Protein hydrolysates from Pacific white shrimp cephalothorax manufactured with different processes: Compositions, characteristics and antioxidative activity

To be submitted to Waste and Biomass Valorization

Sittichoke Sinthusamran [1], Soottawat Benjakul [1,*], Kongkarn Kijroongrojana[1], Thummanoon Prodpran[2], and Hideki Kishimura [3]

[1] Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

[2] Department of Material Product Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

[3] Laboratory of Marine chemical Resource Science Development, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

*To whom correspondence should be addressed. Tel: 66-7428-6334

Fax: 66-7455-8866, e-mail: soottawat.b@psu.ac.th

Abstract

Composition, characteristics and antioxidative activity of the hydrolysate from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) prepared using various hydrolysis processes were studied. Those processes consisted of autolysis (AU), hydrolysis using Alcalase at 0.5% (0.5A) or 1.0% (1.0A) and autolysis, followed by Alcalase hydrolysis at 0.5 and 1.0% (AU+0.5A and AU+1.0A). All hydrolysate samples had the higher protein content (86.04-89.24%) and lower amounts of ash (7.46-11.26%) and lipid (0.43-0.64%), compared to those of cephalothorax ($P<0.05$). The highest yield (54.04%) and protein recovery (84.15%) were observed in AU+1.0A sample, which had the maximum degree of hydrolysis (DH) (44.93%) ($P<0.05$). All hydrolysates had glutamic acid/glutamine (115.80-121.69 mg/g dry sample), aspartic acid/asparagine (84.04-90.28 mg/g dry sample), arginine (63.27-68.62 mg/g dry sample) and leucine (58.67-68.07 mg/g dry sample) as the dominant amino acids. Based on gel filtration chromatography, the hydrolysate with higher DH showed higher amount of smaller peptides with MW lower than 1,355 Da. When antioxidant activities of hydrolysates were determined, AU+1.0A sample had the highest ABTS radical scavenging activity, ferrous ion chelating activity and ORAC value, compared to others ($P<0.05$). However, the highest ferric reducing antioxidant activity and DPPH radical scavenging activity were obtained for 1.0A and AU samples, respectively ($P<0.05$). Furthermore, AU+1.0A sample showed higher protective effect against DNA damage induced by peroxyl radical than 1.0A and AU samples. Therefore, different hydrolysis processes directly affected the protein recovery, chemical composition and antioxidant activities of hydrolysate from the cephalothorax of Pacific white shrimp.

Keywords: Cephalothorax, Protein hydrolysate, Autolysis, Alcalase hydrolysis, Antioxidative activities

Statement of Novelty

The present study provides the newly developed process in utilization of cephalothorax using two-step hydrolysis, in which prior autolysis, followed by hydrolysis using Alcalase under the optimal condition was used. A new hydrolysis process potentially recovered protein from shrimp cephalothorax. The resulting hydrolysate with high yield exhibited the antioxidative activities with different modes of actions. It was also shown to prevent the DNA damage induced by radicals. Thus, the protein hydrolysate from aforementioned byproduct as the new functional ingredient can increase the revenue for the industry via the production of value added products with high nutrition. Additionally, it can reduce the disposal of byproducts, which may cause the environmental problem.

1. Introduction

Pacific white shrimp and its products have become economically important for Thailand [1]. It accounts for 90% of the global aquaculture shrimp production [2,1]. By the year 2012, frozen Pacific white shrimp and products were manufactured and exported over 500,000 tons, particularly to the USA and Japan. During shrimp processing, approximately 40-50% of its total weight are generated as by-products. Those contained 71.4% cephalothorax and 28.6% shell, which are rich in amino acids, peptides, proteins and other components [1,3]. Those leftovers generally have been transformed to animal feed and aquaculture diets [4]. Shrimp cephalothorax is an essential source of protein (50-65%, dry weight basis) and also serves as a source of lipid (11% dry weight basis), chitin (11% dry weight basis), enzymes and other nutritive components [5,4]. The utilization of shrimp cephalothorax by conversion to protein hydrolysate can be a promising approach to eliminate harmful environmental aspects as well as earn the revenue for shrimp processing industry [6].

Protein hydrolysates have attracted increasing interest as potential ingredients for many health-promoting functional foods due to biologically active peptides [5,7]. The recovery of proteins or peptides from shrimp waste by hydrolysis has been widely investigated [8,5,3,9-11]. Endogenous proteases were reported in hepatopancrease [12]. Under optimal condition, those proteases could autolysis [8]. Autolysis and enzymatic hydrolysis of cephalothorax depend on several factors including pH, temperature, time as well as enzyme/substrate ratio [6,1]. Cao et al. [8] reported that optimum autolysis condition for protein recovery of cephalothorax from *Penaens vannamei* included 50 ºC, pH 7.85 and a substrate concentration at 23% (w/v), in which the highest degree of hydrolysis (45%) was obtained. The autolysis of shrimp head by gradual increase in temperature (40-60 ºC) at 5 ºC/30 min resulted in the higher protein recovery (87.4%), compared with autolysis performed at different single temperature (40, 50, and 60 ºC) (43.6-73.6%) [5]. Additionally, a number of commercial proteases have been used for the production of protein hydrolysis from seafood processing by-products. Dey, Dora [6] suggested that protein recovery of hydrolysate from shrimp waste using Alcalase was higher than those using Neutrase, Protamex and Flavourzyme. Sila et al. [11] also documented that the protein hydrolysate from shrimp waste of deep-water pink shrimp was produced by Alcalase hydrolysis. Hydrolysate contained 80.8% protein, 2.74% lipid, 14.4% ash, 1.13% chitin and 1.08 μg carotenoid/g sample.

To maximize the hydrolysis of proteins from cephalothorax, autolysis could be exploited. The liberated protein or peptides more likely served as the proteinaceous substrate for subsequent hydrolysis by commercial proteases. The hydrolysates containing varying peptides from different hydrolysis processes might possess different compositions as well as bioactivities, especially antioxidant activity. Nevertheless, a little information regarding the prior autolysis in combination with subsequent enzymatic hydrolysis for production of

100 protein hydrolysate from shrimp cephalothorax exists. Thus, this study aimed to develop the
101 hydrolysis process rendering the hydrolysate with increased yield and antioxidative activity.
102
103 **2. Materials and methods**
104 2.1 Chemicals
105 Alcalase from *Bacillus licheniformis* (20 unit/g dry matter) was obtained from
106 Novozyme (Bagsvaerd, Denmark). 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-
107 diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
108 diammonium salt (ABTS), 2,4,6-tripyridyl-triazine (TPTZ), 6-hydroxy-2,5,7,8-
109 tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-
110 4′,4″-disulfonic acid sodium salt (ferrozine) 2,2′-azobis (2-methylpropionamidine) (AAPH)
111 and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St.
112 Louis, MO, USA). Plasmid DNA (pUC 18) and SYBR™ Gold DNA Gel Stain was
113 purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Sephadex^{TM} G-25, blue
114 dextran and gel filtration calibration kit (vitamin B12, flavin adeninedinucleotide and
115 glycine-tryrosine) were obtained from GE Healthcare (Uppsala, Sweden). All chemicals were
116 of analytical grade.
117
118 2.2 Collection and preparation of cephalothorax from Pacific white shrimp
119 Cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) were obtained from
120 the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand. Cephalothorax of shrimp
121 with the size of 50–60 shrimp/kg (5 kg) were placed in a polyethylene bag. The bag was
122 imbedded in a polystyrene box containing ice with a shrimp/ice ratio of 1:2 (w/w) and
123 transported to the Department of Food Technology, Prince of Songkla University, Hat Yai,

Songkhla, within approximately 2 h. The sample was stored at -20 ºC until used, but the storage time was not longer than 2 weeks.

2.3 Production of hydrolysate from shrimp cephalothorax

2.3.1 Preparation of cephalothorax

Frozen shrimp cephalothorax were ground using a blender (Phillips, Guangzhou, China) for 3 min. Ground sample was placed in a polyethylene bag and embedded in ice until used.

2.3.2 Hydrolysis of cephalothorax using different processes

Hydrolysis was performed following the method of Holanda, Netto [13] with a slight modification. Ground sample was mixed with distilled water at a ratio of 1:1 (w/v) and then homogenized at 11000 rpm for 2 min using a homogenizer model T25 digital (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The pH of homogenate was adjusted to 8.0 using either 1.0 M NaOH or 1.0 M HCl. Autolysis was conducted at 50 ºC for 3 h in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany). Autolysis was terminated by submersing the mixture in hot water (90 ºC) for 15 min.

