Obtaining of functional components from cooked shrimp (*Penaeus vannamei*) by enzymatic hydrolysis

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ABSTRACT

Non-commercial cooked Whiteleg shrimp (*Penaeus vannamei*) was valorized by application of protein hydrolysis treatments with different proteases (Giant catfish (*Pangasianodon gigas*) viscera proteases, commercial trypsin, and Alcalase[®]) for the obtainment of functional components (protein hydrolysates and carotenoids). Functional properties and *in vitro* inhibitory effect of the resulting protein hydrolysates on Dipeptidyl

Peptidase-IV (DPP-IV) and Prolyloligopeptidase (PO) were evaluated. After hydrolysis, more than 73% of protein was recovered in soluble form, whereas 11-15% was insoluble. Carotenoids, determined as astaxanthin, were mainly present (>97%) in the insoluble protein fraction, presumably in form of complexes of high molecular weight. Protein hydrolysates showed excellent solubility (>97%) in a wide pH range (3-10), good oil binding capacity (0.86-1.83 g oil/g hydrolysates) and discrete inter-facial properties. Besides, all shrimp hydrolysates at concentration of 1 mg/mL provided DPP-IV inhibition activity (22.7-61.7%) and those prepared with trypsin and Alcalase[®] also inhibited PO (inhibition values of 35-40%). The enzymatic processing of non-commercial boiled shrimp could be a useful way to valorize it, obtaining soluble proteins/peptides, potential hypoglycemic and antidepressant compounds (DPP-IV and PO inhibition peptides) and astaxanthin with interesting functional properties for food applications.

Keywords:

Carotenoids, Dipeptidyl peptidase-IV, Giant catfish, Prolyloligopeptidase, Shrimp

1. Introduction

Global shrimp production has increased rapidly in response of increasing demand by consumers for seafood. Whiteleg shrimp (*Penaeus vannamei*), or generally known as Pacific white shrimp, is a variety of prawn of the eastern Pacific Ocean ordinarily captured or raised for food. Pacific white shrimp is the major species for farmed shrimp which globally accounted for over 3.1 million tons in 2012 (FAO, 2012). Today, it is widely farmed in Thailand, Indonesia, Malaysia, Vietnam, India, Ecuador, Mexico and Brazil. Especially in Thailand, more than 500,000 tons of frozen fresh or cooked shrimp are exported each year to

other countries (FiFAD, 2015). However, products that do not meet quality control standards or expire before sale after long frozen storage periods are not suitable for consumption. Instead of trading or discard, these products could be interesting sources of high value substances such as carotenoids, chitin or protein hydrolysates with functional or bioactive properties (Arancibia et al., 2014; Babu et al., 2008; Sila et al., 2015). Crustacean species such as crab, shell, crayfish, shrimp and lobster etc. have been investigated as a potential source of proteins, chitin and carotenoids. In crustaceans, stable complexes of carotenoids bounding to high-density lipoproteins are generally recognized as carotenoproteins (Shahidi & Brown, 1998). Astaxanthin is the predominant pigment isolated from shrimp and is mainly found in the carapace and internal organs, which are usually discarded after industrial processing. Natural astaxanthin is a better and cheaper alternative to that synthetically produced, which contains a mixture of stereoisomers.

Astaxanthin has interesting applications in aqua-feeds and in surimi-based products. Different techniques have been used to recover carotenoids from shrimp by-products in the form of carotenoprotein, such as organic solvents extraction (Shachindra & Mahendrakar, 2005; Babu et al., 2008), lactic fermentation (Armenta-López & Guerrero-Legarreta, 2002), oil process extraction (Shachindra et al., 2006; Handayani et al., 2008; Pu et al., 2010), supercritical carbon dioxide extraction (Babu et al., 2008), alkaline treatment (Khaniki et al., 2013) and autolytic process (Sowmya et al., 2012; Cahú et al., 2012).

Apart from aforementioned techniques, carotenoproteins have also been isolated from crustacean processing discards by enzymatic process (Chakrabarti, 2002; Babu et al., 2008; Klomklao et al., 2009; Sila et al., 2012; Cahú et al., 2012; Khaniki et al., 2013; Senphan & Benjakul, 2014; Sila et al., 2014). Hence, the recovery of these valuable components from by-products or underutilized parts would not only help the crustacean manufacturers, but also

would minimize the environmental pollution problems. Protein hydrolysis is a regular mean to treat fish or shrimp by-products for recovering of bioactive molecules while obtaining protein hydrolysates show a wide range of functional of physiological activities (Kim & Mendis, 2006). Hydrolysis is easily scalable to industrial practice, as well as it provides highly nutritive protein hydrolysates. Different studies have shown that protein hydrolysis is productive to isolate soluble protein out from the lipid fraction and insoluble residues. As well, protein hydrolysis may make possible the obtaining of peptides with interesting nutritional and functional properties (Kristinsson & Rasco, 2000). Additionally, other bioactive molecules, such as chitin (for chitosan production) and carotenoids from crustacean by-products can be successfully extracted by protein hydrolysis. Hence, the recovery of biomolecules from shrimp processing waste by commercial proteases has been widely reported (Babu et al., 2008; Sila et al., 2014; Sila et al., 2015). However, one of the important hurdles is the high cost of commercial enzymes. Alternatively, digestive enzymes from shrimp or fish viscera can be recovered and be successfully used as seafood processing aids (Senphan et al., 2014; Bougatef, 2013), i.e. for the acceleration of fish sauce fermentation (Klomklao et al., 2006), or the extraction of carotenoprotein from shrimp waste (Klomklao et al., 2009; Sila et al., 2012).

