Study on Angiotensin I-Converting Enzyme Inhibitory Activity and Antioxidative Activity of Peptides Derived from Some Thai Traditional Fermented Fishery Products

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ชื่อวิทยานิพนธ์	การศึกษากิจกรรมยับยั้ง angiotensin I-converting enzyme และกิจกรรมต้านอนุมูลอิสระของเพปไทด์ที่ได้จากอาหารหมักจากสัตว์น้ำของไทย
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บทคัดย่อ

อาหารหมักพื้นเมืองจากสัตว์น้ำของไทยบางชนิดเป็นแหล่งของเพปไทด์ที่มีกิจกรรมยับยั้ง angiotensin I-converting enzyme (ACE) และต้านอนุมูลอิสระ ซึ่งสามารถใช้สำหรับควบคุมโรคความดันโลหิตสูงได้ พบว่า สารสกัดจากปลาส้มมีกิจกรรมยับยั้ง ACE สูงสุด ในขณะที่สารสกัดจากบูดู กุ้งจ่อม และเค็มบักนัดแสดงกิจกรรมกำจัดอนุมูลอิสระ 2,2-diphenyl-1-picryl hydrazyl (DPPH) และ 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) และมีกิจกรรมรีดิวซ์เฟอริก (FRAP) สูงสุดตามลำดับ (p<0.05) จากการวิเคราะห์โดยวิธี Principle component analysis (PCA) พบว่า เพปไทด์ที่มีกิจกรรมยับยั้ง ACE สูงมักพบในอาหารหมักที่มีพีเอชและปริมาณเกลือต่ำ และน่าจะสอดคล้องกับการใช้กระเทียมและชนิดของสัตว์น้ำที่ใช้เป็นวัตถุดิบ โดยไม่มีความสัมพันธ์กับอัตราส่วนของกรดอะมิโนอิสระต่อกรดอะมิโนอิสระทั้งหมด สำหรับกิจกรรมต้านอนุมูลอิสระ เพปไทด์ที่มีกิจกรรมรีดิวซ์เฟอริก (FRAP) สูงมักพบในอาหารหมักที่มีอัตราส่วนของกรดอะมิโนอิสระต่อกรดอะมิโนอิสระทั้งหมดสูง อาหารหมักที่มีกิจกรรมกำจัดอนุมูลอิสระ DPPH สูงมักมีกิจกรรมกำจัดอนุมูลอิสระABTS สูงแต่ทั้งสองกิจกรรมไม่มีความสัมพันธ์กับพีเอช ปริมาณเกลือ และอัตราส่วนของกรดอะมิโนอิสระต่อกรดอะมิโนอิสระทั้งหมด นอกจากนี้กิจกรรมยับยั้ง ACE ไม่มีความสัมพันธ์กับกิจกรรมต้านอนุมูลอิสระ อย่างไรก็ตาม การยับยั้ง ACE เป็นผลหลักที่ทำให้ความดันโลหิตลดลง ดังนั้น จึงเลือกปลาส้มที่ผลิตจากปลาตะเพียนซึ่งแสดงกิจกรรมยับยั้ง ACE สูงสุดสำหรับการศึกษาในลำดับต่อไป

การเติมหัวเชื้อ *Pediococcus acidilactici* BCC 9545 และ *Staphylococcus xylosus* BCC 3710 ในปลาส้มที่ผลิตจากปลาตะเพียน มีผลทำให้ระยะเวลาการหมักลดลงแต่ไม่ทำให้เกิดการปลดปล่อยเพปไทด์ที่มีกิจกรรมยับยั้ง ACE ได้มากขึ้น โดยปลาส้มที่เติมหัวเชื้อ *P. acidilactici* BCC 9545 ที่ระดับ 10^4 CFU/กรัม และ *S. xylosus* BCC 3710 ที่ระดับ 10^6 CFU/กรัม มีอัตราการหมักสูงกว่าชุดควบคุม ซึ่งสามารถบ่งชี้ได้จากอัตราการลดลงของพีเอช การผลิตกรดอินทรีย์ ปริมาณกลุ่มของกรดอะมิโนอิสระ และระดับการย่อยสลายโปรตีนที่สูงกว่า (p<0.05) อ้างอิงจากค่าพีเอชที่

ต้องการ (พีเอช 5) ชุดควบคุม และปลาส้มที่เติมหัวเชื้อสามารถหมักสมบูรณ์ได้ภายในเวลา 96 และ 72 ชั่วโมง ตามลำดับ กิจกรรมยับยั้ง ACE ของสารสกัดจากปลาส้มมีความคงตัวต่อความร้อนแต่ไม่คงตัวต่อพีเอชที่สูงขึ้น นอกจากนี้กิจกรรมยับยั้ง ACE ของสารสกัดจากปลาส้มลดลงหลังจากผ่านการย่อยด้วยเปปซินและแพนครีเอติน

การแยกและศึกษาเพปไทด์ที่มีกิจกรรมยับยั้ง ACE ที่พบในสารสกัดของปลาส้มหมักตามธรรมชาติที่ 96 ชั่วโมง ซึ่งแสดงกิจกรรมยับยั้ง ACE สูงสุด ทำโดยการกรองผ่านอัลตราฟิวเตชันเมมเบรนร่วมกับเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรีเวิร์สเฟส พบว่า มี 7 แฟรกชั่นที่มีกิจกรรมยับยั้ง ACE มากกว่า 2 ยูนิต โดยที่แฟรกชั่นเหล่านี้ประกอบด้วยเพปไทด์ต่างๆ 300 เพปไทด์ ที่มีองค์ประกอบของกรดอะมิโนจำนวน 2-16 ตัว และมีมวลโมเลกุลอยู่ในช่วง 300 ถึง 1800 ดาลตัน แม้ว่าองค์ประกอบและลำดับกรดอะมิโนของเพปไทด์นี้แตกต่างกัน แต่มีลักษณะเด่นที่สอดคล้องกับเพปไทด์ที่มีกิจกรรมยับยั้ง ACE 3 ประการ ได้แก่ 1) เป็นเพปไทด์ที่มีลิวซีนตั้งแต่ 1 ตัว เป็นองค์ประกอบภายในสาย 2) เป็นเพปไทด์ที่ประกอบด้วยกรดอะมิโนที่เป็นเบสที่ส่วนปลายที่มีหมู่คาร์บอกซิลเป็นอิสระ และ 3) เป็นเพปไทด์ที่ประกอบด้วยโพรลีน ไทโรซีน ฟรีนิลอะลานีน หรือกรดอะมิโนที่ไม่ชอบน้ำที่ส่วนปลายที่มีหมู่คาร์บอกซิลเป็นอิสระ

การศึกษากิจกรรมยับยั้ง ACE ของเพปไทด์สังเคราะห์จำนวน 20 เพปไทด์ ซึ่งออกแบบจากเพปไทด์ที่พบในสารสกัดเพปไทด์ที่ได้จากปลาส้ม พบว่า Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro เป็นเพปไทด์ที่มีกิจกรรมสูงสุด ตามด้วย Ala-Pro-Val-Tyr-Leu-Arg และ Gln-His-Asn-Asp-Glu-Pro-Pro ตามลำดับ พบว่าการมีโพรลีนที่ส่วนปลายที่มีหมู่คาร์บอกซิลเป็นอิสระหรือ ภายในสายเพปไทด์ มีความสำคัญต่อกลไกการยับยั้ง ACE ของเพปไทด์ที่มีอยู่ในปลาส้ม อย่างไรก็ตาม ระดับความเข้มข้นของเพปไทด์นี้ที่สามารถยับยั้ง ACE ได้ร้อยละ 50 มีค่าสูงกว่า Val-Pro-Pro และ Ile-Pro-Pro ถึงแม้ว่าเพปไทด์ที่มีกิจกรรมยับยั้ง ACE จากปลาส้มมีฤทธิ์น้อยกว่า เพปไทด์ที่พบในการศึกษานี้จัดเป็นเพปไทด์ชนิดใหม่ที่พบในอาหารหมักซึ่งรับประทานในชีวิตประจำวัน ซึ่งอาจจะมีประโยชน์ในการเตรียมผลิตภัณฑ์อาหารเสริมสำหรับผู้ป่วยโรคความดันโลหิตสูง

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ABSTRACT

Some of Thai traditional fermented fishery products were found to be a source of angiotensin I-converting enzyme (ACE) inhibitory and antioxidative peptides which could be used for the hypertension control. The highest ACE inhibitory activity was found in the extract from *Pla-som*. For antioxidative activities, the extracts from *Bu-du, Kung-chom* and *Kem-buk-nud* exhibited the highest 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and ferric reducing antioxidant power (FRAP) ($p<0.05$), respectively. Based on principal component analysis (PCA), peptides with high ACE inhibitory activity were more likely present in products with low pH and low salt content. The presence of these peptides was probably governed by the use of garlic and raw material used but not affected by the ratio of free and total free α-amino acids (F/T ratio). For antioxidative activities, peptides with high FRAP were more likely found in products with high F/T ratio. Products with high DPPH radical scavenging activity more likely had high ABTS radical scavenging activity but both activities showed no correlation to pH, salt content and F/T ratio. In addition, ACE inhibitory activity exhibited no correlation to antioxidative activities. Since ACE inhibition is the main effect on blood pressure lowering, *Pla-som* produced from *Puntius gonionotus* which showed the highest ACE inhibitory activity was further studied.

Inoculation of *Pediococcus acidilactici* BCC 9545 and *Staphylococcus xylosus* BCC3710 in *Pla-som* produced from *Puntius gonionotus* resulted in a reduction of fermentation time but could not increase the release of ACE inhibitory

peptides. *Pla-som* inoculated with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g exhibited a higher rate of fermentation than the control as indicated by the greater rate of pH drop, organic acid production, free amino group content and degree of hydrolysis during fermentation ($p<0.05$). Based on pH desired (pH 5), the fermentation was completed within 96 and 72 h for the control and starters-inoculated *Pla-som*, respectively. The ACE inhibitory activity of the extract from *Pla-som* showed heat stability but was not stable with increasing pH. In addition, this activity of the extract from *Pla-som* decreased after incubation with pepsin and pancreatin.

ACE inhibitory peptides were isolated from the crude extract of *Pla-som* naturally fermented at 96 h that possessed the highest ACE inhibitory activity. The crude extract was purified by ultrafiltration and RP-HPLC, respectively. Seven fractions with ACE inhibitory activity more than 2 units were collected for further identification by LC-MS/MS. These fractions contained 300 peptides which contained 2-16 amino acid residues with the nominal mass ranging from 300 to 1800 Da. Although the amino acid compositions and sequences of these peptides were different, there were 3 distinctive features that could be attributed to the peptides known to exhibit ACE inhibitory activity. These 3 distinctive features were peptides with one or more Leu residues in the sequence, peptides with basic amino acids at C-terminal and peptides with Pro, Tyr, Phe or hydrophobic amino acids at C-terminal.

ACE inhibitory activities of 20 synthetic peptides, which were designed based on peptides derived from *Pla-som* were studied. Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro was the most active peptide in this study, followed by Ala-Pro-Val-Tyr-Leu-Arg and Gln-His-Asn-Asp-Glu-Pro-Pro, respectively. The presence of Pro residues at C-terminal or in the sequence plays important roles in the ACE inhibitory activity of the peptides derived from *Pla-som*. However, the IC_{50} values of these peptides were relatively higher than Val-Pro-Pro and Ile-Pro-Pro. Although they were weaker in ACE inhibition, they are characterized as novel peptides which were derived from a food source that is consumed daily and may be useful in the preparation of antihypertensive functional foods.

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CHAPTER 1

INTRODUCTION

The definition of health has no longer been restricted to the absence of disease but includes physical fitness as well as mental and physiological well-being. For the development, growth and maintenance of the body, food is required, but food is also recognized to play a key role in the quality of life. Functional foods are those foods that positively affect one or more target functions in the human body, beyond the basic nutritional function, in a way that it is relevant to either an improved state of health and well-being and/or reduction of risk of disease (Arvanitoyannis and van Houwelingen-Koukaliaroglou, 2005). Recognized as one of the components of functional foods, bioactive peptides exert a physiological effect in the body (Shahidi and Zhong, 2008). These peptides may naturally occur in food or may be inactive within the sequence of parent protein but can be released during gastrointestinal digestion or food processing.

Fermentation is believed to enhance the neutraceutical value of fermented foods. As a consequence of fermentation, food components are modified by microorganisms that not only grow and consume part of the substrate but also enrich them with the products of their metabolism. Breakdown of food proteins by either endogenous or microbial proteases may release peptides with biological activities potentially used as nutraceuticals and functional food ingredients for health promotion and disease risk reduction, depending on their structural, compositional and sequential properties.

Hypertension is a major health issue, estimated to be affecting 15-20% of the world's adult population. Angiotensin-I converting enzyme (ACE*)*, reactive oxygen species (ROS) and free radical can cause hypertension which is the most common serious chronic health problem and carries a high risk factor for cardiovascular diseases (Jung *et al*., 2006). Thus, the combination of ACE inhibition and antioxidative activity in one multifunctional preparation could be very useful for the control of cardiovascular and chronic diseases (Vercruysse *et al*., 2009).

ACE performs an important role in the regulation of blood pressure and hypertension because it catalyzes the conversion of inactive angiotensin I into angiotensin II, a potent vasoconstrictor and inactives bradykinin, a potent vasodilator (Dzau and Safer, 1988). Inhibition of ACE reduces angiotensin II activity, but increases bradykinin levels, and thus can result in a lowering of blood pressure (Koike *et al*., 1980). Synthetic ACE inhibitors have been developed as antihypertensive drugs but the use of such inhibitors can cause serious side effects (Antonios and Macgregor, 1995).

ROS and free radicals seem to play an important role in the complex and multicausal process that leads to essential hypertension, particularly in the local regulation of capillary bed (Dajas *et al*., 2004). Formation of free radicals such as superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable, and react rapidly with the other groups or substances in the body, leading to cell or tissue injury. Under pathological conditions, the balance between the generation and the elimination of ROS is disturbed; as a result of these events, biomacromolecules, including DNA, membrane lipids and proteins, are damaged by ROS-mediated oxidative stress. Therefore, antioxidants are important for bodily protection against many diseases including hypertension and chronic diseases. The use of synthetic antioxidants is under strict regulation due to potential risks related to health (Vercruysse *et al*., 2009).

The peptides derived from food proteins are considered to be milder and safer, compared with synthetic drugs. Several fermented fuctional foods derive their activities from bioactive peptides that are released after enzymatic digestion. Release of regulatory peptides by enzymatic proteolysis of food proteins may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. Numerous studies found ACE inhibitory and antioxidative peptides from various fishery hydrolysates such as tuna, shrimp and oyster (Hsu, 2010; Qian *et al*., 2008; Lee *et al*., 2010; Cao *et al*., 2010; Wang *et al*., 2008a). Therefore, fermented fishery products might be a good source of bioactive peptides including ACE inhibitory and antioxidative activities.

Review of Literature

1. Fermented foods

Fermented food is foodstuff, which has gone through the fermentation process by microorganisms such as mold, bacteria, or yeast, so that it can be stored for an extended period of time. Fermented foods are of great significance because they provide and preserve vast quantities of nutritious foods in a wide diversity of flavors, aromas and textures which enrich the human diet. Besides the valuable protein and fat nutrients, flavor compounds are abundant and can vary, depending on raw materials, starter cultures and processing conditions.

The fermentation of aquatic products is primarily concerned with the controlled degradation of proteinaceous materials into more stable or at least different tasting products. Degradation in traditional processes is controlled primarily by the addition of salt. Control of the oxygen content during fermenting also helps in determining the final product characteristics. The most common putrefactive microorganisms on fish are inhibited at salt contents above 6 to 8 percent. However, the overall process is also influenced by several other variables including (1) the microflora present in the fish and salt, (2) the proteolytic activity of enzymes characteristic of the fish species, (3) condition of the product entering the fermentation process, (4) presence or absence of oxygen, (5) nutritional state of the fish, (6) temperature, (7) pH of the fermentation mixture, (8) the presence of visceral or vegetable enzymes, (9) the presence and, or concentration of carbohydrates, and (10) the duration of the fermentation process. Process duration is of particular importance in products containing fish or parts of fish, because these will eventually liquefy if the process is allowed to proceed too long (Mackie *et al*., 1971).

1.1 Classification of Thai traditional fermented foods

The classification of Thai traditional fermented foods can be done according to various methods based on final appearance, main processing techniques and ingredients used (Phitakpol *et al*., 1995; Saisithi, 1994) and type of raw materials (Tanasupawat and Visessanguan, 2008).

Phitakpol *et al*. (1995) classified Thai traditional fermented products according to process, ingredients used and the final appearance into 3 different groups including:

1) Fermented with a large portion of salt

This includes hydrolyzed products, e.g. fish sauce (*Nam-pla*, *Bu-du*) and partially hydrolyzed products, e.g. fermented clam (*Hoi-dong*), shrimp or krill paste (*Ka-pi*), fermented shrimp products (*Kung-dong*) and fermented fish viscera (*Tai-pla*), etc.

2) Fermented with salt and carbohydrate source

This group of products includes fermented fish roe (*Khai-plaa-dong*), fermented shrimp products (*Kung-chao, Kung-chom, Kung-som),* fermented fish (*Pla-chao*, *Pla-chom*, *Pla-paeng-daeng*, *Pla-ra*, *Pla-som*, *Som-fug*), etc. Carbohydrate sources, such as cooked rice, roasted rice etc., are added as the carbon source of microorganisms, mostly lactic acid bacteria. These products have the sour taste, due to lactic acid and other acids formed during fermentation.

3) Fermented with salt and fruit

Kem-buk-nud belongs to this group. The fruit, mainly pineapple, is the source of proteolytic enzyme. These products have the unique characteristics due to the presence of fruit.

Saisithi (1994) classified traditional fermented products based on the type of substrate and source of enzyme into 3 groups including:

1) Group I products

Products are processed from fish/salt mixture and hydrolyzed by the combined action of fish and bacterial enzymes. The examples are *Nam-pla*, *Pla-ra*, *Ka-pi*. Both fish sauce and fish paste are prepared from whole fish. The addition of salt to fish is to prevent the spoilage caused by microorganisms. The enzymes from both fish and microorganisms are responsible for hydrolysis. Firstly, fermentation starts with fish enzymes, especially digestive proteinases, resulting in the small peptides. The resulting peptides are used as the substrate for the microorganisms, which play an important role in further hydrolysis as well as flavor development.

2) Group II products

Products, belonging to this category, are produced from fish/salt/carbohydrate mixture partially hydrolyzed by bacterial enzyme. Products include *Pla-chom*, *Pla-som* and *Som-fug*. Lactic acid bacteria play an essential role in flavor and acid taste development in the products. There are a variety of fermented products in this category. Fish can be prepared in different forms, such as the whole fish, pieces and mince. Carbohydrate is usually added in the form of cooked rice. The ratio of salt to fish is 1:3 or 1:4. Salting and fermenting time vary from 1 to 2-3 days. After salting and fermenting, cooked rice is mixed and fermentation is allowed to take place, generally for 2-3 days. Coloring such as *Ang-kak* is sometimes added for some products such as *Plaa-paeng-daeng*. Therefore, carbohydrate is the carbon source for microorganisms, especially lactic acid bacteria. The acids formed are the main preservatives and contribute to the flavor and taste.

3) Group III products

Products under this category are prepared from fish/salt mixture, hydrolyzed mostly by fish enzymes in the presence of carbohydrate fermented by yeast and mold. *Pla-chao* or *Kung-chao* are the examples of this group. Prior to processing, fish or shrimp are prepared in the same manner with *Pla-chom* and *Kung-chom*. However, the fish or shrimp is mixed with salt with the ratio of 3:1 and the mixture is left for 2 days. The liquid formed is drained and the fish (3 parts) is mixed with *Khao-mak* (alcoholic fermented steamed rice). The mixture is left for 10-20 days for fermentation. The products have a sweet, sour and salty taste with slightly alcoholic flavor.

Tanasupawat and Visessanguan (2008) broadly classified Thai fermented food based on raw materials used including:

1) Fishery products

Nam-pla (fish sauce), *Ka-pi* (shrimp paste) and *Bu-du* are produced from fish with a large proportion of salt. Most of them are made from marine fish in the coastal provinces, especially the east coast. There are many kinds of fermented fish with salt and carbohydrate, such as *Pla-ra, Pla-som, Pla-chao, Som-fug,* and *Pla-chom*. The fish used are mainly fresh water but freshwater shrimps are also used. Carbohydrate, such as cooked rice, roasted rice etc., is added as the carbon source of

microorganisms, mostly lactic acid bacteria. These products have the sour taste, due to lactic acid and other acids formed during fermentation. The roasted rice also gives a brown colour. There are two types of fermented rice used, *Khao-mak*, which is mould and yeast-fermented, and *Ang-kak* rice which is fermented using the mould *Monascus purpureus,* which gives a red colour in *Pla-paeng-daeng*.

2) Meat products

Fermented pork sausage (*Nham*) is made from minced red pork meat mixed with pork rind, garlic, pepper, salt, chilli, and trace of potassium nitrate. The mixture is wrapped with banana leaves or plastic sheets and fermented for a few days. *Sai-krog-prieo* (fermented sausage) and *Mam* (fermented beef or pork sausage or beef/ pork liver) are produced from shredded pork or beef meat with fat, cooked rice, salt, sugar, pepper, and spices. All ingredients were packed in the intestine and tied with string at intervals, and fermented.

3) Plant products

Naw-mai-dong (fermented bamboo shoot) was fermented by adding the brine into the sliced pieces of bamboo shoot which packed in the jars. *Phak-gard-dong* (pickled green mustard) was fermented with the mixture of green mustard and brine and packed tightly in jar. *Miang* (fermented tea leaves) is produced in the northern part of Thailand. The steamed tea leaves are wrapped tightly in individual bundles and packed into containers (small baskets for young tea leaves and large underground cement wells for mature tea leaves). The tea leaves are pressed tightly, weighted down, covered with banana leaves or plastic sheets, and fermented for a week or many months.

4) Grains and cereal products

Fermented glutinous rice (*Khao-mak*) is produced by *Loog-pang* (a starter cake containing mould and yeast) as a starter and lactic acid bacteria (LAB) are found in this food. The starter of *Aspergillus oryzae* is used for production of *Koji* in soy sauce fermentation. LAB also contribute to fermentation of rice noodle (*Khanom-jeen*) and soy sauce. The rice wine, such as *Sa-to, Nam-khao*, *Kra-chae*, and *Ou*, are produced with *Loog-pang* (Lotong, 1998) by *Saccharomyces cerevisiae, Saccharomycopsis fibuligera, Aspergillus oryzae, Amylomyces rouxii, Rhizopus* spp. and *Mucor* spp. (Lotong, 1998; Sukhumavasi *et al*., 1975). In soy sauce fermentation,

Aspergillus oryzae is also important for producing *koji* (Lotong, 1998). Although yeasts are found in these fermented products (Suzuki *et al*., 1987), LAB are responsible for their souring and ripening (Tanasupawat and Komagata, 2001). Red yeast rice, red Koji or Angkak obtained as a *Monascus* culture on rice that has long been used as a natural food colorant (Jongrungruangchok *et al*., 2004). Alkaline-fermented soybean foods are very common in northern Thailand. *Thua-nao* fermentation mainly relies on adventitious microorganism in which a gram-positive, endosporeforming bacteria, has usually been found as the predominant microorganism (Sundhagul *et al*., 1972; Leejeerajumnean *et al*., 2001). Microorganisms involved in *Thua-nao* fermentation have been isolated and characterized to be *Bacillus subtilis* (Sundhagul *et al*., 1972; Inatsu *et al*., 2006). Hara *et al*. (1986) reported that four strains of aerobic, gram-positive and spore-forming rods isolated from *Thua-nao* produced in Thailand were taxonomically similar to *Bacillus subtilis* (natto). Protease and amylase produced by the bacteria decompose protein and insoluble sugar in the raw soybeans, thus increasing the nutritional value as well as the availability of the soybean foods (Visessanguan *et al*., 2005; Steinkraus, 1995).

1.2 Types of Thai fermented fishery products

Thai traditional fermented foods are mostly produced according to family tradition and local geographic preferences. Therefore, large differences exist in type of raw materials, ingredients and production methods. Fermented fishery products can be categorized according to main processing techniques and ingredients used (Phitakpol *et al*., 1995). Although some products have the similar processing, some ingredients used can be varied, leading to the different characteristics, especially flavor and taste. Different raw materials including fish, shrimp, mollusk, etc. have also provided the variety of products with differences in appearance, flavor, taste as well as other sensorial properties. This classification based on type of raw material is divided into 3 groups including fermented shrimp, fermented fish and fermented mussel (Table 1).

Table 1. Raw materials and ingredients of Thai fermented fishery products.

Types of fermented fishery products	Name	Raw materials and ingredients
Fermented shrimp or krill	*Ka-pi*	Shrimp or krill and salt
	Kung-chom	Shrimp, salt or salt and fish sauce and mixed roasted rice
	Kheeuy-naam	Krill and salt
Fermented fish	*Bu-du* (fish sauce)	Marine fish and salt
	Tai-pla	Viscera of fish and salt
	Pla-ra	Freshwater fish, salt, roasted rice and rice bran
	Pla-chao	Freshwater fish and *Khao-mak* (alcoholic fermented steamed rice)
	Pla-som	Freshwater fish, salt, steamed rice and/or sticky rice and garlic
	Som-fug	Freshwater fish mince, salt, ground steamed rice and minced garlic
	Pla-chom	Freshwater fish, salt and roasted rice
	Kem-buk-nud	Freshwater fish mince, salt and pine apple
Fermented mussel	*Hoi-dong*	Mussel, salt, tamarind, sugar and coconut juice

Source: Phitakpol *et al*. (1995)

1.3 Microbiology of fermented fishery products

Fish in its natural environment has its own micro-flora in the slime on its body, in its gut and in its gills. These microorganisms, as well as the enzymes in the tissues of the fish, bring about putrefactive changes in fish when it dies. Furthermore, the microorganisms generally present in the salt used for salting also contribute to the degradative changes in the fish.

