands further investigation especially with regards to its phytochemical complement related to possible health benefits as antioxidants (Mariod *et al*., 2010). Arab *et al*. (2011) found that Iranian rice extract have high antioxidant activities. Sirikul *et al*. (2009) reported that both conventional and organic defatted rice bran of *Oryza stiva* L. CV. Khao Dawk Mali-105 possessing high antioxidant activity. Wang *et al*. (2009) studied the scavenging activity of enzymatic hydrolysates of wheat bran by *Bacillus subtilis* xylanase that cleaved the xylan backbone and demonstrated effective *in vitro* antioxidant properties from wheat bran hydrolysates. Chanput *et al*. (2009) have ever been produce protein hydrolysates from rice bran by pepsin and trypsin that had high antioxidant activities.

Inflammation is biodefense mechanism against external stimuli such as bacterial infection or internal stimuli such as biometabolic product (Hye Kim *et al*., 2013) and many mediators are involved such as intracellular inflammatory controllers and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8), prostagrandin, lysosomal enzyme, and free radicals (Seo *et al*., 2002). In particular, transcription factors of inflammatory response become activated in the macrophage by stimuli such as cytokines, tumor necrosis factor (TNF-α), and lipopolysaccharide (LPS), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) are expressed, and nitric oxide (NO) and prostaglandin E2 (PGE2) are produced, resulting in inflammation

(Kwqamata *et al*., 2000). In addition, excessive NO production causes exasperation in inflammatory response, septic shock by excessive blood vessels dilation, inhibited healing, and nerve tissue injury, suggesting harmful action on the body (Hye Kim *et al*., 2013).

The objective of this study was to investigate the effect of different hydrolysis condition on antioxidative activity and anti-inflammatory activity of the RBH.

4.3 Materials and Methods

4.3.1 Materials and Chemicals

Testing chemicals, including chemicals for analysis of antioxidative activity, anti-inflammatory activity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Murine TNF, IL-1β and IL-6 ELISA Development Kit were purchased from Peprotech (Peprotech, Rocky Hill, USA).

The mouse macrophage cell lines, RAW 264.7, were obtained from Nutraceutical and Functional Food Research and Development Center, Prince of Songkla University, Songkhla, Thailand. The other materials required for culturing of the cells were purchased from Gibco BRL, Lift Technology (Thailand).

Rice bran hydrolysate (RBH) was prepared by enzymatic hydrolysis with tilapia viscera enzyme extract selected from the previously studied in chapter 2 in different contents of these enzyme, 1%, 2% and 3% v/w, at 0.5, 1 and 2 h described in chapter 3.

4.3.2 Antioxidative activity of rice bran hydrolysate

4.3.2.1 ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Khantaphant and Benjakul (2010). ABTS radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (4 mM 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid);

ABTS) with 2.5 mM potassium persulphate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 16 h at room temperature. Prior to assay, $ABTS^{\bullet+}$solution was diluted with methanol to obtain an absorbance of 1.1 ± 0.02 at 734 nm. To initiate the reaction, 20 µl of sample was mixed with 280 µl of $ABTS^{\bullet+}$ solution. The absorbance was then read at 734 nm after 2 h dark incubation at room temperature. The percent reduction of $ABTS^{\bullet+}$ to ABTS was calculated according to the following equation:

$$\text{ABTS (\%)} = \left[1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right] \times 100$$

4.3.2.2 DPPH radical scavenging activity assay

The method of DPPH radical scavenging activity was described by (Orhan *et al.*, 2007) with a slight modification. The 100 µl RBH sample were dissolved in distilled water and mixed with 100 µl of 0.2 mM DPPH that was dissolved in methanol. The mixture was then shaken and kept in the dark for 30 min. The absorbance of the resultant solution was recorded at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{DPPH (\%)} = \left[\frac{\text{absorbance of control - absorbance of sample}}{\text{absorbance of control}} \right] \times 100$$

4.3.2.3 Metal chelating activity

The metal chelating activity of the RBH was assessed using the method of Dinis *et al.* (1994) with slight modification. The RBH sample (1 ml) was first mixed with distilled water (3.7 ml). Then it was reacted with a solution containing 2 mM $FeCl_2$ (0.1 ml) and 5 mM ferrozine (0.2 ml). After 10 min, the absorbance of the reaction mixture was measured at 562 nm. The metal chelating ability of the RBH was calculated as a percentage using the equation:

$$\text{Metal chelating activity (\%)} = \left[1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}} \right] \times 100$$