Recently, a large number of studies have been published reporting different biological activities of peptides obtained from crustacean wastes including antioxidant (Guerard et al., 2007) and antihypertensive activities (inhibiting angiotensin I-converting enzyme, ACE) (Cheung & Li-Chan, 2010). Antioxidant peptides obtained from *Penaeus japonicus* (Suetsuna et al., 2000) and ACE-inhibitory peptides obtained from *Acetes chinensis* (Hai-Lun et al., 2006) have been identified. Moreover, fish protein hydrolysates with ability to inhibit dipeptidyl peptidase IV (DPP-IV, potential anti-glycaemics) and prolyloligopeptidase (PO,

potential anti-depressors or memory enhancers) have been recently reported (Sila et al., 2015).

Lately, type 2 diabetes (T2D) is one of the fastest growing health problems. Incretins such as glucagon like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) were cleaved by DPP-IV resulting in a loss in their insulintropic activity. To increase the half-life of the active GLP-1 and GIP, synthetic DPP-IV drug inhibitors are used. Thus, blocking DPP-IV by DPP-IV inhibitors represent alternative agent of anti-diabetes for *T2D treatment* (McIntosh et al., 2005; Power et al., 2014). Furthermore, major depression, mania, schizophrenia and senile dementia of the Alzheimer's type are important neuropathological disorders related to abnormal PO levels. In the last years, different inhibitors of PO have been developed as potential therapeutic drugs treating neurological disorders (Lawandi et al., 2010).

In this study, functional protein hydrolysates and carotenoids were obtained from cooked shrimp of low commercial value by using different proteases (Giant catfish viscera protease, commercial trypsin or Alcalase[®]). Solubility, oil binding capacity, foaming and emulsifying properties of the hydrolysates were determined, as well as their anti-DPP-IV and anti-PO activities.

2. Materials and Methods

2.1. Preparation of giant catfish viscera proteases.

Giant catfish (*Pangasianodon gigas*) viscera proteases (GT) were prepared according to Ketnawa et al. (2013). The viscera from 3-year-old (25-30 kg/fish) were obtained from

Charun Farm, Chiang Rai, Thailand. They were packed in polyethylene bag, kept in ice and transported to the Food Technology Laboratory, Mae Fah Luang University within 60 min. The whole fish viscera were cut into small pieces and homogenized with extraction buffer (10 mM Tris-HCl pH 8.0, containing 10 mM CaCl_2) in the ratio of 1:5 (w/v) for 2 min. The mixture was centrifuged at $10,000\times g$ (Avanti J-30I, Beckman Coulter, Brea, CA, USA) for 10 min at 4°C . The pellet was discarded and the supernatant was collected and used as raw material for extracting visceral proteases by aqueous two phase system (ATPS). The ATPS was prepared in the total volume of 40 grams and using 50 mL graduated centrifuge tubes. The supernatant (70%, w/w) and polyethylene glycol molecular weight of 2000 Da (PEG2000, 15%, w/w) were firstly added to the tubes and finally 15% (w/w) of sodium citrate was added. The mixtures were mixed thoroughly for 15 min using a Vortex mixer. Phase separation was achieved by centrifuging at $4000\times g$ (LegendX1R, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 4°C . After the upper phase of 1st ATPS cycle was obtained, another 10% (w/w) sodium citrate was added to generate the 2nd ATPS cycle, mixed and centrifuged as previously mentioned. The upper phase from the 2nd ATPS included the viscera enzymes and was freeze-dried and stored at -20°C until used.

2.2. Sample hydrolysis and degree of hydrolysis.

The enzymes employed for sample hydrolysis were an extract from giant catfish viscera (G), commercial porcine trypsin (T), and Alcalase[®] (A) (T and A from Novozymes Company, Bagsvaerd, Denmark). An amount of 1 Kg of boiled shrimp (Angulas Aguinaga Factory, Burgos, Spain) stored at -20°C for 20 months was minced with a blender and aliquots of 250 g were further mixed with three volumes of 0.1 M Tris-HCl buffer at pH 8.0 and previously warmed at 50°C . The samples were then hydrolyzed by the enzymes (8 U/g

protein sample) at 50°C, pH 8 for 3 hours. The hydrolysis processes were always controlled by a pH-stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). For comparative purposes, a control sample was processed in the same way without adding enzymes. At the end of the experiment, the mixtures were heated at 95°C for 20 min to inactivate proteases. The samples were then filtered through two layers of sheet cloth to separate the big particles out. The big particles obtained from filtering (mainly carapace and/or meat) were referred to “solid material”. Then, the obtained liquid was centrifuged at 12,000×g at 5°C for 30 minutes. The supernatants and precipitates were then collected and referred to hydrolysates and precipitate, respectively. One aliquot of each supernatant was lyophilized. The liquid and dried hydrolysates, as well as the precipitates, were stored at -20°C (2 days) for further analysis. The degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved with respect to the total number of peptide bonds, was calculated by the method described by Adler-Nissen (1979) as follows:

$$DH = \left(\frac{B \times N_b}{\alpha \times M_p \times h_{tot}} \right) \times 100$$

where B is the amount of NaOH consumed to keep the pH constant during the reaction, N_b is the normality of NaOH, M_p is the mass of the protein substrate in the reaction (determined as $N \times 6.25$), α is the average degree of dissociation of the α -NH₂ groups released during hydrolysis ($\alpha = 10^{(pH-pK_a)}/1 + 10^{(pH-pK_a)}$) and h_{tot} is the total number of peptide bonds per unit weight of shrimp, calculated from the amino acid composition.

