CHAPTER 1

CHEMICAL COMPOSITIONS, FUNCTIONAL PROPERTIES AND ANTIOXIDATIVE ACTIVITY OF PROTEIN HYDROLYSATES FROM TOOTHED PONYFISH MUSCLE TREATED WITH VISCERA EXTRACT FROM HYBRID CATFISH

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Chemical compositions, functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with viscera extract from hybrid catfish

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Abstract

Chemical compositions, functional properties and antioxidative activities of a protein hydrolysate prepared from toothed ponyfish (*Gazza minuta*) muscle, using viscera extract from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*), with a degree of hydrolysis (DH) of 70%, were investigated. Freeze-dried hydrolysate had a high protein content (89.02%, dry weight basis) and it was brownish yellow in color (L*=63.67, a*=6.33, b*=22.41). The protein hydrolysate contained a high amount of essential amino acids (48.22%) and had arginine and lysine as the dominant amino acids. The protein hydrolysate had a good solubility. It was soluble over a wide pH range (3-9), in which more than 77% solubility was obtained. The emulsifying activity index of the protein hydrolysate decreased with increasing concentration ($p<0.05$). Conversely, the foaming abilities increased as the hydrolysate concentrations increased ($p<0.05$). Protein hydrolysate exhibited the increases in 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-Azino-bis(3-ethylbenzo-thiazothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities, ferric reducing power (FRAP) and metal chelating activity as hydrolysate concentration increased ($p<0.05$). ABTS radical scavenging activity of protein hydrolysate was stable when heated at 100°C for 180 min and subjected to a wide pH range (1-11). Therefore, protein hydrolysate from the muscle of toothed ponyfish produced by viscera extract from hybrid catfish can be used as a promising source of functional peptides with antioxidant properties.

Keywords: Hydrolysates; Trypsin; Functionalities; Antioxidant; Fish protein

1. Introduction

Adding enzymes to hydrolyze food proteins is a process of considerable importance that can improve the physicochemical, functional, and sensory properties of the native protein without prejudicing its nutritive value. Hydrolyzing protein can also improve intestinal absorption (Nalinanon *et al*., 2011). Proteases from different sources are commonly used to obtain a more selective hydrolysis since they are specific for peptide bonds adjacent to certain amino acid residues. Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides (Thiansilakul *et al.,* 2007). Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications. Numerous fish protein hydrolysates have been shown to have antioxidant activities such as protein hydrolysate from the muscle of round scad (Thiansilakul *et al.,* 2007), yellow stripe trevally (Klompong *et al.,* 2008) and ornate threadfin bream (Nalinanon *et al.,* 2011). Fish protein hydrolysates can be used in food systems, comparable to other pertinent protein hydrolysates (Nalinanon *et al*., 2011).

The Pla-duk-ra, Thai dry fermented fish product, processing industry is becoming increasingly important since it is one of the income generators for Phatthalung province (Klomklao *et al*., 2010a). Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) is one of the raw materials commonly used for Pla-duk-ra production. During processing, by-products such as the viscera, head, bones and frames are generated in the large quantity. These solid wastes constitute 60-70% of original raw material which pose the great disposal problems. However, these abundant underutilized materials are a rich source of

enzymes, especially proteinases (Klomklao *et al.,* 2009). Hence, recovery of proteinases from hybrid catfish viscera is an approach to minimize the economic and ecological problems of this processing discard. Additionally, fish protein hydrolysate with bioactivity treated with the aid of fish proteinases can be obtained as a new value-added product with high market value. Recently, Klomklao *et al.* (2011) found that hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) viscera contained high proteolytic activity, and a trypsin-like serine proteinase was identified as the dominant proteinase.

Toothed ponyfish (*Gazza minuta*) belongs to the order Perciformes and is a member of the Leiognathidae family. In Thailand, toothed ponyfish has not been used for human consumption due to its small size and low meat content. Normally, it is widely used as fish meal. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. The use of fish proteinase extract recovered from hybrid catfish viscera, a by-product from Pla-duk-ra processing, for hydrolysate production, could lower the cost of commercial proteinases. Furthermore, the production of protein hydrolysate from fish muscle using hybrid catfish viscera extract has also not been investigated. Therefore, the objectives of this investigation were to study the functionalities and antioxidant properties of protein hydrolysate prepared from toothed ponyfish muscle using hybrid catfish viscera extract.

2. Material and Methods

2.1 Chemicals

L-leucine, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine) and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulfite, potassium persulphate, ferrous chloride and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) were procured from Bio-Rad Laboratories (Hercules, CA, USA). All of chemicals used were of analytical grade.

2.2 Fish sample preparation

Viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) were obtained from a local market in Phatthalung province, Thailand. Those samples (5 kg) were packed in polyethylene bag, kept in ice and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung within 30 min. Pooled internal organs were immediately frozen and stored at -20°C until used.

Toothed ponyfish (*Gazza minuta*) with the length of 10 cm were purchased from the dock in Songkhla, Thailand. The fish, off-loaded approximately 18-24 h after capture, were placed in ice at a fish/ice ratio of 1:2 (w/w) and transported to

the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon the arrival, fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (100 g) was placed in a polyethylene bag and stored under vacuum at -20°C until used.

2.3 Preparation of viscera extract

Frozen viscera were thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1-1.5 cm and homogenized with three volumes of acetone at -20°C for 30 min using an IKA homogenizer (Model T25, Selangor, Malaysia) according to the method of Klomklao, Benjakul, Kishimura, Osako, and Tanaka (2010b). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was air-dried at room temperature. Defatted viscera powder obtained was stored at -20°C until used.

To prepare the viscera extract, viscera powder was suspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g (H-200, Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilized. Before use, the lyophilized sample (10 g) was dissolved with 50 ml of cold distilled water (4°C) and referred to as “catfish viscera extract”.

2.4 Production of toothed ponyfish protein hydrolysate

Toothed ponyfish mince was mixed with 0.2 M borate buffer (pH 9.0) at a ratio of 1:3 (w/v) and pre-incubated at 50°C for 10 min. The enzyme hydrolysis was started by adding hybrid catfish viscera extract in the amount required to gain 70% DH, as described by Benjakul and Morrissey (1997). The reaction was conducted at pH 9.0 and 50°C for 15 min. After 15 min of hydrolysis, the enzyme was inactivated by heating at 90°C for 15 min in a water bath. The mixture was then centrifuged at 5,000×g at 4°C for 10 min using a Sorval Model RC-5B Plus centrifuge (Newtown, CT, USA) and the supernatant was collected. Toothed ponyfish protein hydrolysate was freeze-dried using a Dura-Top^{TM}µp freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried toothed ponyfish protein hydrolysate obtained was subjected to analyses.