Microorganisms require water in an available form for growth and metabolism. All microbial growth is inhibited at water activity (a_w) below 0.60. Halophiles grow optimally at high salt concentrations but are unable to grow in salt-free media. Halophiles have the capacity to balance the osmotic pressure of the environments and resist the denaturing effect of salts (Grant *et al*., 2001). Kushner (1993) classified microorganisms based on their requirement for NaCl to five groups, which are non-halophiles (<0.2 M, ~1% salt), slight halophiles (0.2-0.5 M, ~1-3% salt), moderate halophiles (0.5-2.5, ~3-15% salt), borderline extreme halophiles (1.5-4.0 M, ~9-23% salt) and extreme halophiles (2.5-5.2 M, ~15-32% salt). Halotolerant organisms grow best without significant amounts of salt but can also grow in concentrations higher than that of sea water. In addition, halophilic bacteria can be found in fermented fish and fish products, such as fish sauce, fermented fish pastes and salted fish. Xerophiles are those organisms which grow rapidly under relatively dry conditions or below a_w of 0.85 while osmophiles can grow under high osmotic pressure. Most food-borne bacterial pathogens are not able to grow in an a_w range of 0.98-0.93 (Table 2) (Essuman, 1992).

Proteolytic bacteria played an important role during fishery fermentation. There are several groups of proteolytic bacteria in fermented fishery products, including halophilic bacteria, halotolerant and lactic acid bacteria. These bacteria hydrolyze fish protein to peptides and amino acids (Lopetcharat *et al*., 2001). Some amino acids can be used as substrates for lactic acid bacteria.

Lactic acid bacteria dominate fish fermentations (Table 3), similar to other food fermentations, as a consequence of their natural presence on raw materials, their dominance in anaerobic environments and their antagonistic activites against other bacteria, including spoilage and pathogenic bacteria. Traditional fermented fish with salt—*Nam-pla*, *Bu-du*, *Tai-pla*, *Pla-ra*, *pla-chom*, *Kung-chom*, and *Hoi-dong*—are found in different parts of Thailand (Tanasupawat and Komagata, 1995). Lactic acid bacteria *Tetragenococcus halophilus*, *Enterococcus faecalis*, and *Enterococcus hirae*, and the catalase positive cocci *Staphylococcus carnosus* and *Staphylococcus piscifermentans* were reported to be distributed in these products (Tanasupawat and Daengsubha, 1983; Tanasupawat *et al*., 1991, 1992a, b, c).

Table 2. Growth of microorganisms in salted fish.

Water activity (a_w)	Sodium chloride concentration (%)	Microorganisms grooving	
		Pathogens	Spoilage organisms
0.98	< 3.5	All known food-borne	Most micro-organisms pathogens of concern in foods particularly the gram negative rods
0.98-0.93	3.5-10	*Bacillus cereus, Clostridium botulinum, Salmonella spp,. Clostridium perfringens, Vibrio parahaemolyticus*	Lactobacilliaceae, Enterobacteriaceae, Bacilliaceae, Micrococcaceae, moulds
0.93-0.85	10- 17	Staphylococcus	Cocci, yeasts, moulds
0.85-0.60	> 17	Mycotoxic, xerophilic moulds (no mycotoxin is produced at a_w less than 0.80)	Halophilic bacteria, yeasts, moulds

Staphylococcus aureus grows poorly in competition with the natural microflora of most foods at high levels of water activity.

Source: ICMSF (1980)

Table 3. Diversity of lactic acid bacteria in Thai fermented fishery products.

Species	Fermented Products
L. pentosus	*Pla-chom, Kung-chom, Pla-som, Som-fug*
L. plantarum	*Kung-chom, Pla-som, Som-fug*
L. brevis	*Sour fish spawn, Som-fug*
L. fermentum	*Pla-som, Pla-chao*
L. sakei	*Som-fug*
L. farciminis	*Pla-ra, Pla-chom, Kung-chom, Hoi-dong*
L. alimentarius/farciminis	*Pla-som*
L. acidipiscis sp. nov	*Pla-ra, Pla-chom*
P. pentosaceus	*Pla-som, Som-fug*
T. halophilus	*Nam-pla, Ka-pi, Pla-ra, Pla-chom, Kung-chom, Bu-du, Taipla, Pla-paeng-daeng, Hoi-dong*
T. muriaticus	*Nam-pla, Ka-pi*
Lactococcus garviae	*Pla-som*
E. hirae	*Pla-chom, Pla-som*
E. faecalis	*Pla-ra*
W. thailandensis sp. nov	*Pla-ra*
W. confusa	*Pla-som*

Source: Tanasupawat (2009)

1.4 Proteolysis

Proteolysis (protein digesting) is an important biochemical change occurring during the ripening of fermented fishery products. It influences both texture and flavour development due to the formation of several low molecular weight compounds, including peptides, amino acids, aldehydes, organic acids and amines, which are important flavour compounds, or precursors of flavour compounds (Demeyer *et al.,* 1995; Díaz *et al.,* 1993; Naes *et al.,* 1995).

Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. These constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile. The specificity of proteolytic enzymes is governed by the nature of the amino acid and other functional groups (aromatic or aliphatic or sulphur-containing) close to the bond being hydrolyzed. Proteases are present in all living beings and play an important role in normal and abnormal physiological conditions, catalyzing various metabolic reactions (Sandhya *et al.,* 2004). Enzymes catalyze the degradation of proteins and strongly determine the characteristics of a finished fermented product. Important enzymes in fermentation may originate from four general sources (1) viscera and digestive system, (2) muscle tissue, (3) plants added to the fermentation, and (4) microorganisms active in the fermentation.

1) Viscera and digestive enzymes

There is a wide various proteases present in the viscera and digestive tract of aquatic animals; trypsin, chymotrypsin and pepsin are three of the more important ones. Trypsin is present in the pyloric caeca (Mackie *et al*., 1971). Pepsin is usually found in the stomach of fish and is a principal enzyme of the digestive juices. Since the variety of enzymes are found in different locations in the fish viscera and digestive tract, the dressing procedure is important in determining the rate and type of enzymatic degradation occurring. Fermented products manufactured from whole fish will have different characteristics than those manufactured from headed and gutted fish. The enzymatic activity of most visceral and digestive tract enzymes from fish tends to be the greatest at near neutral pH values. This is in contrast with many mammalian digestive enzymes which are most active at a relatively low pH range

(Mackie *et al*., 1971). Additionally, season and feeding activity at the time of capture will influence the enzyme activity. Heavily feeding fish will generally deteriorate more rapidly because the enzyme concentration often is higher in the digestive tract of the fish during feeding. The effect of season on enzyme activity varies with the spawning cycle, water temperature, feeding cycle and other variables. The activity and thermal stability of fish enzymes varies from one species to another. For example, the activity and thermal stability of tryptic enzymes from horse mackerel (*Trachurus mediterraneus ponticus*) are greater than those from sprat (*Sprattus nostamus*). The pepsin from plaice (*Pleuronectes platessa*) is ten times more active than that from horse mackerel. Generally, white fish have less proteolytic activity than do pelagic species (Mackie *et al*., 1971). The effect of digestive and visceral mass enzymes will depend on the method of dressing the fish, fish species, fermentation mixture pH, and other factors. Because there is a large group of enzymes from these sources, and each enzyme will require different conditions for optimal activity, it is difficult to predict the total effect in a specific situation. Thus, experience is a definite advantage in producing the desired product (Wheaton and Lawson, 1985).

2) Muscle tissue enzymes

Muscle tissue enzymes are generally found within the cell. Digestive enzymes, on the other hand, are exocellular secretions. This fact tends to confine muscle tissue enzymes more and reduce their effectiveness during fermentation. Although there are reports of muscle tissue enzymes having optimal activity at neutral pH values, the preponderance of evidence indicates low pH increases muscle tissue enzyme activity. With the exception of fish silage and some other products, most fermented fish products are prepared at pH values above 4. Thus, the pH is not optimum for most muscle tissue enzymes. The above two factors tend to combine to relegate muscle tissue enzymes to a secondary role in fermentations (Mackie *et al*., 1971).

3) Plant enzymes

Several plant enzymes are well known for their ability to tenderize meats. Bromelain found in pineapple juice, papain from papaya latex, and ficin from figs are well-known examples. These enzymes are optimally active at near neutral pH and are quite heat stable, both factors making them well suited to helping hydrolyze

protein in fermented fish products (Mackie *et al*., 1971). Plant enzymes are often added to the fermenting process by simply adding the plant material to the fermenting container. However, it is sometimes advantageous to purchase the concentrated enzymes and add them to the fermenting container (Wheaton and Lawson, 1985).

4) Microbial enzymes

Microorganisms are well known for excretion of proteolytic enzymes capable of degrading proteins. Many types of microbes excrete proteolytic enzymes, including the fungi *Aspergillus oryzae*, the bacteria *Bacillus subtillis*, the actinomycetes *Streptomyces griseus*, and the yeast *Saccharomyces* spp. (Mackie *et al*., 1971). Careful selection by seeding or controlling the growth environment within the fermentation chamber enables the desired microbes to flourish and produce significant quantities of proteolytic enzymes which help hydrolyze the fish protein (Wheaton and Lawson, 1985).

2. Hypertension

Hypertension is a major health issue, estimated to be affecting 15-20% of the world's adult population. Hypertension, or high blood pressure as the disease is known medically, is our most common chronic illness. Hypertension increases the risk for a variety of cardiovascular disease, including stroke, coronary artery disease, heart failure and peripheral vascular disease. Cardiovascular disease is the major cause of death for both males and females in many countries in the world. It usually is in the top five causes of death in lesser-developed countries. The World Health Organization (WHO) estimates that heart disease and stroke will have surpassed infectious diseases to become the leading cause of death and disability worldwide by 2020 (Lopez and Murray, 1998). Attempts to prevent vascular diseases often imply modifications and improvement of causative risk factors such as high blood pressure, obesity, an unfavorable profile of blood lipids or insulin resistance. Recent evidence suggests that an increased consumption of protein, particularly plant protein, may further lower the risk of hypertension and cardiovascular disease (Stamler *et al*., 1996; Hu *et al*., 1999). The mechanisms by which protein could exert its beneficial effects include an

increased intake of biologically active amino acids or peptides (Appel, 2003; Elliott, 2003).

2.1 Hypertensive constitution

Blood does not flow in a steady stream; instead, it moves through the circulatory system in spurts that correlate with the heart's beats. The heart beats about 60 to 70 times a minute at rest and may speed up to 120 to 140 or higher during vigorous exercise. It is not contracting or squeezing all the time, however; after each contraction, the heart muscle rests and gets ready for the next beat. Blood pressure rises and falls with each beat. Thus, blood pressure is expressed in two numbers, such as 120 over 80, or 120/80. Blood pressure when the heart beats is called systolic pressure (higher number). Blood pressure when the heart is at rest is called diastolic pressure (lower number). When blood pressure is measured, the systolic pressure is stated first and the diastolic pressure second. Normal blood pressure provides sufficient blood flow to the vital organs, including the brain, heart, kidneys, intestine and skeletal muscle. High blood pressure is thus a disease when its value is very high and a risk factor throughout its distribution. For diagnostic purposes, blood pressure is considered high when persistently above 140/90 mmHg (Zaret *et al*., 1992).

2.2 Causes of hypertension

In the majority of cases—over 90 percent—no specific cause for the elevated blood pressure can be identified. In this case, the elevated blood pressure is referred to as primary or essential hypertension. Some researchers believe that this type of high blood pressure may be due to hormonal factors relating to the handling of salt by the kidneys and/or to the elaboration of certain substances that cause constriction of blood vessels. These are probably genetically determined, but certain environmental factors, such as a high-salt, low-potassium diet and chronic stress, may play some role.

In up to 10 percent of patients, high blood pressure may be a consequence of another disorder, or a side effect of medication. This type of hypertension is referred to as secondary hypertension. It is important to remember that these cases are relatively uncommon. However, some of the more common causes of

secondary hypertension including kidney disorders, renovascular hypertension, adrenal tumors, pheochromocytoma, drugs and hypertension in pregnancy (Zaret *et al*., 1992).

2.3 Treatment of hypertension

High blood pressure can be controlled. Mild cases are treated by losing excess weight and reducing the intake of sodium and alcohol. More serious cases are treated with drugs. Treatment can usually assure a normal life (Zaret *et al*., 1992). Angiotensin I-converting enzyme (ACE) inhibitors are used as antihypertensive drugs. ACE has been associated with the rennin-angiotensin system, which regulates peripheral blood pressure. ACE inhibitors are now the first-line choice of treatment of hypertension in younger patients and a second-line choice in all patient groups. Moreover, antioxidants may have a positive effect on human health as they can protect our body against damage by reactive oxygen species (ROS), which attack membrane lipids, protein and DNA and play an important role in many diseases such as hypertension, cardiovascular diseases, diabetes mellitus, cancer and Alzheimer (Aruoma, 1998; Valko, *et al*., 2007).

2.3.1 Synthetic drugs

2.3.1.1 Angiotensin I-converting enzyme inhibitors

ACE (dipeptidyl carboxpeptidase, EC 3.4.15.1) is a zinc-metallopeptidase that needs zinc and chloride for its activity (Erdos and Skikgel, 1987). It is widely distributed in mammalian tissues, predominantly as a membrane-bound ectoenzyme in vascular endothelial cells and also in several other cell types including absorptive epithelial, neuroepithelial, and male germinal cells (Steve *et al.,* 1988; Sibony *et al.,* 1993). ACE exists in two distinct isoforms, the somatic and smaller testicular types, which are transcribed from a single gene at alternative initiation sites (Howard *et al.,* 1990; Kumar *et al.,* 1991). In the renin-angiotensin system (RAS), ACE plays a crucial role in the regulation of blood pressure (Figure 1) as well as cardiovascular function.

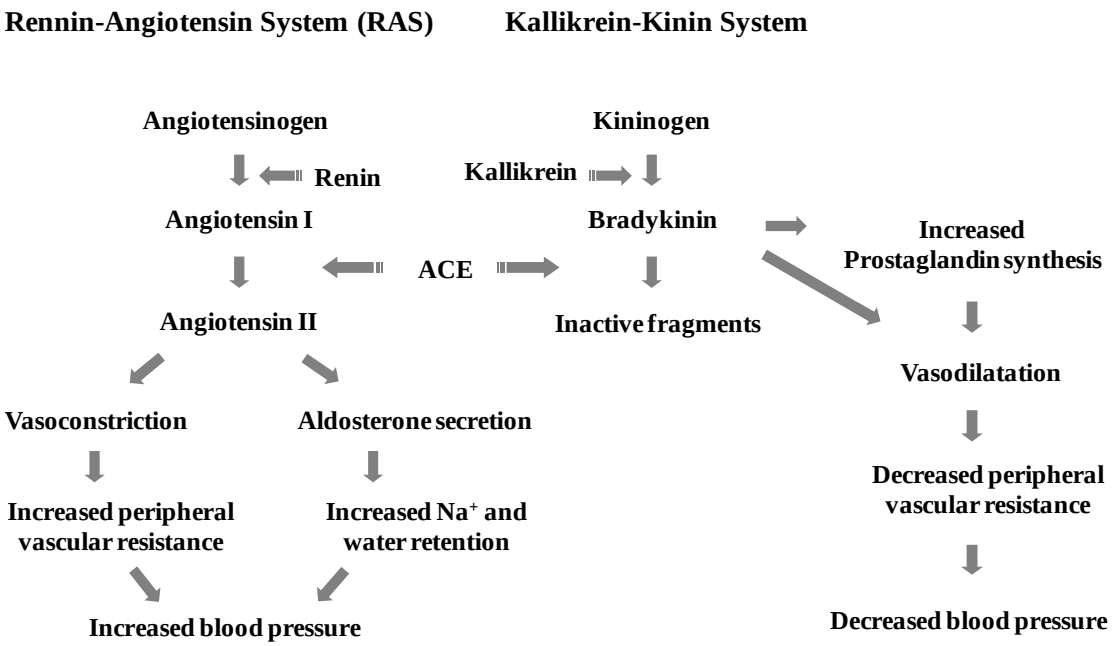

Figure 1. Role of angiotensin converting enzyme (ACE) in blood pressure regulation.
Source: Li *et al*. (2004)

Within the enzyme cascade of the RAS, ACE converts the inactive angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by cleaving dipeptide from the C-terminus into the potent vasoconstricting angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase blood pressure. ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin, a blood pressure lowering onapeptide in the kallikrein-kinin system (Erdos and Skikgel, 1987; Johnston, 1992). Moreover, ACE has been shown to degrade neuropeptides including enkephalins, neurotensin, and substance P, which may interact with the cardiovascular system (Wyvratt and Patchett, 1985).

Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important target. A large number of highly potent and specific ACE inhibitors have been developed as orally active drugs that are used in the treatment of hypertension and congestive heart failure (Xie, 1990). Synthesis of ACE inhibitors, such as captopril, enalapril, alacepril, and lisinopril,

which are currently used extensively in the treatment of essential hypertension and heart failure in humans (Ondetti, 1977; Patchett *et al*., 1980). However, these synthetic drugs are believed to have certain side effects, such as cough, taste disturbances and skin rashes angio-oedema and many other disfunctions of human organs (Atkinson and Robertson, 1979).

2.3.1.2. Antioxidants

Reactive oxygen species (ROS) attack membrane lipids, protein and DNA and play an important role in many diseases such as cardiovascular diseases, diabetes mellitus, cancer and Alzheimer (Aruoma, 1998; Valko *et al.*, 2007). ROS and free radicals are derived from the metabolism of molecular oxygen (Halliwell, 1999). Formation of free radicals include superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (·OH). The deleterious effects of oxygen are said to result from its metabolic reduction to these highly reactive and toxic species (Buechter, 1988).

ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidants depletion, or both. To counteract the oxidant effects and to restore redox balance, cells must reset important homeostatic parameters. ROS are not always harmful metabolic byproducts; when tightly regulated, ROS can act as intracellular signaling molecules (Klein and Ackerman, 2003; Scandalios, 2002). In living cells, the major source of endogenous ROS are hydrogen peroxide and superoxide anion, which are generated as by products of cellular metabolism such as mitochondrial respiration (Nohl *et al.,* 2003). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. Variability or inductive changes in the expression of these enzymes can significantly influence cellular redox potential. ROS can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA (Ahsan *et al*., 2003), sulphydryl groups in protein (Knight, 1995) and cross-linking/ fragmentation of ribonucleoproteins (Waris and Alam, 1998). The relatively unreactive superoxide anion radical is converted by superoxide dismutase (SOD) into H_2O_2, which in turn take part in the "Fenton reaction", with transition metal ion (copper or iron) as catalysts, to produce the very reactive hydroxyl radical (Aruoma *et al*., 1989; Halliwell and Gutteridge, 1990, 1992;

Halliwell, 1994). In addition, ROS promote oxidative damage to many cellular constituents, including amino acids, lipids, and nucleic acids, and play critical roles in aging and senescence-associated disorders (Gilbert and Colton, 1999; Halliwell and Gutteridge, 1999; Stadtman and Berlett, 1998). *In vivo*, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intracellular signaling (Halliwell and Gutteridge, 1999). On the other hand, ROS are also capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids (Farber, 1994). ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases including aging, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Cerutti, 1985; Harman, 1980; Moskovitz *et al.,* 2002).

Antioxidant refers to a compound that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions and which can thus prevent or repair damage done to the body's cells by oxygen. They act by one or more of the following mechanisms: reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen (Tachakittirungrod *et al*., 2007). The efficiency of the antioxidant defense system is altered under pathological conditions and, therefore, the ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damages (Aruoma, 1994; Halliwell, 1994). Cells have several antioxidant defense mechanisms that help to prevent the destructive effects of ROS. These defense mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase and of small molecules such as glutathione and vitamins C and E (Fridovich, 1999). Additionally, synthetic antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone and propyl gallate, have been tested with success in various disease models as well as in clinics (Wanita and Lorenz, 1996). However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compounds (Hettiarachchy *et al*., 1996; Park *et al*., 2001).

2.3.2. Bioactive peptides

Protein components in food are containing bioactive peptides sequences, which could exert a physiological effect in the body. These short chains of amino acids are inactive within the sequence of the parent protein, but can be released by either microorganisms or endogenous proteases during gastrointestinal digestion, food processing or fermentation (Wijesekara and Kim, 2010). Peptides usually contain 2–20 amino acid residues per molecule, but in some cases may consist of more than 20 amino acids. Following digestion, bioactive peptides can either be absorbed through the intestine to enter the blood circulation intact and exert systemic effects, or produce local effects in the gastrointestinal tract. Depending on the amino acids sequence, these peptides can exhibit diverse activities (Erdmann *et al*., 2008). Bioactive peptides exhibit various regulatory effects such as antioxidant (Je *et al*., 2007), immune defense (Tsuruki *et al*., 2003), opioid (Pihlanto, 2000) and antihypertensive activities (Yike *et al*., 2006). Fishery-derived bioactive peptides have been obtained widely by enzymatic hydrolysis of fishery protein. In fermented fishery products such as fish sauce and blue mussel sauce, enzymatic hydrolysis has already been done by microorganisms and bioactive peptides can be purified without further hydrolysis (Ichimura *et al*., 2003; Je *et al*., 2005c; Jung *et al*., 2005). Fishery-derived bioactive peptides have been shown to possess ACE inhibition and antioxidant.

2.3.2.1 Angiotensin I-converting enzyme inhibitory peptides

Fermented fishery products are good source of peptide and amino acid, and rich sources of structurally diverse bioactive compounds. Thus, fishery proteins are of particular interest especially promising due to their high protein content and diverse physiological activities in the human organism, including an antihypertensive effect. Fishery-derived antihypertensive peptides have shown potent ACE inhibitory activities (Table 4). ACE inhibitory peptides are liberated depending on their structural, compositional and amino acid sequence.

Table 4. ACE inhibitory peptides derived from fishery sources.

Source	Enzyme	Peptide sequence	IC_{50} value*	References
Tuna frame	peptic enzymes	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Tyr-Lys-Asp-Thr-Pro	11.28 μM	Lee *et al.* (2010)
Shark meat	protease	Cys-Phe Glu-Tyr Phe-Glu	1.96 μM 2.68 μM 1.45 μM	Wu *et al*. (2008)
Bigeye tuna dark muscle	pepsin	Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro	21.6 μM	Qian *et al.* (2007a)
Anchovy	natural fermentation	Arg-Pro Lys-Pro Ala-Pro	21 μM 22 μM 29 μM	Ichimura *et al*. (2003)
Salmon	thermolysin	Val-Trp Ile-Trp Met-Trp Leu-Trp	2.5 μM 4.7 μM 9.9 μM 17.4 μM	Ono *et al*. (2003)
Cuttlefish	bacterial protease	Ala-His-Ser-Tyr Gly-Asp-Ala-Pro Ala-Gly-Ser-Pro Asp-Phe-Gly	11.6 μM 22.5 μM 37.2 μM 44.7 μM	Balti *et al*. (2010a)
Cuttlefish	proteases	Val-Tyr-Ala-Pro Val-Ile-Ile-Phe Met-Ala-Trp	6.1 μM 8.7 μM 16.32 μM	Balti *et al*. (2010b)
Shrimp	peptide enzymes	Leu–His–Pro	1.6 μM	Cao *et al*. (2010)
Shrimp	*Lactobacillus fermentum* enzymes	Asp-Pro Gly-Thr-Gly Ser-Thr	2.15 μM 5.54 μM 4.03 μM	Wang *et al.* (2008b)
Hard clam	protamex	Tyr-Asn	51 μM	Tsai *et al*. (2008)
Oyster	pepsin	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	66 μM	Wang *et al.* (2008a)
Freshwater clam	protamex and flavourzyme	Val-Lys-Pro Val-Lys-Lys	3.7 μM 1045 μM	Tsai *et al*. (2006)
Blue mussel	natural fermentation	Glu-Val-Met-Ala-Gly-Asn-Leu-Tyr-Pro-Gly	19.34 μg/ml	Je *et al*. (2005c)

*IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Binding to ACE appears to be strongly influenced by the C-terminal sequence of the peptides. It has been postulated that proline, lysine or arginine is preferred as C-terminal residue and thus contributes to the ACE inhibitory potency (Meisel, 1997). In addition, peptides containing tryptophan, tyrosine, proline or phenylalanine at the C-terminal and branched-chain aliphatic amino acids at the N-terminal generally act as competitive inhibitors by binding with ACE (Cheung and Chushman, 1971). ACE inhibitory peptides are generally short chain peptides, often carrying polar amino acid residues like proline. Furthermore, structure-activity relationships among variety of peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides, which contain hydrophobic amino acids at these positions, are potent inhibitors (Qian *et al*., 2007b). Byun and Kim (2002) reported that the synthetic peptide Leu-Gly-Pro showed the high ACE inhibitory activity (IC_{50} value, 0.72 μM). Because of the potent inhibitory activity, peptides should have a branched aliphatic amino acid at the N-terminal, such as leucine, and a proline at the C-terminal (Figure 2).