2.3. Compositional analysis.

The moisture and ash content of the different fractions (solid material, hydrolysates and precipitate) of each sample were determined following AOAC 950.46 and 923.03 methods, respectively (AOAC, 2000). Protein in solid materials was extracted by 3% (w/v) NaOH (ratio 1:12.5, w/v) and incubated at 95°C in water bath for 10 min before adjustment of volume up to 50 mL using distilled water. The extracted protein solution was neutralized by 3% (v/v) HCl before determination of total nitrogen content with a LECO FP-2000 nitrogen/protein analyzer (LECO Corp., St. Joseph, MI, USA). The total protein content was calculated by using a nitrogen-to-protein conversion factor of 6.25. Fat content was determined according to Bligh and Dyer (1989). The amount of PEG2000 that left in enzyme was determined by using a hand refractometer (Atago N1-E, Japan) and calculated based on standard curve of PEG2000. All measurements were expressed on a dry weight basis and were performed in triplicate.

2.4. Astaxanthin extraction and quantification.

Solid materials, freeze-dried hydrolysates and precipitates were mixed with ethyl acetate in 1:1 ratio and then vigorously shaken at 180 rpm/min by a rotary shaker (platform type, Model G2, New Brunswick Scientific, Edison, NJ, USA) for 1 hour, at room temperature and kept protected from light. The solvent was then removed by filtering through Whatman[®] filter paper No.1 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and the residue repeatedly extracted with fresh solvent by shaking for 15 min until colorless. The extracts were pooled together in the same round flask and the ethyl acetate was further evaporated under vacuum at 40°C using a rotary evaporator (Rotary evaporator, Stuark, UK). In triplicate, appropriate dilutions of the extracts in hexane were measured at 470 nm by

using an UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Kyoto, Japan). The concentration of astaxanthin was calculated according to the following equation:

$$\text{Astaxanthin (mg)} = (A \times V \times 597) / 125100$$

where A is the absorbance at 470 nm, V is the dilution volume (mL), 597 is the molecular weight of astaxanthin and 125100 is the molar absorption coefficient reported by Britton (1995).

2.5. Functional properties of hydrolysates

2.5.1. Solubility

Solubility of the protein in the hydrolysates as a function of pH (3, 6, 8 and 10) was determined as described by Zeng et al. (2013) with some modifications. Briefly, 0.25 mg of lyophilized hydrolysates was suspended in 20 mL of buffer solution (0.1 M sodium acetate pH 3.0, 0.1 M phosphate buffer pH 6 or 8 and 0.1 M carbonate-bicarbonate pH 10) and then vortexed for 30s every 5 min, at room temperature (25°C±1). After 30 minutes, the mixtures were centrifuged at 5000×g/15min/20°C, the soluble fraction drained and the tubes containing the insoluble fraction further dried in a hot air oven (90°C) (SNB-100, Memmert, Schwabach, Germany) until constant weight. Solubility was calculated according to the formula.

$$\text{Solubility (\%)} = \left(\frac{W_0 - W_1}{W_0} \right) \times 100$$

where W_0 is the sample weight and W_1 is the weight of the dry precipitate after centrifuging. All tests were carried out in triplicate.

2.5.2. Foaming properties

Foam expansion (FE) and foam stability (FS) of the hydrolysates were tested using a slightly modified version of the method described by Gimenez et al. (2009). The dried hydrolysates were dissolved in 25 mL of water at different concentrations (0.5%, 2% and 4%, w/v) and homogenized with an Ultraturrax T25 blender (Janke and Kunkel IKA-Labortechnik, Staufen, Germany) at a speed of 14,000 rpm for 1 min, in a 50 mL cylinder, to form foam. The total volume was measured after whipping. Foam capacity was expressed as foam expansion, which was calculated according to the following equation:

$$FE(\%) = \frac{(V_T - V_0)}{V_0} \times 100$$

where V_T refers to the total volume after whipping (mL) and V_0 to the volume before whipping. The experiment was performed at least in triplicate.

Foam stability was calculated as the volume of foam remaining after 30 and 60 min using the following equation:

$$FS(\%) = \frac{(V_t \times 100)}{V_{0min}}$$

Where V_t refers to the total volume after leaving at room temperature for different times (0.5, 1, 5, 10, 20, 30 and 60 min) and V_{0min} refers to the volume after whipping at time 0. The experiment was performed at least in triplicate.

2.5.3. Emulsifying properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) of the hydrolysates were determined according to Pearce and Kinsella (1978) with slight modifications. Sunflower oil (Coosol[®], Seville, Spain, 1 mL) and different concentrations (0.1%, 0.5% and 1%, w/v) of dried hydrolysates in water (3 mL) were mixed and homogenized at a speed of 18,000 rpm for 1 min using a T25 Ultraturrax blender. Aliquots of the emulsion (100 μ L) were taken from the bottom of the container at 0 and 10 min after homogenization and diluted to 10 mL with 0.3% SDS solution. The absorbance of the diluted solution was measured at 500 nm. The absorbance measured immediately (A_0) and 10 min (A_{10}) after emulsion formation was used to calculate the emulsifying activity index (EAI) as follows:

$$EAI \left(\frac{m^2}{g} \right) = \frac{(2 \times 2.303 \times DF \times A)}{(C \times \theta \times 10,000 \times \Phi)}$$

Where DF is the dilution factor (200); A is the absorbance at 500 nm; C is the protein concentration (g/mL); θ is the disperse phase volume fraction (0.25); and Φ is the optical path (0.01 m).

The absorbance measured immediately (A_0) and 10 min (A_{10}) after emulsion formation was also used to calculate the emulsion stability index (ESI), as follows:

$$ESI(\text{min}) = \frac{A_0}{\Delta A} \times \Delta t$$

where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min. All determinations were means of at least five measurements.