2.5 Determination of α-amino acids and DH

The α-amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The

absorbance was read at 420 nm and α-amino acid was expressed in term of L-leucine. DH was calculated as follows:

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

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 toothed ponyfish muscle homogenate. L_{max} is total α-amino acid in the original toothed ponyfish muscle homogenate obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

2.6 Determination of functional properties

2.6.1 Solubility

To determine protein solubility, hydrolysate samples (200 mg) were dispersed in 20 ml of deionised water and pH of the mixture was adjusted to 3, 5, 7 and 9 with either 1 M HCl or 1 M NaOH. The mixture was stirred at room temperature for 30 min. The volume of solutions was made up to 25 ml by distilled water, previously adjusted to the same pH as the sample solution, prior to centrifugation at 5,000×g for 15 min. Protein content in the supernatant was determined using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as a standard. Total protein content in the same was determined after solubilisation of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein content in sample}} \times 100$$

2.6.2 Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) were used to measure the emulsifying properties of protein hydrolysate. EAI and ESI were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and protein hydrolysate solutions (0.5%, 1.0%, 2.0% and 3.0%, 6 ml) were homoginised (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 μl) was pipette from the middle portion of the container at 0 and 10 min after homogenization and subsequently diluted 100-fold using 0.1% sodium dodecyl sulphate (SDS) solution. The mixture was mixed thoroughly for 10 s using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formulae:

$$EAI\ (m^2/g) = (2 \times 2.303 \times A \times DF) / l\phi C$$

where $A = A_{500}$, DF = dilution factor (100), l = path length of cuvette (m), ϕ = oil volume fraction and C = protein concentration in aqueous phase (g/m^3);

$$ESI\ (min) = A_0 \times \Delta t/\Delta A$$

where $\Delta A = A_0 - A_{10}$ and Δt = 10 min. A_0 and A_{10} are the absorbances measured immediately and 10 min, respectively.

2.6.3 Foaming properties

Foam expansion (FE) and foam stability (FS) of hydrolysate solutions were determined according to the method of Shahidi, Han, and Synowiecki (1995) with slight modification. Hydrolysate solutions (20 ml) with 0.5%, 1.0%, 2.0% and 3.0% protein concentrations were transferred into a 100-ml cylinder. The solutions were homogenized at 13,400 rpm for 1 min at room temperature. The samples were allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T - V_0/V_0) \times 100$$

$$FS\ (\%) = (V_t - V_0/V_0) \times 100$$

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for different times (30 and 60 min).

2.7 Effect of concentration of protein hydrolysate on antioxidant activities

Toothed ponyfish protein hydrolysate with 70%DH was dissolved in distilled water to obtain the concentration of 5, 10, 20, 30 and 40 mg protein/ml. Antioxidant activity of protein hydrolysates at different concentrations was measured by monitoring the DPPH radical scavenging activity, ABTS radical scavenging activity, Ferric reducing antioxidant power (FRAP) and metal chelating activity.

2.8 Determination of antioxidant activity

2.8.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Nalinanon et al. (2011) with a slight modification. To the sample (1.5 ml), 1.5 ml of 0.15 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol were added. The mixture was then mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of resulting solution was measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu). The blank was prepared in the same manner, except that the distilled water was used instead of sample. DPPH radical scavenging activity was calculated according to the following equation (Yen, & Wu, 1999):

$$\text{DPPH radical scavenging activity} = \left(1 - \frac{A_{517} \text{ of sample}}{A_{517} \text{ of control}}\right) \times 100$$

2.8.2 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay, as described by Binsan et al. (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol, in order to obtain an absorbance of 1.1±0.02 units at 734 nm using spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150µl) was mixed with 2,850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The

absorbance was then measured at 734 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. ABTS radical scavenging activity was calculated according to the following equation:

$$\text{ABTS radical scavenging activity} = \left(1 - \frac{A_{734} \text{ of sample}}{A_{734} \text{ of control}}\right) \times 100$$

2.8.3 Ferric reducing antioxidant power (FRAP) assay

FRAP was assayed according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 6H_2O$ solution. A working solution was prepared fresh by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 6H_2O$ solution. The mixed solution was incubated at 37°C for 30 min and was referred to as the FRAP solution. A sample (150 μl) was mixed with 2,850 μl of FRAP solution and kept for 30 min in the dark. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Increased absorbance of the reaction mixture indicates the increasing ferric reducing antioxidant power.

2.8.4 Metal chelating activity

The chelating activity on Fe^{2+} was measured using the method of Boyer and McCleary (1987) with a slight modification. Diluted sample (4.7 ml) was

mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then measured at 562 nm. The blank was conducted in the same manner but distilled water was used instead of sample. The chelating activity was calculated as follows:

$$\text{Chelating activity} = = (1- \frac{A_{562} \text{ of sample}}{A_{562} \text{ of control}}) \times 100$$

2.9 Proximate analysis

Moisture, protein, fat and ash contents were determined according to the method of AOAC (2000) with the analytical No. of 950.46, 928.08, 960.39 and 920.153, respectively.

2.10 Amino acid analysis

Amino acid compositions of freeze-dried hydrolysate were determined according to the method of Cohen, and Michaud (1993). Hydrolysate (10 μg) was dissolved in 10 mM HCl (10 μl) and treated with 0.2 M borate buffer pH 9.3 (30 μl). The sample solution was reacted with 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (10 μl) to form stable unsymmetric urea derivatives. Amino acids were separated by reverse phase HPLC (AccQ Tag column, Waters, Milford, MA, USA) at 37°C, using gradient mobile phase: deionized water, acetonitrile and eluent A (sodium acetate, phosphoric acid, triethylamine) and detected by a UV detector (Waters 486, Milford, MA, USA) at

254 nm and fluorescence detector (Jasco FP-920, Great Dunmow, Essex, UK) with excitation wavelength at 250 nm and emission wavelength at 395 nm. The amount of amino acids was calculated, based on the peak area in comparison with that of standard. The amino acid content was expressed as a percentage of total amino acids in the sample.

2.11 Color measurement

The color of freeze-dried hydrolysate was measured by Hunter lab color meter and reported by the CIE system. L*, a* and b* parameters, indicating lightness, redness and yellowness, respectively.

2.12 Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.13 Statistical Analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel, & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

3. Results and discussion

3.1 Proximate compositions of protein hydrolysate

The proximate compositions of the freeze-dried toothed ponyfish protein hydrolysate and toothed ponyfish muscle are shown in Table 1. The freeze-dried hydrolysate and toothed ponyfish muscle contained 7.56 and 80.01% moisture, respectively. On dry weight basis, freeze-dried hydrolysate contained higher protein content but lower lipid content than toothed ponyfish muscle. The high protein content was a result of the solubilization of protein during hydrolysis, the removal of insoluble undigested non-protein substances, and partial removal of lipid after hydrolysis (Thiansilakul et al., 2007). Removal of the fat layer after hydrolysis caused a low lipid content in hydrolysate. During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipids (Shahidi et al., 1995). A reduced lipid content was reported in the protein hydrolysate from capelin (Shahidi et al., 1995), herring (Sathivel et al., 2003), salmon (Gbogouri, Linder, Fanni, & Parmentier, 2004) and round scad (Thiansilakul et al., 2007). The protein hydrolysate had a higher ash content (10.01%) than did whole mince (6.50%). The high content of inorganic substances found in the hydrolysate was possibly due to the use of borate buffer during the enzymatic reaction. A high ash content has been recognized as a drawback of protein hydrolysate, making applications limited (Shahidi et al., 1995; Thiansilakul et al., 2007). Since toothed ponyfish protein hydrolysate had a high protein content, it could be used as a source of protein ingredient for human consumption or industrial applications.