4.3.2.4 Hydroxyl radical scavenging activity (Halliwell *et al*.,1987)

Scavenging of hydroxyl free radical was measured by the method of Halliwell *et al.*,(1987) with a slight modification by Mandal *et al*., (2009). 1 mM of EDTA, 10 mM of deoxyribose, 10 mM of hydrogen peroxide, 1mM of ascorbic acid and 1 mM $FeCl_3$ were mixed to form the reaction mixture. After an incubation period of 1 h at 37°C the extent of deoxyribose degradation was measured by the TBA reaction. 10% TCA was added in the reaction mixture and kept for 15 min in 90°C. The hydroxyl radical scavenging activity of the RBH was calculated as a percentage using the equation:

$$\text{scavenging (\%)} = \left(\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \right) \times 100$$

4.3.2.5 Superoxide anion scavenging activity

The superoxide anion scavenging activity was assay as describe by Su *et al*., (2009) with 80 µl of the RBH sample was added to 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA. The mixture was incubated at 25°C for 10 min and 1.5 mM of pyrogallol was added. The absorbance of the reaction mixture was measured at 420 nm at 0 and 4 min. The superoxide anion scavenging activity of the RBH was calculated as a percentage using the equation:

$$\text{Activity (\%)} = \frac{(\Delta \text{ absorbance of control} - \Delta \text{ absorbance of sample})}{\Delta \text{ absorbance of control}} \times 100$$

4.3.2.6 Reducing power

The reducing power of the RBH was measured according to the method described by Chen *et al*. (2007). Different concentrations of RBH were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 1.0% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. After incubation, the reaction was terminated by the addition of 10% trichloroacetic acid (TCA) and then followed by centrifugation at 5000×g for 10 min. The upper layer was mixed with distilled water and 0.1% (w/v)

ferric chloride ($FeCl_3$) and then the absorbance of the mixture was measured at 700 nm against a blank. Increase of absorbance of the reaction mixture at a wave length of 700 nm indicates an increase of reducing power.

4.3.3 Anti-inflammatory activity

4.3.3.1 Cell culture

The mouse macrophage-like cell line RAW 264.7 (the American Type Culture Collection, USA) was cultured in RPMI Medium 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin sulfate at 37°C and 5% CO_2 in humidified incubator. For all experiments, the cells were sub-cultured until a 70%-80% confluent monolayer was achieved.

4.3.3.2 Cell viability assay

The cell viability was determined by the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay (or MTT assay) as described by Szliszka *et al*. (2011) and Bronikowska *et al*. (2012). This test is based on the cleavage of the tetrazolium salt MTT to a blue formazan dye by viable cells. Briefly, RAW264.7cells (1×10^6 cell/well) were seeded in a 96 well plate for 2 h and treated with soluble fraction of rice bran (RB) 500 μg/ml and 1, 5, 10, 100 and 500 μg/ml of RBH diluted in RPMI medium. After 24 h the medium was removed, and 10 μl MTT solutions (5 mg/ml) was added to each well for 2 h. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Controls included native RAW 264.7 cells and medium alone. The spectrophotometric absorbance was measured at 570 nm using a microplate reader. The cytotoxicity as percentage of cell death was calculated by the formula:

$$\frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

4.3.3.3 Inhibitory of nitric oxide

The effect of RBH on nitric oxide (NO) production by RAW 264.7 cell lines was determined using a method modified from Wang *et al*., (2007). Briefly, RAW 264.7 cells were seeded in 96-well plates at a density of 1.0×10^6 cells/well and allowed to adhere for 2 h. After that the cell were pretreated with RBH (1, 5, 10, 100, 500 μg/ml) containing 0.5 μg/ml of lipopolysaccharide (LPS) for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min, the absorbance was measured at 570 nm using the microplate reader. Cytotoxicity was also determined by MTT assay. The NO inhibition was calculated by the following formula, then plot graph between % inhibition and RBH concentration to obtain IC_{50}.

$$\text{Inhibition (\%)} = \frac{[(\text{OD control} - \text{OD blank control}) - (\text{OD sample} - \text{OD blank sample})]}{(\text{OD control} - \text{OD blank control})} \times 100$$

4.3.3.4 Inhibitory of cytokine (TNF-α, IL-1β, IL-6)

RAW 264.7 cells were seeded in 96-well plates at $1x10^6$ cells/well and cultured for 2 h. After LPS media were treated for 6 h, RBH sample were added and incubated until 48 h. Then cytokine (TNF-α, IL-1β, IL-6) content was determined by a quantitative sandwich enzyme-linked immune-sorbent assay (ELISA) using the Murine TNF, IL-1β and IL-6 ELISA Development Kit (Peprotech, Rocky Hill, USA) according to the manufacturer's instructions.