For one-step hydrolysis processes, 0.5 or 1.0% Alcalase (w/w, solid content) was added into the homogenate (pH 8.0), pre-incubated at 60 ºC for 15 min. The mixture was mixed well and hydrolysis was carried at 60 ºC for 2 h. Enzyme inactivation was performed by subjecting the mixture to heating at 90 ºC for 15 min.

For two-step hydrolysis processes, the homogenate (pH 8.0), was firstly incubated at 50 ºC for 3 h for initial autolysis. After autolysis, the mixture was pre-incubated at 60 ºC for 15 min. Subsequently, Alcalase (0.5 or 1.0% w/w) was added into the mixture. Hydrolysis was conducted at 60 ºC for 2 h. Enzymatic reaction was terminated as previously described.

All the obtained mixtures were cooled down to room temperature (25 ºC) using a running water. The mixtures were filtered with two layers of cheesecloth to remove the undigested carapace and pereiopods (solid phase). The resulting filtrates were centrifuged at 4000x*g* at 4 ºC using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) for 15 min. Then, the supernatants were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) for 72 h. The hydrolysate from autolysis was named "AU", while those from one-step hydrolysis process with the aid of 0.5 and 1.0% Alcalase were referred to as "0.5A" and "1.0A", respectively. The hydrolysates attained from two-step hydrolysis process using autolysis, followed by hydrolysis with 0.5 and 1.0% Alcalase were termed "AU+0.5A" and "AU+1.0A", respectively. All the hydrolysate samples were then subjected to analyses.

2.4 Analyses

2.4.1 Proximate analysis

Ground cephalothorax and hydrolysate samples were analyzed for moisture, protein, fat and ash contents using the analytical method no. of 950.46, 920.153, 960.39 and 928.08, respectively [14]. The conversion factor used for calculation of protein content was 6.25. Chitin content was determined according to the method of Senphan et al. [9] with a slight modification. Samples (2 g) were mixed with 30 ml of 1.25 M NaOH at 100 ºC for 3 h. The mixture was filtered under vacuum using a Whatman No.1 filter paper. The residue was shaken with 30 ml of 1 M HCl for 30 min at 25 ºC, filtered and washed with distilled water. The washed residue was then homogenized with cold acetone (-20 ºC) at a speed of 13,000 rpm using a homogenizer for 3 min to remove lipids and pigments. After washing with 3 volumes of distilled water, 30 ml of 0.5% NaOCl were mixed with the sample and stirred for 30 min at 25 ºC. The mixture was then filtered and washed with distilled water. The residue

174 was dried at 60 ºC for 24 h using an oven (Memmert, Schwabach, Germany). The dried
175 matter referred to as “chitin” was weighed.
176
177 2.4.2 Yield and protein recovery
178 The yield of hydrolysate was calculated based on dry weight of initial shrimp
179 cephalothorax after drying at 105 ºC for 12 h in a hot air oven.

180 $$\text{Yield (\%)} = \frac{\text{weight of dry hydrolysate (g)}}{\text{weight of dried initial sample used (g)}} \times 100$$

181 Protein recovery of hydrolysate was calculated based on the initial amount of protein
182 present in the initial cephalothorax as determined by the method of AOAC [14].
183 Protein recovery

184 $$(\%) = \frac{\text{protein content of supernatant (g/ml)} \times \text{volume of supernatant (ml)}}{\text{protein content of initial sample used (g/g)} \times \text{weight of initial sample used (g)}} \times 100$$

185
186 2.4.3 Determination of total carotenoid content
187 Total carotenoid content was determined according to the method of Senphan et al.
188 [9] with a slight modification. After being extracted and properly diluted, the absorbance of
189 samples was read at 468 nm. The content of carotenoid in hydrolysate sample was calculated
190 using the equation given by Saito, Regier [15]:

191 $$\text{Total carotenoid (µg/g sample)} = \frac{A_{468} \times \text{volume of extract} \times \text{dilution factor}}{0.2 \times \text{weight of sample used (g)}}$$

192 where 0.2 is the A_{468} of 1 µg/ml standard astaxanthin.
193
194 2.4.4 Amino acid analysis
195 Amino acid composition of the cephalothorax and hydrolysate samples was analyzed
196 as described by Benjakul et al. [16]. The samples were hydrolyzed under reduced pressure in
197 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115 °C for 24

198 h. The hydrolyzed samples were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate
199 buffer (pH 2.2). An aliquot of 0.04 ml was applied to an amino acid analyzer (MLC-703;
200 Atto Co., Tokyo, Japan).
201
202 2.4.5 Determination of degree of hydrolysis (DH)
203 DH of hydrolysate was determined according to the method of Benjakul, Morrissey
204 [17]. Hydrolysate samples were determined for free amino group content using 2,4,6-
205 trinitrobenzenesulfonic acid (TNBS). DH was calculated as follows:

206 $$DH=\left[\frac{L_H - L_0}{L_{max} - L_0}\right]\times 100$$

207 where L_H corresponded to the amount of free amino acid in the hydrolysate. L_0 was the
208 amount of free amino acid in original shrimp cephalothorax. L_{max} was the maximum amount
209 of free amino acid in cephalothorax obtained after acid hydrolysis using of 6 M HCl. The
210 hydrolysis was run at 105 ºC for 24 h in an oil bath (BUCHI Labortechnik AG, Tokyo,
211 Japan).
212
213 2.4.6 Determination of color
214 The color of hydrolysate powders (5 g) was measured by a Hunter lab colorimeter
215 (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* values indicating
216 lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded.
217 The colorimeter was warmed up for 10 min and calibrated with a white standard. Total
218 difference in color (ΔE^*) was calculated according to the following equation [18]:

219 $$\Delta E^* = \sqrt{(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2}$$

220 where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the
221 sample and that of white plate standard (L^* = 92.84, a^* = -1.26 and b^* = 0.47).

222

223 2.4.7 Molecular weight distribution

224 Molecular weight distribution of hydrolysate samples was performed using a
225 Sephadex G-25 gel filtration column (2.5 x 50 cm) (17-0032-01, GE Healthcare Bio-Science
226 AB, Uppsala, Sweden). The sample (50 mg) was dissolved in distilled water (2 ml) and the
227 mixture was loaded onto a column. After being loaded, the elution was performed using a
228 ÄKTA chromatography system (ÄKTAprim plus, GE healthcare Bio-Science AB, Uppsala,
229 Sweden) coupled with a fraction collector. Distilled water was used as eluent at a flow rate of
230 0.5 ml/min. The fractions of 3 ml were collected and their absorbance was recorded at 220
231 and 280 nm. Blue dextran (2,000,000 Da) was used for void volume measurement. The
232 molecular weight (MW) markers, including insulin chain B (3495.89 Da), vitamin B12
233 (1355.4 Da), glycine–tyrosine (238.25 Da) and tyrosine (181.2 Da) were used. MW of
234 fraction was estimated from the plot between available partition coefficient (K_{av}) and the
235 logarithm of the molecular weight of the protein standards.

236

237 2.4.8 Determination of bitterness

238 Bitterness in the hydrolysates was examined by 5 female and 6 male panelists with the
239 ages of 25 and 33. The panelists were trained using a caffeine as a standard for a period of
240 one month, twice a week. The standard solutions at different concentrations (0, 25, 50 and 75
241 ppm) were presented. Distilled water was used to represent score of 0, while 75 ppm caffeine
242 represented the score of 15. For evaluation, 15-cm line scale anchored from “none” to
243 “intense” was used.