Table 1

Proximate composition of freeze-dried hydrolysate produced from toothed ponyfish muscle and toothed ponyfish muscle*

Compositions	Freeze-dried hydrolysate	Toothed ponyfish muscle
Protein**	89.02±0.12b***	83.21±1.20a
Lipid**	1.00±0.27a	9.32±0.05b
Ash**	10.01±0.05b	6.50±0.01a
Moisture	7.56±0.28a	80.01±0.27b

*Mean±SD from triplicate determination
**Dry weight basis
***The different letters in the same row denote the significant differences ($p<0.05$)

3.2 Amino acid composition

The amino acid compositions of freeze-dried toothed ponyfish protein hydrolysates are presented in Table 2. Protein hydrolysate was rich in arginine, lysine and histridine, which accounted for 14.6%, 13.5% and 10.5% of the total amino acids, respectively. Protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.93. Round scad protein hydrolysate showed high essential amino acid/non-essential amino acid ratio (0.92) (Thiansilakul et al., 2007). Iwasaki, and Harada (1985) reported that fish and shellfish contained the high essential amino acid/non-essential amino acid ratio. Therefore, the obtained toothed ponyfish protein hydrolysate could possibly be a dietary protein supplement to poorly balanced dietary proteins. Fish byproducts have a high percentage of essential amino acids and can be used to produce nutritious products

(Shahidi, 1994). Toothed ponyfish protein hydrolysate contained a low level of proline (0.41%). The presence of proline residues in the centre of the peptides generally contributes to the bitterness. Thus the peptidase, which can cleave the hydrophobic amino acids and proline, is capable of debittering protein hydrolysate (Capiralla, Hiroi, Hirokawa, & Maeda, 2002). Additionally, the protein hydrolysate consisted of hydrophobic amino acids, such as leucine (9.97%), valine (6.42%), phenylalanine (4.60%), and isoleucine (3.55%). Bitterness was one of the main contributors to off-flavor of fish hydrolysate. Thiansilakul et al. (2007) reported that bitter peptide from round scad protein hydrolysate contained leucine, valine, phenylalanine and isoleucine.

Table 2

Amino acid composition of freeze-dried hydrolysate produced from toothed ponyfish muscle

Amino acids	Content (%)
Asp+Asn	1.91
Ser	7.78
Glu+Gln	3.55
Gly	1.31
His	10.5
Arg	14.6
Thr*	5.17
Ala	4.84
Pro	0.41
Cys-S-S-Cys	0.90
Tyr	6.08
Val*	6.42
Met*	5.01
Lys*	13.5
Ile*	3.55
Leu*	9.97
Phe*	4.60

*Essential amino acids

3.3Color

Freeze-dried toothed ponyfish protein hydrolysate was brownish yellow in color (L^* = 63.67, a^* = 6.33, b^*= 22.41), as shown in Table 3. The dark color of fish protein hydrolysate was probably from the oxidation of myoglobin and the melanin pigment of the raw material (Benjakul, & Morrissey, 1997). The result was in agreement with Thiansilakul et al. (2007) who reported that round scad protein hydrolysate, prepared using Flavozyme, was brownish yellow in color (L^* = 58.00, a^* = 8.38, b^*= 28.32). Sathivel et al. (2003) reported that the color of whole herring and herring byproduct hydrolysate, produced by Alcalase, varied with substrates. Herring gonad hydrolysate was darkest (L* = 74.6) and most yellowish (b* = 18), whereas whole herring hydrolysate was the lighter (L* = 89.4) and least yellowish (b* = 8.0). Freeze-dried hydrolysate from Pacific whiting solid wastes prepared using Alcalase was brownish yellow in color (L^* = 54.59, a^* = 6.70, b^*= 27.89) (Benjakul, & Morrissey, 1997). Therefore, the varying color of fish protein hydrolysate depended on the composition of the raw material and the hydrolysis condition.

Table 3

L*, a* and b*-values of freeze-dried hydrolysate produced from toothed ponyfish muscle

Color characteristics	Freeze-dried hydrolysate
L*	63.67
a*	6.33
b*	22.41

3.4 Solubility

The solubilities of the freeze-dried toothed ponyfish protein hydrolysates at various pHs are shown in Table 4. The hydrolysates were soluble over a wide pH range, in which more than 77% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent, compared with acidic pH. Nalinanon et al. (2011) reported that protein hydrolysates from ornate threadfin bream muscle prepared using pepsin from skipjack tuna stomach showed high solubility (>70%) in the pH range of 3-9. The lowest solubility was found in the hydrolysate at pH 5.0 ($p<0.05$). The result suggested that proteins or peptides remaining after hydrolysis were precipitated at this pH, which was close to the isoelectric point (pI) of myofibrillar proteins. The pH affects the charge on the weakly acidic and basic side chain groups and hydrolysates generally show low solubility at their isoelectric points (Linder, Fanni, & Parmentier, 1996). Solubility variations could be attributed to both net charge of peptides, that increase as pH moves away from pI, and surface hydrophobicity, that promotes the aggregation via hydrophobic interaction (Sorgentini, & Wagner, 2002). Due to the high

solubility of the muscle hydrolysate over a wide pH range, it was presumed that products had a low molecular weight and were hydrophilic in nature (Klompong et al., 2007).

Table 4

Solubility of protein hydrolysate from toothed ponyfish muscle prepared using proteinases from hybrid catfish viscera at various pHs.

pH	Solubility (%)*
3	85.05±0.50b**
5	77.11±2.50a
7	87.65±2.54b
9	92.55±1.56c

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences ($p<0.05$).

3.5 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of toothed ponyfish protein hydrolysates at various concentrations (0.5%, 1.0%, 2.0% and 3.0%) are shown in Table 5. EAI of protein hydrolysates decreased with increasing concentrations ($p<0.05$). However, no differences in ESI were found when the hydrolysate concentrations increased ($p>0.05$). Thiansilakul et al. (2007) reported that EAI of protein hydrolysate from round scad meat decreased with increasing concentrations (0.1-3.0%) and the decrease in ESI of protein hydrolysate was noticeable when the hydrolysate concentration increased up to 0.5%. EAI estimates the ability of the protein to aid in the formation and

stabilization of newly-created emulsion by giving units of area of the interface that is stabilized per unit weight of protein; this is determined by the turbidity of the emulsion at a wavelength of 500 nm (Thiansilakul et al., 2007). Protein hydrolysates are surface-active materials and promote an oil-in-water emulsion because of their hydrophilic and hydrophobic groups and their charge (Gbogouri et al., 2004). The dependence of emulsifying activity on the concentration of protein has been explained by adsorption kinetics (Kinsella, 1976). At low protein concentrations, protein adsorption at the oil-water interface is diffusion controlled. At high protein concentration, the activation energy barrier does not allow protein migration to take place in a diffusion-dependent manner that leads to accumulation of proteins in the aqueous phase. Thus, protein or peptides were most likely localized in the aqueous phase and a lower amount of proteins or peptides migrated to the interface (Nalinanon et al., 2011). The increase in protein-protein interaction resulted in a lower protein concentration at the interface, in which a thinner film stabilizing the oil droplet is formed. Therefore, emulsifying properties of hydrolysate were governed by the molecular properties and the concentration employed.