Numerous *in vivo* studies of fishery-derived antihypertensive peptides in spontaneously hypertensive rats have shown potent ACE inhibition activity (Lee *et al*., 2010; Zhao *et al*., 2009; Qian *et al*., 2007a) and their systolic blood pressure has reduced significantly after oral administration of peptides. Lee *et al*. (2010) reported that a single oral administration (10 mg/kg of body weight) of peptide from tuna frame protein has shown a strong suppressive effect on systolic blood pressure of spontaneously hypertensive rats and this antihypertensive activity was similar with captopril, a commercial antihypertensive drug. Moreover, no side effect was observed on rats after administration of antihypertensive peptide. In addition, these fishery bioactive peptides exhibit antihypertensive activity *in vivo* than *in vitro*. The exact mechanisms underlying this phenomenon have not yet been identified. However, it was suggested that bioactive peptides have higher tissue affinities and are subjected to a slower elimination than captopril. The antihypertensive peptide isolated from bonito fish hydrolysate product, has found to be hydrolyzed by ACE to produce a smaller peptide than the initial one, which had 8-fold ACE inhibitory activity compared with the initial peptide (Fujita and Yoshikawa, 1999).

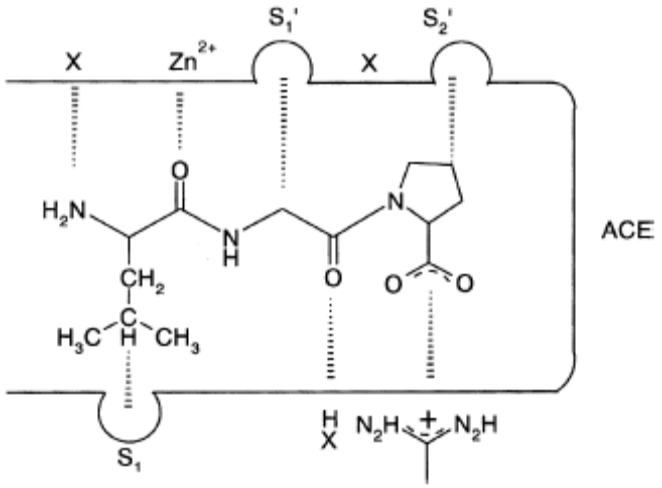

Figure 2. Binding model for interactions between ACE inhibitory peptide (Leu-Gly-Pro) and the active site of ACE.

Source: Byun and Kim (2002)

2.3.2.2. Antioxidative peptides

Protein hydrolysates prepared from various fishery sources such as sardinelle, tuna, grass carp and jumbo squid contained peptides with antioxidant activity (Table 5). Additionally, antioxidant activity has been found in a number of fermented fishery products such as fermented blue mussels (Jung *et al*., 2005), fish sauces (Harada *et al.,* 2003; Michihata, 2003), and fermented shrimp paste (Peralta *et al*., 2005). The antioxidant activity has been attributed to certain amino acid sequences (Suetsuna *et al*., 2000). Peptides with the molecular weight of approximately 1400 Da and with 5–16 amino acid sequences showed strong inhibition activities on the autoxidation of linoleic acid (Chen *et al*., 1995; Wu *et al*., 2003). Several amino acids, such as Tyr, Met, His, Lys and Trp, were generally accepted as antioxidants despite their occasionally pro-oxidative effects (Chen *et al*., 1996). High amounts of histidine and some hydrophobic amino acids are related to the antioxidant potency (Pena-Ramos *et al*., 2004). The activity of histidine-containing peptides is thought to be connected to hydrogen-donating ability, lipid peroxyradical trapping and/or the metal ion chelating ability of the imidazole group (Chan and Decker, 1994). The addition of a leucine or proline residue to the N-terminus of a histidine–histidine dipeptide would enhance antioxidant activity.

Table 5. Antioxidant peptides derived from fishery sources.

Source	Enzyme	Molecular weight (Da)	Peptide sequence	References
Sardinelle (*Sardinella aurita*)	crude extract from sardine viscera	538.2 471.3 431.2 403.1 263.08	Gly-Ala-Leu-Ala-Ala-His Leu-Ala-Arg-Leu Leu-His-Tyr Gly-Ala-Trp-Ala Gly-Gly-Glu	Bougatef *et al.* (2010)
Tuna dark muscle	orientase	978	Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr	Hsu (2010)
	protease XXIII	756	Pro-Met-Asp-Tyr-Met-Val-Thr	
Tuna cooking juice	orientase (*Bacillus subtilis*)	1305 938 584	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr Pro-Ser-Asp-His-Asp-His-Glu Val-His-Asp-Tyr	Hsu *et al.* (2009)
Tuna backbone protein	peptic enzymes	1519	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser	Je *et al.* (2007)
Grass carp	alcalase 2.4L	966.3	Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val	Ren *et al.* (2008)
Hoki (*Johnius belengerii*)	pepsin	1801	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn	Kim *et al.* (2007)
Alaska Pollack frame	mackerel intestine crude enzyme	672	Leu-Pro-His-Ser-Gly-Tyr	Je *et al.* (2005b)
Blue mussel	natural fermentation	620	Phe-Gly-His-Pro-Tyr	Jung *et al*. (2005)
Blue mussel	natural fermentation	962	His-Phe-Gly-Asp-Pro-Phe-His	Rajapakse *et al.* (2005)
Oyster	pepsin, typsin and α-chymotrypsin	1600	Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu	Qian *et al.* (2008)
Jumbo squid	trypsin, α-chymotrypsin and pepsin	1241.59 880.18	Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu	Mendis *et al.* (2005)
Marine rotifer (*Brachionus rotundiformis*)	peptic enzymes	1076 1033	Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His	Byun *et al.* (2009)

According to Chen *et al*. (1996), His and Pro play an important role in the antioxidative activity of peptides designed peptides tests, among which Pro-His-His showed the greatest antioxidant activity among all tested peptides and had synergistic effects with nonpeptidic antioxidants. The hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets (e.g., lipophilic fatty acids) (Chen *et al*., 1998). Moreover, protein hydrolysates and peptides with an increase in hydrophobicity would increase their solubility in lipid and therefore enhances their antioxidative activity (Rajapakse *et al*., 2005; Zhu *et al*., 2006).

Objectives

1. To investigate ACE inhibitory activity and antioxidative activity of the extracts from some Thai traditional fermented fishery products.
2. To study the change in ACE inhibitory activity of the extract from *Pla-som* with and without starter inoculation during fermentation.
3. To purify and identify ACE inhibitory peptides extracted from *Pla-som*.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Samples

Thirty samples of some Thai tradition fermented fishery products including *Kapi* (*Kapi* I, II, III, IV), *Kung-chom* (*Kung-chom* I, II), *Kheeuy-naam* (*Kheeuy-naam* I, II), *Bu-du* (*Bu-du* I, II), *Tai-pla* (*Tai-pla* I, II), *Pla-ra* (*Pla-ra* I, II, III, IV), *Pla-chao* (*Pla-chao* I, II), *Pla-som* (*Pla-som* I, II, III, IV), *Som-fug* (*Som-fug* I, II), *Pla-chom* (*Pla-chom* I, II), *Kem-buk-nud* (*Kem-buk-nud* I, II) and *Hoi-dong* (*Hoi-dong* I, II) were collected from local producers in Thailand (Table 6). Samples were thoroughly mixed, packaged in polyethylene bag and stored at -20^{o}C until used.

For production of *Pla-som*, Thai sliver barb (*Puntius gonionotus*), purchased from Thai market of Thailand were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand within 1 h.

Table 6. Description of some fermented fishery products in Thailand.

Types of fermented fishery products	Thai name	Raw material and ingredient	Fermentation period	Label	Species raw material	Local province
Fermented shrimp or krill	*Kapi*	Shrimp or krill and salt	4-6 months	KP-I	-	Trat
				KP-II	-	Satun
				KP-III	-	Pattani
				KP-IV	*Mesopodopsis orientalis*	Samut Sakhon
	Kung-chom	Shrimp, salt and roasted rice	3-5 days	KC-I	-	Buriram
				KC-II	-	Buriram
	Kheeuy-naam	Krill and salt	3-7 days	KN-I	-	Trang
				KN-II	-	Trang
Fermented fish	*Bu-du*	Whole marine fish and salt	3-6 months	BD-I	-	Pattani
				BD-II	-	Pattani
	Tai-pla	Viscera of fish and salt	10-20 days	TP-I	-	Rayong
				TP-II	-	Pattani
	Pla-ra	Gutted freshwater fish, salt, roasted rice and rice bran	6 months	PR-I	*Channa striatus*	Ayutthaya
				PR-II		Nakhonsawan
				PR-III	*Trichogaster richopterus or Pallas*	Ayutthaya
				PR-IV		Nakhonsawan
	Pla-chao	Gutted freshwater fish, salt and *Khao-maak* (sweet fermented glutinous rice)	10-20 days	PCO-I	*Puntius gonionotus*	Ayutthaya
				PCO-II		Lopburi
	Pla-som	Gutted freshwater fish, salt, cooked rice and/or sticky rice and minced garlic	5-7 days	PS-I	*Cirrhina microlepis*	Phetchaboon
				PS-II	*Tilapia nilotica*	Phetchaboon
				PS-III	*Puntius gonionotus*	Phetchaboon
				PS-IV		Lopburi
	Som-fug	Minced freshwater fish, cooked rice, garlic and salt	3–5 days	SF-I	-	Lopburi
				SF-II	-	Bangkok
	Pla-chom	Gutted freshwater fish, salt and roasted rice	3-5 days	PCM-I	*Rasbora argyrotaenia*	Lopburi
				PCM-II		Pathumthani
	Kem-buk-nud	Filleted freshwater fish, salt and pine apple	3 months	KBN-I	-	Ubonratchathani
				KBN-II	-	Ubonratchathani
Fermented mussel	*Hoi-dong*	Mussel and salt	4-5 days	HD-I	*Perna viridis*	Lopburi
				HD-II		Samut Prakan

1.2 Chemicals

Ethanol was obtained from Carlo erba (Val de Reuil, France) and trifluoroacetic acid (TFA) was obtained from Applied Biosystems (Warrington, Cheshire, U.K.). Methanol and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). 2,2–azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH) were purchased from Wako (Osaka, Japan). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Dojindo (Kumamoto, Japan). Hippuryl–histidyl–leucine (HHL), 2,4,6-trichloro-s-triazine (TT), dioxane, L-tyrosine, L-leucine, PMSF and trinitrobenzenesulphonic acid (TNBS) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Sodium sulfite, formic acid and calcium carbonate were purchased from Fluka (Buchs, Switzerland). MRS agar, Baird Parker Agar (BPA), Egg Yolk Tellurite Emulsion, TCA, potassium phosphate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). All chemicals for electrophoresis were obtained from Bio-Rad (Richmond, CA, USA).

1.3 Enzymes

Angiotensin I-converting enzyme of rabbit lung, pepstatin, pepsin and pancreatin were procured from Sigma (St. Louis, MO, USA).

2. Instruments

Instruments	Model	Company/Country
pH meter	SevenEasy	Mettler Toledo, Germany
Homogenizer	T 25 basic	IKA labortechnik, Germany
Refrigerated centrifuge	5810R	Eppendorf, Germany
Refrigerated centrifuge	Avanti J-E	Beckman Coulter, USA
Freeze dryer	Supermodulyo	Savant, USA
Water bath	WB22+SV1422	Memmert, Germany
UV-Vis spectrophotometer	Helios Alpha	Thermo Spectronic, UK
Sonicator	DK 514 BP	Bandelin, Germany
Magnetic stirrer	RO 15 powder	IKA labortechnik, Germany
Vortex mixer	G-560E	Vortex Genie 2, USA
MALDI-TOF/TOF	Ultraflex III	Bruker Daltonik GmbH, Germany
Grinder	MX-T2G	National, Japan
Stomacher	Lab-blender 400	Seward Medical, UK
Rotary evaporator	R-114, B-480, B-169	Büchi, Germany
	F25	Julabo, Germany
Electrophoresis	AE-6530 Dual mini-slab system	ATTO, Japan
HPLC	2690, 996	Waters, USA
LC-MS/MS	HDMS SYNAPT	Waters, USA

3. Methods

3.1 Study on ACE inhibitory activity and antioxidative activity of the extracts from some Thai traditional fermented fishery products

3.1.1 Determination of salt content and pH

Salt content was determined according to the method of AOAC (2000). Sample (0.5-1 g) was treated with 10-20 ml of 0.1 N $AgNO_3$ and 10 ml of Conc. HNO_3. The mixture was boiled on a hot plate for 10 min and then cooled using running water. The mixture was filtered through filter paper (Whatman No. 1). The filtrate was adjusted to the volume of 50 ml by distilled water and 5 ml of ferric alum indicator were added. The mixture was titrated with standardized 0.1 N KSCN until the solution became permanent brownish-red. The salt content was then calculated and expressed as %NaCl. pH was determined by a pH meter (Mettler Toledo, Germany) as described by Benjakul *et al*. (1997).

3.1.2 Determination of F/T ratio

Contents of free and total free α-amino acids of samples were measured by the method of Benjakul and Morrissey (1997). The sample (1 g) was mixed with 9 ml of 1% (w/v) SDS and homogenized with a homogenizer (IKA, T 25 basic, Germany) at a speed of 9,500 rpm for 1 min. The homogenate was heated at 85^{o}C for 15 min and then was centrifuged at 12,000*xg* for 10 min using a centrifuge (Eppendorf, 5810R, Germany) at room temperature. To the supernatant obtained (125 µl), 2 ml of 0.2 M phosphate buffer, pH 8.2 and 1 ml of 0.01% (w/v) TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled-water bath (Memmert, WB22+SV1422, Germany) at 50^{o}C for 30 min in dark. The reaction was stopped by addition of 2 ml of 0.1 M sodium sulfite. The mixture was then cooled at room temperature for 15 min. The absorbance of the resulting solution was measured at 420 nm using a UV-Vis spectrophotometer (Thermo Spectronic, Helios Alpha, UK). A standard curve was prepared using L-leucine. F/T ratio was calculated by

$$\text{F/T ratio} = (L/L_{max}) \times 100$$

Where L is the amount of free α-amino group in the product, L_{max} is the total free α-amino group obtained after acid hydrolysis (6 M HCl at 100^{o}C for 24 h).

3.1.3 Peptide extraction

Peptides were extracted by method of Gibbs *et al*. (2004) with modifications. The samples were added with mixed solution (49.5% water, 49.5% acetonitrile and 1% trifluoroacetic acid) at the ratio of sample to mixed solution of 1:4 (w/v). The mixture was homogenized at 9,500 rpm for 1 min, sonicated for 5 min using a sonicator (Bandelin, DK 514 BP, Germany), vortexed for 2 min, and then centrifuged at 15,000*xg* for 10 min. The extracts were filtered through a filter paper (Whatman No. 4) and the pellets were twice re-extracted under the same conditions. The pooled supernatants were condensed with a rotary evaporator under reduced pressure at 45^{o}C and lyophilized. Dry extracts were stored at -20^{o}C until analysis.

3.1.4 Determination of peptide content

The lyophilized samples were dissolved in 50% (v/v) acetonitrile. Peptide contents were determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as standard and expressed as mg peptide/g sample.

3.1.5 MALDI-TOF-MS analysis

Peptide masses were determined using Matrix Assisted Laser Desorption Ionization - Time of Flight tandem with Time of Flight (MALDI-TOF/TOF) (Ultraflex III, Bruker Daltonik GmbH, Germany). MALDI-TOF equipped with a delayed extraction source and a 337 nm pulsed nitrogen laser. An instrument Bruker Ultraflex III MALDI-TOF/TOF was run in the positive refraction mode using 20 kV acceleration. Samples (25 mg) were dissolved with 0.1% TFA (50 µl) and desalted with a ZipTip C18. Desalted samples were mixed with matrix solutions (2,5-dihydroxybenzoic acid (DHBA)) at ratio of 1:1, 2:1 and 3:1 (v/v) and spotted onto the MALDI target plate. All spectra were the results of signal averaging of 1000 shots.

3.1.6 Determination of ACE inhibitory activity

The ACE inhibitory activity was determined following the method described by Hayakari *et al*. (1978) with modifications. This method is based on the colorimetric reaction of hippuric acid with 2,4,6-trichloro-s-triazine (TT). The lyophilized samples were dissolved in distilled water. Each 0.1 ml assay mixture contained the following components at the indicated final concentrations: 30 mg dried extraction/ml sample, 100 mM potassium phosphate buffer (pH 8.3), 600 mM sodium chloride, 3 mM Hippuryl-L-histidyl-L-leucine (HHL), and 72.5 mU/ml ACE (rabbit

lungs) or 20 U/ml ACE (pig lungs). This mixture was incubated at 37^{o}C for 15 min. The reaction was terminated by addition of 0.3 ml of TT (3% w/v) in dioxane and 0.6 ml of 0.2 M phosphate buffer, pH 8.3. Enzymatic activity was determined by measuring absorbance at 382 nm using a UV-Vis spectrophotometer (Thermo Spectronic, Helios Alpha, UK). To compare ACE inhibitory activity among all samples tested, the activity was expressed as percent inhibition of the enzyme activity based on the same amount of dry extract used under the same assayed condition.

3.1.7 Determination of antioxidative activity

3.1.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay as described by Wu *et al*. (2003) with modifications. The lyophilized samples were dissolved in 50% (v/v) acetonitrile. The sample solution (1.5 ml) was added with 1.5 ml of 0.15 mM DPPH in 95% ethanol. The mixture was shaken and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Thermo Spectronic, Helios Alpha, UK). The DPPH radical scavenging activity of the sample was compared with that of a reference standard, Trolox. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

3.1.7.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as per the method of Arnao *et al*. (2001) with modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-Vis spectrophotometer (Thermo Spectronic, Helios Alpha, UK). Fresh ABTS solution was prepared for each assay. The lyophilized samples were dissolved in 50% (v/v) acetonitrile. The sample solution (150 µl) was mixed with 2,850 µl of ABTS solution. The mixture was incubated at room temperature for 2 h in dark. The reaction mixture was measured at 734 nm using the spectrophotometer. A standard curve of Trolox was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

3.1.7.3 Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was assayed following the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer (pH 3.6), 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37^{o}C for 30 min and was referred to as FRAP solution. The lyophilized samples were dissolved in 50% (v/v) acetonitrile. The sample solution (150 µl) was added to 2,850 µl of FRAP solution and kept for 30 min in dark. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. The standard curve was prepared using Trolox. The activity was expressed as µmol Trolox equivalents (TE)/g protein.

3.1.8 Principle component analysis

Principal component analysis (PCA) with varimax rotation was performed, using the Statistical Package for Social Science (SPSS version 10.0, SPSS Inc., Chicago, IL, USA).

3.2 Study on effect of starter cultures (*Pediococcus acidilactici* BCC 9545 and *Staphylococcus xylosus* BCC 3710) on properties and ACE inhibitory activity of *Pla-som* during fermentation

3.2.1 Starter preparation

Pure culture of *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 were obtained from the BIOTEC Culture Collection, Thailand. One loopful of a stock culture of *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 kept in 20% glycerol at -80^{o}C was cross-streaked on MRS agar containing calcium carbonate (1%) and nutrient agar (NA), respectively. MRS agar containing calcium carbonate (1%) and nutrient agar (NA) with starters were incubated for 48 h at 30 and 37^{o}C, respectively. A single colony of *P. acidilactici* BCC 9545 on MRS agar containing calcium carbonate (1%) was transferred into 5 ml of MRS and incubated at 30^{o}C for 16 h. A single colony of *S. xylosus* BCC 3710 on NA was transferred into 5 ml of nutrient broth (NB) and incubated at 37^{o}C for 18 h. Cells were harvested by centrifugation at 10,000 rpm for 15 min using a centrifuge (Eppendorf, 5810R, Germany) at 4^{o}C and washed with 5 ml

of sterile deionized water. Finally, the cell concentrations of *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 were adjusted to 10^7 and 10^9 CFU/ml with sterile deionized water, respectively.

3.2.2 Production of *Pla-som*

The fish, Thai sliver barb (*Puntius gonionotus*), were washed and filleted. The fillets were soaked in water from washing rice for 5 min. The drained fillets were mixed with 6% salt and 0.1% sodium nitrate followed by incubation at room temperature for 12 h in closed package. Sticky rice (6%), garlic (11%), monosodium glutamate (0.1%) and the mixed starters of *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g were added into the mixture and mixed thoroughly for 5 min. The mixture containing no bacterial starters was used as the control. The products were packed in vacuum bags and fermented at 30^{o}C for 120 h. Samples were randomly taken and determined for further analysis at 0, 24, 48, 72, 96 and 120 h. Prior to analyses, the vacuum bags were removed. Samples were cut and ground in a meat grinder (MX-T2G National, Tokyo, Japan) for 1 min and kept in ice for further analyses.

3.2.3 Microbiological analysis

Lactic acid bacteria count was determined using De Man Rogosa and Sharpe (MRS) agar according to the method of AOAC (2000). The sample (25 g) was aseptically transferred to a sterile plastic pouch and pummeled for 1 min in a stomacher Lab-blender 400 (Seward Medical, London, UK) with 225 ml of sterile peptone water (0.1 g/100 ml). Appropriate decimal dilutions of the samples were made using the same diluents. Aliquots of 0.1 ml of each dilution were plated in duplicate on MRS agar containing calcium carbonate (1%) and Baird Parker Agar (BPA) supplemented with Egg Yolk Tellurite Emulsion and incubated for 1–2 days at 30^{o}C and 37^{o}C, respectively. The loads were expressed as log CFU/g sample.

3.2.4 Determination of pH and total acidity

Direct pH measurement was taken using a standard pH meter (Mettler Toledo, Germany). The total acidity of samples was determined according to the method of AOAC (2000). To the sample (5 g), 40 ml of CO_2-free distilled water was added and the mixture was homogenized at 9,500 rpm for 1 min using an IKA homogenizer (T 25 basic, Germany). The homogenate was then centrifuged at 3,000*xg*

for 15 min using a centrifuge (Eppendorf, 5810R, Germany) at room temperature. The supernatant was filtered through a filter paper (Whatman No. 4). The filtrate was titrated with the standardized 0.1 M NaOH using phenolphthalein as an indicator. The total acidity was calculated as lactic acid and expressed as percentage (w/w).

3.2.5 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Samples (3 g) were homogenized with 27 ml of 5% SDS. The homogenate was heated at 85^{o}C for 1 h, followed by centrifuging at 10,000*xg* for 15 min at room temperature. The protein concentration of supernatant was determined by the Lowry *et al*. (1951) method. The samples (20 µg) were loaded on the gel made of 4% stacking and 12.5% running gels and subjected to electrophoresis at a constant current of 20 mA per gel using an ATTO AE-6530 Dual mini-slab system. After separation, protein bands were stained using Coomassie Brillant Blue R-250 (0.125%) in 45% methanol and 10% acetic acid. Destaining was performed using 30% methanol and 10% acetic acid.

3.2.6 Determination of TCA-soluble peptide

TCA-soluble peptide content was determined according to the method of Green and Babbitt (1990). Sample (3 g) was homogenized with 27 ml of 15% (w/v) TCA. The homogenate was kept in ice for 1 h and centrifuged at 12,000*xg* for 5 min. The soluble peptides in the supernatant were measured by the method of Lowry *et al.* (1951) using tyrosine as standard and expressed as µmole tyrosine/g sample.

3.2.7 Determination of free amino group content and degree of hydrolysis

Degree of hydrolysis (DH) of sample was measured by the method of Benjakul and Morrissey (1997). The sample (1 g) was mixed with 9 ml of 1% (w/v) SDS and homogenized with a homogenizer (IKA, T 25 basic, Germany) at a speed of 9,500 rpm for 1 min. The homogenate was heated at 85^{o}C for 15 min and then was centrifuged at 12,000*xg* for 10 min using a centrifuge (Eppendorf, 5810R, Germany) at room temperature. To the supernatant obtained (125 µl), 2 ml of 0.2 M phosphate buffer, pH 8.2 and 1 ml of 0.01% (w/v) TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled-water bath (Memmert, WB22+SV1422, Germany) at 50^{o}C for 30 min in dark. The reaction was stopped by addition of 2 ml 0.1 M sodium sulfite. The mixture was then cooled at room

temperature for 15 min. The absorbance of the resulting solution was measured at 420 nm using a UV-Vis spectrophotometer (Thermo Spectronic, Helios Alpha, UK). A standard curve was prepared using L-leucine. Free amino group content was expressed in terms of L-leucine. DH was calculated according the method of Benjakul and Morrissey (1997) with a slight modification.

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

Where L_t is the amount of α-amino acid released at time *t*. L_0 is the amount of α-amino acid in the original *Pla-som* (fermentation time 0 h). L_{max} is the total free α-amino acid in the original *Pla-som* obtained after acid hydrolysis (6 M HCl at 100^{o}C for 24 h).

3.2.8 Peptide extraction

The *Pla-som* was extracted as previously described. Dry extracts were stored at -20^{o}C until analysis.

1.2.9 Determination of ACE inhibitory activity

The water soluble fraction of dry extracts of *Pla-som* was determined for ACE inhibitory activity as previously described. The ACE inhibitory activity of *Pla-som* was expressed as units/mg peptide. One unit of ACE activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay condition. One unit of ACE inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

3.2.10 Characterization of ACE inhibitory peptide from *Pla-som*

3.2.10.1 Effect of temperature on ACE inhibitory activity

Lyophilized sample (50 mg) was dissolved in 1 ml distilled water and 20-fold diluted with distilled water. The solution was subsequently incubated at different temperatures (25, 37, 50, 60, 70, 80, 90 and 100^{o}C) for 1 h, followed by sudden cooling in iced water. After the temperature was acclimated to room temperature (25^{o}C), the residual ACE inhibitory activity was determined.