244 The hydrolysate samples, at a protein concentration of 2 g/100 ml, were served at
245 ambient temperature coded with three-digital random number together with reference
246 standard caffeine solution. Panelists then evaluated for bitterness of samples, compared to the

247 reference using a 15-cm line scale. Between samples, panelists were asked to eat a piece of
248 un-salted cracker and rinse their mouths thoroughly with distilled water [19].
249
250 2.4.9 Determination of antioxidative activities
251 Prior to analysis, hydrolysates samples were dissolved in distilled water to obtain the
252 concentration of 5 mg/ml. The solutions of hydrolysates were determined for antioxidative
253 activities as follows: ABTS radical scavenging activity [20], DPPH Radical Scavenging
254 Activity (DPPH) [7], ferrous ion chelating activity [7], ferric reducing antioxidant power
255 (FRAP) Sae-leaw et al. [7] and oxygen radical absorbance capacity (ORAC) [7]. Activities
256 were expressed as μmol Trolox (TE) equivalent/g sample, except for metal chelating activity,
257 which was reported as μmol EDTA equivalent/g sample.
258
259 2.4.10 Measurement of inhibitory activity toward peroxyl radical induced supercoiled
260 plasmid DNA strand scission
261 Peroxyl radical induced supercoiled plasmid DNA strand scission inhibitory activity
262 was determined as described by Kittiphattanabawon et al. [21] with slight modifications.
263 Supercoiled plasmid DNA (pUC 18) (0.025 μg/μl; 4 μl) dissolved in 10 mM Tris–HCl
264 containing 1 mM EDTA (pH 7.8) was mixed with 2 μl of different hydrolysates to obtain the
265 final concentrations of 1, 3 and 5 mg/ml. To initiate the oxidation reaction, 4 μl of 10 mM
266 AAPH were added. The mixture was incubated at 37 °C for 1 h in the dark. The controls
267 were prepared in the same manner by using distilled water instead of oxidants. After
268 incubation, 2 μl of the loading dye (0.25% bromophenol blue, 50% glycerol) were added to
269 the reaction mixture. Then, the mixture (6 μl) was loaded onto 1% agarose gel, and the DNA
270 bands were stained with SYBR gold. Electrophoresis was conducted at 100 V for 50 min
271 using a horizontal gel electrophoresis system (Mini-Sub® cell GT, Biorad, Hercules, CA,

USA) equipped with PowerPac™ basic power supply (Biorad, Hercules, CA, USA). The DNA Sub cell®bands were visualized under transillumination of UV light using Uvitec chemiluminescence Documentation System (Uvitec, Cambridge, UK).

2.5 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using the Duncan's multiple range test [22]. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Yield and protein recovery

Yield and protein recovery of the hydrolysates from cephalothorax prepared using different hydrolysis processes are shown in Table 1. Generally, yield of hydrolysate samples correlated well with protein recovery. The highest yield (54.04%) and protein recovery (84.15%) were found for AU-1.0A sample, compared to other hydrolysates ($P<0.05$). Yield of hydrolysates prepared by one-step hydrolysis process was lower than that of hydrolysates prepared using two-step hydrolysis processes ($P<0.05$). However, no difference in the yield between 1.0A and AU+0.5A samples was observed ($P>0.05$). For hydrolysate prepared using single process, in which cephalothorax were hydrolyzed by Alcalase without prior autolysis, there were no differences in yield and protein recovery between 0.5A and 1.0A samples ($P>0.05$). The result indicated that proteins of cephalothorax could be hydrolyzed to higher degree when two-step process including autolysis, followed by hydrolysis with 1.0% Alcalase was conducted. Shrimp cephalothorax contained hepatopancreas, which was an excellent source of proteases, especially trypsin and chymotrypsin [12]. Proteases in

hepatopancreas actively cleaved various proteinaceous substrates during autolysis [9]. In the present study, autolysis at 50 ºC for 3 h used in the first step of hydrolysis resulted in the cleavage of proteins to some degree by endogenous proteases. Subsequently, the partially hydrolyzed protein substrates could be more effectively hydrolyzed by Alcalase in combination with endogenous proteases at the second step of hydrolysis, particularly when higher level of Alcalase was used. This was evidenced by the higher yield and protein recovery of AU+1.0A sample. Alcalase has been widely used in hydrolysate production because of its broad specificity with the high hydrolysis efficacy [7]. Karnjanapratum, Benjakul [23] reported that the higher hydrolysis of gelatin from unicorn leatherjacket skin was observed when autolysis was conducted before hydrolysis by papain from papaya latex. Therefore, the use of two-step hydrolysis process including autolysis at 50 ºC for 3 h, followed by hydrolysis by 1.0% Alcalase for 2 h, could increase the cleavage of peptide bands in shrimp cephalothorax proteins. As a result, protein hydrolysate with the higher yield and protein recovery was obtained.

3.2 Degree of hydrolysis (DH)

DH of protein hydrolysates obtained from various hydrolysis processes is presented in Table 1. All hydrolysate samples had DH in the range of 35.31-44.93%. AU-1.0A sample showed the highest DH, compared with other samples ($P<0.05$), while the lowest DH was found in AU sample (35.31%). Cao et al. (2008) documented that DH of hydrolysate from *Penaens vannamei* was 45.06% when autolysis was conducted at pH 7.85, 50 ºC for 3 h. In the present study, autolysis process was carried out at 50 ºC and pH 8.0 for 3 h. When autolysis proceeded, proteins were cleaved by endogenous proteases including trypsin, chymotrypsin and elastase. This resulted in the release of peptides from protein matrix during hydrolysis [12]. When Alcalase was added after autolysis, DH continuously increased,

322 especially with increasing Alcalase levels ($P<0.05$). The result reconfirmed that autolysis in
323 conjunction with Alcalase effectively hydrolyzed proteins in shrimp cephalothorax. The
324 partially hydrolyzed peptides or loosen protein matrix could favor the migration of Alcalase
325 to substrate. Subsequently, the exposed substrates were readily available for hydrolysis by
326 both endogenous proteases and Alcalase [23]. Overall, the increase in DH of hydrolysate was
327 in accordance with the increases in yield and protein recovery (Table 1). Therefore, two-step
328 hydrolysis showed the high efficacy in protein hydrolysis, particularly when Alcalase at 1.0%
329 was used after autolysis.
330
331 3.3 Chemical compositions
332 Chemical compositions of the cephalothorax and its hydrolysates prepared by
333 different processes are shown in Table 2. Cephalothorax of Pacific white shrimp comprised
334 58.43% protein, 15.75% ash and 17.26% lipid. The protein content of cephalothorax of
335 Pacific white shrimp was higher than that of black tiger shrimp (*Penaeus monodon*), which
336 was 52.3% [24] or 42.02% [6] and that of *Metapenaeus dobsoni* (40.06%) [25]. However, it
337 was lower than that of Pacific white shrimp (*Peanaeus vannamei*) (61.61%) as reported by
338 Cao et al. [5]. Differences in shrimp species and the living environments were associated with
339 varying chemical compositions [26]. Chitin and total carotenoid contents of cephalothorax
340 were 8.32% and 150.58 μg/g sample, respectively.
341 High protein contents (86.04-89.18%) of all the protein hydrolysates were observed.
342 There were no differences in protein content among all hydrolysate samples ($P>0.05$). The
343 high protein content was a result of the solubilization of proteins during hydrolysis, the
344 removal of insoluble undigested non-proteinaceous substances and the partial removal of
345 lipid after hydrolysis [27]. All the hydrolysate samples had no difference in lipid contents
346 ($P>0.05$), except AU sample, which showed slightly lower content. It was noted that lipid

contents in all hydrolysates were much lower than that of cephalothorax. This indicated the effective removal of lipids from the hydrolysates. Lipid contents of all the hydrolysate were lower than that of hydrolysates from *Penaeus monodon* (2.4-3.0%) [6] and deep-water pink shrimp (2.74%) [11]. The low amount of lipid in the hydrolysate samples might lead to the stability towards lipid oxidation [11]. AU+0.5A and AU+1.0A samples had the higher ash content, compared with others ($P<0.05$). The higher ash content of hydrolysate samples was most likely caused by the release of mineral during the longer hydrolysis process. Cao et al. [5] reported that the most abundant mineral in protein hydrolysate was potassium, followed by calcium and magnesium. Although proteins could be effectively recovered from cephalothorax using two-step hydrolysis process involving autolysis with subsequent Alcalase hydrolysis, minerals were plausibly co-extracted.

Additionally, all hydrolysate samples had lower chitin content (0.67-1.08%) than shrimp cephalothorax (8.32%). It indicated that chitin was not liberated during hydrolysis by endogenous proteases or/and Alcalase. As a result, chitin was still retained in carapace or pereiopods. Carotenoid contents of protein hydrolysate samples were in the range of 3.37-6.11 μg/g sample. Senphan et al. [9] reported that the carotenoid content in hydrolysate from shrimp shell increased with increasing enzyme levels. Carotenoproteins from Pacific white shrimp waste were more hydrolyzed with combination of protease and lipase, compared to those hydrolyzed by only protease [28]. When proteins associated with carotenoid, e.g. astaxanthin were hydrolyzed, carotenoids, which were lipid soluble, were more likely localized in lipid phase or pellet associated with lipids. As a consequence, lower carotenoid content was obtained in the resulting hydrolysates.