4.4 Results and Discussion

4.4.1 Antioxidative activity of RBH

The ability to scavenge the ABTS radical (Figure 19), DPPH radical (Figure 20), hydroxyl radical (Figure 21), superoxide radical (Figure 22), reducing power (Figure 23) and metal chelating activity (Figure 24) of RBHs were observed by

increasing of hydrolysis time and the content of tilapia viscera enzyme extract. The strongest activity was achieved by hydrolysis for 30 min with 1% tilapia enzyme. It was important to note that this condition showed the lowest DH value. It is well documented that biological activity of protein hydrolysate is associate with DH (Ismail and Hasni, 2014). Therefore, it can be indicated that the RBH with lower DH has more ability to donate hydrogen atom to the free radicals than higher DH. The result of this study is correspond with the finding of Klompong *et al*., (2007) which stated that at low DH (5%) the protein hydrolysate exhibited the highest DPPH radical scavenging activity. In addition, Ismail and Hasni (2014) found Green mussel hydrolysate with low DH showed higher DPPH radical scavenging activity than that of the higher DH. Li *et al*. (2007) reported that when the DH exceed 85%, most of the peptides have been converted into free amino acids and the radical scavenging activity decreased. Therefore, the antioxidant activity (ABTS, DPPH, and hydroxyl) of RBH was found to depend on the DH and enzyme used.

The highest metal chelating activity of the RBH was observed if the hydrolysis was performed by using 1% tilapia enzyme for 30 min. Its activity was, however, not significant difference from that RBHs obtained by using higher enzyme concentration. Extension of hydrolysis duration caused significant reduction on the metal chelating activity.

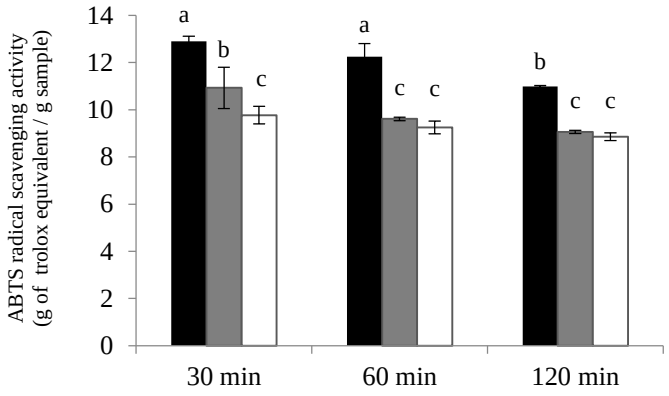


Figure 19 Effect of hydrolysis condition on ABTS scavenging activity of RBH (■: 1% tilapia viscera extract in hydrolysis reaction, ▩: 2% tilapia viscera extract in hydrolysis reaction, □: 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

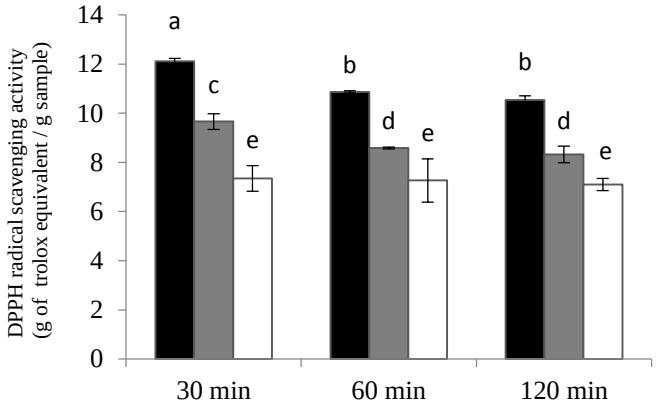


Figure 20 Effect of hydrolysis condition on DPPH scavenging activity of RBH (■: 1% tilapia viscera extract in hydrolysis reaction ▩: 2% tilapia viscera extract in hydrolysis reaction, □: 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