Table 5

Emulsifying properties of toothed ponyfish protein hydrolysate at various concentrations

Hydrolysate concentrations (%)	Emulsifying activity index* (m^2/g)	Emulsion stability index* (min)
0.5	42.71±0.28d**	19.86±1.85a
1.0	22.82±2.39c	18.30±2.09a
2.0	11.90±1.36b	17.35±2.61a
3.0	8.43±0.05a	17.93±1.34a

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences ($p<0.05$).

3.6 Foaming properties

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul et al., 2007). Foam expansion (FE) and foam stability (FS) of hydrolysates from toothed ponyfish muscle at various concentrations (0.5%, 1.0%, 2.0% and 3.0%) are shown in Table 6. Protein concentrations had impact on FE ($p<0.05$). The increase in FE was observed when concentration of hydrolysate increased ($p<0.05$). Sanchez and Patino (2005) revealed that an increase in protein concentration resulted in a higher rate of diffusion. Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air-water interface (Nalinanon et al., 2011). In general, proteins, which rapidly adsorb at the newly-created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, exhibit better foaming ability than proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). FE after

whipping for 30 and 60 min was monitored to indicate the FS of protein hydrolysates. At 30 min, FS of hydrolysate was improved by increasing concentration and the protein hydrolysate with a concentration of 2 and 3% showed the highest foam stability ($p<0.05$). However, slight differences in FS after whipping for 60 min were found among hydrolysates with different concentration. A similar result was also reported in protein hydrolysates prepared from round scad mince, using Flavourzyme, with 60% DH (Thiansilakul et al., 2007). Lawal (2004) postulated that an increase in foam stability with increasing concentration was a result of formation of stiffer foams. FS is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips, Whitehead, & Kinsella, 1994).

Table 6

Foaming properties of toothed ponyfish protein hydrolysate at various concentrations

Hydrolysate concentrations (%)	Foaming expansion* (m^2/g)	Foaming stability* (%)	
		30 min	60 min
0.5	110.00±10.00a**	95.00±8.66a	90.00±8.66a
1.0	135.00±5.00b	116.67±7.64b	113.33±5.77b
2.0	143.33±2.87b	128.33±2.89c	121.67±2.89b
3.0	158.33±7.64c	131.67±2.89c	123.33±2.89b

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences ($p<0.05$).

3.7 Effect of concentration of fish protein hydrolysate on antioxidant activity

3.7.1 DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysate from toothed ponyfish muscle prepared using hybrid catfish viscera extract with the DH of 70% is depicted in Fig. 1a. The DPPH radical scavenging activity increased as the concentration of protein hydrolysate increased up to 40 mg/ml ($p<0.05$). This result was in accordance with Jao and Ko (2002) who reported that the DPPH radical scavenging activity of protein hydrolysates from tuna cooking juice, increased when the concentration increased from 17% to 75%. DPPH is a stable free radical that shows a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged by changing color from purple to yellow and the absorbance is reduced (Thiansilakul *et al*., 2007). The results so obtained suggest that the hydrolysates contained amino acids or peptides that were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

3.7.2 ABTS radical scavenging activity

The ABTS radical scavenging activity of toothed ponyfish protein hydrolysate increased sharply at the concentration ranging from 0 to 20 mg/ml. Thereafter, the slight increase in ABTS radical scavenging activity was observed up to 40 mg/ml (Fig. 1b). Similar results of ABTS radical scavenging activity of protein hydrolysate from toothed ponyfish muscle were generally observed, compared with those of DPPH radical scavenging activity. Nevertheless, slight

differences in activities determined by both assays were observed. The result suggested that those protein hydrolysates might scavenge two different radicals, $ABTS^{\cdot}$ and DPPH, differently. $ABTS^{\cdot}$ assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavengers of lipid peroxyl radicals) (Thiansilakul *et al.,* 2007). Therefore, the protein hydrolysate from tooth ponyfish muscle treated with the viscera extract from hybrid catfish was able to scavenge free radicals, thereby, preventing lipid oxidation via a chain breaking reaction.