3.2.10.2 Effect of pH on ACE inhibitory activity

Lyophilized sample (50 mg) was dissolved in 1 ml distilled water and 20-fold diluted with distilled water. The solution was also adjusted to different pHs (2 to 12) using HCl and NaOH and incubated at 37^{o}C for 1 h. After the pH values were adjusted to 8.3, the residual ACE inhibitory activity was determined.

3.2.10.3 Effect of gastrointestinal protease on ACE inhibitory activity

Stability against gastrointestinal protease was assayed *in vitro* (Lo *et al.*, 2006). Lyophilized sample (50 mg) was dissolved in 1 ml distilled water. The solution was adjusted to pH 2.0 with 1 M HCl and pepsin dissolved in 10 mM HCl was added to obtain the final concentration of 4% pepsin (w/w protein). The mixture was incubated at 37^{o}C for 1 h with continuous shaking (Memmert, WB22+SV1422, Germany). Thereafter, the pH of reaction mixture was raised to 5.3 with 1 M NaOH. The mixture was randomly taken and centrifuged at 10,000*xg* for 15 min. The supernatant was used for determination of ACE inhibitory activity. The remaining mixture was digested further by pancreatin (2% w pancreatin/w protein) and the pH of mixture was adjusted to 7.5 with 1 M NaOH. The mixture was incubated at 37^{o}C for 3 h with continuous shaking. The digestion was terminated by submerging the mixture in boiling water for 10 min. The mixture was centrifuged at 10,000*xg* for 15 min and the supernatant was assayed for residual ACE inhibitory activity. The ACE inhibitory activity was expressed as IC_{50} value. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

3.3 Identification of ACE inhibitory peptides from *Pla-som*

3.3.1 Determination of peptide content

The peptide content of *Pla-som* was measured by the method of Church *et al.*, (1983) with some modifications. Reagent (50 ml) was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of o-phthaldialdehyde solution (dissolved in 1 ml of methanol) and 100 µl of β-mercaptoethanol and then adjusted to 50 ml with distilled water. Sample (50 µl) was mixed with 2 ml of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (Thermo Spectronic, Helios Alpha, UK). The peptide content was quantified using casein tryptone (Difco Laboratories, Sparks, MD, USA) as standard.

3.3.2 Purification of ACE inhibitory peptides

3.3.2.1 Ultrafiltration

The lyophilized sample (0.5 g) was dissolved in 10 ml of nanopure water. The water soluble fraction of lyophilized sample was passed through ultrafiltration membrane with molecular weight cut-off (MWCO) of 3 kDa (Millipore, Bedford, MA, USA). The permeate was lyophilized and stored at -20^{o}C.

3.3.2.2 Reversed-phase HPLC system

The lyophilized permeate with MW smaller than 3 kDa was dissolved in nanopure water. This solution was then separated by RP-HPLC on Alltima C_{18} column (4.6 mm x 250 mm, 5 µm). A HPLC Waters Separation Module 2690 was operated to give a flow rate of 1 ml/min. Separation was performed under linear gradient elution conditions using acetonitrile as the organic modifier. Eluent A was 0.1% (v/v) trifluoroacetic acid (TFA) in nanopure water and eluent B was 0.1% (v/v) TFA in 100% (v/v) acetonitrile (CH_3CN) solution. The chromatographic column was conditioned with 100% of eluent A. After 20 µl of solution with MW smaller than 3 kDa were injected into the C_{18} column, eluent B concentrations were increasing: 0-40 min, 0-50% (v/v); 40-50 min, 50% (v/v). A photo diode array (Model 996, Waters Associates, Milford, MA, USA) set at the wavelength of 220 nm and 280 nm, was used as the detector. Data was processed and analysed using Millennium 32 software (Waters Associates, Milford, MA, USA). The active fraction was concentrated by lyophilization.

3.3.3 Determination of molecular weight and amino acid sequence of the purified peptides

The active fraction from RP-HPLC was analyzed by LC-MS/MS. Nanoscale LC separation of peptides was performed with a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 µm, 180-µm x 200-µm Trap column and a BEH130 C_{18} 1.7 µm, 100-µm x 100-µm analytical reversed phase column (Waters Corp., Milford, MA). The active fraction was initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 3 µl/min for 3 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a gradient of 2–40% mobile phase B over 30 min at a flow rate of 1000 nl/min followed by a 10-min rinse

with 80% of mobile phase B. The column temperature was maintained at 35°C. The lock mass was delivered from the auxiliary pump of the NanoAcquity pump with a constant flow rate of 350 nl/min at a concentration of 200 fmol/µl of [Glu^1]fibrinopeptide B to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analyzed in triplicate. Analysis of peptides was performed using a SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer was operated in the V-mode of analysis with a resolution of at least 10,000 full-width half-maximum. All analyses were performed using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with [Glu^1]fibrinopeptide B from m/z 50 to 1600 with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1]fibrinopeptide B. The reference sprayer was sampled with a frequency of 20 sec. Accurate mass LC-MS data were acquired with expression mode. The spectral acquisition time was 0.6 sec. In MS expression mode, low energy of trap was set at a constant collision energy of 6 V. In elevated energy of MS expression mode, the collision energy of trap was ramped from 15 to 40 V during each 0.6-s data collection cycle with one complete cycle of low and elevated energy. In transfer collision energy control, low energy was set at 4 V for low energy and 7 V for high energy. The quadrupole mass analyzer was adjusted such that ions from m/z 200 to 1990 were efficiently transmitted. Data obtained from the LC-MS/MS analysis were converted into Mascot generic format (*mgf*) and subjected to analyze *de novo* peptide sequencing by PepNovo program (Frank and Pevzner, 2005).

3.3.4 Peptide synthesis

Peptides were synthesized by ChinaPeptides Co., Ltd, Shanghai, China. Solid phase peptide synthesis (SPPS) uses chemical synthesis way to make peptides. The purity of peptides synthesized was more than 95% by HPLC analysis. The peptides synthesized were assayed for ACE inhibitory activity. The ACE inhibitory activity was expressed as IC_{50} value. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

3.4 Statistical analysis

The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS version 10.0, SPSS Inc., Chicago, IL, USA).

CHAPTER 3

RESULTS AND DISCUSSION

1. ACE inhibitory activity and antioxidative activity of the extracts from some Thai traditional fermented fishery products

1.1 Salt content of fermented fishery products

Contents of salt in the samples tested in this study varied from 2 to 21% (Table 7). The contents were complied with the Thai Industrial Standard Institute (TISI) standards and those previously reported by Phitakpol *et al*. (1995). The importance of salt in food production arises from its roles in preservation and contributions to flavor, texture and colour. The level of salting will also affect the growth and selection of microorganisms during fermentation and properties of food products. Products with high salt content, including *Kapi*, *Bu-du and Tai-pla*, are widely used as condiment, whereas products with low salt content, including *Pla-som* and *Som-fug*, can be served as a main dish. The high salt concentration (>5%) can inhibit the growth of pathogenic and spoilage microorganisms by lowering the water activity (a_w). The mechanism of microbial growth inhibition by salt appears related to plasmolysis or water loss from the cell. When the a_w of the food is reduced through salt addition, bacterial cells experience osmotic shock and plasmolysis, loosing turgor, leading to cessation of growth, death or cells entering a dormant state (Davidson, 2001). In contrast, the low salt concentration (2-3%) can enhance protein functional properties and textural properties of finished product. Salt has many functional attributes; activation of proteins to improve hydration and water binding capacity; increasing binding properties of proteins to improve texture and increasing viscosity of meat thereby facilitating the formation of a stable batter (Desmond, 2006). It is also used for colour maintenance, improves flavor and mouthfeel of products (Brandsma, 2006).

Table 7. Chemical properties, F/T ratio and peptide content of some traditional fermented fishery products.

Fermented fishery products	Salt (% w/w)	pH	F/T ratio (%)	Peptide content (mg peptide/g sample)
Kapi I	20.64 ± 0.17 ^{b}	7.78 ± 0.02 ^{b}	19.46 ± 0.06 ^{kl}	66.60 ± 0.71 ^{b}
Kapi II	16.39 ± 0.13 ^{h}	7.48 ± 0.01 ^{d}	19.67 ± 0.03 ^{jkl}	73.22 ± 0.90 ^{a}
Kapi III	17.71 ± 0.20 ^{g}	7.68 ± 0.01 ^{c}	21.38 ± 0.13 ^{ijk}	62.03 ± 1.15 ^{c}
Kapi IV	15.82 ± 0.15 ^{i}	7.50 ± 0.01 ^{d}	19.02 ± 0.06 ^{l}	55.34 ± 0.36 ^{d}
Kung-chom I	9.04 ± 0.02 ^{o}	4.35 ± 0.02 ^{t}	34.70 ± 0.50 ^{f}	21.54 ± 0.47 ^{i}
Kung-chom II	8.83 ± 0.02 ^{p}	5.14 ± 0.03 ^{l}	32.13 ± 0.52 ^{g}	19.02 ± 0.00 ^{k}
Kheeuy-naam I	7.46 ± 0.09 ^{r}	7.88 ± 0.01 ^{a}	31.35 ± 0.24 ^{g}	25.33 ± 0.74 ^{gh}
Kheeuy-naam II	7.44 ± 0.20 ^{r}	6.22 ± 0.02 ^{e}	35.53 ± 0.13 ^{f}	20.83 ± 0.92 ^{ij}
Bu-du I	20.39 ± 0.06 ^{c}	5.87 ± 0.01 ^{g}	40.32 ± 0.27 ^{e}	20.08 ± 0.30 ^{jk}
Bu-du II	19.84 ± 0.03 ^{d}	5.57 ± 0.02 ^{i}	41.44 ± 0.07 ^{de}	21.04 ± 0.29 ^{ij}
Tai-pla I	21.34 ± 0.12 ^{a}	4.96 ± 0.01 ^{m}	50.25 ± 0.84 ^{b}	19.90 ± 0.08 ^{jk}
Tai-pla II	18.79 ± 0.07 ^{e}	5.30 ± 0.01 ^{k}	26.88 ± 1.05 ^{h}	37.10 ± 1.44 ^{e}
Pla-ra I	15.45 ± 0.28 ^{j}	5.97 ± 0.02 ^{f}	21.44 ± 0.24 ^{ijk}	29.15 ± 1.62 ^{f}
Pla-ra II	18.33 ± 0.13 ^{f}	5.76 ± 0.01 ^{h}	21.69 ± 3.57 ^{ij}	19.76 ± 0.22 ^{jk}
Pla-ra III	11.38 ± 0.06 ^{m}	4.95 ± 0.02 ^{m}	25.84 ± 0.43 ^{h}	13.73 ± 0.00 ^{mn}
Pla-ra IV	15.88 ± 0.13 ^{i}	5.38 ± 0.01 ^{j}	41.20 ± 0.44 ^{de}	11.56 ± 0.07 ^{p}
Pla-chao I	5.96 ± 0.14 ^{u}	4.38 ± 0.02 ^{t}	18.13 ± 1.95 ^{l}	28.92 ± 0.13 ^{f}
Pla-chao II	7.72 ± 0.00 ^{q}	4.70 ± 0.05 ^{op}	8.57 ± 0.94 ^{o}	20.93 ± 0.45 ^{ij}
Pla-som I	5.10 ± 0.02 ^{v}	4.73 ± 0.02 ^{o}	9.17 ± 0.73 ^{o}	15.11 ± 0.28 ^{l}
Pla-som II	4.67 ± 0.04 ^{w}	5.32 ± 0.01 ^{k}	11.29 ± 0.70 ^{mn}	12.23 ± 0.03 ^{op}
Pla-som III	4.70 ± 0.01 ^{w}	5.36 ± 0.02 ^{j}	9.48 ± 0.37 ^{no}	15.63 ± 0.06 ^{l}
Pla-som IV	4.01 ± 0.17 ^{x}	5.55 ± 0.01 ^{i}	4.47 ± 0.06 ^{q}	2.70 ± 0.11 ^{r}
Som-fug I	2.06 ± 0.03 ^{y}	5.35 ± 0.02 ^{j}	6.50 ± 0.10 ^{p}	8.85 ± 0.05 ^{q}
Som-fug II	1.87 ± 0.20 ^{y}	4.83 ± 0.01 ^{n}	11.98 ± 0.05 ^{m}	11.21 ± 0.01 ^{p}
Pla-chom I	10.91 ± 0.14 ^{n}	4.98 ± 0.03 ^{m}	27.76 ± 0.38 ^{h}	26.21 ± 0.13 ^{g}
Pla-chom II	7.19 ± 0.08 ^{s}	4.49 ± 0.01 ^{r}	23.22 ± 0.42 ^{i}	13.53 ± 0.15 ^{no}
Kem-buk-nud I	13.66 ± 0.10 ^{k}	4.43 ± 0.01 ^{s}	52.93 ± 0.73 ^{a}	24.55 ± 1.09 ^{h}
Kem-buk-nud II	13.07 ± 0.21 ^{l}	4.64 ± 0.03 ^{q}	48.66 ± 0.35 ^{b}	25.86 ± 0.14 ^{g}
Hoi-dong I	6.40 ± 0.05 ^{t}	4.68 ± 0.04 ^{p}	42.71 ± 1.05 ^{d}	12.98 ± 0.06 ^{no}
Hoi-dong II	7.84 ± 0.04 ^{q}	4.42 ± 0.01 ^{s}	46.47 ± 1.83 ^{c}	14.86 ± 0.17 ^{lm}

Means ± SD from triplicate determinations.

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

1.2 pH of fermented fishery products

The pH values varied from 4 to 8 (Table 7). Products with high salt content, such as *Kapi*, had neutral pH (pH 6-8), whereas those added with carbohydrate substrates, including *Kung-chom*, *Pla-ra*, *Pla-chao*, *Pla-som*, *Som-fug*, *Pla-chom* and *Hoi-dong* were slightly acidic (pH 4-6). The differences in pH among products could be influenced by the differences in buffering capacity of muscle proteins (Srikorski *et al*., 1990), indigenous microflora (Østergaard *et al*., 1998) and carbohydrate sources used (Owens and Mendoza, 1985). Increasing amount of carbohydrate, such as cooked rice, roasted rice, and garlic, in products might reduce the buffering capacity of the fish muscle (Owens and Mendoza, 1985). The high pH might be a result of formation of volatile base compounds such as ammonia and the degradation products generated during fermentation (Faithong *et al*., 2010). The low pH was generally associated with lactic acid and other acids formed during fermentation. The formation of lactic acid and the decrease in pH of products can consequently inhibit the growth of spoilage bacteria and pathogens (Valyasevi *et al*., 2001). Lactic and acetic acids play an important role in imparting a tangy acidic character and may enhance saltiness, possibly by masking other flavors (Visessanguan *et al*., 2004).

1.3 F/T ratio and peptide content of fermented fishery products

F/T ratio and peptide content were in the range of 4-53% and 2-67 mg peptide/g sample, respectively (Table 7). By definition, F/T ratio represents the amount of free α-amino acid present in the sample with respect to their total quantity. Higher ratio indirectly indicated the occurrence and extent of protein degradation during processing and fermentation. Among all fermented products tested, *Kem-buk-nud* and *Ka-pi* exhibited the highest F/T ratio and peptide content, respectively. The high peptide content was observed in products with high salt concentration, such as *Kapi, Bu-du* and *Tai-pla*. Additionally, products with low salt content and pH, such as *Pla-som* and *Som-fug*, showed the lowest F/T ratio and peptide content. The differences in F/T ratio and peptide content among products could be influenced by many factors, such as ingredients, indigenous microorganism and processing condition. Pineapples added in *Kem-buk-nud* could increase degree of protein hydrolysis because they

contain a proteolytic enzyme called bromelain. In addition, free amino acids might be from some ingredients used, such as monosodium glutamate and garlic (Rhodes *et al*., 1991; Lee and Harnly, 2005). High salt concentration can inhibit spoilage bacteria originally present in fish, leaving only the halotolerant and halophilic microorganisms to grow in product. These microorganisms could produce proteinases which rapidly degraded proteins to peptides (Kanlayakrit *et al*., 2004). Carbohydrate, such as cooked rice, roasted rice, and garlic, were substrates for growth of lactic acid bacteria. These bacteria could produce organic acids and cause the pH drop to the acidic range. This acidic condition might maximize proteolytic activities. Cathepsins are generally active at pH range of 5.0-5.5 (Visessanguan *et al*., 2003). In addition, during the growth of lactic acid bacteria, peptidases are produced and slowly degrade the proteins to small peptides and free amino acids (Molly *et al*., 1997). However, the resulting peptides and free amino acids were lost during fermentation because they are used as the substrate for the microorganisms. Moreover, processing condition and fermentation time have an important influence on proteolytic activities and the growth of microorganisms during fermentation.

1.4 MALDI-TOF-MS analysis

The MALDI-TOF mass spectra of the extracts from all samples are depicted in Figure 3. DHBA was a relatively efficient matrix for the ionization and desorption of lower molecular mass peptides. Intense signals were generally found in the mass range of *m/z* 700-6000, suggesting the presence of mixture of small peptide in the crude extracts. Products containing a lower molecular weight, such as *Kem-buk-nud* and *Bu-du*, had higher F/T ratio, indicating the greater degradation. For *Kung-chom*, *Pla-ra*, *Pla-som*, *Pla-chom* and *Kem-buk-nud*, differences in MALDI-TOF mass spectra were obtained, possibly due to difference in raw material, ingredients and processes as well as fermentation time used. In addition, a wide variety of smaller peptides was generated. Enzymes involved in fermentation may originate from 4 sources, including (1) viscera and digestive system, (2) muscle tissue, (3) plants added to the fermentation, and (4) microorganisms active in the fermentation (Mackie *et al*., 1971).

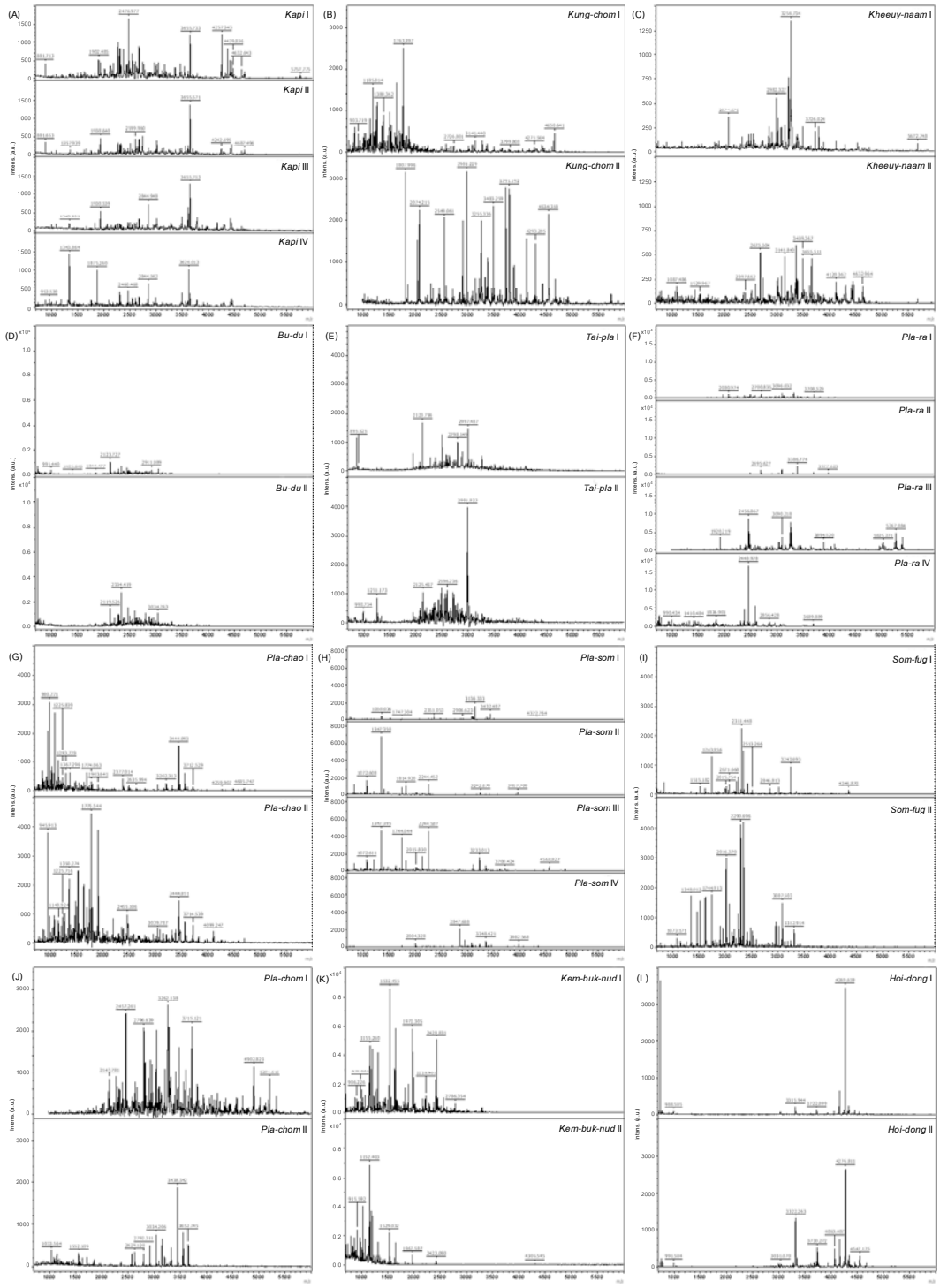

Figure 3. MALDI-TOF mass spectra of the extracts of different fermented fishery products. (A) *Kapi*, (B) *Kung-chom*, (C) *Kheeuy-naam*, (D) *Bu-du*, (E) *Tai-pla*, (F) *Pla-ra*, (G) *Pla-chao*, (H) *Pla-som*, (I) *Som-fug*, (J) *Pla-chom*, (K) *Kem-buk-nud* and (L) *Hoi-dong*.

Bioactive peptides were found to have molecular weight below 6000 Da and their bioactivity is based on amino acid composition and sequence (Pihlanto, 2000). Thus, peptides with molecular weight below 6000 Da were interested in this study. Recently, it has been recognized that many peptides that are released *in vitro* or *vivo* from animal protein are bioactive and have regulatory functions as ACE inhibitory activity and antioxidants (Hartmann and Meisel, 2007; Korhonen, 2009). Several ACE inhibitory and antioxidant peptides were found in fermented fishery products such as blue mussel sauce and oyster sauce (Je *et al*., 2005a, 2005c; Jung *et al*., 2005). Therefore, these fermented products tested might be a good source of ACE inhibitory and antioxidative peptides.

1.5 ACE inhibitory activity of fermented fishery products

ACE inhibitory activity of extracts from all fermented fishery products was found in the range of 0-98% (Table 8). Extracts from all *Pla-som* exhibited the highest ACE inhibitory activity, compared with other products. Among all *Pla-som*, the extracts from *Pla-som* III and IV, produced from *Puntius gonionotus*, had the highest ACE inhibitory activity. In addition, the extracts from *Kapi, Kung-chom, Kheeuy-naam, Bu-du, Tai-pla, Pla-ra, Pla-chao, Pla-som, Som-fug, Pla-chom, Kem-buk-nud* and *Hoi-dong* also exhibited ACE but the activities varied, possibly due to differences in raw material, ingredients and processing employed according to local producers. He *et al*. (2007) reported ACE inhibitory peptides produced from different marine protein materials, including fish, shrimp, seashell, algae and seafood waste. Each material and some ingredients were presumed to contain different substrates, such as proteins and peptides, and proteolytic enzymes (Faithong *et al*., 2010). Peptides present in enzymatically digested proteins exhibited different physicochemical properties and biological activities depending on their molecular weights and amino acid sequences (Kim and Mendis, 2006).

Table 8. ACE inhibitory activity and antioxidative activities of the extracts from different traditional fermented fishery products determined by different assays.