372 3.4 Amino acid composition

373 Amino acid compositions of the cephalothorax and hydrolysate from shrimp
374 cephalothorax prepared by various hydrolysis processes are presented in Table 3.
375 Cephalothorax of Pacific white shrimp consisted of glutamic acid/glutamine (54.36 mg/g dry
376 sample), aspartic acid/asparagine (42.28 mg/g dry sample) and arginine (30.66 mg/g dry
377 sample) as dominant amino acids. Lower contents of cysteine (0.12 mg/g dry sample),
378 hydroxylysine (0.33 mg/g dry sample) and tryptophan (4.27 mg/g dry ample) were found in
379 initial dried cephalothorax, compared with those of hydrolysates.

380 When cephalothorax was hydrolyzed, the major amino acids of all the hydrolysate
381 samples were glutamic acid/glutamine, aspartic acid/asparagine, arginine and leucine, which
382 accounted for 113.08-121.69, 85.53-90.28, 63.27-68.62 and 58.67-68.07 mg/g dry sample,
383 respectively. The result was in accordance with Cao et al. [5] who reported that the abundant
384 amino acids in hydrolysate from shrimp head of *Penaeus Vannamei* were glutamic acid,
385 asparagine, and leucine. Sila et al. [11] also found glutamic acid/glutamine, aspartic
386 acid/asparagine and alanine as predominant amino acids in hydrolysate from shrimp
387 (*Parapenaeus longirostris*) by-products prepared using Alcalase. Nevertheless, negligible
388 contents of cysteine (0.15-0.25 mg/g dry ample), hydroxylysine (0.96-1.21 mg/g dry sample)
389 and tryptophan (9.28-9.64 mg/g dry sample) were observed in all the hydrolysates samples.
390 Large amounts of isoleucine, leucine, lysine, phenylalanine, valine and threonine, essential
391 amino acids for human being diet [6], were also found in all hydrolysates. Among all the
392 hydrolysates, AU+1.0A sample (346.65 mg/g dry sample) had the highest essential amino
393 acids, compared with others. Sila et al. [11] found that the hydrolysate from shrimp by-
394 products had a high content of essential amino acids, such as arginine, lysine, histidine and
395 leucine (53, 75, 22 and 34 residues per 1000 residues, respectively). Most amino acids of
396 hydrolysate from *Penaens vannamei* heads prepared at different autolysis time were not

changed when hydrolysis time was higher than 3 h [8]. Those hydrolysates had a high content of essential amino acids and extremely high content of flavoring amino acids (glutamic acid, aspartic acid, glycine and alanine) [8]. Several amino acids, such as tyrosine, methionine, histidine, lysine, and tryptophan, may significantly contribute to the antioxidant activity of the hydrolysates [11]. The hydrophobic amino acids of all hydrolysate samples, including alanine, isoleucine, leucine, methionine, phenylalanine, proline, tyrosine and valine, were in the range of 358.83-377.56 mg/g dry sample. Nevertheless, hydrophobic amino acid residues in hydrolysates contributed to the bitterness of protein hydrolysate [29]. The results suggested that the hydrolysates from shrimp cephalothorax could serve as an excellent source of amino acids.

3.5 Color

The color has the direct influence on the appearance of a product, which has the impact on the overall acceptability of consumers. The color values of the hydrolysates from different hydrolysis processes expressed as L^*, a^*, b^* and ΔE^* are shown in Table 1. AU sample had the higher lightness (L^*-value) with lower redness (a^*-value) and yellowness (b^*-value), compared with other hydrolysate samples ($P<0.05$). However, no differences in a^*-value were obtained between AU and 0.5A samples ($P>0.05$). L^*-values of hydrolysate samples decreased when Alcalase was used in hydrolysis processes ($P<0.05$). AU+1.0A sample had the lowest L^*-values but showed the highest a^* and b^*-values ($P<.05$). Higher a^* and b^*-values in hydrolysate were associated with the increase in carotenoid content during hydrolysis process as shown in Table 2. The difference in color was therefore governed by the different pigment contents [30]. Moreover, enzymatic browning reactions occurring during hydrolysis at temperature range of 50-60 ºC plausibly contributed to the decrease in lightness, leading to darker color of protein hydrolysates. Two-step hydrolysis processes had

422 a longer hydrolysis time (5 h) than other processes (AU, 0.5A and 1.0A), leading to higher
423 browning reaction. Generally, the hydrolysates obtained from two-step hydrolysis processes
424 had a slightly higher redness and yellowness than those from one-step hydrolysis process.
425 Overall, AU+0.5A and AU+1.0A samples showed the higher total difference in color (ΔE^*-
426 value) along with higher b^*-values than others. Thus, the varying colors of the hydrolysates
427 were more likely related with pigments present in hydrolysates. Additionally, it depended on
428 the composition of the raw material and hydrolysis processes used.
429
430 3.6 Molecular weight distribution
431 Elution profiles of different shrimp cephalothorax hydrolysates on the Sephadex G-25
432 gel filtration chromatography are illustrated in Fig. 1. A_{220} was used to monitor peptide bonds,
433 while A_{280} indicated the peptides or proteins, mainly containing aromatic amino acids. AU
434 samples had three major peaks of both A_{220} and A_{280}, representing peptides having MW of
435 4350.8, 496, and 220 Da, respectively. The first peak of A_{220} and A_{280} peak, which contained
436 peptides with MW of 8970-4350.8 Da, had lower peak area, compared with other fractions
437 possessing lower MW. The result indicated that the hydrolysate had a larger proportion of
438 peptides or proteins with MW lower than 1355 Da. The increase in DH (Table 1) of resulting
439 hydrolysates was agreement with increasing peak area of fractions with low MW peptides.
440 When Alcalase was used, the peak, especially the third peak, was slightly shifted to the lower
441 MW. Coincidentally, the first peak of A_{280} was lower. This suggested that the hydrolysis was
442 more pronounced with increasing Alcalase level used. For two-step hydrolysis (AU+0.5A
443 and AU+1.0A) processes, the higher amount of small peptide fraction with MW lower than
444 220 Da was obtained, particularly when 1.0% Alcalase was used after autolysis (AU+1.0A).
445 The result indicated that Alcalase could provide a hydrolysate with higher DH as indicated by
446 the formation of smaller peptides. Two-step hydrolysis process thus showed higher efficacy

447 in hydrolysis. This was indicated by the higher yield with maximum DH when autolysis was
448 conducted before the hydrolysis using Alcalase. Karnjanapratum, Benjakul [23] also
449 documented that the varying MW profiles revealed the differences in the degree of protein
450 hydrolysis, which was governed by the hydrolysis process and the enzyme used. Thus, the
451 hydrolysis process and enzyme used directly affected the MW distribution of resulting
452 hydrolysate samples.
453
454 3.7 Bitterness score
455 The bitterness scores were 4.16±1.52, 3.04±1.63, 2.66±1.49, 2.14±1.55 and 2.12±1.14
456 for AU, 0.5A, 1.0A, AU+0.5A and AU+1.0A samples, respectively. Caffeine standard
457 solution (25 ppm) had the score of 5.44±1.34. AU+1.0A sample showed the lowest bitterness
458 score, compared with others ($P<0.05$). However, no difference in bitterness score between
459 0.5A, 1.0A, AU+0.5A and AU+1.0A samples were observed ($P>0.05$). There was also no
460 difference in bitterness between AU, 0.5A and 1.0A samples ($P>0.05$). Bitterness might be
461 associated with the formation of peptides containing hydrophobic amino acids such as valine,
462 isoleucine, leucine, phenylalanine, tryptophan and tyrosine at C-terminal [29]. Bitterness was
463 one of the main contributors to off-flavor of protein hydrolysate [27]. Although higher
464 hydrophobic amino acids (377.56 mg/g dry sample) (Table 3) were present in AU+1.0A, low
465 bitterness score was attained. Bitter taste of a peptide was governed by several factors such as
466 DH, concentration and location of bitter taste residues and number of carbons on the R-group
467 of branched chain amino acid [29]. When DH was increased, hydrophobic amino acids
468 became more exposed. This led to the increased bitterness of hydrolysate [31]. Aspevik et al.
469 [32] also found that the hydrophobic peptide fraction with MW between 500 and 2000 Da
470 from Atlantic salmon showed positive correlation with bitterness and astringent flavor.
471 However, two-step hydrolysis process with the highest DH (Table 1) (AU+0.5A and

AU+1.0A) had the lower bitterness, compared with the hydrolysate prepared using autolysis process alone (AU). This was plausibly governed by differences in amino acid sequences of hydrolysates. The hydrophobic amino acid residues in the peptides more likely determined the bitterness of hydrolysates [33]. Yarnpakdee et al. [29] reported that the two-step hydrolysis with Alcalase, followed by papain reduced the bitter taste of hydrolysate from Nile tilapia, compared to those produced using a single hydrolysis (Alcalase hydrolysis). Therefore, two-step hydrolysis process could reduce bitterness of hydrolysate.