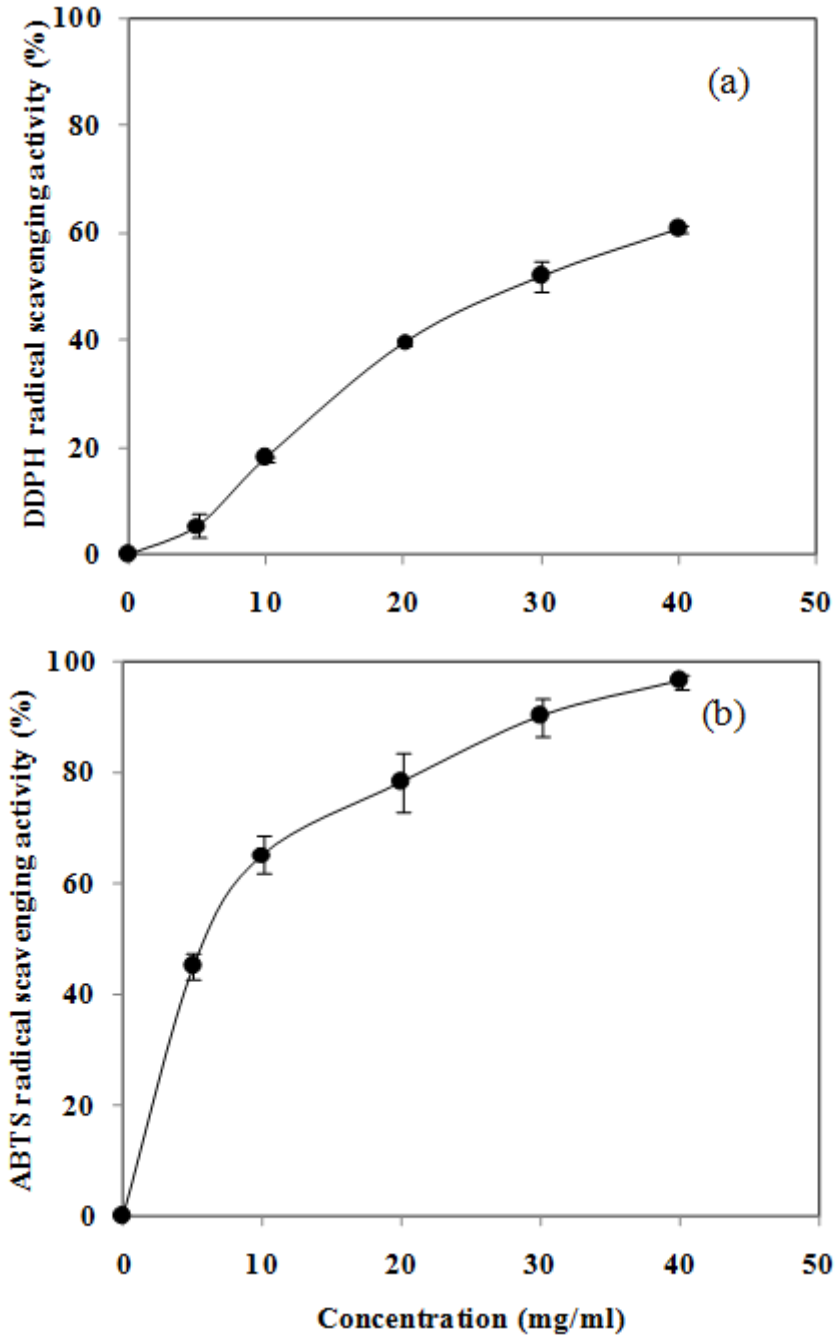

Fig. 1. DPPH (a) and ABTS (b) radical scavenging activity of toothed ponyfish protein hydrolysate at different concentrations.

3.7.3 Ferric reducing antioxidant power (FRAP)

Antioxidant potential of toothed ponyfish protein hydrolysate was estimated from its ability to reduce TPTZ-FE (III) complex to TPTZ-FE (II) complex. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Thiansilakul *et al.,* 2007). With increasing hydrolysate concentration, toothed ponyfish protein hydrolysate showed increased FRAP ($p<0.05$) (Table 7). Therefore, toothed ponyfish protein hydrolysate exhibited the FRAP in the concentration-dependent manner. The results suggested that antioxidative compounds in protein hydrolysate tested showed higher FRAP when higher concentrations were used. Klompong *et al.* (2008) reported that the reducing power of protein hydrolysate from yellow stripe trevally mince increased as the hydrolysate concentration increased. From the results, protein hydrolysates prepared with viscera extract from hybrid catfish had the pronounced effect of donating electrons that led to the retardation of propagation of lipid oxidation (Binsan *et al.,* 2008).

3.7.4 Metal chelating activity

Metal ion chelating activity of toothed ponyfish protein hydrolysate is shown in Table 7. The continuous increase in metal chelating activity of protein hydrolysate was found with increasing concentrations up to 40 mg/ml. Metal ion is an effective prooxidant, which can accelerate the initiation process (Thiansilakul *et al.,* 2007). As a consequence, the ability of toothed ponyfish

protein hydrolysate in chelating those metal ions could lead to the prevention of lipid oxidation.

Protein hydrolysates from aquatic species contain both antioxidative and prooxidative components, and their final effect depends on their concentration (Klompong *et al.,* 2008). From the results, toothed ponyfish protein hydrolysate showed the antioxidative effect in the concentration ranges used in this study. Moreover, toothed ponyfish protein hydrolysate could function as both primary and secondary antioxidant via scavenging the free radical and chelating the metal ions.

Table 7

FRAP and metal chelating activity of toothed ponyfish protein hydrolysate at different concentrations.

Hydrolysate concentrations (%)	FRAP* (A593)	Metal chelating activity* (%)
0	0a**	0a**
5	0.14±0.01b	12.36±1.04b
10	0.29±0.01c	26.77±0.67c
20	0.59±0.01d	41.33±1.39d
30	0.98±0.01e	57.33±1.05e
40	1.43±0.08f	77.54±0.85f

*Mean ± SD from triplicate determinations.
**Different letters in the same column indicate significant differences ($p<0.05$).

3.8 pH and thermal stability of antioxidant hydrolysate

The effects of pH on the stability of antioxidant protein hydrolysate are depicted in Fig. 2a. ABTS radical scavenging activity of the antioxidant protein hydrolysate remained constant over the pH range of 1-10. At pH 11, ABTS radical-scavenging activity slightly decreased ($p<0.05$). The results suggested that antioxidant hydrolysate exhibiting ABTS radical scavenging activity might lose its activity to some extent at high pH. Due to the stability over a wide pH range, antioxidant protein hydrolysate from the muscle of toothed ponyfish have potential for application in any food system at extream pH.

Thermal stability of antioxidant protein hydrolysate as monitored by ABTS radical scavenging activities is shown in Fig. 2b. ABTS radical scavenging activities of antioxidant hydrolysate were quite stable when heated at 100°C up to 180 min, where activities of more than 98% were retained (data not shown). From the results, protein hydrolysate from toothed ponyfish muscle with 70%DH can be incorporated in cooked food systems without significant loss of its antioxidant activity.