Type of products	ACE inhibitory activity (%)	DPPH (μmol TE/g protein)	ABTS (μmol TE/g protein)	FRAP (μmol TE/g protein)
Kapi I	3.36 ± 0.79 ^{n}	53.67 ± 0.53 ^{bc}	94.40 ± 0.00 ^{d}	42.01 ± 0.34 ^{m}
Kapi II	42.25 ± 1.92 ^{kl}	49.49 ± 1.19 ^{c}	75.30 ± 2.17 ^{fg}	42.23 ± 0.15 ^{m}
Kapi III	0.00 ± 0.00 ^{n}	43.25 ± 3.64 ^{d}	55.70 ± 3.70 ^{l}	33.39 ± 0.07 ^{no}
Kapi IV	0.00 ± 0.00 ^{n}	26.40 ± 1.25 ^{jkl}	150.55 ± 5.60 ^{c}	21.98 ± 0.26 ^{r}
Kung-chom I	53.82 ± 4.70 ^{i}	37.61 ± 0.69 ^{ef}	264.17 ± 3.72 ^{a}	31.02 ± 0.19 ^{op}
Kung-chom II	52.40 ± 0.08 ^{i}	36.55 ± 0.66 ^{ef}	186.40 ± 8.13 ^{b}	27.53 ± 0.09 ^{pq}
Kheeuy-naam I	66.38 ± 0.26 ^{gh}	28.89 ± 0.42 ^{ijk}	63.89 ± 2.14 ^{ijk}	14.15 ± 0.08 ^{s}
Kheeuy-naam II	0.00 ± 0.00 ^{n}	27.07 ± 0.90 ^{ijkl}	59.21 ± 1.36 ^{jkl}	188.10 ± 2.67 ^{e}
Bu-du I	65.83 ± 2.69 ^{h}	74.14 ± 7.54 ^{a}	183.49 ± 1.70 ^{b}	60.86 ± 0.55 ^{k}
Bu-du II	0.00 ± 0.00 ^{n}	55.12 ± 0.49 ^{b}	81.98 ± 2.24 ^{ef}	450.22 ± 5.86 ^{c}
Tai-pla I	68.17 ± 0.43 ^{gh}	31.57 ± 2.50 ^{hi}	86.14 ± 0.63 ^{e}	22.81 ± 0.52 ^{r}
Tai-pla II	45.51 ± 4.96 ^{kj}	40.63 ± 0.63 ^{de}	189.10 ± 5.43 ^{b}	24.69 ± 0.26 ^{qr}
Pla-ra I	76.66 ± 1.13 ^{de}	23.44 ± 3.46 ^{lm}	51.79 ± 3.54 ^{l}	8.43 ± 0.02 ^{tu}
Pla-ra II	44.04 ± 1.00 ^{kl}	25.40 ± 0.13 ^{klm}	28.82 ± 0.00 ^{n}	148.43 ±2.51 ^{g}
Pla-ra III	74.74 ± 0.09 ^{ef}	27.18 ± 0.25 ^{ijkl}	43.05 ± 2.96 ^{m}	10.96 ± 0.26 st
Pla-ra IV	47.10 ± 1.33 ^{kj}	40.68 ± 1.03 ^{de}	40.35±5.27^{m}	363.90 ± 4.18 ^{d}
Pla-chao I	65.77 ± 3.46 ^{h}	16.42 ± 2.40 ^{m}	25.66 ± 3.79 ^{no}	3.54 ± 0.09 ^{v}
Pla-chao II	87.54 ± 1.55 ^{b}	13.17 ± 0.16 ^{m}	16.92 ± 0.95 ^{p}	35.84 ± 2.55 ^{n}
Pla-som I	77.31 ± 1.40 ^{cde}	20.76 ± 0.56 ^{l}	57.14 ± 6.70 ^{kl}	10.32 ± 0.43 st
Pla-som II	80.74 ± 0.37 ^{cd}	30.57 ± 2.55 ^{hij}	71.03 ± 7.27 ^{ghi}	12.98 ± 0.23 st
Pla-som III	97.96 ± 6.24 ^{a}	15.56 ± 3.54 ^{m}	53.06 ± 0.00 ^{l}	5.16 ± 0.29 ^{uv}
Pla-som IV	81.97 ± 0.89 ^{c}	34.62 ± 0.55 ^{fg}	17.48 ± 1.88 ^{op}	105.47 ± 1.34 ^{i}
Som-fug I	76.57 ± 1.11 ^{de}	13.49 ± 0.22 ^{m}	22.10 ± 1.11 ^{nop}	53.24 ± 1.74 ^{l}
Som-fug II	73.12 ± 2.77 ^{ef}	15.95 ± 0.31 ^{m}	17.19 ± 3.72 ^{p}	71.54 ± 1.11 ^{j}
Pla-chom I	31.27 ± 3.99 ^{m}	22.78 ± 0.18 ^{lm}	29.52 ± 1.66 ^{n}	112.12 ± 3.56 ^{h}
Pla-chom II	40.28 ± 3.44 ^{l}	25.28 ± 0.38 ^{klm}	22.24 ± 2.29 ^{nop}	181.74 ± 3.54 ^{f}
Kem-buk-nud I	49.14 ± 0.22 ^{ij}	50.28 ± 1.52 ^{c}	65.52 ± 1.06 ^{hij}	527.56 ± 2.28 ^{b}
Kem-buk-nud II	49.69 ± 0.00 ^{ij}	51.58 ± 1.07 ^{c}	78.84 ± 2.69 ^{efg}	582.06 ± 4.61 ^{a}
Hoi-dong I	28.92 ± 0.78 ^{m}	29.02 ± 0.58 ^{ijk}	18.26 ± 2.27 ^{op}	151.78 ± 1.08 ^{g}
Hoi-dong II	71.11 ± 1.31 ^{fg}	28.89 ± 1.37 ^{ijk}	73.04 ± 4.31 ^{gh}	14.02 ± 0.05 ^{s}

TE: Trolox equivalents.

Mean ± SD from duplicate determinations.

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

Processing conditions, such as oxygen content, pH and time, can be controlled to obtain the desirable final product characteristics and bioactive peptides (van der Ven *et al*., 2002). The presence or absence of oxygen can control the growth of microorganisms and oxidation in products. The pH also controls microorganisms growth and enzymatic activities. High content of small peptides and free amino acids can be found if the process is allowed to proceed too long (Mackie *et al*., 1971).

1.6 Antioxidative activity of fermented fishery products

DPPH, ABTS radical scavenging activities and FRAP of extracts from all products are presented in Table 8. The extracts from all products showed DPPH, ABTS radical scavenging activities and FRAP. DPPH radical scavenging activity varied from 13 to 74 μmol TE/g protein. This indicated that the extracts from all fermented products possessed the ability to donate the hydrogen atom to free radicals, in which the propagation process could be retarded (Faithong *et al*., 2010). The extract from *Bu-du* showed the highest scavenging toward DPPH radical (p<0.05), whereas the extracts from *Pla-ra* (I, II), *Pla-chao* (I, II), *Pla-som* (I, III), *Som-fug* (I, II) and *Pla-chom* (I, II) showed the lowest DPPH radical scavenging activity, compared with those of other products.

The result showed that ABTS radical scavenging activities of the extracts from all products were generally similar to those observed for DPPH radical scavenging activity. ABTS radical scavenging activity is an excellent tool for determining the activity of electron-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavenger of lipid peroxyl radicals) (Leong and Shui, 2002). Among all products, the extract from *Kung-chom* exhibited the highest ABTS radical scavenging activity (p<0.05). The lowest ABTS radical scavenging activity was found in the extracts from *Pla-chao* (II), *Pla-som* (IV), *Som-fug* (I, II), *Pla-chom* (II) and *Hoi-dong* (I).

FRAP varied in different products. Among all products, the highest FRAP was found in the extract from *Kem-buk-nud*, whereas the extracts from *Pla-chao* (I) and *Pla-som* (III) showed the lowest FRAP. Therefore, protein was probably hydrolyzed to peptide or free amino acids, which were able to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex.

The result was in accordance with Virtanen *et al*. (2006), who reported that antioxidative activities depend on certain fermented product types. The antioxidative activities of products were most likely governed by the type and amino acid composition of resulting peptides (Faithong *et al*., 2010). Several amino acids, such as Tyr, Met, His, Lys and Trp, were generally accepted as antioxidants despite their occasionally pro-oxidative effects (Chen *et al*., 1996). The antioxidant activity of histidine-containing peptides was reported and attributed to the chelating and lipid radical-trapping ability of the imidazole ring (Murase *et al*., 1993; Park *et al*., 2001; Uchida and Kawakishi, 1992). Low molecular weight peptides (1-3 kDa) have higher scavenging activity than high molecular weight counterpart (Chang *et al*., 2007; Wu *et al*., 2003). Peptides with the molecular weight of approximately 1400 Da and with 5–16 amino acid sequences showed strong inhibition activities on the autoxidation of linoleic acid (Chen *et al*., 1995; Wu *et al*., 2003). The highest antioxidative activity of isolated peptides from of tuna cooking juice hydrolysates by orientase was found in the fractions with molecular weight of 400-1500 Da. Three antioxidative peptides comprising 4–10 amino acids residues were observed, and the sequences of the peptides were Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (1305 Da), Pro-Ser-Asp-His-Asp-His-Glu (938 Da), and Val-His-Asp-Tyr (584 Da) (Hsu *et al*., 2009).

1.7 Principal component analysis

Principal component analysis (PCA) is utilized to determine the important factors explaining correlation of variables and samples which the data are decomposed into separate sets of loading and scores for each of the two modes of interest (variables and samples). Whole variability of the data is explained in order to provide a clear and more interpretable visualization of data structure in a reduced dimension. PCA was performed on the 7 variables including salt content, pH, F/T ratio, ACE inhibitory activity, DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP of 30 fermented fishery products (Tables 7 and 8). Seven variables were reduced into 3 components explaining 81% of the variation in all samples. PC1 described 29% of the variation, while PC 2 and PC 3 described an additional 28% and 24%, respectively (Figure 4).

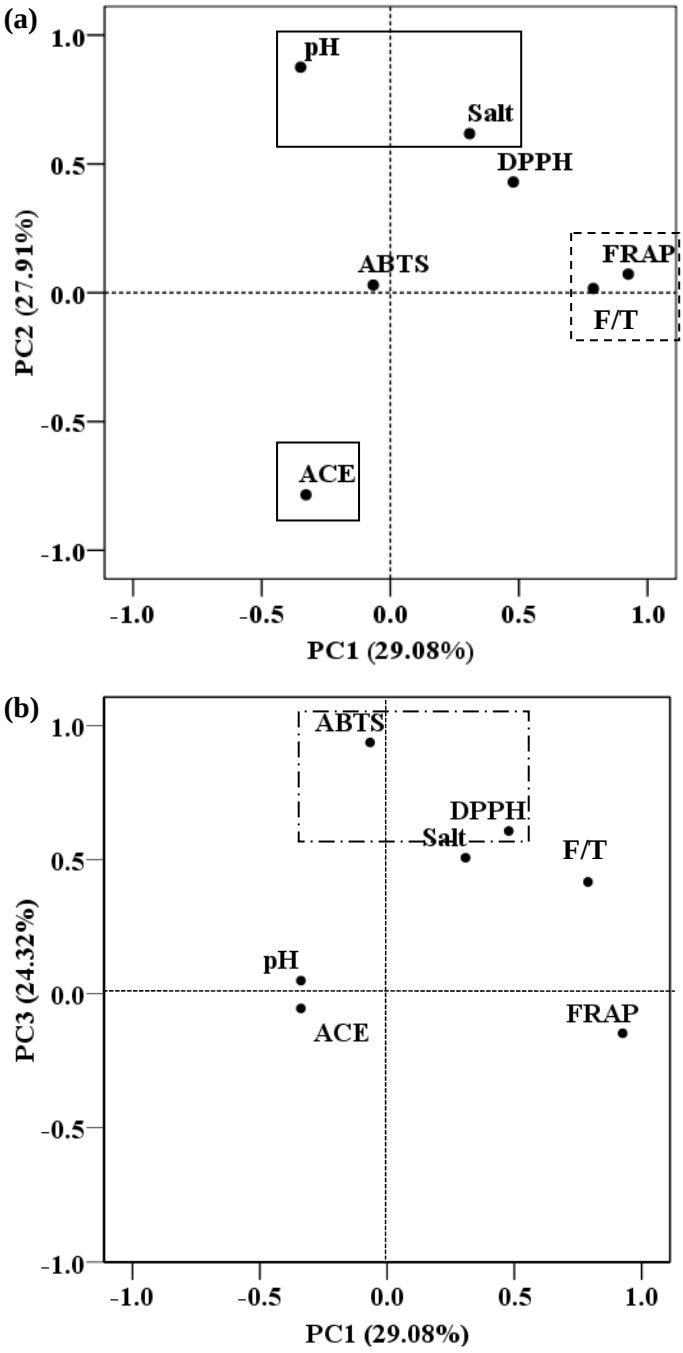

Figure 4. Loading plots of all formulations of fermented fishery products on the PC1-PC2 space (a) and the PC1-PC3 space (b).

PC1 loading showed a significant contribution of FRAP and F/T ratio with high positive loadings. The result indicated that peptides with high FRAP were more likely found in products with high F/T ratio such as *Kem-buk-nud* (I, II), *Bu-du* (II) and *Pla-ra* (IV). PC2 loading described that pH and salt content were negatively correlated to ACE inhibitory activity. Therefore, peptides with high ACE inhibitory activity were more probably found in products with low pH and low salt content. Moreover, products with high ACE inhibitory activity, including *Pla-chao, Pla-som* and *Som-fug*, were characterized by garlic added (Figure 5a and Table 7). Suetsuna (1998) suggested that garlic contains dipeptides possessing ACE inhibitory properties. These dipeptides were identified as Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe and Asn-Phe. Furthermore, the result indicated that fermented fish had higher ACE inhibitory activity than fermented shrimp or krill (Figure 5b and Table 6). PC3 loading described that products with high DPPH radical scavenging activity more likely had high ABTS radical scavenging activity. However, both activities showed no correlation to pH, salt content and F/T ratio. The result reconfirmed that F/T ratio or free amino group content was not the major factor affecting DPPH and ABTS radical scavenging activity. Faithong *et al*. (2010) reported that antioxidative activities of *Kapi, Jaloo* and *Kung-som* were not correlated with degree of hydrolysis. The degree of hydrolysis was most likely depended on specific characteristics of the bacterial enzyme (Virtanen *et al*., 2006). In addition, high degree of hydrolysis might cause the loss in activity of some antioxidants. Wu *et al*. (2003) reported that during hydrolysis, the antioxidant activity increased initially but decreased gradually thereafter.

In addition, ACE inhibitory activity exhibited no correlation to antioxidative activity. In this study, no products were found to exhibit the high both activities. The extract from *Pla-som* showed the highest ACE inhibitory activity. The extracts from *Bu-du*, *Kung-chom* and *Kem-buk-nud* had the highest DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP, respectively. Since ACE inhibition is the main effect on blood pressure lowering, the following studies were paid on *Pla-som* produced from *Puntius gonionotus* which showed the highest ACE inhibitory activity.

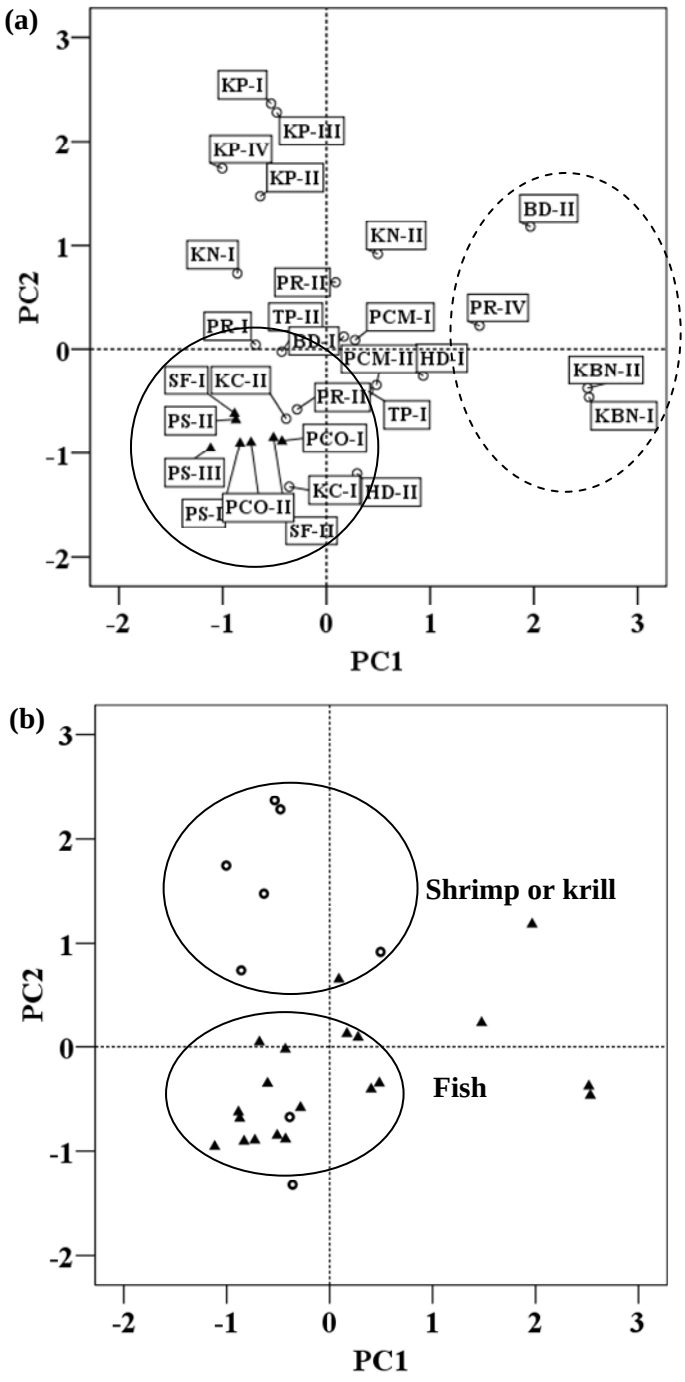

Figure 5. Score plots of the variables in the plane defined by principal components for fermented fishery products consisted of garlic (▲) and no garlic (○) [a]. Fermented fishery products produced from fish (▲) and shrimp or krill (○) [b].

2. Effect of starter cultures on properties and ACE inhibitory activity of *Pla-som* during fermentation

2.1 Effect on lactic acid bacteria and micrococci-staphylococci counts of *Pla-som* during fermentation

Changes in lactic acid bacteria (LAB) and micrococci-staphylococci counts in *Pla-som* fermented with and without inoculation of *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 are shown in Figure 6. Initial counts of LAB were within the range of 10^5-10^6 CFU/g. Visessanguan *et al.* (2006) reported that the initial flora of the Nham derived mainly from the raw materials. The number of LAB in control and starter-inoculated *Pla-som* increased continuously up to 48 h and remained unchange during 48 and 120 h of fermentation. The starter-inoculated *Pla-som* had higher LAB count than the control during fermentation. Generally, LAB produce lactic acid as their main fermentation end-product via glycolysis (the Embden-Meyerhof pathway) or the 6-phosphoglyconate/phosphoketolase pathway and they are named homo- or heterofermentative LAB, respectively (Axelsson, 1998). A rapid growth of LAB associated with the rapid decrease in pH to below 5-4.5 is essential to prevent spoilage and pathogenic bacteria in fishery fermented products (Østergaard *et al*., 1998). Various metabolic products of LAB, such as short-chain organic acids, carbon dioxide, hydrogen peroxide, diacetyl, and bacteriocin, are known as antimicrobial agents (Rowan *et al.,* 1998).

The initial micrococci-staphylococci counts of the control and starter-inoculated *Pla-som* were 10^6 CFU/g. The number of micrococci-staphylococci increased to 10^7 CFU/g at 24 h and decreased to 10^6 CFU/g at 96 h in control and starter-inoculated *Pla-som*. At fermentation time of 120 h, micrococci-staphylococci counts in control increased, whereas those in starter-inoculated *Pla-som* decreased. The starter-inoculated *Pla-som* showed lower micrococci-staphylococci count than control during fermentation. A decrease in micrococci-staphylococci count was probably due to rapid acidification of growth of LAB. The result was in accordance with Dalmış and Soyer (2008). Therefore, the addition of *P. acidilactici* BCC 9545 in *Pla-som* might contribute to decrease number of micrococci-staphylococci.

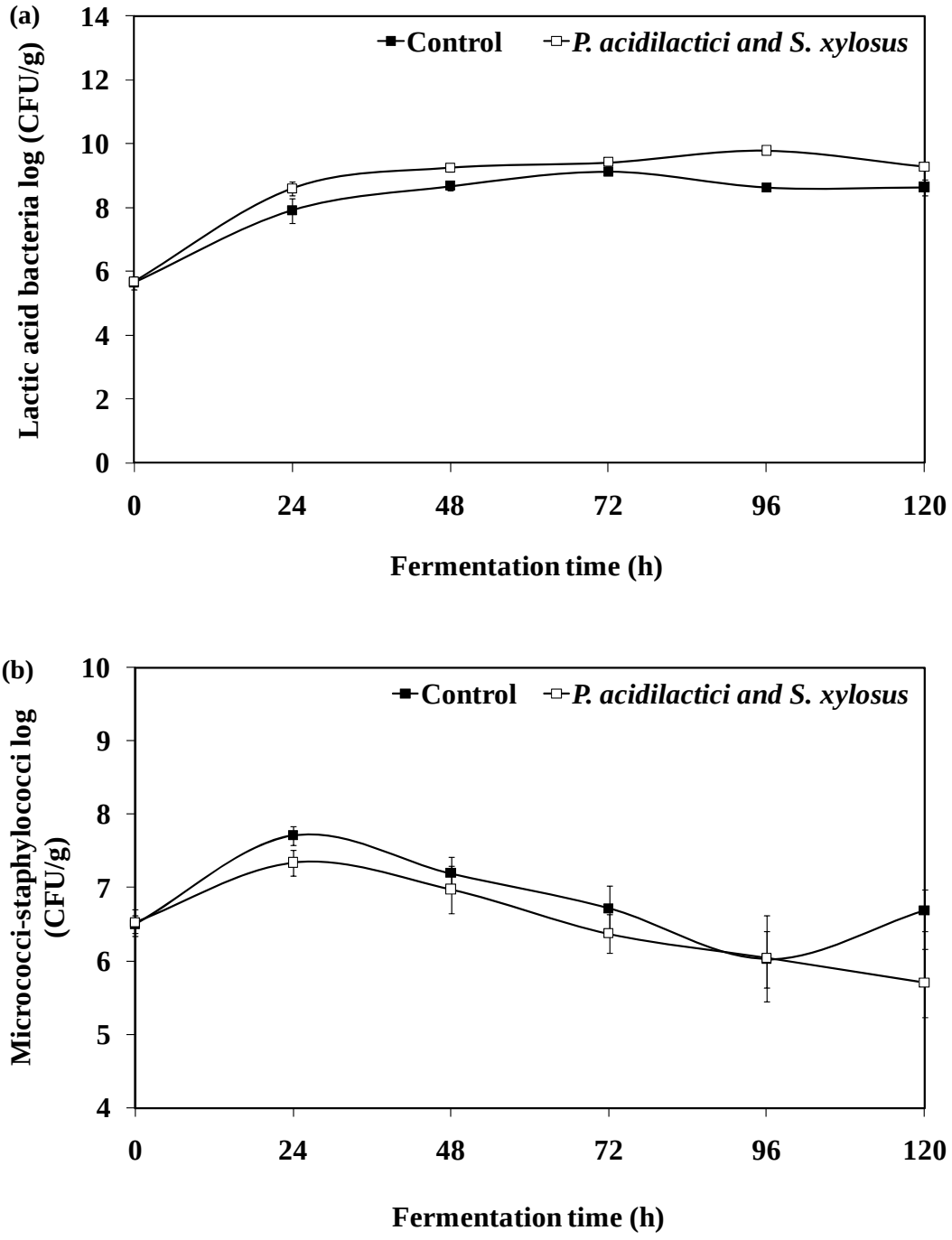

Figure 6. Changes in lactic acid bacteria (a) and micrococci-staphylococci (b) of *Plasom* fermented with and without inoculation with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g during fermentation at 30°C. Values represent the means ± SD (n = 3).

Generally, *Staphylococcus* contribute to the development of colour by reducing nitrate to nitrite and participate in the development of flavours of dry fermented sausages (Demeyer *et al*., 1986; Schleifer, 1986). Proteinases from *S. xylosus* were responsible for breakdown of fish protein into peptides and free amino acids (Kaban and Kaya 2009). In addition, *Staphylococcus* influence the composition of non-volatile and volatile compounds mainly by degrading free amino acids and inhibiting the oxidation of unsaturated free fatty acids (Hammes and Hertel, 1998; Søndergaard and Stahnke, 2002). Free fatty acids are the products of lipolysis and are the precursors of the lipid oxidation (Toldra *et al*., 2001).

2.2 Effect on pH and total acidity of *Pla-som* during fermentation

Changes in pH and total acidity of control and starter-inoculated *Pla-som* are shown in Figure 7. The averages initial pH of all samples tested was 6.21. In general, pH of fermented fishery products should be below 5-4.5 in order to inhibit pathogenic and spoilage bacteria (Owens and Mendoza, 1985; Østergaard *et al*., 1998). Therefore, the pH of *Pla-so*m in this study was found to be in an acceptable range for safety aspects. The pH of *Pla-som* inoculated with starters decreased to 5 within 72 h, whereas the pH of control decreased to 5 within 96 h. The total acidity of control and starter-inoculated *Pla-som* increased until 96 h and was constant up to 120 h. In addition, starter-inoculated *Pla-som* showed a higher rate of fermentation than the control, particularly during the first 24-48 h, as indicated by the greater rate of pH drop and organic acid production ($p<0.05$). Therefore, the inoculation of *P. acidilactici* BCC 9545 could accelerate the fermentation process, approximately 72 h faster than the control.