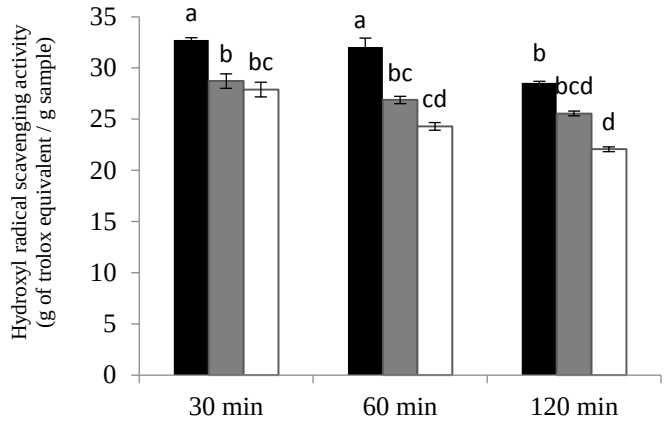


Figure 21 Effect of hydrolysis condition on hydroxyl radical scavenging activity of RBH (■ : 1% tilapia viscera extract in hydrolysis reaction, ■: 2% tilapia viscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

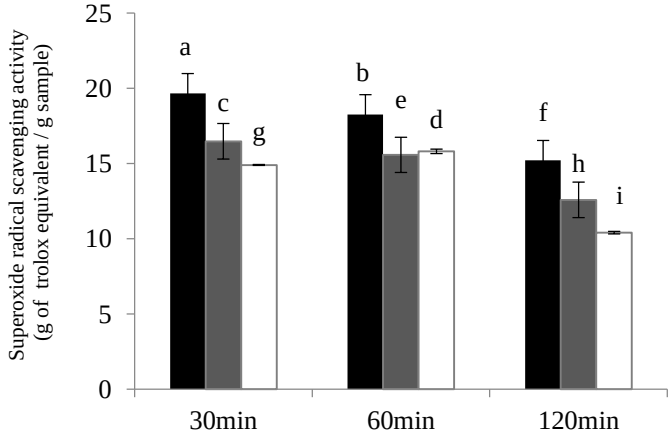


Figure 22 Effect of hydrolysis condition on superoxide radical scavenging activity of RBH (■ : 1% tilapia viscera extract in hydrolysis reaction, ■: 2% tilapia viscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

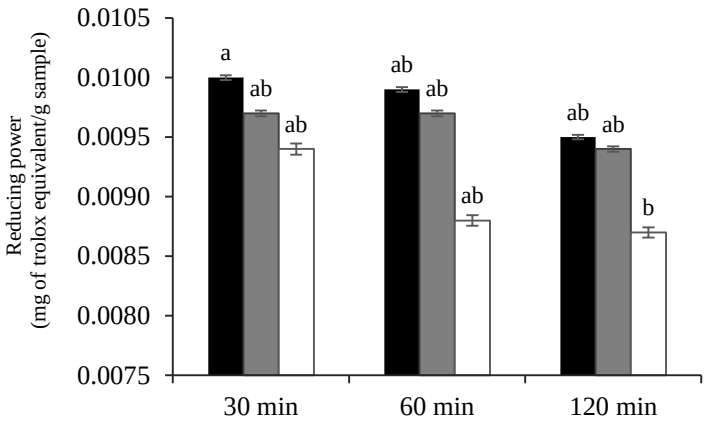


Figure 23 Effect of hydrolysis condition on reducing power of RBH (■ : 1% tilapia viscera extract in hydrolysis reaction, ■ : 2% tilapiaviscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

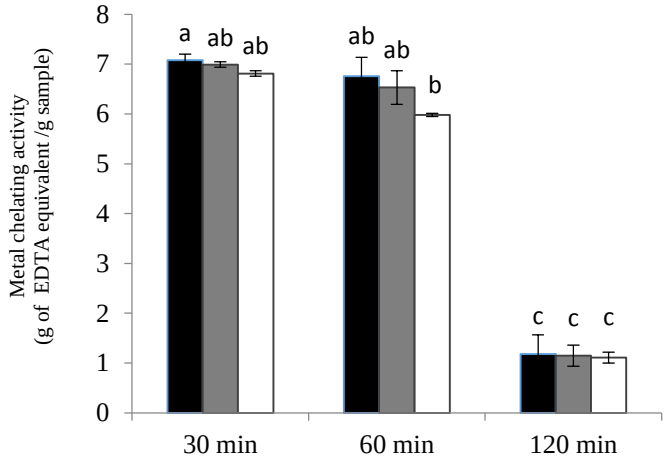


Figure 24 Effect of hydrolysis condition on metal chelating activity of RBH (■ : 1% tilapia viscera extract in hydrolysis reaction, ■ : 2% tilapia viscera extract in hydrolysis reaction, □ : 3% tilapia viscera extract in hydrolysis reaction). The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