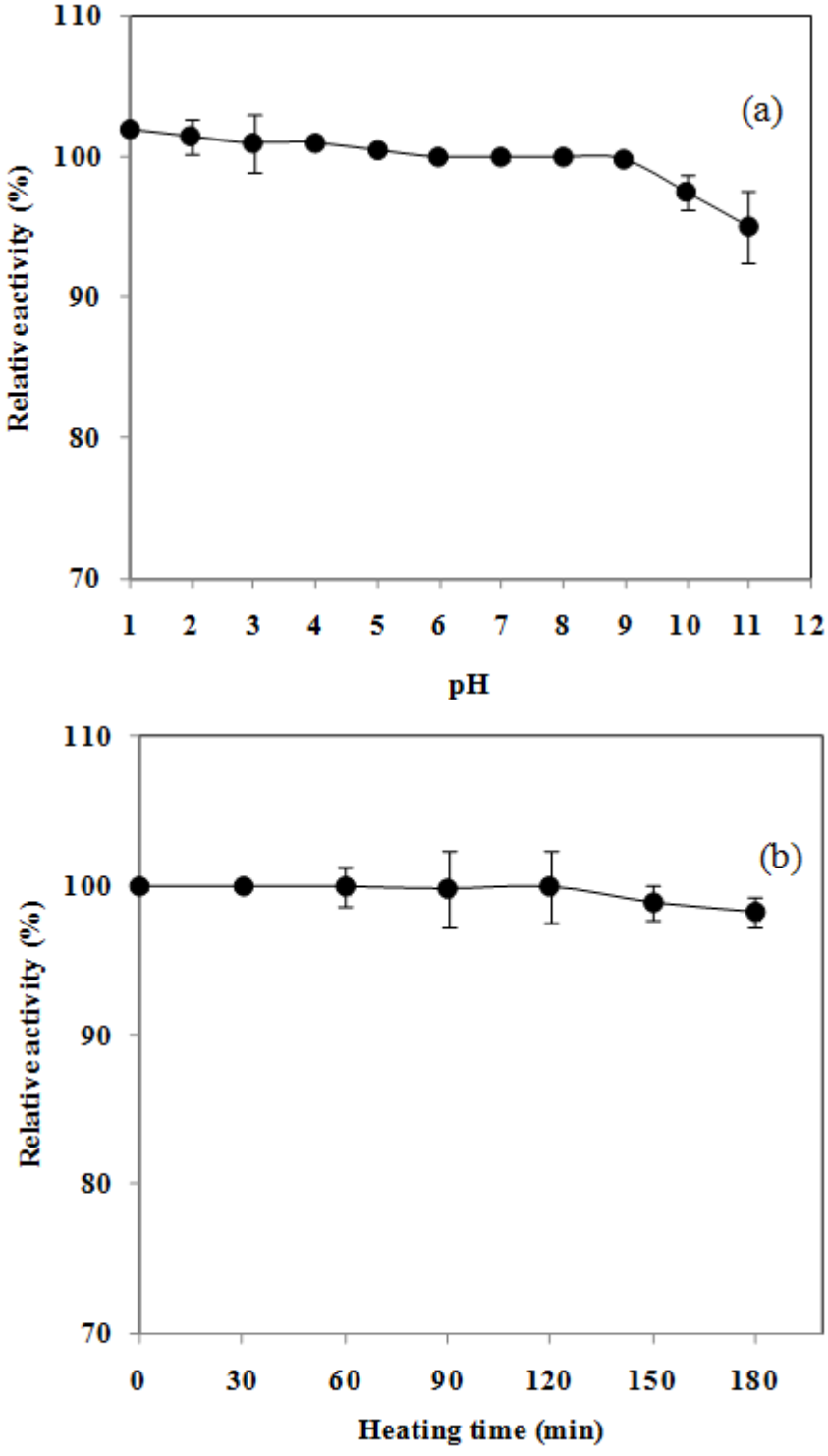

Fig. 2. Effect of pHs (a) and heating time (b) on ABTS radical scavenging activity of toothed ponyfish protein hydrolysate prepared using hybrid catfish viscera extract. Bars represent standard deviation from triplicate determinations.

4. Conclusion

Production of protein hydrolysates from toothed ponyfish muscle could be achieved by hybrid catfish viscera extract hydrolysis. The protein hydrolysate could be used as an emulsifier and as a foaming agent with antioxidant activities. Therefore, toothed ponyfish protein hydrolysate can be used as a natural additive, possessing functionalities and antioxidative properties in food system.

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โครงการส่งเสริมการวิจัยในอุดมศึกษาและพัฒนามหาวิทยาลัยวิจัยแห่งชาติ

สำนักงานคณะกรรมการอุดมศึกษา

มหาวิทยาลัยทักษิณ

โครงการ: การศึกษาองค์ประกอบทางเคมี สมบัติเชิงหน้าที่และกิจกรรมการต้านออกซิเดชันของโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาแป้นเขี้ยวที่ผลิตโดยใช้ส่วนสกัดจากเครื่องในปลาดุกบิ๊กอุย

ชื่อหัวหน้าโครงการวิจัยผู้รับทุนรองศาสตราจารย์ ดร. สรรพสิทธิ์ กล่อมเกล้า

รายงานในช่วงตั้งแต่วันที่ 1 ตุลาคม 2555 **ถึงวันที่** 30 กันยายน 2556

<u>รายจ่าย</u>

หมวด	งบประมาณรวมทั้งโครงการ	ค่าใช้จ่ายงวดปัจจุบัน	คงเหลือ (หรือเกิน)
1. ค่าตอบแทน	15,000	15,000	0
2. ค่าจ้าง	110,160	110,160	0
2. ค่าวัสดุ	157,000	160,962	-3,962
3. ค่าใช้สอย	34,506	35,003	-497
รวม	**316,666**	**321,125**	**-4,459**

<u>จำนวนเงินที่ได้รับและจำนวนเงินคงเหลือ</u>

จำนวนเงินที่ได้รับ	จำนวนเงิน	วันที่ได้รับ
งวดที่ 1	**158,333**	**-**
งวดที่ 2	**126,666**	**-**
งวดที่ 3	**31,667**	**-**
รวม	**316,666**	

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(รศ.ดร.สรรพสิทธิ์ กล่อมเกล้า) (รศ.ดร.สรรพสิทธิ์ กล่อมเกล้า)

หัวหน้าโครงการวิจัยผู้รับทุน เจ้าหน้าที่การเงินโครงการ

วันที่ 1 พฤศจิกายน 2556 วันที่ 1 พฤศจิกายน 2556

ภาคผนวก

ประวัติคณะผู้วิจัย

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5. ประวัติการศึกษา

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2545	ปริญญาตรี	วิทยาศาสตรบัณฑิต (อุตสาหกรรมเกษตร) เกียรตินิยมอันดับสอง	มหาวิทยาลัยสงขลานครินทร์
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การทำบริสุทธิ์โปรตีน(Protein Purification)

เอนไซม์ในอาหาร (Food Enzyme)

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7.1 ผลงานตีพิมพ์

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arabesque greenling (*Pleurogrammus azonus*). Eur. Food Res. Technol. 232: 381-388.

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34. **Klomklao, S**., Kishimura, H. and Benjakul, S. 2013. Use of viscera extract from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) for the production of protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle. Food Chem. 136: 1006-1012.

35. **Klomklao, S**., Benjakul, S. and Kishimura, H. 2013. Functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with the viscera extract from hybrid catfish. Int. J. Food Sci. Tech.48: 1483-1489**.**

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37. **Klomklao, S**., Kishimura, H. and Benjakul, S. 2013. Anionic trypsin in Pacific saury (*Cololabis Saira*) viscera: Purification and biochemical characteristics. J. Aquatic Food Prod. *Accepted.*

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7.2 ผลงานประชุมเชิงวิชาการ

1. **Klomklao, S**., Benjakul, S. and Visessanguan, W. 2003. Characterization of proteinases from tuna spleen. 29th Congress on Science and Technology of Thailand, October 20-22, 2003. Golden Jubilee Convention Hall, Khon Kean, Thailand.

2. **Klomklao, S**., Benjakul, S., Visessanguan, W. and Simpson, B.K. 2004. Partitioning and recovery of proteinases from yellowfin tuna (*Thunnus albacores*) spleen by aqueous two-phase systems. The 6th Agro-Industrial Conference, May 28-29, 2004. Impact, Bangkok, Thailand.

3. **Klomklao, S**., Benjakul, S., Visessanguan, W. and Simpson, B.K. 2004. Partitioning and recovery of proteinases from yellowfin tuna (*Thunnus albacores*) spleen by aqueous two-phase systems. RGJ Seminar XXX: Biosciences and Biotechnology for Development of Southern Thailand, August 13, 2004. Faculty of Agro-Industry, PSU, Songkhla, Thailand.

4. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K. and Saeki, H. 2005. Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. The Joint Meeting of the Tohoku-Hokkaido Branches of JSFS, November 4-5, 2005. Faculty of Agriculture, TohokuUniversity, Sendai, Japan.

5. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K.

2006. Enzymatic hydrolysis of sardine proteins by trypsin from tuna spleen. 2006 CIFST/AAFC Joint Conference, May 28-30, 2006. Delta Hotel, Downtown, Montreal, Canada.

6. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2006. Purification and characterization of trypsins from skipjack tuna (*Katsuwonus pelamis*) spleen. IUFoST 13th World Congress of Food Science and Technology, September 17-21, 2006. Nantes, France.
7. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007. A 29 kDa protease from the digestive glands of Atlantic bonito (*Sarda sarda*): Recovery and characterization. Food Innovation Asia 2007: The 9th Agro-Industrial Conference "Q" Food for Good Life, June 14-15, 2007. BITEC, Bangkok, Thailand.
8. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007. Extraction of carotenoprotein from black tiger shrimp shell with the aid of bluefish trypsin. IFT 2007 Annual Meeting & Food Expo, July 28- August 1, 2007. Chicago, USA.
9. **Klomklao, S**., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007. Trypsin from tongol tuna (*Thunnus tonggol*) spleen: Purification and characterization. The 33rd Congress on Science and Technology of Thailand (STT. 33), October 18-20, 2007. WalailakUniversity, Nakhon Si Thammarat, Thailand.
10. **Klomklao, S**., Benjakul, S.and Kishimura, H. 2008. Biochemical characteristics of proteinases from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) viscera. Food Innovation Asia Conference 2008: FoSTAT-The 10th Agro-Industrial Conference, June 12-13, 2008. BITEC, Bangkok, Thailand.
11. **Klomklao, S**., and Kishimura, H. 2008. Properties of trypsin purified from the pyloric ceca of Pacific saury (*Cololabis saira*). The 34rd Congress on Science and Technology of Thailand (STT. 34), October 31- November 2, 2008. QueenSirikitNationalConvention Center, Bangkok, Thailand.
12. Amemiya, H., Tanaka, M., **Klomklao, S.** and Osako, K.Studies on the gelation property of deep-sea water shrimp (*Pandalus borealis*) meat. Spring Meeting 2009 of the

Japanese Society of Fisheries Science, March 27-31, 2009. TokyoUniversity of Marine Science and Technology, Tokyo, Japan.

13. **Klomklao, S**., Benjakul, S., Osako, K. and Tanaka, M. 2009. Extraction, purification and biochemical properties of trypsin inhibitor in adzuki bean (*Vigna angularis*). Food Innovation Asia Conference 2009: FoSTAT-The 11th Agro-Industrial Conference, June 18-19, 2009. BITEC, Bangkok, Thailand.
14. **Klomklao, S**., Kishimura, H. and Benjakul, S. 2009. Endogenous proteinases in Japanese sandfish (*Arctoscopus japonicus*): Autolysis and biochemical characteristics. Food Innovation Asia Conference 2009: FoSTAT-The 11th Agro-Industrial Conference, June 18-19, 2009. BITEC, Bangkok, Thailand.
15. **Klomklao, S**., Benjakul, S., and Kishimura, H. 2009. Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. 9th the Annual Thailand Research Fund Meeting, October 15-17, 2009. HolidayInnResortReagentBeach, Cha-Am, Petchburi, Thailand
16. Chamsai, P., Chaijan, M. and **Klomklao, S**. 2009. Antioxidative activity of Maillard reaction products from porcine sarcoplasmic protein-sugar model system. The 35th Congress on Science and Technology of Thailand (STT 35), October 15 – October 17, 2009. The Tide Resort (BansaenBeach), Chonburi, Thailand.
17. **Klomklao, S**., Kishimura, H. and Benjakul, S. 2009. Two isoforms of trypsin from the intestine of skipjack tuna (*Katsuwonus pelamis*): Purification and characterization. 11th ASEAN Food Conference 2009, October 21-23, 2009. The Rizqun International Hotel, Bandar Seri Begawan, Brunei Darussalam.
18. **Klomklao, S**., Kishimura, H. and Benjakul, S. 2010. Two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*): Purification and biochemical properties. Food Innovation Asia Conference 2010: The 12th Agro-Industrial Conference, June 17-18, 2010. BITEC, Bangkok, Thailand.
19. **Klomklao, S**. and Benjakul, S. 2010. Endogenous proteinases in true sardine (*Sardinops melanostictus*). Food Innovation Asia Conference 2010: The 12th Agro-Industrial Conference, June 17-18, 2010. BITEC, Bangkok, Thailand.

20. **Klomklao, S**., Benjakul, S. and Simpson, B.K. 2010. Trypsin from the pyloric ceca of bluefish: Purification and biochemical properties. International Conference on Agriculture and Agro-Industry 2010, November 19-20, 2010. MaeFahLuangUniversity, Chiang Rai, Thailand.

21. **Klomklao, S**. and Benjakul, S. 2010. Production and acceleration of fish sauce fermentation by adding skipjack tuna spleen and reducing the salt content. 2nd ChiangMaiUniversity Agro-Industrial Conference, November 22-23, 2010. ChiangMaiUniversity, Chiang Mai, Thailand.

22. Amemyia, H., Tanaka, M., **Klomklao, S.** and Osako, K. 2010. The effects of egg white on the aptitude of pink shrimp as a raw material for kamaboko product. 5th International Conference on Innovations in Food & Bioprocess Technology, December 7-9, 2010. Asian Institute of Technology, Pathumthani, Thailand.

23. **Klomklao, S.** and Benjakul, S. Isolation of trypsin from hybrid catfish viscera by partitioning in aqueous two-phase systems of polyethyleneglycol-phosphate. 12th ASEAN Food Conference 2011, June 16-18, 2011. BITEC, Bangkok, Thailand.

24. **Klomklao, S**., Benjakul, S. and Kishimura, H. Purification and biochemical properties of trypsin from the pyloric ceca of pectoral rattail (*Coryphaenoides pectoralis*). 12th ASEAN Food Conference 2011, June 16-18, 2011. BITEC, Bangkok, Thailand.

25. Chaijan, M., Panpipat, W., Benjakul, S., **Klomklao, S.** and Riebroy, S. 2012. Characterization of dark and ordinary muscles from frigate mackerel (*Auxisthazard*). International Conference on Food and Applied Bioscience, February 6-7, 2012. Kantary Hills Hotel, Chiang Mai, Thailand.

26. Wongwichian, C., Chaijan, M. and **Klomklao, S.** 2012. Physicochemical instability of muscles from two species of scad during iced storage. International Conference on Food and Applied Bioscience, February 6-7, 2012. Kantary Hills Hotel, Chiang Mai, Thailand.

27. **Klomklao, S.,** Chaijan, M. and Benjakul, S. 2012. Purification and biochemical characteristics of trypsin from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). International Conference on Food and Applied Bioscience, February 6-7, 2012. Kantary Hills Hotel, Chiang Mai, Thailand.