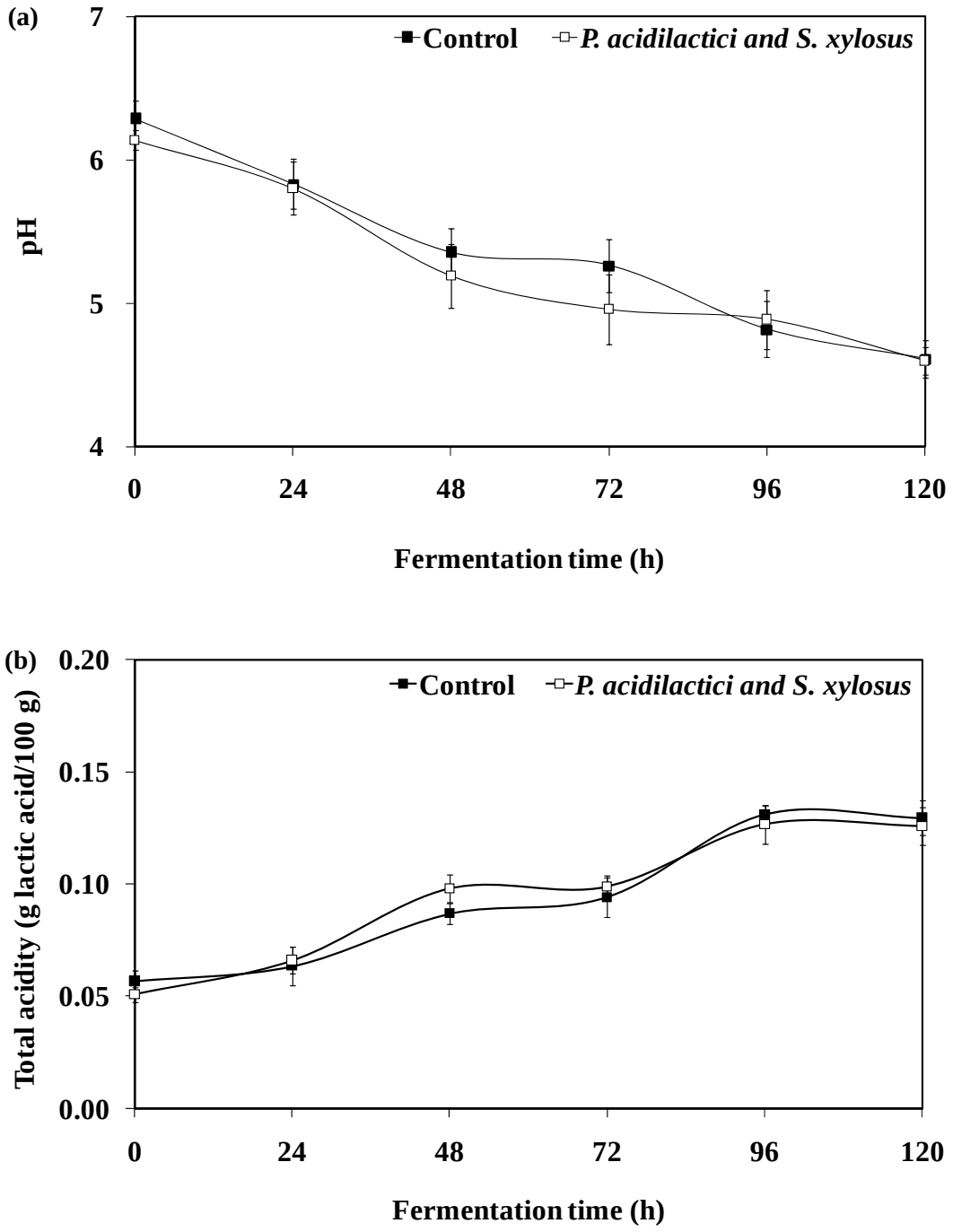

Figure 7. Changes in pH (a) and total acidity (b) of *Pla-som* fermented with and without inoculation with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g during fermentation at 30°C. Values represent the means ± SD (n=3).

2.3 Effect on proteolysis of *Pla-som* during fermentation

Electrophoretic patterns of proteins in control and starter-inoculated *Pla-som* during fermentation revealed an intense degradation of proteins throughout the fermentation up to 120 h (Figure 8). With increasing fermentation time, the intensity of the bands of 205 kDa (myosin heavy chain, MHC), 45 kDa (actin) and 38 kDa (tropomyosin T1), 36 kDa (sarcoplasmic protein) and 16 kDa decreased progressively with the coincidental appearance of protein bands of 120-60, 30-20 and 15-10 kDa. Degradation of proteins resulted in an increase in peptides and free amino acids (Visessanguan *et al*., 2004). TCA-soluble peptide, free amino group content and degree of hydrolysis (DH) in *Pla-som* increased with increasing fermentation time (Figure 9 and 10). TCA-soluble peptide content in the supernatant was measured according to the Lowry method (Lowry *et al*., 1951). The method combines the reactions of copper ions with the peptide bonds under alkaline condition with the oxidation of aromatic residues. Free amino group content and DH were similarly tested by trinitrobenzenesulfonic acid hydrate (TNBS) which reacts only with amino groups in their unprotonated state (Benjakul and Morrissey, 1997). DH is defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis (Adler-Nissen, 1986). Increased TCA-soluble peptide, free amino group content and DH were coincidental with the continuous decreased band intensity of the major proteins during fermentation (Figure 8). During fermentation, no significant differences were observed in TCA-soluble peptide of control and starter-inoculated *Pla-som* ($p>0.05$). Free amino group content and DH showed significant difference between control and starter-inoculated *Pla-som* ($p<0.05$). The result indicated that free amino group contents were increased by addition of starter but peptide content was not affected by starter added. The higher free amino group content indicates the greater degradation in *Som-fug*, a fermented fish paste (Riebroy *et al*., 2008). The proteolysis in *Pla-som* could be influenced by many factors such as endogenous and microbial enzymes. The initial hydrolysis of muscle proteins is attributed mainly to endogenous enzyme and is followed by the action of microbial peptidases, which further degraded the protein fragments to small peptides and free amino acids.

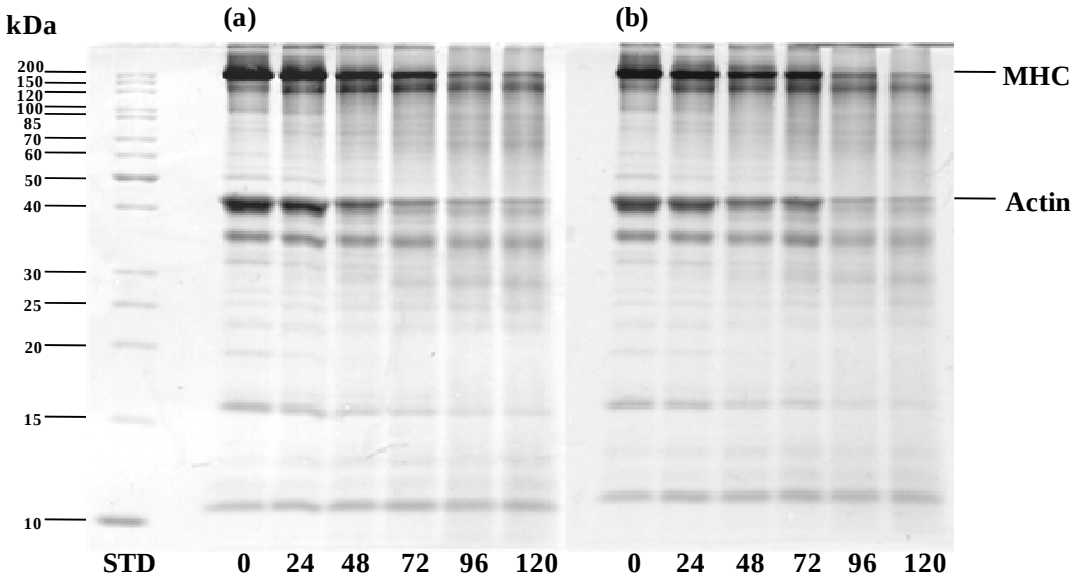

Figure 8. SDS-PAGE pattern of protein in traditional *Pla-som* (a) and *Pla-som* inoculated with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g (b) during fermentation at 30°C.

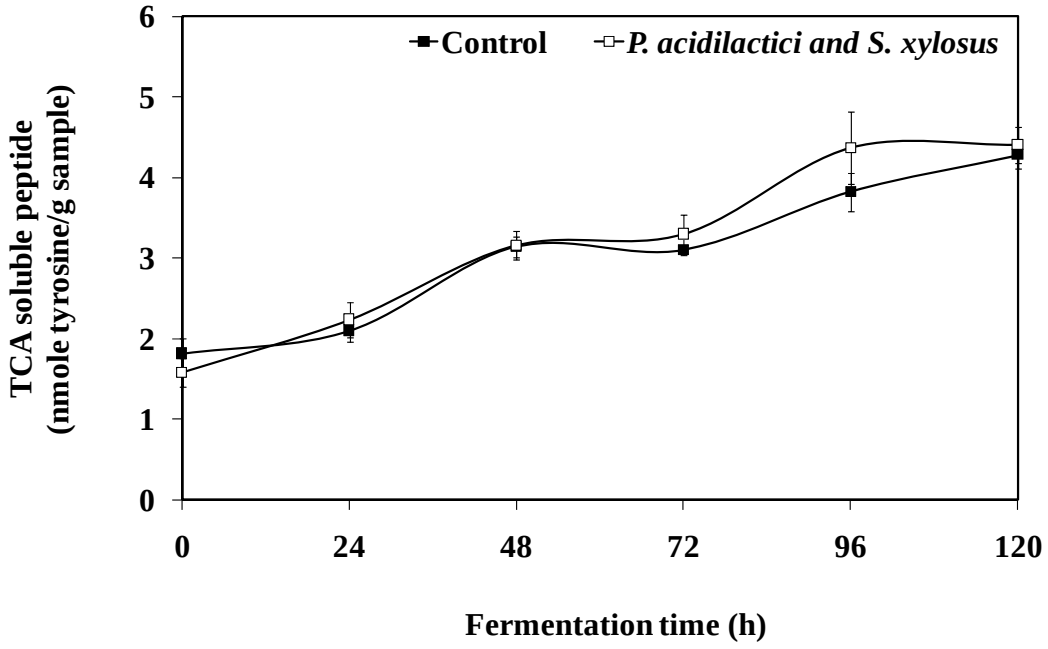

Figure 9. Change in TCA-soluble peptide of *Pla-som* fermented with and without inoculation with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g during fermentation at 30°C. Values represent the means ± SD (n = 3).

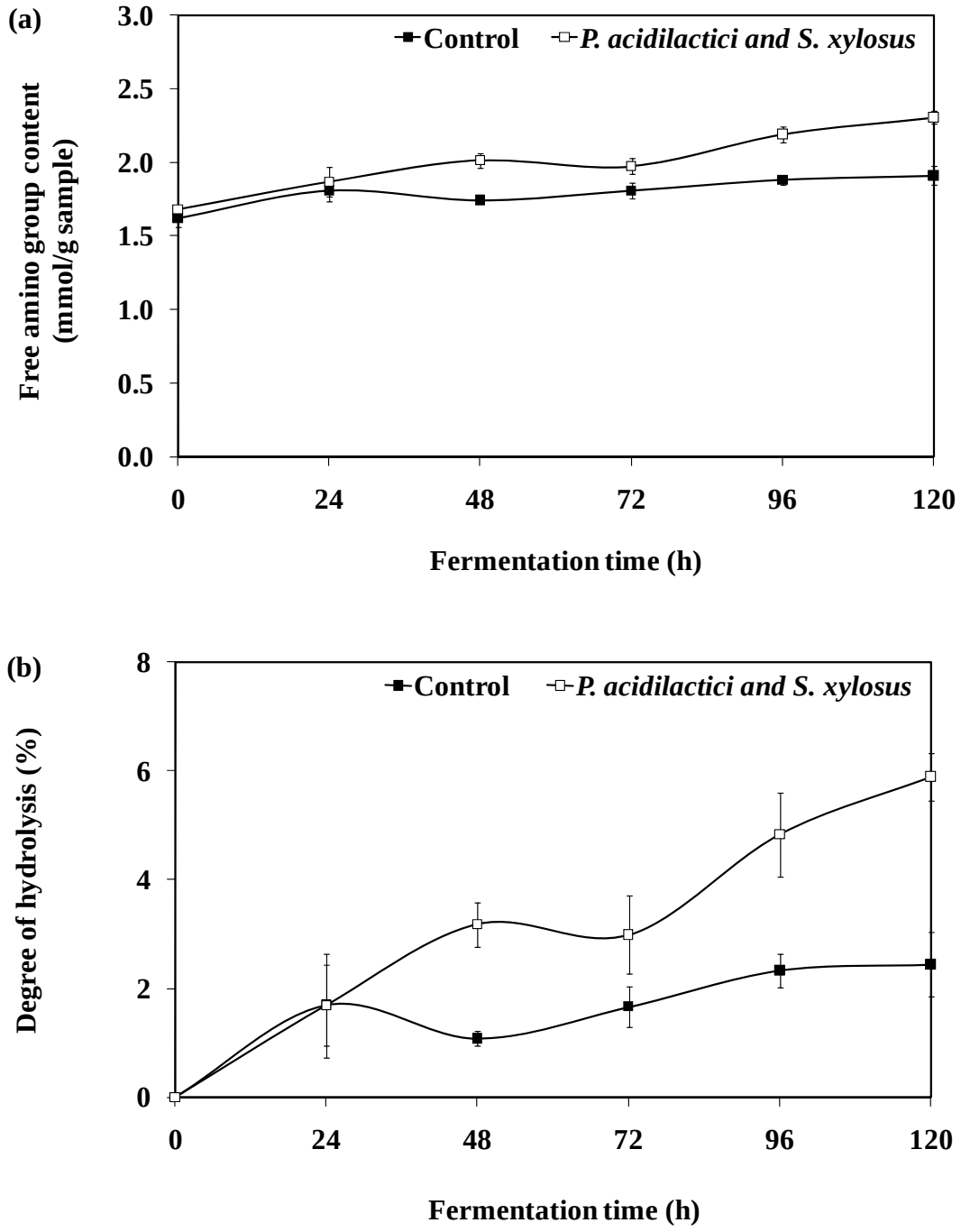

Figure 10. Change in free amino group content (a) and degree of hydrolysis (b) of *Pla-som* fermented with and without inoculation with *P. acidilactici* BCC 9545 at 10^4 CFU/g and *S. xylosus* BCC 3710 at 10^6 CFU/g during fermentation at 30°C. Values represent the means ± SD (n = 3).

Fish muscle serine proteases might contribute to hydrolysis at the beginning of fermentation, where pH was in the neutral range. Fish muscle serine proteases have been reported from the muscle soluble fraction of several fish species (Folco *et al*., 1984; Kinoshita *et al*., 1990b; Yanagihara *et al*., 1991; Benjakul *et al*., 2003; Ohkubo *et al*., 2004a). Further myofibril-bound serine proteases were also found in the myofibril fraction of fresh water fish (Osatomi *et al*., 1997; Ohkubo *et al*., 2004b). Strong proteinase activity, produced by staphylococci and bacilli, is responsible for breakdown of fish protein into peptides and free amino acids (Valysevi and Rolle, 2002). In addition, lactic acid bacteria generally have little proteolytic activity against meat proteins. Nevertheless, lactic acid fermentation of pork enhanced meat protein degradation (Kato *et al*., 1994). Williams *et al*. (1998) reported that peptidases, such as aminopeptidase and serine dipeptidyl peptidase, were produced by lactic acid bacteria. During fermentation, a wide variety of smaller peptides and free amino acids was generated, depending on enzyme specificity (Faithong *et al.*, 2010). The peptides produced as a result of enzymatic degradation of proteins have an important influence on biological activities (Jamdar *et al*., 2010).

2.4 Changes in ACE inhibitory activity of *Pla-som* during fermentation

The release of ACE inhibitory peptides of *Pla-som*, produced from *Puntius gonionotus*, garlic and sticky rice, was likely to depend on fermentation time (Table 9). However, the inoculation of *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 in *Pla-som* could not increase the release of ACE inhibitory peptides. Some materials, including *Puntius gonionotus* and garlic, for production of *Pla-som*, were found to have ACE inhibitory activity with 2,532 and 486 units/g sample, respectively, whereas sticky rice showed no ACE inhibitory activity. Suetsuna (1998) suggested that garlic contains ACE inhibitory peptides, such as Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe and Asn-Phe. At 0 h, *Pla-som* showed inhibitory activity on ACE with 800–1,000 units/g sample. As the fermentation proceeded, the increases in peptide content and ACE inhibitory activity of control and starter-inoculated *Pla-som* were observed (Table 9).

Table 9. Peptide content and ACE inhibitory activity of raw materials used for production of *Pla-som* and their changes during fermentation at 30°C.

Raw material		Peptide content (mg peptide/g sample)	ACE inhibitory activity (units/g sample)
Puntius gonionotus		25.57 ± 1.44	2532 ± 119
Garlic		1.76 ± 0.17	486 ± 54
Sticky rice		ND	-
Pla-som naturally	at 0 h	3.46 ± 0.85 ^{bc}	869 ± 175 ^{cd}
fermented	at 24 h	3.07 ± 0.44 ^{c}	563 ± 235 ^{d}
	at 48 h	4.44 ± 0.64 ^{b}	1060 ± 189 ^{bc}
	at 72 h	4.58 ± 0.15 ^{b}	1060 ± 9 ^{bc}
	at 96 h	4.31 ± 0.95 ^{b}	1382 ± 53 ^{ab}
	at 120 h	6.95 ± 0.59 ^{a}	1579 ± 362 ^{a}
Pla-som inoculated with	at 0 h	3.76 ± 1.72 ^{b}	968 ± 211 ^{bc}
P.acidilactici and	at 24 h	3.11 ± 0.08 ^{b}	743 ± 157 ^{c}
S.xylosus	at 48 h	4.72 ± 0.66 ^{ab}	1241 ± 264 ^{b}
	at 72 h	4.58 ± 0.84 ^{ab}	1062 ± 250 ^{bc}
	at 96 h	4.31 ± 0.72 ^{ab}	1334 ± 102 ^{ab}
	at 120 h	5.85 ± 0.51 ^{a}	1673 ± 97 ^{a}

Different letters (a,b) in the same column indicate significant differences (p<0.05).
Values represent the means ± SD (n=3).
ND: Not detected

The extract from the complete fermentation of control (at 96 h) exhibited higher ACE inhibitory activity than the extract from the complete fermentation of starter-inoculated *Pla-som* (at 72 h). At the same fermentation time, peptide content and ACE inhibitory activity showed no significant difference between control and starter-inoculated *Pla-som* (p>0.05). Therefore, the peptide content correlated to ACE inhibitory activity. Peptides were released during fermentation which might contribute to activity of ACE inhibition. Tsai *et al*. (2006) reported that

the ACE inhibitory activity was affected by hydrolysis time and correlated to peptide content. ACE inhibitory activity is mainly dependent on a specific peptide structure. Although starter-inoculated *Pla-som* showed higher free amino group content and DH than control during fermentation (Figure 10), no difference in ACE inhibitiory activity was observed between control and starter-inoculated *Pla-som* ($p>0.05$). Therefore, ACE inhibitory activity may not be affected by free amino acid.

2.5 Stability study of ACE inhibitory activity

The extracts from control and starter-inoculated *Pla-som* still had ACE inhibitory activity after treatment at different temperatures (Figure 11a). More than 85% of activity were retained after heat treatments. The slight increase in ACE inhibitory activity of the extract from control was noticeable when heated at 60^{o}C ($p<0.05$). In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and exposure of hydrophobic domain (Sikorski and Naczk, 1981). The increases in ACE inhibitory activity of the extract from *Pla-som* heated at 60^{o}C were probably due to the exposure of the hydrophobic domain. However, ACE inhibitory activity of the extract from *Pla-som* slightly decreased when heated up to 100^{o}C for control and 90^{o}C for starter-inoculated. The denaturation of ACE inhibitory peptides might be enhanced when heated at higher temperature. Accumulated energy or enthalpy might be sufficient for ACE inhibitory peptides to undergo denaturation, thereby losing in activity. These results indicated that ACE inhibitory activity of the extracts from control and starter-inoculated *Pla-som* showed heat stability.

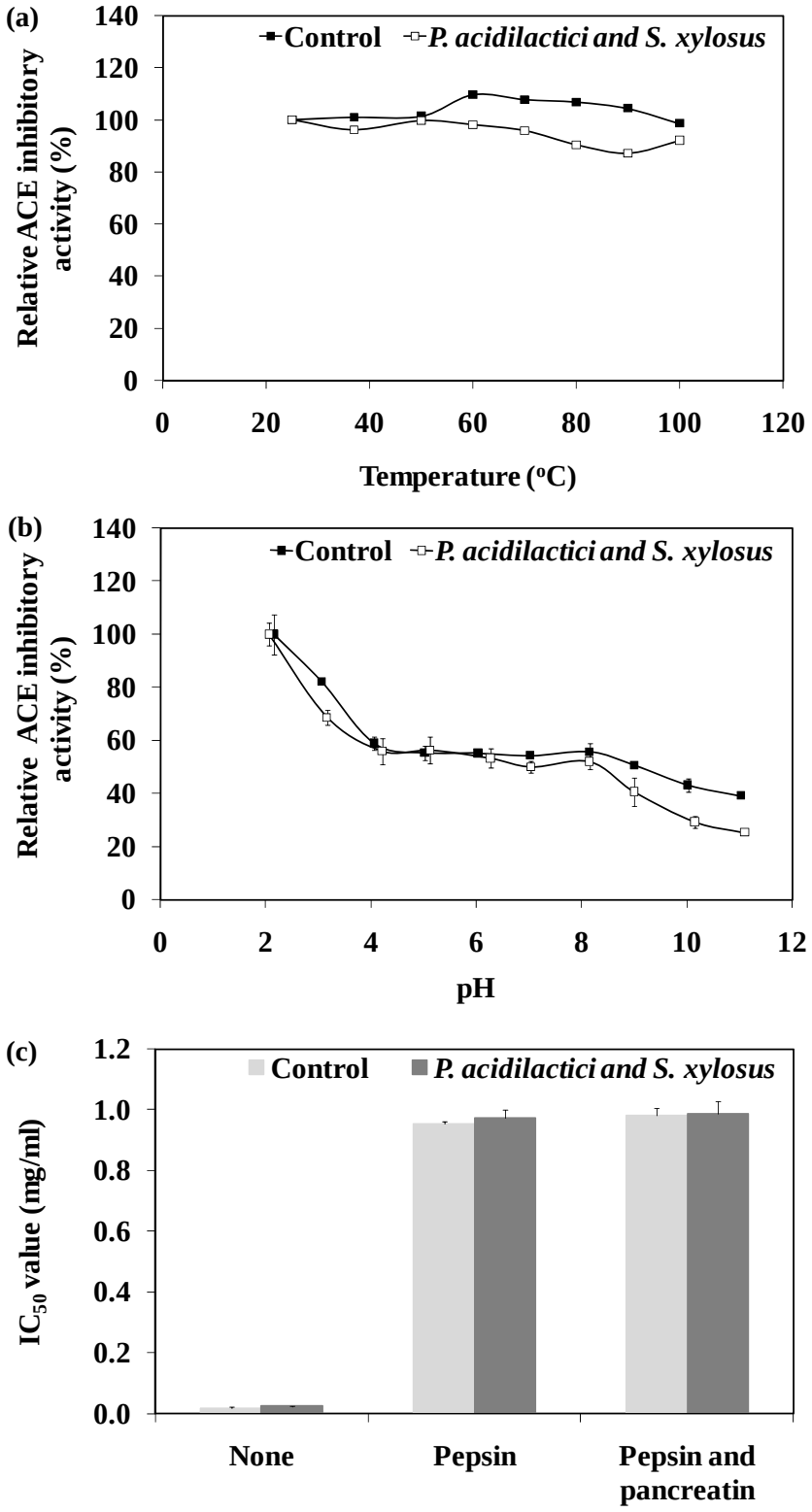

Figure 11. Stability of ACE inhibitory activity of the extracts from control and starter-inoculated *Pla-som* after 1 h incubation at different temperature (a), pH (b) and after digestion with gastrointestinal proteases (c).

The influences of pH on ACE inhibitory activity of the extracts from control and starter-inoculated *Pla-som* are shown in Figure 11b. The extracts from control and starter-inoculated *Pla-som* exhibited the similar activity at different pH. The extract from *Pla-som* showed high relative activity at pH 2. ACE inhibitory activity decreased at pH 3 and approximately 50% of relative activity was found in pH range of 4-8, followed by decline at pH above 8. When pH is increased, away from the isoelectric point, the ionizable groups in proteins become increasingly charged to a point, where charge repulsion causes the protein molecules to unfold and may bring about different protein structures that could modify functionalities, which are different from the native protein (Dill and Shortle, 1991). The repulsion between charged residues of peptide molecules might be associated with conformational changes. Subsequently, those active peptides might undergo aggregation, thereby losing their activity. Therefore, increase in pH might cause the losing activity of ACE inhibitor.

The stability of the extracts from control and starter-inoculated *Pla-som* against gastrointestinal proteases was assessed *in vitro*. The extracts from both control and starter-inoculated *Pla-som* exhibited the similar activity. The ACE inhibitory activity decreased markedly by hydrolysis with pepsin, simulating stomach digestion and slightly decreased to after further digestion with pancreatin, simulating small intestine digestion (Figure 11c). Most ACE inhibitory activity of peptides was lost during hydrolysis with pepsin. Pepsin cleaves peptide bonds where N-terminal is donated by tryptophan, tyrosine, phenylalanine, leucine, aspartic acid and glutamic acid. Pancreatin consists of lipase, amylases and proteases, such as trypsin and chymotrypsin. It is therefore able to break down fats, starch and proteins. Trypsin and chymotrypsin are serine proteases. 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 non polar amino acids such as tyrosine, phenylalanine, tryptophan and leucine (Simpson, 2000). Therefore, amino acid composition and sequence of peptide plays an important role in ACE inhibitory activity. In addition, size of peptides affected the ACE inhibitory activity and survival of ACE inhibitory peptides in simulated gastrointestinal digestion. The extract from *Pla-som* consisted of short-chain and long-chain peptides. Other authors have reported that the bioactivity of short-chain peptides may be

preserved during the gastrointestinal digestion process, but the longer molecules would need to be protected to escape the action of digestive enzymes and exert their physiological effects in the organism (Rohrbach *et al.,* 1981). Therefore, the decreased activity after digestion was probably due to degradation of many long-chain peptides with ACE inhibitory activity in *Pla-som*. The result indicated that high pH and gastrointestinal proteases cause the inactivation of ACE inhibitory peptides of *Pla-som*.