3.8 Antioxidative activities of shrimp cephalothorax hydrolysate produced with different hydrolysis processes

3.8.1 ABTS radical scavenging activity

ABTS radical scavenging activities of all hydrolysates were in range of 443.87-857.89 μmol TE/g sample (Table 4). The highest ABTS radical scavenging activity (857.89 μmol TE/g sample) was obtained for AU+1.0A sample ($P<0.05$). On the other hand, AU sample exhibited the lowest activity. In general, ABTS radical scavenging activities increased with increasing DH of resulting hydrolysates ($P<0.05$). Alcalase is endopeptidase capable of hydrolyzing proteins with broad specificity for peptide bonds. Proteins or peptides released during autolysis could be more cleaved to short chain peptides by Alcalase. Those peptides were able to scavenge ABTS radicals effectively. Senphan, Benjakul [1] reported that gelatin hydrolysates from seabass skin with DH ranging from 10% to 30% prepared using Alcalase had the increased ABTS scavenging activity with increasing DH. ABTS radical scavenging activity is used to determine the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) [34]. Binsan et al. [20] reported that the water fraction of extracted Mungoong from cephalothorax of white shrimp showed the highest ABTS radical scavenging activity, compared with ethanol fraction. The result suggested that antioxidative

peptides in hydrolysate were most likely hydrophilic as indicated by high proportion of hydrophilic amino acids (Table 3). Thus, shrimp cephalothorax hydrolysates with higher DH had the higher ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

3.8.2 DPPH radical scavenging activity

Hydrolysate produced by autolysis process (AU sample) exhibited the highest DPPH radical scavenging activity ($P<0.05$) (Table 4). However, there was no difference in DPPH radical scavenging activity between 0.5A and 1.0A samples ($P>0.05$). Also, no differences in activity between AU+0.5A and AU+1.0A samples were found ($P>0.05$). Overall, the increase in DH was shown to decrease DPPH scavenging radical activity of the resulting hydrolysates. Senphan, Benjakul [1] documented that hydrolysate prepared using Alcalase with DH of 10, 20 and 30% had no difference in DPPH radical scavenging activity. Sila et al. [11] reported that the hydrolysate from shrimp waste had the lower DPPH radical scavenging activity than BHA at the same concentration. The DPPH radical scavenging assay has been widely used to determine antioxidant properties of compounds. DPPH is a stable free radical, which can be reduced by a proton donating substrate such as an antioxidant, causing the decolorization of DPPH and reducing the absorbance at 517 nm in ethanol [11]. The decrease in DPPH radical scavenging activity of hydrolysate with higher DH (AU+0.5A and AU+1.0A) might be caused by the increased hydrophilicity of peptides. As a consequence, those peptides had lower ability to scavenge lipophilic DPPH radicals. DPPH radical scavenging assay is widely used for measurement of radical scavenging capacities of lipophilic antioxidant [35]. The result indicated that hydrolysates from cephalothorax of Pacific white shrimp contain peptides possessing the potential to prevent or retard lipid oxidation via a chain breaking

521 reaction. Nevertheless, capacity in hydrogen donation of peptides produced was governed by
522 process used for hydrolysis.
523
524 3.8.3 Ferric reducing antioxidant power (FRAP)
525 All the samples had FRAP values in the range of 5.38-9.83 μmol TE/g sample. The
526 highest FRAP was obtained in 1.0A sample ($P<0.05$) (Table 4). It was noted that
527 hydrolysates from two-step process showed the lower FRAP than those from one-step
528 process (0.5A and 1.0A) or autolysis (AU). No difference in the FRAP between AU+0.5A
529 and AU+1.0A samples were observed ($P>0.05$). FRAP is commonly used to measure the
530 capacity of substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex [20]. The
531 result demonstrated that two-step hydrolysis processes, which rendered higher DH, might
532 produce small peptides with low ability in reducing TPTZ-Fe(III) complex. FRAP of swollen
533 seabass skin hydrolysate prepared using Alcalase increased with increasing DH up to 20%.
534 However, no difference in FRAP of hydrolysates was noticeable when DH was higher than
535 20% [1]. Nevertheless, Karnjanapratum, Benjakul [36] reported that the increases in FRAP
536 were in accordance with increasing α-amino group content of gelatin hydrolysate from
537 unicorn leatherjacket skin. The higher reducing power indicated that the hydrolysate samples
538 could donate the electron to free radicals to higher extent, leading to the prevention or
539 retardation of propagation. Thus, the shrimp cephalothorax hydrolysate prepared by
540 hydrolysis using 1% Alcalase contained high amounts of peptides, which potentially donated
541 electron to free radicals, thereby terminating the chain reaction.
542
543 3.8.4 Ferrous ion chelating activity
544 AU+1.0A sample showed the highest ferrous chelating activity, while AU sample
545 exhibited the lowest activity ($P<0.05$) (Table 4). The ferrous ion chelating activities of the

546 hydrolysates were 6.50, 11.56, 14.26, 15.24 and 17.95 μmol EDTA/g sample for AU, 0.5A,
547 1.0A, AU-0.5A and AU+1.0A samples, respectively. The results of ferrous ion chelating
548 activity were similar to those of ABTS radical scavenging activity (Table 4). The transition
549 metals such as Fe, Cu and Co in foods participate in the formation of free radicals or reactive
550 oxygen species, which directly affect both rates of autoxidation and breakdown of
551 hydroperoxide to volatile compounds [1]. Thus, chelation of transition metal ions by certain
552 peptides in hydrolysates could retard or interrupt the oxidation process. Ferrozine
553 quantitatively forms complexes with the Fe^{2+} ion. In the presence of chelating agents, the
554 complex formation is disrupted, resulting in the decrease in color formation [1]. The peptides
555 present in the hydrolysates more likely had different metal ion chelating capacity, depending
556 on the amino acid sequences and chain length of peptides [7]. Sila et al. [11] reported that the
557 ferrous chelating capacity of the hydrolysate from shrimp (*Parapenaeus longirostris*) waste
558 by Alcalase treatment was higher than that of butylated hydroxyanisole (BHA). However,
559 gelatin hydrolysate from seabass skin prepared with different DHs (10% to 40%) had no
560 differences in ferrous ion chelating activity [1]. In the present study, smaller peptides might
561 be able to interact or bind with metal ion more effectively, compared with those with the
562 larger sizes. Thus, the shrimp cephalothorax hydrolysates prepared under the optimal
563 condition could act as the secondary antioxidant, which was able to chelate the prooxidative
564 metals, leading to the decreased lipid oxidation.
565
566 3.8.5 Oxygen radical absorbance capacity (ORAC)
567 The ORAC values of hydrolysate prepared by different hydrolysis processes ranged
568 from 339.90 to 391.81 μmol TE/g sample (Table 4). The highest ORAC value was observed
569 in AU+1.0A sample ($P<0.05$), whereas the AU sample showed the lowest value ($P<0.05$).
570 However, no difference in ORAC values between AU+0.5A and AU+1.0A samples were

observed (P>0.05). There was no difference in ORAC values between 0.5A and 1.0A samples (P>0.05). The higher ORAC value of AU+1.0A was in accordance with the higher ABTS scavenging radical activity and ferrous ion metal chelating activity. ORAC is the assay, which determines the antioxidant activity of compounds by scavenging peroxyl radical [7]. Nevertheless, ORAC assay is limited to measurement of chain breaking antioxidant capacity against only peroxyl radicals [37]. Kittiphattanabawon et al. [38] reported that gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme with 40% DH showed the highest ORAC value than those having DH of 10-30%. The result demonstrated that the hydrolysate prepared using two-step hydrolysis process, especially that using autolysis with subsequent hydrolysis by 1% Alcalase (AU+1.0A) had the highest ability in donating a hydrogen atom to the peroxyl radical, compared with the others.