4.4.2 Cytotoxicity of RBH to RAW 264.7 cells

Effect of the soluble fraction of RB and RBH on cytotoxicity in RAW 264.7 cells is shown in Figure 25. The data was presented only those of the lowest and highest concentration studied range; 1 and 500 μg/ml, respectively. It was found that RB and RBH was not only exhibited no cytotoxicity against RAW 264.7 cells but also exerted the cell growth with about 1.5 times higher than that of the control. The stimulus effect on the cell growth was likely decreased by increasing the concentration of the RBH. At high concentration both samples probably cause a rapid cell growth and due to limited of resources such as nutrients and oxygen thereafter may trigger cell death. Ethanolic extract of defatted rice bran was also found no cytotoxicity in RAW264.7 cells at concentration range of 10-1000 μg/ml as well (Kim *et al.*,2013).

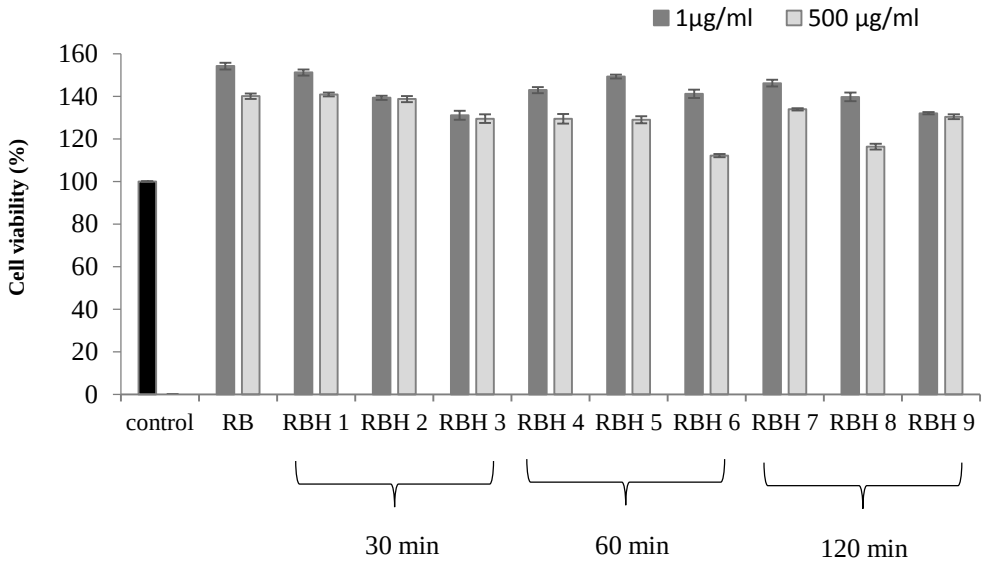


Figure 25 Effect of RBH (1 and 500 µg/ml) on cytotoxicity in Raw 264.7 cells. (RBH1, RBH4 and RBH7 produced by 1% tilapia viscera extract, RBH2, RBH5 and RBH8 produced by 2% tilapia viscera extract and RBH3, RBH6 and RBH9 produced by 3% tilapia viscera extract.) The data are means ± SD (n =3).

4.4.3 Inhibitory effect of RBH on nitric oxide production in LPS-stimulated RAW264.7 cell

In murine macrophage RAW 264.7 cells, LPS only induced the transcription and protein synthesis of iNOS (inducible nitric oxide synthase) and increased nitric oxide production (Yoon *et al*., 2009). Inhibition of RB and various RBHs against NO production in RAW 264.7 cell treated with LPS is shown in Figure 26. It was found that RBH showed higher anti-inflammatory activity than that of RB. The RBH1 and RBH2 exhibited the promising anti-inflammatory activity (IC_{50} = 48 and 49 µg/ml, respectively). It was noted that the viability of cells was higher than 80% in all RBH tested. The result also suggested that anti-inflammatory activity of RBH was decreased with increasing of hydrolysis time.