3. Identification of ACE inhibitory peptides from *Pla-som*

3.1 Purification of ACE inhibitory peptides

ACE-inhibitory peptides were isolated from the crude extract of *Pla-som* naturally fermented at 96 h that possessed the highest ACE inhibitory activity. For the purification of ACE inhibitory peptides, the crude extract was first filtered through a membrane with an ultrafiltration system. About 79 % of activity was recovered in the fraction with MW below 3 kDa with the inhibitory activity of 67.95 units/mg peptide, suggesting that ACE inhibition of *Pla-som* extract was mainly attributable to peptide components with molecular masses lower than 3 kDa. Many studies have shown that ACE inhibitors derived from food proteins are usually short peptides with molecular weight lower than 1500 Da (Zhao *et al*., 2007; Oshima *et al*., 1979; Zhang *et al*. 2009; Guo *et al*. 2009). The permeate was then lyophilized and further purified by a RP-HPLC on Alltima C_{18} column and then eluted with acetonitrile containing 0.1% TFA as the eluent under gradient elution conditions as described in materials and methods. The elution profiles of peptides in the fraction <3 kDa MWCO are shown in Figure 12. Of 50 fractions, 12 fractions exhibited the ACE inhibitory activity in which only 7 fractions with ACE inhibitory activity more than 2 units (designated as fractions A to G) were further analyzed (Table 10). These fractions contributed to about 70% of total activity. Among all fractions, fraction G exhibited the highest ACE inhibitory activity, followed by fractions B and D, respectively.

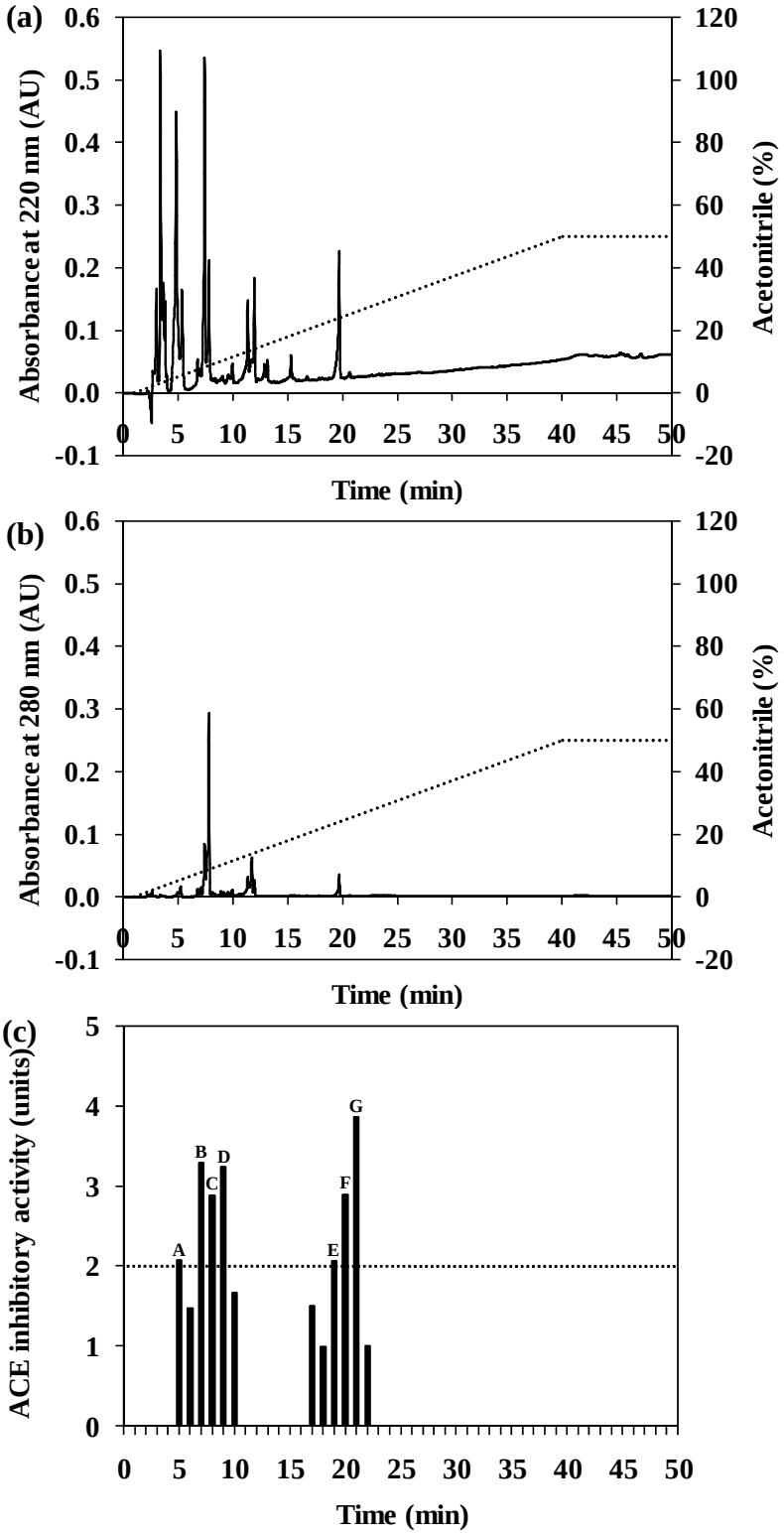

Figure 12. RP-HPLC chromatogram on Alltima C_{18} column of active fraction isolated from the 3 kDa permeate of *Pla-som* extract monitoring by A_{220} (a) and A_{280} (b). Fractions were determined for ACE inhibitory activity (c).

Table 10. Purification of ACE inhibitory peptide from *Pla-som* extract using RP-HPLC.

Fraction	Total peptide content[a] (mg)	Total ACE inhibitory activity (units)	Specific activity (units/mg peptide)	Purification fold	Yield (%)
<3 kDa[b]	0.423 ± 0.005	28.77 ± 0.08	67.95 ± 0.19	1	100
A	0.036 ± 0 [b]	2.07 ± 0.05 [d]	56.81 ± 1.27 [c]	0.84	7.2
B	0.022 ± 0.001 [d]	3.28 ± 0.28 [b]	150.67 ± 12.83 [c]	2.22	11.4
C	0.032 ± 0 [c]	2.88 ± 0.02 [c]	91.34 ± 0.74 [c]	1.35	10.0
D	0.043 ± 0.003 [a]	3.23 ± 0.17 [bc]	75.36 ± 4.04 [c]	1.11	11.2
E	0.006 ± 0 [f]	2.07 ± 0.20 [c]	368.77 ± 36.40 [b]	5.43	7.2
F	0.011 ± 0.002 [e]	2.89 ± 0.01 [d]	274.79 ± 0.67 [b]	4.04	10.0
G	0.001 ± 0 [g]	3.86 ± 0.18 [a]	2758.59 ± 128.04 [a]	40.6	13.4

[a]Total peptide content: Total peptide of sample was determined by the method of Church *et al*. (1983).

[b]<3kDa: Fraction with molecular weight below 3 kDa

3.2 Identification of ACE inhibitory peptides

Peptides in fractions A to G were further purified with LC-MS/MS and identified using *de novo* sequencing by PepNovo program. As shown in Table 11, total 300 peptides were identified (Appendix). Except fraction G which consisted of 245 peptides, the number of peptides identified in each fraction varied from 5 to 16 peptides. These peptides were constituted with 2-16 amino acid residues with the nominal mass ranging from 300 to 1800 Da. Although the amino acid compositions and sequences of these peptides were different, there were 3 distinctive features that could be attributed to the peptides known to exhibit ACE inhibitory activity (Guang and Phillips, 2009; Alemán *et al*., 2011; Gobbetti *et al*., 2000).

Table 11. Molecular weight range, amino acid residues and number of peptides identified in fractions A-G.

Fraction	Molecular weight range (Da)	Amino acid residues*	Number of peptides	Number of peptides with Leu residues in sequence	Number of peptides with amino acid residues at C-terminal															
					Ala	Cys	Asp	Glu	Phe	Gly	His	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Tyr
A	434-862	2-7	6	3	0	0	0	0	0	0	0	2	0	0	0	1	0	3	0	0	0	0
B	480-861	4-7	5	4	0	0	0	0	0	0	0	1	0	0	0	0	0	3	0	0	0	1
C	415-862	3-7	10	5	0	1	0	0	0	0	1	1	0	0	0	1	0	6	0	0	0	0
D	434-862	3-7	7	6	0	0	0	0	1	0	0	2	0	0	0	0	0	4	0	0	0	0
E	618-1681	6-14	16	11	0	0	0	0	0	0	0	4	1	0	0	1	0	6	0	4	0	0
F	679-1584	7-14	14	8	0	0	0	0	0	0	0	1	0	0	0	0	0	12	0	0	0	1
G	397-1798	3-16	245	199	4	0	5	4	4	3	8	96	8	1	5	6	2	82	5	3	6	3

*Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* are shown in Appendix.

Firstly, about 87% of the peptides identified contained one or more Leu residues in the sequence. The presence of Leu residues in the peptide sequence seems to play an important role in ACE inhibitory activity (Alemán *et al*., 2011). According to previous studies, the presence of Leu as the C-terminal amino acid helps to inhibit ACE (Cheung *et al*., 1980; FitzGerald and Meisel, 2000).

Secondly, about 74% of the peptides identified had basic amino acids, particularly Lys and Arg at the C-terminal. The positive charge on the guanidine or ε-amino group of the C-terminal Arg and Lys side-chains seems to contribute substantially to inhibiting potency, which indicates a possible interaction between the inhibitor and an anionic binding site of ACE that is different from the catalytic site (Guang and Phillips, 2009). The removal of the Arg residue at the C-terminus may lead to essentially inactive peptide analogues (FitzGerald and Meisel, 2000; Murray and FitzGerald, 2007). In *Pla-som*, predominant generation of peptides with Arg and Lys residues at the C-terminal might be a result of protein degradation caused by the trypsin-like enzymes endogenously found in fish muscle. Trypsin-like enzyme has a

very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine residues (Simpson *et al*., 2000).

Thirdly, 10% of the peptides identified consisted of Pro, Tyr, Phe or hydrophobic amino acids including Ala, Cys, Met and Val at C-terminal. According to Turner and Hooper (1992), binding to ACE is strongly influenced by the C-terminal amino acid residue. Although the precise substrate specificity is not clear, it seems that ACE prefers to have substrates or competitive inhibitors containing a hydrophobic amino acid residue (aromatic or branched side chains) at the C-terminal position (Gobbetti *et al*., 2000). Li *et al*. (2004) reported that ACE preferred substrates containing hydrophobic amino acid residue, especially Pro residue at C-terminal position. Proline at C-terminal of peptide has been shown to enhance binding to ACE (Cheung *et al*., 1980). It is probably due to the rigid structure of this residue, which may lock the carboxyl group into a conformation favorable for interacting with the positively charged residue at the active site of the enzyme (Cushman *et al*., 1977). Therefore, the higher ACE inhibition capacity of these peptides might relate to their high positive charge or hydrophobic amino acid at the C-terminal**.** However, binding of ACE is influenced not only by amino acid residue at the C-terminal but also the C-terminal tripeptide sequence of the substate. The C-terminal tripeptide residues may interact with subsites S_1, S'_1 and S'_2 at the active site of ACE (Ondetti and Cushman, 1982). The tripeptides with Trp, Tyr, Phe, Pro and a hydrophobic amino acid at the C-terminal were effective for ACE inhibitory activity because of the interaction between three subsites at the active site of ACE (Pihlanto, 2000). Moreover, previous structure-activity relationship studies predict that a tripeptide with hydrophobic amino acid residues at the amino-terminal, a positively charged residue at the middle position and an aromatic residue or Pro at the carboxy-terminus will emerge as potent ACE inhibitor peptides (Wu *et al*., 2006b). Cushman *et al*., (1981) reported that the active sites S_1, S'_1 and S'_2 of ACE had strong affinities for the side chains of tryptophan, alanine and proline, respectively. For long-chain peptides, the C-terminal tetrapeptide residues were more important to their ACE inhibitory activity than the C-terminal tripeptide residues. The most likely preferred amino acid residues starting from C-terminus are Tyr and Cys for the first position, His, Trp and Met for the second

position with Ile, Leu, Val and Met for third position and Trp for the fourth position (Wu *et al*., 2006a).

3.3 ACE inhibitory activity of synthesized peptides

According to Pripp *et al*. (2004), potential peptides in each fraction were selected to explore the structure-activity relationship. Twenty structurally related peptides were synthesized and their activities were compared and summarized in Table 12. Among twenty synthesized peptides, Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro was the most active peptide in this study, followed by Ala-Pro-Val-Tyr-Leu-Arg and Gln-His-Asn-Asp-Glu-Pro-Pro, respectively (Table 12). Since all these peptides were identified in fraction G, they probably contributed to the highest ACE-inhibitory activity of this fraction. However, the IC_{50} values of these peptides were relatively higher than Val-Pro-Pro and Ile-Pro-Pro which are marketed as a dietary supplement for hypertensive patients (Nakamura, 2004). These results indicate that the ACE-inhibitory activity of the peptides depends on their amino acid sequences as well as the constituent amino acids. In this study, it is worth noting that the presence of Pro at the C-terminal or in the sequence was preferred for the ACE-inhibitory activity of the peptide derived from *Pla-som*. Other Pro-containing peptides were also active in ACE inhibition. The differences in the activity of individual peptides are due to the environment surrounding the imidazole group, as indicated by various observations. The hydrophobicity of the compounds was important for the accessibility to the hydrophobic targets.

Table 12. IC_{50} value of synthetic peptides simulating peptides isolated from *Pla-som* and some commercial ACE inhibitors.

Fraction	Sequence	MW (Da)	IC_{50} value* (μM)
A	His-Leu-Asp-Pro	480.53	710
B	Ala-Asn-Leu-Tyr	479.54	-
	Val-His-Pro-Lys	479.58	-
	Phe-His-Ser-Ser-Leu-Asn-Arg	859.95	583
C	Gly-Ala-Leu-Arg	415.50	5,502
	Gly-Glu-Leu-Cys	420.48	-
	Ser-Phe-Ser-Pro	436.47	912
D	Phe-Leu-Arg	434.54	1,335
	Leu-Val-Thr-Phe	478.59	838
	Phe-His-Ser-Ser-Leu-Asn-Arg	859.95	583
E	Ala-Pro-Ser-Pro-Tyr-Lys	661.76	1,167
G	Leu-Leu-Leu-His	494.64	-
	Ala-Leu-Thr-Pro-Pro-Thr	598.70	2,233
	Pro-Val-Leu-Pro-Pro-His	658.80	1,615
	Ala-Pro-Val-Tyr-Leu-Arg	717.87	138
	Phe-Val-Tyr-Gly-Leu-Leu-Pro	808.00	374
	Gln-His-Asn-Asp-Glu-Pro-Pro	835.83	209
	Gln-His-Pro-Phe-Asn-Pro-Pro	835.92	482
	Phe-Val-Ser-Glu-Ser-Asp-Pro-Asn-Leu-Pro	1104.09	248
	Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro	1222.38	124
	Ala-Leu-Gly-Glu-Asp-His-Pro-Leu-Leu-Pro-Asp-Leu-Val-Pro	1485.71	285
	Val-Pro-Pro	311.38	80
	Ile-Pro-Pro	325.41	49

*IC_{50} value identifies the concentration of inhibitor required to reduce the ACE activity by 50%.

For longer peptides more than six amino acids in length, the relationship between C-terminal structure and activity decreased, reflecting the likely influence by steric effects. From the results, it is possible that peptides up to 6 amino acids in length might have more ACE inhibitory activity than peptides with less than 7 amino acid residues. Several peptides with more than tripeptide with ACE inhibitory activity were reported such as Glu-Val-Met-Ala-Gly-Asn-Leu-Try-Pro-Gly (IC_{50} = 2.98 μM) from sauce of fermented blue mussel (Je *et al*., 2005c), Leu-Gly-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe (IC_{50}= 4.92 μM) and Val-Val-Tyr-Pro-Trp (IC_{50}= 6.02 μM) from porcine hemoglobin (Yike *et al*., 2006), Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro (IC_{50}= 21.6 μM) from bigeye tuna dark muscle (Qian *et al*., 2007a) and Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Tyr-Lys-Asp-Thr-Pro (IC_{50}= 11.28 μM) from tuna frame protein (Lee *et al*., 2010).The kinetic study revealed that ACE inhibitory tuna frame protein acts as a non-competitive inhibitor, which means that it cannot bind at active site but at the other exclusive of the active site of ACE. Generally, ACE inhibitors contain one or more molecular functionalities such a zinc binding ligands (usually either a phosphate or carboxylate oxygen, or thiol sulphur), a hydrogen-bond donor and C-terminal group (Andrews *et al*., 1985). Cheung *et al*. (1980) reported that ACE prefers competitive inhibitors that contain hydrophobic amino acid residues such as Pro, Phe and Tyr at the three positions from the C-terminal. Peptide (Val-Leu-Ile-Val-Pro) with a potent competitive inhibitor of ACE was isolated from glycinin (Mallikarjun Gouda *et al*., 2006). The competitive inhibition competes with the substrate for binding to the active site of the enzyme. Therefore, ACE inhibitory potency is influenced by the composition of amino acids and structure of peptide. In addition, the exceptionally potent ACE inhibitory activity in the fried whole egg digest indicates a possible synergistic effect among various peptides (Li *et al*., 2004).

ACE inhibitor peptides from *Pla-som* can be characterized as novel peptides, derived from a food source that is eaten daily. Therefore, the ACE inhibitory peptides from *Pla-som* may be very useful in the preparation of antihypertensive functional foods.

CHAPTER 4

CONCLUSION

1. From 12 types of Thai traditional fermented fishery products, *Pla-som* had the highest inhibition of ACE, whereas *Bu-du*, *Kung-chom* and *Kem-buk-nud* exhibited the highest DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP (p<0.05), respectively. Based on PCA, peptides with high ACE inhibitory activity were more likely found in products with low pH and low salt content. The presence of these peptides was probably related to the use of garlic and raw material used but not affected by F/T ratio. For antioxidative activities, peptides with high FRAP were more likely found in products with high F/T ratio. Products with high DPPH radical scavenging activity more likely had high ABTS radical scavenging activity but both activities showed no correlation to pH, salt content and F/T ratio.

2. *Pla-som* inoculated with *P. acidilactici* BCC 9545 and *S. xylosus* BCC 3710 showed a higher rate of fermentation than the control as indicated by the greater rate of pH drop, organic acid production, free amino group content and degree of hydrolysis during fermentation (p<0.05). Based on pH desired (pH 5), the fermentation was completed within 96 and 72 h for the control and starters-inoculated *Pla-som*, respectively. However, the inoculation of this starter could not increase the release of ACE inhibitory peptides which more likely depend on time of fermentation. The ACE inhibitory activity of the extract from *Pla-som* showed heat stability but lost in activity at high pH. In addition, the ACE inhibitory activity of the extract from *Pla-som* decreased after incubation with pepsin and pancreatin.

3. The crude extract of *Pla-som* was purified by ultrafiltration and RP-HPLC system, respectively. In the first purification step, the fraction with molecular weight below 3 kDa brought about a high ACE inhibitory activity and was used for subsequent purification step. Of 50 fractions, only 7 fractions were found to have ACE inhibitory activity more than 2 units with the contribution of 70% of total activity.

These fractions contained 300 peptides. These peptides were constituted with 2-16 amino acid residues with the nominal mass ranging from 300 to 1800 Da. Although the amino acid compositions and sequences of these peptides were different, there were 3 distinctive features that could be attributed to the peptides known to exhibit ACE inhibitory activity. These 3 distinctive features were peptides with one or more Leu residues in the sequence, peptides with basic amino acids at C-terminal and peptides with Pro, Tyr, Phe or hydrophobic amino acids at C-terminal.

4. ACE inhibitory activities of 20 synthetic peptides, which were designed based on peptides derived from *Pla-som* were studied. Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro was the most active peptide in this study, followed by Ala-Pro-Val-Tyr-Leu-Arg and Gln-His-Asn-Asp-Glu-Pro-Pro, respectively. The presence of Pro residues at C-terminal or in the sequence plays important roles in the ACE inhibition of the peptides derived from *Pla-som*.

5. The IC_{50} values of the peptides derived from *Pla-som* were relatively higher than Val-Pro-Pro and Ile-Pro-Pro. Although they were weaker in ACE inhibition, they are characterized as novel peptides, derived from a food source. Therefore, the ACE inhibitory peptides from *Pla-som* may be very useful in the preparation of antihypertensive functional foods.

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APPENDIX

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som*

Fraction	Mass (Da)	Sequence
a	434.083	Asp-Asp-Gly-Lys
	478.423	Phe-Arg-Arg
	480.670	Phe-Trp-Lys
	481.393	His-Leu-Asp-Pro
	860.083	Phe-Lys-Pro-Ala-Glu-Leu-Arg
	861.735	Leu-Tyr-Pro-Ala-Glu-Leu-Arg
b	480.378	Val-His-Pro-Lys
	480.555	Ala-Asn-Leu-Tyr
	859.463	Glu-Val-Thr-Leu-Glu-Leu-Arg
	860.496	Lys-Glu-Asp-Ala-Glu-Leu-Arg
	860.500	Phe-His-Ser-Ser-Leu-Asn-Arg
c	415.996	Gly-Ala-Leu-Arg
	433.399	Gly-Ser-Ala-Ala-Lys
	434.407	Thr-Thr-Gly-Arg
	437.196	Ser-Phe-Ser-Pro
	478.209	Phe-Arg-Arg
	478.415	Gly-Glu-Leu-Cys
	479.625	His-Ser-Val-His
	859.570	Glu-Asp-Val-Val-Glu-Leu-Arg
	860.496	Leu-Gln-Ser-Asp-Glu-Leu-Arg
	861.503	Phe-His-Leu-Leu-Leu-Leu-Arg
d	434.570	Trp-Thr-Lys
	435.086	Phe-Leu-Arg
	479.522	Leu-Val-Thr-Phe
	719.147	Asn-Ala-Leu-Asp-Met-Arg

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
d	860.263	Phe-His-Ser-Ser-Leu-Asn-Arg
	861.400	Phe-His-Leu-Ser-Glu-Thr-Lsy
	861.503	Tyr-Leu-Pro-Ala-Glu-Leu-Arg
e	618.388	Gly-Ser-Gly-Lys-Gly-Gly-Arg
	632.689	Gly-Thr-Gly-Lys-Ala-Ala-Lys
	662.385	Ala-Pro-Ser-Pro-Tyr-Lys
	808.382	Glu-Ser-Glu-Asp-Glu-Val-Thr
	808.382	Glu-Ser-Met-Leu-Glu-Val-Thr
	808.515	Thr-Asp-Leu-Phe-Leu-Val-Thr
	808.619	Phe-Ala-Glu-Leu-Pro-Met-Thr
	860.780	Trp-Val-Ala-Ser-Glu-Leu-Arg
	1010.461	Ala-Leu-Asp-Gln-Thr-Gly-Val-Asp-Asn-Pro
	1021.535	Gln-Asp-Thr-Gly-Val-Asn-Met-Thr-Arg
	1027.205	Asp-Gly-Glu-Leu-Gln-Thr-Gly-Val-Asp-Leu
	1134.575	Leu-Gly-Ala-Thr-Gly-Val-Asp-Asn-Pro-Tyr-Lys
	1150.523	Glu-Gln-Thr-Asp-Asn-His-Leu-His-Arg
	1583.577	Leu-Val-Asp-Glu-Ser-Phe-Thr-Gly-Val-Asp-Asn-Phe-Leu-Lys
	1583.844	Leu-Asp-Leu-Asp-Ser-Phe-Thr-Gly-Val-Asp-Asn-Thr-Met-Arg
	1680.307	Glu-Ser-Tyr-Leu-Asn-Pro-Leu-Glu-Val-Asp-Asn-Met-Thr-Arg
f	679.234	Ala-Ser-Asn-Ala-Gly-Pro-Tyr
	701.313	Asp-Pro-Leu-Gly-Gly-Ser-Arg
	701.332	Asp-Leu-Gly-Ala-Ala-Val-Arg
	741.116	Pro-Gly-Gly-Gln-Val-Gln-Arg