3.9 Inhibitory activity toward supercoiled plasmid DNA strand scission oxidation

The inhibition of supercoiled plasmid DNA strand scission oxidation induced by AAPH in the presence of hydrolysate from cephalothorax of Pacific white shrimp prepared by different hydrolysis processes is depicted in Fig. 2. The assay has been used to evaluate the antioxidant activity of hydrolysates, based on their protection of supercoiled DNA strand from scission by oxidative stressor into open circular or linear form [21]. The scission of supercoiled DNA strand took place and was converted to the open circular form when exposed to peroxyl radicals [21]. The supercoiled DNA band of sample treated with AAPH was not detectable (lane CD). Peroxyl radical of AAPH has a long half-life, thus having a greater affinity to diffuse into cells. This leads to more macromolecular damage [39]. Oxidative damage to DNA may occur at both the phosphate backbone and the nucleotide bases. There are a wide variety of modifications, including strand scission, sister chromatid exchange, DNA-DNA and DNA-protein cross-links as well as base modification [21].

In the presence of the selected hydrolysates (AU, 1.0A and AU+1.0A) at different levels (1, 3 and 5 mg/ml), the retention of supercoiled DNA strand increased with increasing amount of hydrolysates added. The highest retention of supercoiled DNA was obtained when hydrolysate at 5 mg/ml was incorporated. The result demonstrated that the hydrolysate from cephalothorax of Pacific white shrimp had a protective activity against DNA scission induced by peroxyl radical. This might be associated with their ability to scavenge free radical and metal chelating activity as shown in Table 4. The result was in agreement with Kittiphattanabawon et al. [21] who documented that the ability to scavenge hydroxyl and peroxyl radical and chelating activity of gelatin hydrolysate from blacktip shark skin contributed to the protective ability against DNA damage. Oxidative stress caused by reactive oxygen species (ROS), such as peroxyl and hydroxyl radicals, led to damaged DNA, is involved in mutagenesis and carcinogenesis [40,41]. At the same concentration of hydrolysate incorporated, AU+1.0A had the higher inhibitory effect on DNA scission, compared to AU and 1.0A samples. The higher protective effect on DNA damage of AU+1.0A sample was related with higher ABTS radical scavenging activity and chelating activity (Table 4). Yarnpakdee et al. [39] reported that hydrolysate prepared by two-step processes from Nile tilapia showed higher protection effect against supercoiled DNA damage, compared to that prepared from the one step. Furthermore, Kittiphattanabawon et al. [21] also reported that the retention of supercoiled plasmid DNA induced by peroxyl and hydroxyl radicals increased as DH of hydrolysate increased. Thus, the hydrolysates from cephalothorax of Pacific white shrimp could inhibit DNA oxidation induced by peroxyl radical. It was suggested that hydrolysates could be used functional food ingredient to prevent oxidative stress.

4. Conclusion

The protein hydrolysate produced from cephalothorax of Pacific white shrimp could be used as potential source of nutritive ingredients or additives with high antioxidants. Hydrolysis processes directly influenced the chemical composition and antioxidative activities of the resulting hydrolysate. All hydrolysate samples had high protein content with low amount of lipid. Two-step hydrolysis process including autolysis, followed by Alcalase was an effective means to provide the peptides with small MW and less bitterness. Moreover, the hydrolysate showed high ABTS radical scavenging activity, metal chelating activity and ORAC. The hydrolysate also decreased peroxyl radical-induced supercoiled plasmid DNA strand scission. Therefore, autolysis process followed by Alcalase hydrolysis was an efficient process for protein recovery of cephalothorax of Pacific white shrimp with high yield. The resulting hydrolysates with antioxidant activity could be used as functional ingredient or supplement.

Acknowledgements

This research was supported by Prince of Songkla University. Thailand Research Fund (TRF) Distinguished Research Professor Grant (No. DPG5880002) was acknowledged for the financial support.

References

1. Senphan, T., Benjakul, S.: Antioxidative activities of hydrolysates from seabass skin prepared using protease from hepatopancreas of Pacific white shrimp. Journal of Functional Foods **6**, 147-156 (2014)

644 2. Ketnawa, S., Martínez-Alvarez, O., Gómez-Estaca, J., del Carmen Gómez-Guillén, M.,
645 Benjakul, S., Rawdkuen, S.: Obtaining of functional components from cooked shrimp
646 (*Penaeus vannamei*) by enzymatic hydrolysis. Food Bioscience **15**, 55-63 (2016)
647 3. Dey, S.S., Dora, K.C.: Antioxidative activity of protein hydrolysate produced by Alcalase
648 hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*). Journal of
649 Food Science and Technology **51**, 449-457 (2014)
650 4. Takeungwongtrakul, S., Benjakul, S., H-kittikun, A.: Lipids from cephalothorax and
651 hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*): Compositions and
652 deterioration as affected by iced storage. Food Chemistry **134**, 2066-2074 (2012)
653 5. Cao, W., Zhang, C., Hong, P., Ji, H., Hao, J., Zhang, J.: Autolysis of shrimp head by
654 gradual temperature and nutritional quality of the resulting hydrolysate. LWT - Food
655 Science and Technology **42**, 244-249 (2009)
656 6. Dey, S.S., Dora, K.C.: Optimization of the production of shrimp waste protein hydrolysate
657 using microbial proteases adopting response surface methodology. Journal of Food
658 Science and Technology **51**, 16-24 (2014)
659 7. Sae-leaw, T., O'Callaghan, Y.C., Benjakul, S., O'Brien, N.M.: Antioxidant activities and
660 selected characteristics of gelatin hydrolysates from seabass (*Lates calcarifer*) skin as
661 affected by production processes. Journal of Food Science and Technology **53**, 197-
662 208 (2016)
663 8. Cao, W., Zhang, C., Hong, P., Ji, H.: Response surface methodology for autolysis
664 parameters optimization of shrimp head and amino acids released during autolysis.
665 Food Chemistry **109**, 176-183 (2008)
666 9. Senphan, T., Benjakul, S., Kishimura, H.: Characteristics and antioxidative activity of
667 carotenoprotein from shells of Pacific white shrimp extracted using hepatopancreas
668 proteases. Food Bioscience **5**, 54-63 (2014)

669 10. Sowmya, R., Ravikumar, T.M., Vivek, R., Rathinaraj, K., Sachindra, N.M.: Optimization
670 of enzymatic hydrolysis of shrimp waste for recovery of antioxidant activity rich
671 protein isolate. Journal of Food Science and Technology **51**, 3199-3207 (2014)
672 11. Sila, A., Sayari, N., Balti, R., Martinez-Alvarez, O., Nedjar-Arroume, N., Moncef, N.,
673 Bougatef, A.: Biochemical and antioxidant properties of peptidic fraction of
674 carotenoproteins generated from shrimp by-products by enzymatic hydrolysis. Food
675 Chemistry **148**, 445-452 (2014)
676 12. Senphan, T., Benjakul, S.: Compositions and yield of lipids extracted from
677 hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei)* as affected by prior
678 autolysis. Food Chemistry **134**, 829-835 (2012)
679 13. Holanda, H., Netto, F.: Optimization of the conditions for the enzyme hydrolysis of
680 shrimp residue, using response surface methodology (RSM). In book of abstracts. In:
681 2002 IFT Annual Meeting, Anaheim, Calif., USA. 2002, p. 194
682 14. AOAC: Official method of analysis of association of chemistry. The Association of
683 Official Analytical Chemists, Washigton D.C. (2000)
684 15. Saito, A., Regier, L.W.: Pigmentation of Brook Trout (*Salvelinus fontinalis*) by Feeding
685 Dried Crustacean Waste. Journal of the Fisheries Research Board of Canada **28**, 509-
686 512 (1971)
687 16. Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y., Roytrakul, S.:
688 Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and
689 *Priacanthus macracanthus*. Food Chemistry **116**, 445-451 (2009)
690 17. Benjakul, S., Morrissey, M.T.: Protein hydrolysates from Pacific whiting solid wastes.
691 Journal of Agricultural and Food Chemistry **45**, 3423-3430 (1997)
692 18. Gennadios, A., Weller, C.L., Hanna, M.A., Froning, G.W.: Mechanical and barrier
693 properties of egg albumen films. J. Food Sci. **61**, 585-589 (1996)