The protein hydrolysate and β-glucan have been reported their anti-inflammatory activity. Ndiaye *et al*. (2012) indicated that pea protein hydrolysate

showed significant inhibition of NO production by activated macrophage up to 20%. Ciacci *et al*. (2014) reported that kiwi fruit peptide displays anti-inflammatory activity that was highly effective in preventing the increase of LPS-induced reactive oxygen species (ROS) levels in cell line. While, Xu *et al.* (2012) reported that β-glucan from the fruiting bodies of *Lentinus edodes* resulted in the striking inhibition of NO production in LPS-activated macrophage RAW 264.7 cells.

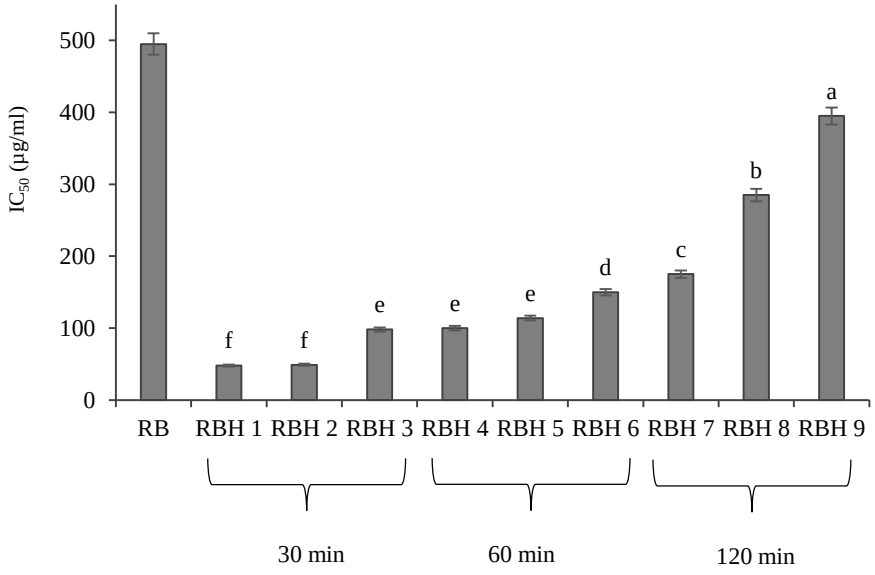


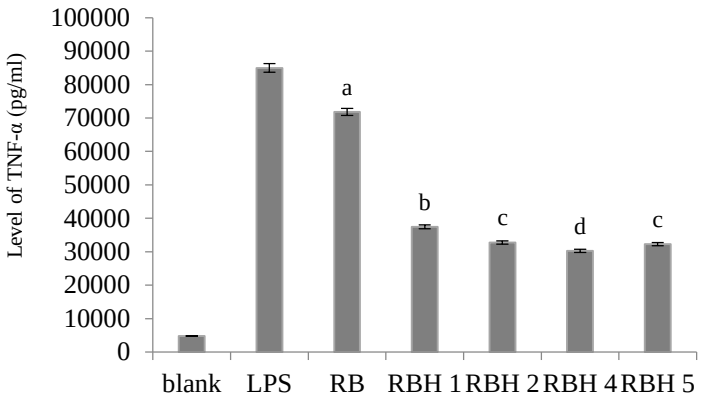
Figure 26 Effect of RBH on NO inhibition in RAW 264.7 cells.
The data are means ± SD (n =3) and shown as IC_{50} (µg/ml). The different letters indicate significant differences ($p \leq 0.05$).

4.4.4 Effect of RBH on cytokine (TNF-α, IL-1β, IL-6) secretion

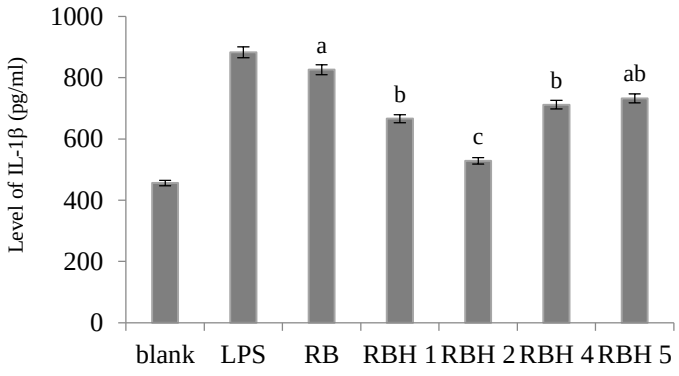
The RBH 1, RBH 2, RBH 4 and RBH 5 were selected for their inhibitory activity against secretion of pro-inflammatory cytokine based on their inhibitory effect against NO production. As shown in Figure 27, secretion of TNF-α, IL-1β and IL-6 induced by LPS were inhibited by all the RBH used with an IC_{50} of > 30269, 528.70 and 6617 pg/ml, respectively. Our data presented that TNF-α is a major pro-inflammatory cytokine response to LPS because of their highest volume released