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
f	756.346	Ala-Val-Leu-Lys-Gly-Leu-Arg
	758.278	Pro-Asp-Ser-Asn-Gly-Leu-Arg
	838.512	His-Asp-Leu-Pro-Asn-Ser-Arg
	959.096	Ser-His-Gly-Asn-Leu-Leu-Tyr-Arg
	960.409	Tyr-Thr-Leu-Val-Leu-Pro-Val-Arg
	1006.507	Leu-Thr-Gly-Val-Asp-Asn-Pro-Tyr-Lys
	1021.451	Gln-Thr-Gly-Asp-Val-Asn-Met-Thr-Arg
	1022.404	Glu-Thr-Asp-Gly-Val-Asn-Met-Thr-Arg
	1583.411	Ser-Val-Phe-Asp-Asn-Gln-Thr-Gly-Val-Asp-Asn-Thr-Met-Arg
	1583.411	Ser-Val-Phe-Asp-Asn-Gln-Thr-Gly-Val-Asp-Asn-Met-Thr-Arg
g	397.199	His-Leu-Lys
	397.199	Leu-His-Lys
	398.159	Ser-Tyr-Glu
	495.320	Leu-Leu-Leu-His
	500.900	Ala-Ala-Ala-Leu-Arg
	545.061	Ala-Ala-Ala-Gly-Gln-Lys
	597.774	Leu-Ala-Ala-Leu-Pro-Asn
	599.346	Ala-Leu-Thr-Pro-Pro-Thr
	601.281	Gly-Gly-Ala-Ser-Pro-Gly-Arg
	601.309	Gly-Glu-Gly-Pro-Gly-Gly-Lys
	650.288	Glu-Asp-Ser-Glu-Gly-Asn
	650.656	Met-Ser-Asp-Leu-Gly-Lys
	654.357	Gly-Gln-Ala-Gly-Pro-Pro-Lys
	659.409	Pro-Val-Leu-Pro-Pro-His

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	673.380	Leu-Met-Ser-Glu-Gly-His
	674.378	Asn-Gly-Ser-Gly-Leu-Val-Lys
	674.391	Asn-Gly-Ser-Gly-Leu-Ala-Arg
	674.396	Asn-Ser-Gly-Gly-Leu-Ala-Arg
	680.395	Ala-Gly-Pro-Leu-Ser-Val-His
	690.303	Phe-Ala-Gly-Asp-Asp-Ala-Asn
	698.341	Asn-Ala-Val-Leu-Thr-Leu-Ser
	699.287	Ala-Ala-Asp-Glu-Ser-Leu-Leu
	708.366	Leu-Ala-Gly-Gly-Glu-Asp-Phe
	709.284	Pro-Glu-Leu-Leu-Gly-Glu-Ala
	709.309	Gly-Phe-Ser-Gly-Val-Asp-Lys
	710.249	Leu-Ala-Gln-Pro-Gly-Pro-Phe
	713.331	Leu-Val-Val-Leu-Ser-Asp-Ser
	714.334	Val-Asn-Val-Leu-Ser-Asp-Ser
	718.110	Ala-Pro-Val-Tyr-Leu-Arg
	719.447	Tyr-Glu-Leu-Ala-Gly-Ala-Asp
	723.346	Val-Leu-Val-Leu-Ala-Leu-Asp
	723.829	Ala-Phe-Ala-Ala-Glu-Ser-Lys
	728.333	Leu-Gln-Thr-Gly-Val-Asp-Asp
	733.360	Gly-Pro-Tyr-Ser-Gly-Pro-Arg
	733.360	Gly-Pro-Tyr-Leu-Ala-Gly-Arg
	737.863	Ala-Ala-Asp-Glu-Ser-Gln-His
	738.361	Gln-Thr-Ser-Ala-Gly-Phe-Lys
	738.366	His-Gly-Ala-Asn-Ala-Leu-Arg
	744.492	Val-Gly-Leu-Asp-Gln-Gly-Arg
	745.328	Gly-Ser-Leu-Phe-Gly-His-Lys

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	746.268	Asp-Glu-Ser-Thr-Gly-Ser-Val-Ala
	746.731	Gly-Gly-Leu-Asp-Asp-Asp-Arg
	750.236	Tyr-Leu-Asn-Ser-Asp-Thr-Gly
	750.389	Leu-Tyr-Val-Gly-Gly-Asn-Lys
	752.280	Asp-Asp-Phe-Gly-Gly-Asn-Lys
	756.389	Val-Leu-Gln-Thr-Ala-Pro-Lys
	758.570	Leu-Ser-Asn-Asp-Pro-Gly-Arg
	762.399	Val-Glu-Asn-Gly-Thr-Ser-Arg
	762.700	Met-Leu-Ser-Thr-Gly-Ser-Val-Ser
	764.351	Asn-Val-Val-Leu-Ser-Asp-His
	765.436	Gly-Pro-Ser-Leu-Val-His-Arg
	766.396	Leu-Leu-Ser-Glu-Pro-Ala-His
	768.342	Asp-Leu-Pro-Ala-Lys-Pro-Lys
	773.394	Val-Phe-Leu-Gly-Leu-Pro-Lys
	780.364	Asn-Gly-Gly-Pro-Thr-Val-Val-His
	781.356	Val-Glu-Leu-Leu-Ala-Ala-Gly-Gln
	781.363	His-Val-Leu-Val-Ala-Ser-Arg
	782.285	Leu-Ser-Tyr-Ala-Ser-Phe-Asn
	782.399	Val-Leu-Val-Leu-Ser-Asp-Arg
	783.440	Asn-Asn-Ser-Asn-Phe-His-Thr
	789.409	Asp-Gly-Gly-Gly-Ser-Gly-Leu-Ala-Arg
	789.814	Phe-Val-Tyr-Gly-Leu-Leu-Pro
	792.595	Gln-Lys-Asp-Ser-Asn-Gly-Tyr
	797.320	Asn-Lys-Leu-Leu-Pro-Gly-Arg
	799.385	His-Phe-Thr-Asp-Val-Glu-Ala
	800.396	Gly-Asp-Asp-Ala-Pro-Val-Val-Lys

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	800.405	Gly-Asp-Asp-Ala-Pro-Val-Val-Lys
	800.409	Gly-Asp-Trp-Pro-Val-Val-Lys
	803.893	Ala-Pro-Ala-Cys-Glu-Glu-Lys
	804.393	Ala-Gly-Asp-Asp-Ser-Ser-Pro-Arg
	805.450	Leu-His-Val-Asp-Pro-Pro-Lys
	812.209	Phe-Ala-Phe-Ser-Gly-Gln-Arg
	813.363	Leu-Asp-Leu-Ser-Pro-Leu-Arg
	815.398	Glu-Glu-Thr-Pro-Ala-Leu-Arg
	815.408	Glu-Thr-Glu-Pro-Leu-Ala-Arg
	817.428	Gln-Ser-Val-Ala-Gly-Asp-Leu-Lys
	817.573	Glu-Ser-Gln-Val-Asn-Leu-Lys
	828.380	Val-Thr-Asp-Val-Leu-Asp-Ala
	836.357	Gln-His-Pro-Phe-Asn-Pro-Pro
	836.357	Gln-His-Asn-Asp-Glu-Pro-Pro
	838.310	Glu-Val-Pro-Gly-Thr-His-Ala-Lys
	839.392	Gln-His-Asn-Thr-Ala-Leu-Arg
	842.336	Glu-Glu-Gly-Thr-His-Asn-Arg
	843.382	Ser-Val-Ala-Gly-Leu-Thr-Ala-Pro-Lys
	845.353	Pro-Phe-Asp-Val-Val-Leu-Arg
	855.544	Ala-Gln-Asp-Val-Asp-Leu-Asp-Val
	860.494	Leu-Val-Val-Leu-Ser-Asp-Ser-Phe
	860.496	Phe-Gln-Pro-Ala-Glu-Leu-Arg
	860.810	Phe-Gln-Leu-Ala-Leu-Leu-Arg
	861.916	Tyr-Glu-Leu-Pro-Ala-Leu-Arg
	873.708	Gln-Leu-Asp-Glu-Leu-Lys-Lys
	874.385	Met-Leu-Ser-Thr-Gly-Ser-Val-Ala-Gln

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	882.528	Pro-Leu-Ser-Asn-Lys-Pro-Val-Lys
	883.480	Ser-Glu-Leu-Asp-Gly-Leu-Gly-Val-Leu
	891.395	Asp-Ser-Leu-Thr-Leu-Thr-Leu-Lys
	891.456	Asp-Val-Leu-Asp-His-His-Arg
	891.504	Leu-Gln-Glu-Asn-Phe-Leu-Lys
	891.666	Asp-Val-Gly-Gly-Phe-Gln-Leu-Arg
	895.278	Glu-Leu-Met-His-Leu-Pro-Arg
	896.392	Asp-Glu-Tyr-Glu-Met-Asp-Leu
	901.457	Pro-Ala-Gly-Asp-Asn-Gly-Ser-Gln-Arg
	905.410	Glu-Asn-Glu-Thr-Gly-Leu-Ser-Arg
	912.467	Leu-Asp-Phe-Thr-Phe-Asn-Arg
	914.570	Thr-Leu-Asn-Gln-Asp-Val-Pro-Lys
	916.382	Leu-Leu-Thr-Glu-Ala-Glu-Leu-Lys
	917.426	Ala-Asn-Val-Leu-Lys-Met-Asn-Lys
	923.421	Phe-Leu-Phe-Glu-Leu-Val-Arg
	924.464	Ser-Glu-Val-Thr-Val-Phe-Ser-Arg
	924.464	Asp-Ser-Leu-Leu-Ser-Pro-His-Arg
	941.463	Tyr-Val-Leu-Ser-Ser-Glu-Asp-Glu
	951.499	Pro-Val-Asn-Ser-Glu-Ala-Pro-Gly-Ser-Leu
	957.486	Pro-Val-Asp-Ser-Thr-Val-Pro-Val-Tyr
	960.450	Asp-Tyr-Asp-Asp-Tyr-Asn-Arg
	960.553	Tyr-Pro-Asn-Tyr-Phe-Glu-Lys
	963.411	Val-Val-Ser-Thr-Asp-Phe-Ser-Glu-Val
	981.526	Val-Asp-Asp-Glu-Tyr-Ser-Cys-Leu
	981.578	Val-Asp-Asp-Glu-Tyr-Asp-Asn-Met
	984.669	Pro-Glu-Leu-Gly-Tyr-Leu-His-Arg

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	986.395	Asp-Leu-Leu-Asn-Gln-Pro-Met-Arg
	1002.578	Tyr-Thr-His-Leu-Phe-Pro-Pro-Lys
	1004.563	Leu-Asn-Gly-Pro-Val-Leu-Tyr-Thr-Lys
	1005.671	Thr-Leu-His-Leu-Val-Val-Val-Pro-Lys
	1012.108	Glu-Tyr-His-Leu-Glu-Gly-His-Lys
	1015.684	Thr-Pro-Glu-Ser-Gln-Val-Asn-Leu-Lys
	1016.640	Asp-Thr-Gly-Asp-Asp-Ala-Pro-Gly-Leu-Arg
	1031.496	Leu-Glu-Leu-Phe-Leu-Gly-Val-Leu-Lys
	1031.496	Leu-Glu-Asp-Gly-Gly-Gly-Ser-Ala-Val-Ala-Arg
	1035.637	Val-Phe-Asp-Asn-Gly-Ser-Gly-Leu-Val-Lys
	1036.645	Cys-Ser-Asp-Asn-Gly-Ser-Gly-Leu-Val-Lsy
	1037.574	Thr-Phe-Asp-Phe-Asp-Phe-Phe-Val
	1045.632	Ala-Leu-Val-Gly-Ala-Phe-Gly-Ala-Ala-Leu-Arg
	1047.255	Gly-Glu-Val-Gln-Glu-Ala-Ala-Thr-Asp-Lys
	1047.462	Gly-Glu-Val-Asp-Thr-Asn-Ser-Leu-Gly-Arg
	1050.736	Tyr-Glu-Leu-Phe-Gly-Phe-Phe-Lys
	1070.520	Phe-Gly-Val-Tyr-Thr-Gly-Val-Phe-Leu-Ser
	1073.798	Leu-Phe-Leu-Asn-Lys-Asp-Val-Pro-Lys
	1075.528	Asp-Phe-Asp-Gln-Val-Pro-Leu-Asn-Lys
	1084.417	Val-Leu-Leu-His-Thr-Thr-Tyr-Gly-Ser-Leu
	1084.490	Val-Phe-Ser-Glu-Thr-Thr-Tyr-Gly-Ser-Leu
	1085.607	Phe-Val-Ser-Glu-Ser-Asp-Pro-Asn-Leu-Pro
	1085.871	Leu-Ala-Val-Leu-Thr-Leu-Thr-Leu-Asn-Lys
	1086.387	Gly-Gln-Val-Leu-Thr-Leu-Ser-Leu-Gln-Lys
	1087.509	Tyr-Asp-Ser-Leu-Gly-Glu-Leu-Tyr-Lys
	1087.597	Ser-Val-Val-Leu-Thr-Leu-Thr-Gly-Leu-Gly-Lys

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	1089.754	Val-Val-Leu-Thr-Leu-Asp-Cys-Asn-Arg
	1103.590	Val-Val-Gly-Phe-Ala-Gly-Asp-Val-Ser-Phe-Arg
	1126.453	Ala-Glu-Thr-Tyr-Asp-Phe-Glu-Asp-Val-Gly
	1130.561	Leu-Asp-Met-Ser-Gly-Tyr-Gly-Leu-Phe-Lys
	1134.518	Leu-Gln-Thr-Gly-Val-Asp-Asn-Glu-Met-Lys
	1134.523	Leu-Gln-Thr-Gly-Val-Asp-Asn-Pro-Tyr-Lys
	1134.683	Gln-Leu-Thr-Gly-Val-Asp-Asp-Gly-Ser-Ser-Arg
	1141.491	Leu-Asp-Asp-Ala-Leu-Gln-Thr-Gly-Val-Asp-Asn
	1141.891	Tyr-Gly-Leu-Asn-His-Tyr-Phe-Val-Glu
	1148.509	Thr-Pro-Glu-Pro-Glu-Gln-Glu-Tyr-Arg
	1149.510	Asp-Glu-Glu-Leu-Glu-Ala-Ser-Asp-Asn-Lys
	1154.265	Ala-Gly-Pro-Leu-Asn-Leu-Thr-Asn-Leu-Asn-Lys
	1161.661	Lys-Glu-Gln-Leu-Asp-Ala-Ser-Asn-Glu-Lys
	1163.624	Phe-Phe-His-Leu-Ala-Ala-Glu-Asn-Ser-Lys
	1164.627	Ala-Thr-Thr-Asp-Leu-Asp-Ala-Glu-Asn-Ser-Lys
	1164.627	Phe-Ala-Ala-Val-Leu-Asp-Ala-Glu-Asn-Ser-Lys
	1165.354	Leu-Val-Ser-His-Leu-Leu-Leu-Asn-Thr-Arg
	1180.926	Leu-Val-His-Ala-Gly-Ala-Ala-Phe-Phe-Phe-Thr
	1181.454	Val-Leu-Phe-Pro-Asp-Leu-Gly-His-Glu-Arg
	1194.616	Leu-Glu-Ser-Thr-Gly-Ser-Val-Ala-Glu-Phe-Arg
	1206.649	Pro-Val-Ser-Asn-Glu-Gln-Asp-Tyr-Val-Arg
	1208.582	Val-Val-Asn-Ser-Glu-Gln-Tyr-Asp-Val-Arg
	1212.519	Ser-Pro-Ala-Asp-Ala-Glu-Leu-His-Val-Pro-Pro-Val
	1212.519	Leu-Ala-Ala-Asp-Ala-Glu-Leu-His-Pro-Val-Pro-Val
	1215.569	Asp-Pro-Val-Leu-Thr-Thr-Ser-Pro-Glu-Glu-Lys
	1215.569	Leu-Val-Val-Leu-Tyr-Glu-Gln-Pro-Val-Arg

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	1216.060	Leu-Val-Val-Leu-Pro-Ala-Asp-Asp-Phe-Ala-Arg
	1216.558	Tyr-Glu-Asp-Glu-Ala-Pro-Ser-His-Leu-Arg
	1216.572	Pro-Asp-Val-Pro-Val-Thr-Ser-Leu-Leu-Leu-Tyr
	1217.535	Thr-Val-Asp-Asp-Glu-Tyr-Pro-Asp-Leu-Ala-Val
	1222.716	Tyr-Val-Gly-Thr-Ala-Leu-His-Leu-His-Asp-Pro
	1239.597	Ala-Glu-Thr-Tyr-Asp-Phe-Glu-Asp-Val-Gly-Leu
	1240.621	Val-Tyr-Gly-Ser-Leu-Phe-Glu-Leu-Trp-Lys
	1246.582	Tyr-Glu-Leu-Val-Thr-Val-Gln-Ala-Pro-Val-Lys
	1256.652	Asp-Leu-Asp-Ala-Leu-Gln-Thr-Gly-Val-Asp-Asn-Asp
	1264.094	Ala-Leu-Leu-Leu-Gly-Leu-His-Pro-Leu-Leu-Gly
	1264.688	Ala-Leu-Asn-Leu-Leu-Asn-Ala-Glu-Ala-His-Ala-Lys
	1264.688	Ala-Leu-Asn-Leu-Leu-Asn-Ala-Gln-Asp-His-Arg
	1296.557	Phe-Asp-Glu-Glu-Leu-Phe-Ala-Leu-Ala-Asn-Lys
	1312.400	Ala-Val-Leu-Leu-Tyr-Pro-Thr-Val-His-Ala-Thr-Phe
	1316.340	Leu-Val-Lys-Ala-Ala-Phe-Gly-Gly-Asp-Pro-Thr-Leu-Lys
	1316.679	Leu-Val-Thr-Pro-Leu-Tyr-Gly-Asp-Asp-Ala-Pro-Arg
	1316.679	Leu-Val-Ser-His-Ser-Tyr-Gly-Asp-Asp-Ala-Pro-Arg
	1316.679	Leu-Val-Gly-Thr-Ala-Ser-Ser-Ala-Gly-Asp-Pro-Leu-Thr-Lys
	1325.626	Tyr-Pro-Val-Val-Thr-Ser-Ser-Thr-Gly-Ala-Ser-Thr-Arg
	1325.665	Val-Leu-Phe-Pro-Glu-Ala-His-Ala-Thr-Ala-Asn-Arg
	1329.756	Glu-Leu-Asp-Ser-Asp-Ser-His-Asp-Ala-Leu-Val-Glu
	1338.205	Ala-Ala-Asp-Glu-Gly-Gly-Val-Val-Asp-Leu-Ala-His-Gly-Lys
	1338.643	Ala-Ala-Asp-Glu-His-Leu-Val-Leu-Glu-Glu-Arg
	1362.635	Leu-Leu-Gly-Asp-Pro-Pro-Asn-Glu-Gly-Glu-Pro-Pro-Lys
	1362.635	Leu-Leu-Gly-Asp-Pro-Gly-Glu-Leu-Leu-Gly-His-Gly-Gly-Lys
	1378.741	Phe-Leu-Leu-Val-Ala-Ser-Asp-Ser-Gly-Gln-Leu-Tyr-Lys

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	1379.670	Phe-Leu-Ala-Gly-Ala-Gly-Asp-Ser-Asp-Gly-Asp-Gly-Gly-Leu-Lys
	1379.670	Phe-Leu-Leu-Phe-Gln-Aln-Asn-Ser-Gln-Ala-Leu-Lys
	1379.670	Phe-Leu-Asn-Val-Ala-Ser-Ser-Gly-Gly-Thr-Ala-Thr-Val-Arg
	1379.670	Phe-Asn-Val-Leu-Ser-Ala-Ser-Asp-Ser-Gln-Ala-Leu-Lys
	1380.678	Phe-Leu-Leu-Thr-Ala-Asp-Glu-Glu-Ser-Leu-Ser-Arg
	1387.612	Phe-Val-Tyr-Gly-Leu-Leu-Phe-Ser-Gly-Glu-Gln-Lys
	1391.657	Val-Asp-Tyr-Ser-Glu-Thr-Leu-His-Glu-Gly-Asn-Lys
	1391.687	Val-Asp-Thr-Phe-Asp-Leu-Gln-Leu-Val-Trp-Arg
	1391.687	Val-Asp-Phe-Val-Pro-His-Val-His-Leu-Ser-Asn-Lys
	1392.690	Thr-Leu-Thr-Phe-Asp-Leu-Asp-Glu-Pro-Ser-Val-Arg
	1397.816	Ala-Phe-Glu-Lys-Glu-Leu-Ala-Gly-His-His-Met-Lys
	1438.959	Phe-His-Gln-Gln-Leu-Leu-Ser-Pro-Gln-Gln-Gly-Arg
	1442.969	Thr-Glu-Val-Ala-Val-Thr-Gln-Pro-Gly-Ala-Lys-Asp-Ala-Arg
	1444.742	Phe-Gly-Leu-Glu-Leu-Ala-Gly-Glu-Ser-Ser-Leu-Pro-Pro-Lys
	1483.686	Gly-Pro-Gln-Asn-Asp-Leu-Thr-Val-Leu-Asp-Ala-Ala-Asn-Arg
	1485.681	Ala-Leu-Gly-Glu-Asp-His-Pro-Leu-Leu-Pro-Asp-Leu-Val-Pro
	1502.389	Pro-Leu-Ala-Ala-Phe-Phe-Gly-Phe-Phe-Phe-Pro-Asn-Lys
	1503.598	Pro-Asp-Leu-Gly-Asp-Asp-Leu-Tyr-Leu-Phe-His-Ala-Lys
	1503.598	Asp-Pro-Tyr-Phe-Glu-Gly-His-Gly-Val-Thr-Leu-Leu-Arg
	1503.727	Pro-Asp-Leu-Gly-Asp-Asp-Leu-Glu-Phe-Phe-His-Ala-Lys
	1503.805	Asp-Pro-Tyr-Leu-Gln-Ser-Pro-Ser-Leu-Asn-Thr-Leu-Arg
	1504.912	Thr-Leu-Tyr-Gln-Leu-Ser-Phe-Phe-Phe-Pro-Asn-Lys
	1504.975	Val-Leu-Asp-Pro-Glu-Ser-Ser-Gly-Met-Ala-His-Phe-Ser-Lys
	1553.745	Leu-Tyr-Leu-Glu-Leu-Thr-Leu-Gly-His-Leu-Leu-Leu-Arg
	1554.748	Tyr-Asn-Glu-Leu-Thr-Glu-Glu-Thr-Gly-Thr-Glu-Leu-Arg

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	1576.880	Phe-Thr-Thr-Ala-Leu-Leu-Thr-Val-Gly-Tyr-Leu-Leu-His-Lys
	1607.754	Ser-Glu-Thr-Glu-Lys-Asp-Leu-Leu-Phe-Ser-Val-Glu-Asp-Asp
	1629.699	Asp-Glu-Tyr-Pro-Tyr-Phe-Asp-His-Phe-Leu-Gln-Arg
	1629.699	Leu-Met-Tyr-Pro-Asn-Leu-Val-Leu-His-Leu-Phe-Leu-Arg
	1630.702	Leu-Met-Tyr-Asp-Phe-Asp-Leu-Leu-Asp-Asp-Ser-Gly-Ala-Arg
	1630.702	Glu-Asp-Tyr-Gln-Asn-Leu-Ala-Lys-Gly-Pro-Leu-Gly-Val-Ala-Arg
	1630.702	Asp-Glu-Tyr-Phe-Asn-Phe-Met-His-Thr-Thr-Val-Ala-Arg
	1635.704	Thr-Val-Gly-Asn-Asp-Thr-Gln-Val-Met-Leu-Asp-Met-Arg-Arg
	1657.941	Thr-Leu-Thr-Gln-Leu-Tyr-Pro-Asp-Val-Ala-Pro-Val-Arg-Gly-Lys
	1660.730	Thr-Val-Asp-Asp-Glu-Tyr-Pro-Asp-Leu-Gly-Pro-Val-Arg-Gly-Lys
	1660.730	Thr-Val-Asp-Asp-Glu-Tyr-Gly-Leu-Leu-Thr-Pro-Pro-Arg-Gly-Lys
	1694.024	Leu-Thr-Leu-Leu-Gln-Gln-Leu-Asn-Thr-Gln-Val-Gly-Thr-His-Lys
	1720.017	Pro-Val-Asp-Asp-Phe-Leu-Asp-Ala-Asp-His-Asn-Asn-Gly-Tyr-Lys
	1742.475	Thr-Leu-Pro-Glu-His-Asp-Lys-Thr-Val-His-Lys-Leu-Val-Pro-Lys
	1743.688	Lys-Thr-Leu-Asp-Lys-Asp-Leu-Asp-Val-Leu-Phe-Ala-Thr-His-Lys
	1743.718	Thr-Glu-Gln-Asn-Leu-Leu-Leu-Asp-Pro-His-Leu-Leu-Pro-Leu-Lys

Table: Amino acid sequences of ACE inhibitory peptides derived from *Pla-som* (continued)

Fraction	Mass (Da)	Sequence
g	1744.722	Thr-Glu-Glu-Asn-Val-Leu-Leu-Val-Glu-Glu-Glu-Val-Leu-Thr-Lys
	1744.722	Thr-Glu-Glu-Leu-Lys-Leu-Glu-Thr-Asp-Leu-Leu-Val-Leu-Thr-Lys
	1745.725	Thr-Glu-Glu-His-Leu-Leu-Glu-His-Ala-Val-Leu-Leu-Glu-Gly-Arg
	1769.188	Ser-His-Leu-Asp-Glu-Asn-Glu-Glu-Val-Asp-Asp-Ala-Asn-Pro-Gly-Lys
	1793.911	Asp-Phe-Pro-Leu-Gln-Gln-Asn-Ser-Thr-Asp-Gln-Ala-Gln-Ser-Ser-Lys
	1796.523	Ser-Leu-Asp-Leu-His-Val-Ala-Glu-Leu-Val-Val-Phe-Leu-Lys-Gly-Arg
	1797.762	Ala-Glu-Asp-Pro-Gly-Glu-Tyr-Phe-Phe-Gly-Phe-Leu-His-Leu-Arg
	1797.762	Ala-Glu-Asn-Glu-Phe-Glu-Thr-Gln-Thr-Gln-Phe-Leu-Gly-Arg-Lys

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