2.5.4. Oil-holding capacity

Oil-holding capacity was determined according to Choi et al. (2009). One gram of dried hydrolysates was transferred into an accurately weighed 50 mL-centrifuge tube. Sunflower oil (20 mL) was added and the mixture thoroughly mixed and vortexed every 5 min, at room temperature ($25^{\circ}\text{C}\pm 1$). After 20 min, the samples were centrifuged ($5000\times g$, 10 min), the free oil decanted and the fat adsorption of the sample was determined from the weight difference. Oil-holding capacity of each sample was assayed in triplicate and the results were calculated according to the following equation:

$$\text{Oil - holding capacity (\%)} = \left(\frac{W_f - W_0}{W_0} \right) \times 100$$

Where W_0 is the initial weight of the samples and W_f is the weight of the samples after test.

2.6. Dipeptidyl peptidase-IV (DPP-IV) inhibiting activity

DPP-IV-inhibiting activity of each hydrolysates was measured according to Tulipano et al. (2011) with slight modifications. Briefly, 10 μL of DPP-IV (10.64 mUnits) from porcine kidney (Sigma-Aldrich, San Luis, MO, United States) were incubated with 190 μL of 100 mM Tris-HCl buffer, pH 8 (control) or 190 μL of sample in buffer (1 $\mu\text{g}/\mu\text{L}$), at 30°C for 15 min. The reaction started after addition of 100 μL of assay buffer containing the chromogenic substrate (H-Gly-Pro-AMC-HBr) at a final concentration of 25 μM . The change in fluorescence at 355 nm/460 nm was monitored at 1-min intervals for 15 min in an Appliskan Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Recorded data were plotted versus time. The DPP-IV inhibitory activity of the samples was expressed as percentage of inhibition. Logarithmic regression was used to calculate the IC₅₀ value, or concentration of sample needed to inhibit 50% of DPP-IV activity.

2.7. *Prolyloligopeptidase (PO) inhibiting activity*

PO-inhibiting activity of each hydrolysates was evaluated according to Yoshimoto et al. (1980) with a slight modification. Ten microliters (500 μ Units) of PO from *Flavobacterium meningosepticum* (Seikagaku Corporation, Tokyo, Japan) were incubated with 190 μ L of 0.1 M phosphate buffer (pH 7.0) with 1 mM EDTA (control) or 190 μ L of sample in buffer (1 μ g/ μ L) at 30°C for 15 min. The reaction was started by adding 100 μ L of 0.01 mM of Z-Gly-Pro-AMC (chromogenic substrate). The change in fluorescence at 355 nm/460 nm was monitored at 1-min intervals for 15 min in an Appliskan Multimode Microplate Reader. Recorded data were plotted versus time. The PO inhibitory activity of the samples was expressed as percentage of inhibition.

2.8. *Statistical analysis*

SPSS Statistic Program (Version 16.0) (SPSS Inc, Chicago, IL, USA) was used for data analysis. All data was subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. Significance of differences was defined at $P < 0.05$.

3. Results and Discussion

3.1. The effect of protease type on protein and astaxanthin recovery

The Alcalase[®] hydrolysis showed the highest DH (5.49%), followed by the hydrolysis with the viscera proteases (2.65%) and with pancreas trypsin (2.28%). After hydrolysis, three different fractions were collected: solid material (mainly constituted by carapace and/or meat), protein hydrolysates (soluble fraction after centrifugation) and precipitate (insoluble fraction after centrifugation). The proximate composition of these fractions is depicted in Table 1. Moisture was the main component of all fractions and lipids were only found in the precipitates and in the solid material. Lipids were absent in hydrolysates because they were most prone to centrifuged out with insoluble protein and precipitants. The ash content of hydrolysates and precipitants was relatively high. Kristinsson and Rasco (2000) also reported that ash content is frequently high in fish protein hydrolysates. The highest values were found in fractions obtained after treatment of shrimp with sample G, mainly because of the amount of viscera extract used (200 g) was considerably higher than that of trypsin (789 mg) or Alcalase[®] (1 mL). It also explains the high amount of PEG2000 found in the supernatant obtained after hydrolysis with the viscera extract.

The highest amount of protein, in relative terms (approx. 48% of total protein) was found in the solid material from control sample. In contrast, the solid material of the hydrolyzed samples was mainly composed of uncolored carapace and showed a low amount of protein (approx. 9-11% of total protein), that is indicating that the proteases were efficient in the extraction of protein from shell. A relevant amount of soluble protein was found in the supernatant from control sample (approx. 40% of the total protein, Figure 1) but always lower than that of the supernatants from hydrolyzed samples (approx. 75-78%). The amount of soluble protein obtained was higher at increasing DH, as also obtained by Kristinsson and Rasco (2000). The amount of non-soluble protein (precipitants) of samples hydrolyzed was

lower than that of the control sample. These results suggest that the enzymatic hydrolysis of cooked shrimp released soluble peptides and free amino acids from insoluble protein, as well as that the intact protein may also be bound to lipids in the mixture and be removed with the lipids during centrifugation.

The relative astaxanthin content in the different fractions obtained after hydrolysis is shown in Figure 2. Astaxanthin was not found in the supernatants, indicating that carotenoids were probably recovered in the form of insoluble complexes of high molecular weight. These complexes remained primarily in the solid material of the control sample (approx. 91%) and in the precipitates (approx. 97-98% of the total content) of the hydrolyzed samples. At the same hydrolysis time, total carotenoid extraction of cooked shrimp was much higher when proteases were used (536-702 $\mu\text{g}/100\text{ g}$ of sample, Table 2). The highest efficiency was achieved with Alcalase[®] and the lowest with the viscera extract. The amount of astaxanthin extracted from cooked shrimp is lower than that reported by different authors from raw shrimp, mainly because of high amounts of astaxanthin were previously extracted during boiling. Senphan and Benjakul (2014) used trypsin from shrimp hepatopancreas (5-30 U/g shrimp shell) for recovery of carotenoproteins from shells of Pacific white shrimp and provided a recovery of 0.50 mg/g sample. Klomklao et al. (2009) used trypsin from bluefish viscera (1.2 U/g) for recovery of carotenoprotein from black tiger shrimp shells and reported a recovery of around 90 $\mu\text{g}/\text{g}$ sample. The increase in the amount of trypsin used for these authors to hydrolyze shells also resulted in a higher recovering of protein.