28. **Klomklao, S**.and Benjakul, S. 2012. Production and characterization of protein hydrolysate from tooth ponyfish (*Gazza minuta*) muscle using hybrid catfish (*Clarias macrocephalus* × *Clarias gariepnus*) viscera extract. Food Innovation ASIA Conference 2012, June 14-15, 2012. BITEC, Bangkok, Thailand.
29. Wongwichian, C., Chaijan, M. and **Klomklao, S**.2012. Characteristics and gel-forming ability of surimi from oxeye scad (*Selar boops*) and shrimp scad (*Alepes djedaba*). Food Innovation ASIA Conference 2012, June 14-15, 2012. BITEC, Bangkok, Thailand.
30. **Klomklao, S**., Benjakul, S., and Kishimura, H. 2012. 24 kDa Trypsin: A predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). 12th the Annual Thailand Research Fund Meeting, October 10-12, 2012. Holiday Inn Resort Reagent Beach, Cha-Am, Petchburi, Thailand.
31. Chaijan, M., Panpipat, W., Wongwichian, C., Riebroy, S., **Klomklao, S.** and Benjakul, S. Prooxidative activity of catfish myoglobin on lipid oxidation. Eurofoodchem XVII, May 7-10, 2013. Istanbul, Turkey.
32. **Klomklao, S**.and Benjakul, S. 2013. Functional properties and antioxidative activity of protein hydrolysates from toothed ponyfish muscle treated with hybrid catfish viscera extract. The 15th Food Innovation ASIA Conference 2013, June 13-14, 2013. BITEC, Bangkok, Thailand.
33. **Klomklao, S**.Pinyo, P. and Heemlah, S. 2013. Recovery and biochemical characteristics of trypsin inhibitor from sangyod rice. The 15th Food Innovation ASIA Conference 2013, June 13-14, 2013. BITEC, Bangkok, Thailand.

7.3 บทความเชิงวิชาการ

Klomklao, S. 2008. Digestive proteinases from marine organisms and their applications. Songklanakarin J. Sci. Technol. 30: 37-46.

7.4 Book Chapter

1. Benjakul, S., **Klomklao, S.** and Simpson, B.K. 2010. Enzyme in Fish Processing. In Enzyme in Food Technology, (R.J. Whitehurst and M.V.,Oort, eds.). pp.211-235, UK: Blackwell Publishing.
2. Simpson, B.K., Rui, X. and **Klomklao, S.** 2012. Enzymes in Food Processing. In Food Biochemistry and Food Processing, (B.K. Simpson, ed). pp. 181-206, UK: John Wiley & Sons, Inc.
3. **Klomklao, S.**, Benjakul, S. and Simpson, B.K. 2012. Seafood Enzymes: Biochemical Properties and Their Impact on Quality. In Food Biochemistry and Food Processing, (B.K. Simpson, ed). pp. 207-284, UK: John Wiley & Sons, Inc.
4. Simpson, B.K., Benjakul, S. and **Klomklao, S.** 2012. Natural Food Pigment. In Food Biochemistry and Food Processing, (B.K. Simpson, ed). pp. 704-722, UK: John Wiley & Sons, Inc.

7.5 การทำวิจัยและการฝึกอบรม

- Participated in workshop on “Quality and Functionality of Meat” at Faculty of Agriculture Technology, King Mongkut’s Institute of Technology Ladkrabang. Bangkok, Thailand (April 2-8, 2004).

- Participated in Seminar on “Optimization techniques and strategic design for biomolecules purification” at Department of Biochemistry, Faculty of Science, Prince of Songkla University, Thailand (October, 2004).

- Participated in international seminar on “Effective utilization of marine food resources” at Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand (December, 2003).

- Received Scholarship from Commission on Higher Education to conduct the research on ‘Purification and Characterization of Proteinases from Tuna Spleen' at Hokkaido University, Hokkaido, Japan (April, 2005 – March, 2006).

- Received Scholarship from Thailand Research Fund under the Royal Golden Jubilee Ph. D. Program (PHD/0216/2546) to conduct the research on ‘Purification and Characterization of Trypsin from the Pyloric Caeca of Bluefish and Bonito and Recovery and

Characterization of Carotenoprotein from Shrimp Waste by Bluefish trypsin' at McGill University, Macdonald Campus, Quebec, Canada (May – November, 2006).

- Received Scholarship from Commission on Higher Education under UMAP Program to conduct the research on 'Heat Stable Trypsin Inhibitor in Adzuki Bean: Isolation and Characterization' at Tokyo University of Marine Science and Technology, Tokyo, Japan (April – May, 2008).

7.6 รางวัล

- Recipient of 3rd place in the 1st FoSTAT Quiz Bowl, 2001

- The excellent poster presentation in The Poster Competition at the 9th Agro-Industrial Conference, June 14-15, 2007. BITEC, Bangkok, Thailand

- Faculty Outstanding Thesis Award, Prince of Songkla University, Year 2007

- University Outstanding Thesis Award, Prince of Songkla University, Year 2007

- The Best Ph.D. Thesis Award, Agro-Industry Academic Council Association (AIAC), Year 2008.

-The best poster presentation in The Poster Competition at the 11th Agro-Industrial Conference, June 18-19, 2009. BITEC, Bangkok, Thailand

- TSU Top Publication Award, Year 2009

- Recipient of 2nd place in The Poster Competition at the 2nd Chiang Mai University Agro-Industrial Conference, November 22-23, 2010. Chiang Mai, Thailand

- TSU Top Publication Award, Year 2010

- Faculty Top Beloved Lecturer Award, Thaksin University, Year 2010

- Outstanding TRF Research Project Year 2011

- Faculty Top Beloved Lecturer Award, Thaksin University Year 2011

- Recipient of 2nd place in The Poster Competition at the International Conference on Food and Applied Bioscience, February 6-7, 2012. Kantary Hills Hotel, Chiang Mai, Thailand.

- Recipient of 2nd place in TSU Best Science and Technology Research Award, Year 2012

- Recipient of 2nd place in TSU Top Citation Research Award, Year 2012

7.7 Ad hoc Reviewer for:

Journal of Food Biochemistry

Journal of the Science of Food and Agriculture

Comparative Biochemistry and Physiology, Part B

Journal of Food Science

Food Hydrocolloids

Food Chemistry

Journal of Agricultural and Food Chemistry

International Journal of Food Science and Technology

Journal of Food and Nutritional Research

Biotechnology and Bioprocess Engineering

Journal of Medicinal Food

Process Biochemistry

Journal of Aquatic Food Product Technology

Fisheries Science

International Aquatic Research

Cereal Chemistry

African Journal of Agricultural Research

African Journal of Biotechnology

Fish Physiology and Biochemistry

International Food Research Journal

Preparative Biochemistry and Biotechnology

Phytochemistry

Marine Drugs

Walailak Journal of Science and Technology

Songklanakarin Journal of Science and Technology

Chiang Mai Journal of Science

7.8 Invited speaker:

Klomklao, S., Benjakul, S. and Simpson, B.K. 2006. Useful enzymes from seafood processing discards. Fishery & Aquaculture By-Products Workshop. Oct 22-24, 2006. Fisheries and Marine Institute, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

7.9 Collaborative universities/institutions

1. Department of Food Technology, Faculty of Agro-Industry, Prince of SongklaUniversity, Hat Yai, Thailand
2. Department of Food Technology, School of Agricultural Technology, WalailakUniversity, Nakhon Si Thammarat, Thailand
3. Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Japan
4. Department of Food Science and Agricultural Chemistry, McGill University, Canada.
5. Department of Food Science and Technology, TokyoUniversity of Marine Science and Technology, Japan.