694 19. Nilsang, S., Lertsiri, S., Suphantharika, M., Assavanig, A.: Optimization of enzymatic
695 hydrolysis of fish soluble concentrate by commercial proteases. Journal of Food
696 Engineering **70**, 571-578 (2005)
697 20. Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M., Kishimura, H.:
698 Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white
699 shrimp (*Litopenaeus vannamei*). Food Chemistry **106**, 185-193 (2008)
700 21. Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Shahidi, F.: Inhibition of
701 angiotensin converting enzyme, human LDL cholesterol and DNA oxidation by
702 hydrolysates from blacktip shark gelatin. LWT - Food Science and Technology **51**,
703 177-182 (2013)
704 22. Steel, R.G.D., Torrie, J.H.: Principles and Procedures of Statistics: A Biometrical
705 Approach., 2nd ed. McGraw-Hill, New York (1980)
706 23. Karnjanapratum, S., Benjakul, S.: Characteristics and antioxidative activity of gelatin
707 hydrolysates from unicorn leatherjacket skin as affected by autolysis-assisted process.
708 Journal of Food Processing and Preservation **39**, 915-926 (2015)
709 24. Phuong, P.T.D., Minh, N.C., Cuong, H.N., Van Minh, N., Han, N.T., Van Hoa, N., Yen,
710 H.T.H., Trung, T.S.: Recovery of protein hydrolysate and chitosan from black tiger
711 shrimp (*Penaeus monodon*) heads: approaching a zero waste process. Journal of Food
712 Science and Technology **54**, 1850-1856 (2017)
713 25. Gunasekaran, J., Kannuchamy, N., Kannaiyan, S., Chakraborti, R., Gudipati, V.: Protein
714 hydrolysates from shrimp (*Metapenaeus dobsoni*) head waste: Optimization of
715 extraction conditions by response surface methodology. Journal of Aquatic Food
716 Product Technology **24**, 429-442 (2015)
717 26. Shahidi, F., Synowiecki, J.: Isolation and characterization of nutrients and value-added
718 products from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*)

719 processing discards. Journal of Agricultural and Food Chemistry **39**, 1527-1532
720 (1991)
721 27. Thiansilakul, Y., Benjakul, S., Shahidi, F.: Compositions, functional properties and
722 antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus*
723 *maruadsi*). Food Chemistry **103**, 1385-1394 (2007)
724 28. Armenta, R.E., Guerrero-Legarreta, I.: Amino acid profile and enhancement of the
725 enzymatic hydrolysis of fermented shrimp carotenoproteins. Food Chemistry **112**,
726 310-315 (2009)
727 29. Yarnpakdee, S., Benjakul, S., Kristinsson, H.G., Kishimura, H.: Antioxidant and sensory
728 properties of protein hydrolysate derived from Nile tilapia (*Oreochromis niloticus*) by
729 one- and two-step hydrolysis. Journal of Food Science and Technology **52**, 3336-3349
730 (2015)
731 30. Pongsetkul, J., Benjakul, S., Vongkamjan, K., Sumpavapol, P., Osako, K., Faithong, N.:
732 Changes in volatile compounds, ATP-related compounds and antioxidative properties
733 of Kapi, produced from Acetes vulgaris, during processing and fermentation. Food
734 Bioscience **19**, 49-56 (2017)
735 31. Hou, H., Li, B., Zhao, X., Zhang, Z., Li, P.: Optimization of enzymatic hydrolysis of
736 Alaska pollock frame for preparing protein hydrolysates with low-bitterness. LWT -
737 Food Science and Technology **44**, 421-428 (2011)
738 32. Aspevik, T., Totland, C., Lea, P., Oterhals, Å.: Sensory and surface-active properties of
739 protein hydrolysates based on Atlantic salmon (*Salmo salar*) by-products. Process
740 Biochemistry **51**, 1006-1014 (2016)
741 33. Spellman, D., O'Cuinn, G., FitzGerald, R.J.: Bitterness in *Bacillus* proteinase
742 hydrolysates of whey proteins. Food Chemistry **114**, 440-446 (2009)

743 34. Nalinanon, S., Benjakul, S., Kishimura, H., Shahidi, F.: Functionalities and antioxidant
744 properties of protein hydrolysates from the muscle of ornate threadfin bream treated
745 with pepsin from skipjack tuna. Food Chemistry **124**, 1354-1362 (2011)
746 35. Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S.I., Chun, O.K.: Comparison of
747 ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US
748 foods. Journal of Food Composition and Analysis **24**, 1043-1048 (2011)
749 36. Karnjanapratum, S., Benjakul, S.: Antioxidative gelatin hydrolysate from unicorn
750 leatherjacket skin as affected by prior autolysis. International Aquatic Research **7**,
751 101-114 (2015)
752 37. Prior, R.L., Wu, X., Schaich, K.: Standardized methods for the determination of
753 antioxidant capacity and phenolics in foods and dietary supplements. Journal of
754 Agricultural and Food Chemistry **53**, 4290-4302 (2005)
755 38. Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Shahidi, F.: Gelatin hydrolysate
756 from blacktip shark skin prepared using papaya latex enzyme: Antioxidant activity
757 and its potential in model systems. Food Chemistry **135**, 1118-1126 (2012)
758 39. Yarnpakdee, S., Benjakul, S., Kristinsson, H.G., Bakken, H.E.: Preventive effect of Nile
759 tilapia hydrolysate against oxidative damage of HepG2 cells and DNA mediated by
760 H_2O_2 and AAPH. Journal of Food Science and Technology **52**, 6194-6205 (2015)
761 40. Chandrasekara, A., Shahidi, F.: Bioactivities and antiradical properties of millet grains
762 and hulls. Journal of Agricultural and Food Chemistry **59**, 9563-9571 (2011)
763 41. Zhong, Y., Shahidi, F.: Lipophilised epigallocatechin gallate (EGCG) derivatives and
764 their antioxidant potential in food and biological systems. Food Chemistry **131**, 22-30
765 (2012)
766
767

Table 1 Yield, protein recovery, degree of hydrolysis (DH) and color values of the hydrolysates from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) prepared by various hydrolysis processes

Samples	Yield (%)	Protein recovery (%)	DH (%)	Color values			
				L^*	a^*	b^*	ΔE^*
AU	46.46±0.94d	73.55±1.32d	35.31±0.81e	78.75±0.28a	1.42±0.05c	19.05±0.76c	23.82±0.87c
0.5A	49.58±0.41c	75.91±1.63cd	37.61±0.83d	78.13±0.34b	1.25±0.08c	17.34±0.06d	23.28±0.20c
1.0A	51.39±0.46bc	77.91±1.54c	40.53±0.63c	77.00±0.29c	1.95±0.09b	21.58±0.03b	25.13±0.46b
AU+0.5A	52.00±0.57b	81.18±1.92b	42.28±0.86b	76.94±0.28c	1.99±0.06b	22.54±0.38a	27.06±0.03a
AU+1.0A	54.04±0.74a	84.15±1.70a	44.93±0.75a	76.17±0.06d	2.41±0.03a	21.83±0.27a	27.09±0.42a

Values are presented as mean ± SD (n = 3) (dry weight basic)

Different lowercase letters within the same column indicate significant difference ($P<0.05$)

AU; autolysis at 50 ºC for 3 h, 0.5A; 0.5% Alcalase for 2 h (55 ºC, pH 8), 1.0A; 1.0% Alcalase for 2 h (55 ºC, pH 8), AU+0.5A; autolysis for 3 h, followed by 0.5% Alcalase for 2 h (55 ºC, pH 8), AU+1.0A; autolysis for 3 h, followed by 1.0% Alcalase for 2 h (55 ºC, pH 8).

Table 2 Chemical compositions, chitin and total carotenoid contents of the cephalothorax and hydrolysates from shrimp celphalothorax of Pacific white shrimp (*Litopenaeus vannamei*) prepared by various hydrolysis processes

Samples	Chemical composition (%)				Chitin (%)	Total carotenoid (μg/g)
	Moisture	Protein	Ash	Lipid		
Cephalothorax	75.86±.16	58.43±1.45	15.75±0.11	17.26±1.74	8.32±0.20	150.58±2.65
AU	5.11±0.09a	89.18±2.16a	7.46±0.20b	0.43±0.04b	0.67±0.06d	3.37±0.05d
0.5A	4.99±0.02b	89.24±2.09a	8.16±0.48b	0.56±0.06a	0.73±0.12cd	3.61±0.27d
1.0A	4.43±0.10c	88.23±1.38a	8.50±0.61b	0.58±0.02a	0.77±0.13cd	4.28±0.22c
AU+0.5A	4.17±0.04d	86.04±1.68a	10.59±1.37a	0.63±0.03a	0.98±0.1bc	5.47±0.23b
AU+1.0A	4.92±0.10b	86.84±2.04a	11.26±1.54a	0.64±0.09a	1.08±0.21a	6.11±0.30a

Values are presented as mean + SD (n = 3)

Different lowercase letters within the same column indicate significant difference ($P<0.05$). Caption: see Table 1.