3.2 Functional properties of hydrolysates

3.2.1. Solubility

It is known that enzymatic methods can improve functional properties of protein. A major structural change caused by enzymatic breakdown of protein leads to gradually cleaved protein into smaller peptide units so increasingly higher solubility than the intact protein. Besides, solubility is one of the most important functional properties of proteins and can also affect other functional properties, such as emulsification, foaming and gelling (Sila et al., 2015). In this work, three different proteases were used to solubilize cooked shrimp protein. The solubility of the protein in the hydrolysates and control obtained after processing of cooked shrimp was tested in the pH range of 3 to 10, as shown in Figure 3. The hydrolysates had in all cases good solubility (>96%), which was in almost all cases higher than that of the control sample, especially at pH 3. The highest solubility of the hydrolysates was observed at pH 8 (>99%), followed by pH 10 (97-99%), 6 and 3 (97-98% for both). *The solubility of Barbel skin gelatin hydrolysates hydrolyzed by Barbel crude acid protease extract was around 80% in acidic conditions (pH 2-5) and at alkaline conditions (pH 7-11)* (Sila et al., 2015). The solubility slightly decreased around the isoelectric point due to the effect of the pH solution on solubility of protein hydrolysates (Kristinsson & Rasco, 2000; Gbogouri et al., 2004). Protein hydrolysates generally show the lowest solubility at their isoelectric points and the highest when maximally charged (Chobert Bertrand-Harb, & Nicolas, 1988; Kristinsson & Rasco, 2000). The balance of hydrophilic and hydrophobic forces of peptides is another important cause of solubility enhancement. The small peptides released from proteins have proportionally more polar residues, with the ability to form more hydrogen bonds with water and therefore increasing solubility (Gbogouri et al., 2004).

3.2.2. Foaming properties

The foaming expansion (FE) and foam stability (FS) of the different supernatants was calculated at 0 min after whipping (Table 2 and 3). The lowest FE was obtained with the

control sample at all concentrations tested and the highest was obtained with the hydrolysates produced by commercial trypsin and/or viscera proteases. It was found that FE increased significantly with increasing concentration (from 0.5 to 4%, w/v) ($P < 0.05$). At a concentration of 2%, the foaming capacity of hydrolysates hydrolyzed by viscera proteases, commercial trypsin and Alcalase[®] was 92%, 92% and 88%, respectively, whereas it was only 60% for non-hydrolyzed sample. This might be due to hydrolyzed proteins in dispersions cause a lowering of the surface tension at the water/air interface, thus creating foam (Ktari et al., 2012). An improvement in foaming capacity for enzymatically modified food protein has been reported. Several works have also reported the increment of foam capacity in fish protein hydrolysates when compared to non-hydrolyzed samples. Besides, foam expansion seems to be a positive relationship with concentration (Foh et al., 2012; Krati et al., 2012). The high foaming capacity suggested an increase in surface activity, due to the initially greater number of polypeptide chains that arose from partial proteolysis, which allowed more air to be incorporated and probably because high concentration of protein produces an increase in the thickness of interfacial films (Zayas, 1997). From the view of practical application, shrimp hydrolysates could be used as good foaming agents due to high ability to adsorb rapidly at air-water interface during bubbling.

The foaming stability (FS) is an important property of food dispersions, since consumer perception of quality is influenced by appearance. The highest FS was achieved with the control sample at concentrations higher than 0.5% (Table 3). At all concentrations used, foaming stability decreased significantly with time. At a concentration of 2%, the foaming capabilities after 30 min were 64.3%, 59.8% and 58.9% for G, T and A, respectively. Besides, the FS of all samples dropped along time at all concentrations tested (Table 3). The lower FS of the hydrolyzed samples could be related to the occurrence of

peptides with smaller molecular sizes, less secondary structure and less surface hydrophobicity than the original protein. These peptides could not have strength to hold stable foam as larger peptides or proteins do microscopic peptides did not have strength to hold stable foam (Kristinsson & Rasco, 2000; Ktari et al., 2012). According to Shahidi and Synowiecki (1995), the reduction of FS was due to the fact that the peptides did not have strength to hold stable foam. Increasing concentration of all samples contributed to higher stability of foam, except in G that provided the opposite trend, probably because of the PEG content could impair intermolecular bonds between proteins or hinder the formation of a stable film around the air bubbles. Similar trend was observed in the study of Tilapia hydrolysates (Foh et al., 2012) and Zebra blenny muscle protein hydrolysates (Ktari et al., 2012). Foam stability could be improved by increasing protein concentration because this could increase viscosity and facilitates formation of a multilayer cohesive protein film at the interface (Jemil et al., 2014). Thus, increasing concentrations of sample may result in stiffer foams and therefore in better foam stability (Lawal, 2004). The success of whipping agents largely depends on how long the whip can be maintained, thus, the hydrolysates could be used as aerating agents in whipped toppings, frozen desserts and bakery products.