(Figure 27). All RBHs effectively and significantly inhibited secretion of TNF-α; 60 % reduction. Bessis *et al.* (1998) reported that TNF-α is one of the major pro-inflammatory cytokines involved in the pathogenesis of chronic inflammatory disease. TNF-α plays an important part in an innate immune response (Kim *et al*., 2013). Moreover, TNF-α is a pleiotropic inflammatory cytokine and can stimulate the production or expression of IL-1β and IL-6 (Aggarwal and Natarajan, 1996). RBH2 was the most effective pro-inflammatory cytokine inhibitory candidate based on its inhibition against generation of IL-1β (Figure 27b). We also found that IL-6 secretion was effectively decreased by about 80% by the RBH1 and RBH2 (Figure 27c). Jo *et al*. (2010) reported that anti-inflammatory compounds exert their activity by inhibiting substances in the body that cause inflammation of prostaglandin E2 (PGE2) and NF-κB. Inhibition of excessive inflammation mediators such as NO and PGE2 maintain and protect inflammation diseases (Srisuk *et al*., 2014).

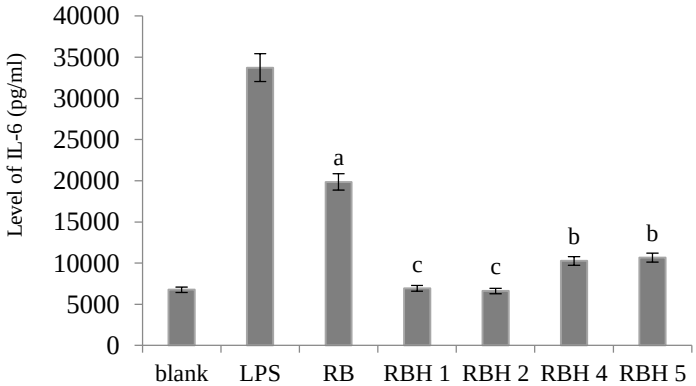
In addition, based on their inhibitory activity on generation of all cytokines, RBH2 was therefore the most outstanding than other RBHs in anti-pro-inflammatory cytokines.



(a)



(b)



(c)

Figure 27 Effect of RBH on cytokine (TNF-α, IL-1β, IL-6) secretion by RAW 264.7 cell induced with LPS. The data are means ± SD (n =3). The different letters indicate significant differences ($p \leq 0.05$).

4.5 Conclusion

Based on the results, the RBHs produced with different condition in this study displayed the efficacy in antioxidant activity and non-toxicity to RAW 264.7 cell line. In addition, RBH showed ability to inhibit NO, especially RBH produced by mild condition, and to reduce pro-inflammatory cytokine secretion. RBH2 exhibited strong anti-pro-inflammatory cytokines with a potent candidate to be developed as an antioxidant and anti-inflammatory ingredient for a functional food product.

CHAPTER 5

SUMMARY AND FUTURE WORKS

5.1 Summary

1. Extraction of crude tilapia viscera enzyme using 5% NaCl solution obtained the higher potential activity of protease and amylase than that of water and acetone extractant.

2. The application of crude tilapia viscera extract in production of defatted rice bran hydrolysate obtained a bioactive hydrolysate with potential free radical scavenging, anti-inflammatory and anti-proinflammatory cytokine (TNF-α, IL-1β and IL-6) secretion properties.

3. Different of hydrolysis conditions have an influence on the quality of RBH. The mild condition, low enzyme content and hydrolysis time, results in better bioactive hydrolysate.

5.2 Future works

1. Antioxidative and anti-inflammatory activities of RBH in animal trial should be studied.

2. Crude tilapia viscera enzyme extract should be scaled up for commercial production.

3. RBH with antioxidative or anti-inflammatory activities should be scaled up for commercial production.

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List of Publication and Proceeding

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