3.2.3. Emulsifying properties.

Emulsifying activity index (EAI) and emulsion stability index (ESI) of the hydrolysates at various concentrations (0.1%, 0.5% and 1.0%, w/v) are shown in Table 4. EAI is a function of oil volume fraction and protein concentration. The mechanism of the emulsification process is the absorption of proteins to the surface of freshly formed oil droplets during homogenisation and form a protective membrane that prevents droplets from coalescing (Ktari et al., 2012). EAI and ESI of all shrimp hydrolysates decreased with increasing concentration. The hydrolysates produced by viscera proteases showed the highest

EAI, with values 4-fold higher than those of commercial trypsin and Alcalase[®]. This might be partially due to the presence of some amount of PEG in the viscera peptidases, which can act as emulsifying agent improving EAI (Castellanos et al., 2003). The non-hydrolyzed sample showed a significant higher EAI than those of Alcalase[®] and commercial trypsin. Discussing the effect of the protein concentration; it is worth noting that it was inversely related to EAI ($P \leq 0.05$). A decrease in the emulsifying ability with increasing protein concentration has been already reported for fish muscle protein hydrolysates (Ktari et al., 2012), fermented fish meat protein hydrolysates (Jemil et al., 2014), sole and squid skin gelatin hydrolysates (Giménez et al., 2009) and round scad protein hydrolysates (Thiansilakul et al., 2007). This is related to protein adsorption at the oil-water interface is diffusion controlled in low protein concentrations whereas at higher concentrations, proteins exhibit characteristic problems including aggregation, precipitation, gelation and especially increased viscosity which can limit protein-protein interactions required to form the interfacial membrane around oil droplets (Lawal, 2004; Ktari et al., 2012). Furthermore, this phenomenon may be due to an increase in protein-protein interactions with increasing concentration, resulting in a lower protein concentration at the oil-water interface. From the results, an increase in the number of peptide molecules and exposed hydrophobic amino acid residues due to hydrolysis of proteins would contribute to an improvement in the formation of emulsions. Therefore, emulsifying properties of hydrolysates were governed by the molecular properties, particularly the size of peptides and the concentration employed (Jemil et al., 2014).

With respect to the emulsion stability, sample G formed emulsion were the most stable ($P < 0.05$), whereas no differences ($P > 0.05$) were observed between the other samples and the control at the lowest protein concentration tested. However, at higher concentrations, ESI of all hydrolysates were higher than that of the control. As for the EAI, the protein

concentration was inversely related with emulsion stability. As discussed above, the presence of PEG is likely to be responsible of the higher ESI of sample G, as compared to the others. Shrimp protein hydrolysates showed are appreciable capability of stabilizing emulsions thus, indicates its potential for use as emulsifying agent in food industry.

3.2.4. Oil-holding capacity

Significant differences ($P < 0.05$) were observed in terms of the oil-holding capacity (OHC) displayed by the hydrolysates (Figure 4), being especially remarkable the OHC of sample G, which was able to bind almost 2-fold of his weight of oil. The literature agrees that hydrolyzed samples present a better oil-holding capacity than the corresponding proteins of origin, due to the release and exposure of hydrophobic residues (Sila et al., 2014). This is especially true in the present work, as the raw material employed was boiled shrimp, in which proteins are denatured and so their functional properties are very limited. OHC of protein hydrolysates can be related to their origins and their processing procedures, which determine their compositions, physical structures, porosities and particle sizes. OHC has special interest particularly for the holding of fat during industrial processing and storage or during culinary preparations, such as frying. Besides, OHC is an important functional characteristic of ingredients used in the meat and confectionery industries. Several factors may affect the ability of hydrolysates to bind fat, such as bulk density of the protein (Kinsella and Melachouris, 1976), degree of hydrolysis (Kristinsson & Rasco, 2000) and enzyme-substrate specificity (Haque, 1993). Kristinsson and Rasco (2000) reported that the fat absorption of salmon protein hydrolysates decreased with increasing DH. The same trend was also found by Tanuja et al. (2014), working with hydrolysates from striped catfish. On the contrary, the results of the present work do not show a correlation between hydrolysis degree and OHC. This can be explained by the fact that hydrolysis can liberate some peptides from the native

protein, which would enhance the flexibility of the peptides in hydrolysates. The extensive hydrolysis would break many peptide bonds, thus contributing to the decrease of the oil binding properties (Souissi et al., 2007). Discussing the absolute values of OHC, Tanuja et al. (2014) found values comparable to those obtained in the present work (0.8 to 1.6 g oil/g sample), whereas Wasswa et al. (2007) and Sila et al. (2014), working with hydrolysates from grass carp skin or shrimp by-products, found OHC values of 3.6 and 2.1 g of oil/g hydrolysates, respectively.

3.3. Assessment of DPP-IV and PO-inhibiting activities

The ability of the hydrolysates to inhibit DPP-IV activity was evaluated and the results obtained are shown in Table 5. Significant differences were found among samples ($P<0.05$). The hydrolysates exhibited higher DPP-IV inhibiting ability than the hydrolysates of the non-hydrolyzed sample. Different DPP-IV inhibiting abilities were found, being the best that of sample A (61.7%). The DPP-IV inhibiting activity of the samples was therefore strongly influenced by the protease used. The IC_{50} value of the most potent inhibitors (A and T) was calculated, being that of the sample A the lowest (0.73 mg/mL). Barbell skin gelatin hydrolysates produced by Esperase provided DPP-IV inhibition with a higher IC_{50} value (2.21 mg of dry weight/mL, Sila et al., 2014). For a trypsin whey protein hydrolysates, Silveira Martínez-Maqueda et al. (2013) reported an IC_{50} value (1.51 mg/mL) also higher than those reported in Table 5.

The samples showed slight PO-inhibiting activity (Table 5). The control sample and sample G were not able to inhibit PO, at least in the concentration tested. The hydrolysates obtained with T or A exhibited similar inhibitory activity ($P<0.05$), although it was below of 40%. The occurrence of PO-inhibiting peptides in protein hydrolysates is scarcely

documented; in fact, PO-inhibiting peptides have been documented only in salmon, cod, trout and cheese hydrolysates (Sørensen et al., 2004). The enzymes used to hydrolyze shrimp yielded peptides showing different inhibitory characteristics against both DPP-IV and PO, even though both serine proteases belong to the PO-family. A key factor of these differences in the inhibitory activity could be attributed to the impact of the enzyme's specificity influencing both the nature and composition of resulting peptides and the characteristics of hydrolysates (Sila et al., 2014).

4. Conclusion

Protein hydrolysis is an effective means to valorize non-commercial boiled shrimp, as it is possible to obtain not only protein hydrolysates with adequate functional properties for food applications and DPP-IV and PO-inhibiting peptides, but also astaxanthin. Giant catfish viscera has been shown to be a good source of enzymes, as results obtained are comparable to those of commercial trypsin and Alcalase[®] and could be used as an economic source of enzymes with applications in carotenoid extraction.

Acknowledgements

This study was financed by the Spanish Ministry of Economy and Competitiveness through project AGL2011-2760. Ms. Sunantha Ketnawa was funded by the Royal Golden Jubilee Ph.D. Program (Grant No. PHD 0113/2554) from the Thailand Research Fund. We are grateful to the Analysis Service Unit facilities of ICTAN for the analysis of total nitrogen.

Conflict of Interest

Sunantha Ketnawa, Soottawat Benjakul, Oscar Martinez-Alvarez, Joaquín Gómez-Estaca, María del Carmen Gómez-Guillén, and Saroat Rawdkuen declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects.

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Fig. 1 - Relative protein content (%) in the different fractions obtained after hydrolysis of boiled shrimp with giant catfish viscera proteases (G), trypsin (T) or Alcalase (A), as compared to those from a non-hydrolyzed control sample (N) (non-significant differences ($P > 0.05$) are highlighted with an asterisk).

Fig.2 - Relative astaxanthin content (%) in the different fractions obtained after hydrolysis of boiled shrimp with giant catfish viscera proteases (G), trypsin (T) or Alcalase (A), as compared to those of a non-hydrolyzed control sample (N). Results are expressed as mean $\pm$ SD ($n=3$). Different letters indicate significant differences ($P \leq 0.05$) in the same fraction by difference enzymes.

Fig. 3 - Solubility of the freeze-dried supernatants (mean $\pm$ SD) derived from hydrolysis of boiled shrimp as a function of pH. N: control sample; G: shrimp hydrolysate produced by giant catfish viscera proteases; T: shrimp hydrolysate produced by commercial trypsin; A: shrimp hydrolysates produced by Alcalase (non-significant differences ($P > 0.05$) are highlighted with an asterisk).

Fig. 4 - Oil-holding capacity of the freeze-dried supernatants (mean $\pm$ SD) after enzymatic processing of boiled shrimp. N: control sample; G: shrimp hydrolysate produced by giant catfish viscera proteases; T: shrimp hydrolysate produced by commercial trypsin; A: shrimp hydrolysate produced by Alcalase. Bars represent SD ($n=3$).

Table 1- Chemical composition of fractions obtained after enzymatic processing of cooked shrimp.

Fractions	Composition (%)						
	Enzyme	Protein	Lipids	Moisture	Ash	PEG	Ast*
Solid	N	17.7 $\pm$ 0.6 ^a	0.6 $\pm$ 0.0 ^c	71.0 $\pm$ 0.7 ^a	4.1 $\pm$ 0.0 ^d	NM	582.3 $\pm$ 3.0 ^a

Composition (%)							
Fractions	Enzyme	Protein	Lipids	Moisture	Ash	PEG	Ast*
materials	G	5.2±0.1 ^d	0.2±0.0 ^d	56.9±0.5 ^d	10.9±0.1 ^c	NM	13.1±0.0 ^d
	T	5.8±0.3 ^c	1.3±0.1 ^b	67.9±0.2 ^b	11.7±0.1 ^b	NM	14.9±0.1 ^c
	A	6.1±0.2 ^b	1.9±0.1 ^a	64.4±0.4 ^c	14.1±0.1 ^a	NM	22.8±0.5 ^b
	N	2.0±0.0 ^d	ND	96.6±0.0 ^a	1.0±0.0 ^c	0.0±0.0 ^b	0
	G	3.8±0.0 ^c	ND	78.9±0.0 ^d	3.2±0.2 ^a	19.4±0.5 ^a	0
Hydrolysates	T	4.6±0.0 ^a	ND	93.9±0.0 ^c	1.0±0.1 ^b	0.0±0.0 ^b	0
	A	4.0±0.1 ^b	ND	94.7±0.0 ^b	1.0±0.0 ^c	0.0±0.0 ^b	0
	N	17.3±0.0 ^a	1.2±0.0 ^c	77.4±0.6 ^c	2.1±0.1 ^c	NM	56.2±1.3 ^d
Precipitants	G	10.3±0.0 ^c	2.9±0.0 ^a	68.8±0.1 ^d	4.5±0.2 ^a	NM	536.4±1.7 ^c
	T	9.7±0.0 ^d	1.1±0.0 ^d	83.4±0.4 ^a	1.1±0.1 ^d	NM	685.7±5.9 ^b
	A	10.7±0.0 ^b	1.6±0.0 ^b	81.9±0.3 ^b	2.7±0.1 ^b	NM	702.2±1.8 ^a

Different letters in the same column mean significant differences in the composition per fraction ($P<0.05$). N: control; G: giant catfish viscera proteases; T: commercial trypsin; A: Alcalase[®]. ND: not detectable. NM: no determination. The amount of carbohydrates in shrimp shells (as chitin) was not determined. Ast*: astaxanthin ($\mu\text{g}/100\text{ g}$ of cooked shrimp). PEG: polyethylene glycol molecular weight of 2000 Da.

Table 2- Foaming expansion (%) of protein fraction from each supernatant at different concentrations.

Enzyme	Foaming expansion (%)		
	0.5	2	4
N	56±0.0 ^{cC}	60±0.0 ^{cB}	63±1.9 ^{cA}
G	80±0.0 ^{aC}	92±0.0 ^{aB}	100±0.0 ^{aA}
T	68±0.3 ^{bC}	92±0.5 ^{aB}	100±0.0 ^{aA}
A	52±0.0 ^{dC}	88±0.3 ^{bB}	99±1.9 ^{bA}

Different letters in the same column mean significant differences between hydrolysates ($P<0.05$). Different capital letters in the same line mean significant differences at different concentration ($P<0.05$). Values are given as mean $\pm$ SD from triplicate determinations ($n = 3$).

N, control shrimp hydrolysates; G, shrimp hydrolysates produced by giant catfish viscera proteases; T, shrimp hydrolysates produced by commercial trypsin; A, shrimp hydrolysates produced by Alcalase[®].

Table 3- Foaming stability (%) of protein fraction from each supernatant at different concentrations and different times.

Enzyme	Concentration	Foaming stability (%)						
	(%)	0.5	1	5	10	20	30	60

Time (min)		
N		83±2 ^a 801±1 ^a 78±2 ^a 74±2 ^a 70±1 ^a 68±1 ^a 66±1 ^a
G	0.5	84±2 ^a 75±1 ^b 69±1 ^a 64±1 ^c 63±1 ^b 62±1 ^c 60±1 ^c
T		83±3 ^a 75±1 ^a 71±2 ^a 67±1 ^b 66±2 ^b 64±1 ^c 63±0 ^b
A		83±1 ^a 80±3 ^a 75±1 ^a 75±1 ^a 73±1 ^a 72±1 ^a 68±2 ^a
N		88±2 ^a 77±2 ^a 72±2 ^a 72±2 ^a 71±1 ^a 69±1 ^b 67±1 ^a
G	2	71±2 ^c 70±2 ^b 69±1 ^b 67±1 ^b 67±1 ^b 64±1 ^b 64±0 ^b
T		89±4 ^a 82±0 ^a 72±3 ^b 61±2 ^c 61±1 ^c 60±2 ^c 58±2 ^c
A		85±4 ^a 84±3 ^a 78±4 ^a 66±4 ^b 61±2 ^c 59±3 ^c 56±2 ^c
N		96±1 ^a 95±1 ^a 86±1 ^a 82±2 ^a 74±1 ^a 70±2 ^a 65±2 ^a
G	4	65±2 ^c 64±2 ^c 64±2 ^d 61±2 ^c 62±2 ^b 62±2 ^b 61±2 ^b
T		97±1 ^a 89±2 ^b 75±1 ^c 63±1 ^c 60±1 ^c 59±1 ^b 55±1 ^d
A		94±0 ^b 92±0 ^a 84±2 ^b 71±2 ^b 64±0 ^b 60±1 ^b 58±1 ^c

Different letters in the same column mean significant differences between hydrolysates at the same time ($P<0.05$). Values are given as mean $\pm$ SD from triplicate determinations ($n = 3$). N, control shrimp hydrolysates; G, shrimp hydrolysates produced by giant catfish viscera proteases; T, shrimp hydrolysates produced by commercial trypsin; A, shrimp hydrolysates produced by Alcalase[®].

Table 4- Emulsifying properties of protein fraction from each hydrolysate at different concentrations.

Enzyme	Concentration of supernatant (%)					
	0.1	0.5	1.0	0.1	0.5	1.0

	Emulsifying activity index (m ² /g)			Emulsion stability index (min)		
N	2.11±0.02 ^{bA}	1.09±0.01 ^{cB}	0.94±0.02 ^{cC}	11.5±0.0 ^{bA}	10.0±0.0 ^{dB}	10.0±0.0 ^{cB}
G	8.05±0.08 ^{aA}	7.44±0.02 ^{aB}	6.63±0.07 ^{aC}	12.1±0.1 ^{aA}	11.6±0.1 ^{aB}	11.4±0.1 ^{aC}
T	1.93±0.01 ^{cA}	1.76±0.04 ^{bB}	1.12±0.01 ^{bC}	11.6±0.1 ^{bA}	11.4±0.1 ^{bA}	11.1±0.0 ^{bB}
A	1.24±0.03 ^{dA}	0.77±0.01 ^{dB}	0.65±0.02 ^{dC}	11.2±0.0 ^{bA}	11.1±0.0 ^{cB}	10.0±0.0 ^{cC}

Different letters in the same column mean significant differences between hydrolysates ($P<0.05$). Different capital letters in the same line mean significant differences at different concentration ($P<0.05$). Values are given as mean $\pm$ SD from triplicate determinations ($n = 3$).

N: control shrimp hydrolysates; G, shrimp hydrolysates produced by giant catfish viscera proteases; T, shrimp hydrolysates produced by commercial trypsin; A, shrimp hydrolysates produced by Alcalase[®].

Table 5- DPP-IV and PO-inhibiting activity (%) of supernatants at the concentration of 1 mg/mL.

Enzyme	DPP-IV inhibition (%)	IC ₅₀ DPP-IV (mg/mL)	PO inhibition (%)
N	14.9±3.2 ^d	-	-
G	22.7±2.1 ^c	-	-
T	44.9±2.1 ^b	0.96	39.5±3.0 ^a
A	61.7±3.3 ^a	0.73	35.2±3.9 ^a

Different letters in the same column indicate significant differences among samples ($P < 0.05$).

N: control shrimp hydrolysates; G, shrimp hydrolysates produced by giant catfish viscera proteases; T, shrimp hydrolysates produced by commercial trypsin; A, shrimp hydrolysates produced by Alcalase[®].

Accepted manuscript