Table 3 Amino acid compositions of the celphalothorax and hydrolysates of shrimp celphalothorax of Pacific white shrimp (*Litopenaeus vannamei*) prepared by various hydrolysis processes

Amino acids	Samples					
(mg/g dry sample)	Cephalothorax	AU	0.5A	1.0A	AU+0.5A	AU+1.0A
Alanine	24.95	58.25	57.65	56.05	54.38	55.27
Arginine	30.66	65.95	68.62	64.44	63.27	65.92
Aspartic acid/asparagine	42.28	86.88	90.28	85.53	84.04	86.67
Cysteine	0.12	0.25	0.18	0.22	0.15	0.19
Glutamic acid/Glutamine	54.36	118.54	121.69	115.80	113.08	116.50
Glycine	25.86	59.79	62.24	59.25	57.97	59.01
Histidine	11.69	25.13	25.48	24.00	23.84	24.57
Isoleucine	17.05	43.03	38.55	39.93	39.44	41.29
Leucine	29.80	68.07	58.67	61.97	61.59	65.11
Lysine	29.90	64.27	65.74	62.47	61.17	62.20
Hydroxylysine	0.33	1.08	1.21	0.96	0.98	1.05
Methionine	9.65	2.41	19.37	19.92	19.48	20.52
Phenylalanine	20.65	45.85	42.63	43.12	43.19	44.56
Proline	23.90	54.36	54.69	53.67	51.92	51.17
Serine	18.92	40.22	41.97	40.28	39.56	40.22
Threonine	18.46	40.49	41.10	39.63	38.67	39.16
Tryptophan	4.27	9.28	9.34	9.29	9.64	9.51
Tyrosine	18.63	26.92	36.47	36.75	39.88	40.70
Valine	20.80	50.41	49.51	49.03	47.96	49.25
Total amino acid	402.28	861.19	885.39	862.32	850.22	872.87
Hydophobic amino acids	169.82	358.83	367.06	369.95	367.64	377.56
Essential amino acids	158.00	339.66	341.05	340.07	335.36	346.65

Caption: see Table 1.

Table 4 ABTS and DPPH radical scavenging activites, ferric reducing antioxidant power (FRAP), ferrous ion chelating activity and oxygen radical absorbance capacity (ORAC) of hydrolysates from cephalothorax of Pacific white shrimp prepared by different hydrolysis processes

Samples	ABTS (μmol TE/g smple)	DPPH (μmol TE/g smple)	FRAP (μmol TE/g smple)	Chelating (μmol EDTA/g smple)	ORAC (μmol TE/g smple)
AU	443.87±30.16e	4.84±0.26a	7.51±0.08c	6.50±0.12e	349.67±11.21d
0.5A	611.71±6.92d	4.13±0.23b	8.51±0.33b	11.56±0.39d	343.27±17.40bc
1.0A	693.79±31.01c	3.96±0.22b	9.83±0.54a	14.26±0.25c	358.47±25.04bc
AU+0.5A	737.34±6.94b	3.07±0.35c	5.38±0.22d	15.24±0.82b	371.63±7.83ab
AU+1.0A	857.89±26.51a	2.69±0.19c	5.40±0.50d	17.95±0.28a	391.81±8.15a

Values are presented as mean + SD (n = 3)

Different lowercase letters within the same column indicate significant difference ($P<0.05$).

Caption: see Table 1.

Figure legend

Figure 1 Elution profile by Sephadex G-25 size exclusion chromatography of hydrolysates from cephalothorax of Pacific white shrimp prepared by different hydrolysis processes. Absorbance at 280 nm (filled circle), 220 nm (open circle). AU; autolysis at 50 ºC for 3 h, 0.5A; 0.5% Alcalase for 2 h, 1.0A; 1.0% Alcalase for 2 h, AU+0.5A; autolysis for 3 h, followed by 0.5% Alcalase, AU+1.0A; autolysis for 3 h, followed by 1.0% Alcalase.

Figure 2 Agarose gel electrophoresis of DNA treated with peroxyl radicals in the absence and presence of AU, 1.0A and AU+1.0A hydrolysate samples at different concentrations (1, 3 and 5 mg/ml). Caption: see Fig. 1. C and CD denote control (DNA alone) and control damage (DNA+oxidative stressors), respectively. 1, 3 and 5 denote DNA + radicals + hydrolysates at concentrations of 1, 3 and 5 mg/ml, respectively.

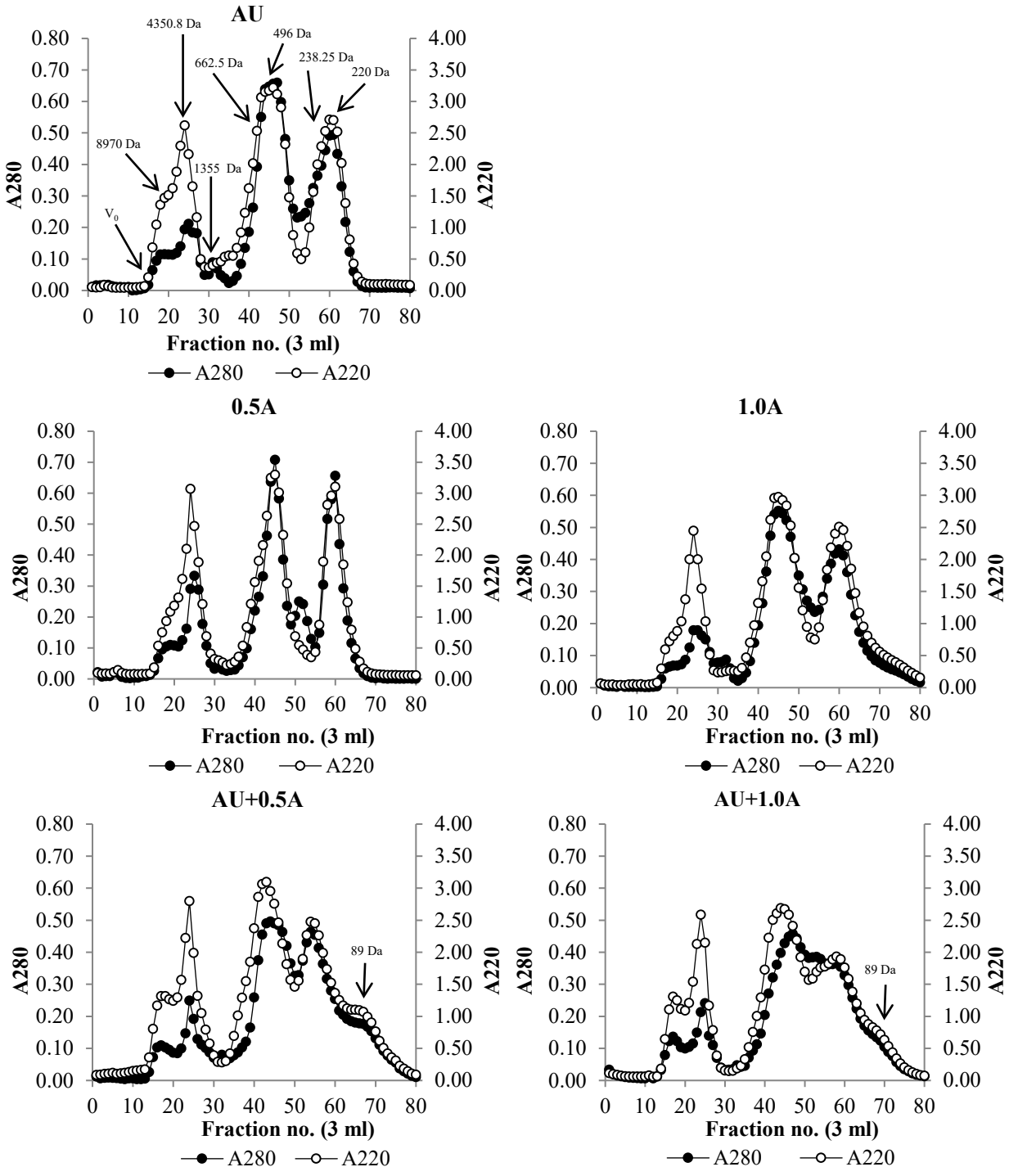
AU
4350.8 Da
496 Da
662.5 Da
238.25 Da
220 Da
8970 Da
1355 Da
V0
A280
A220
Fraction no. (3 ml)
A280
A220
0.5A
Fraction no. (3 ml)
A280
A220
1.0A
Fraction no. (3 ml)
A280
A220
AU+0.5A
89 Da
Fraction no. (3 ml)
A280
A220
AU+1.0A
89 Da
Fraction no. (3 ml)
A280
A220

Figure 1

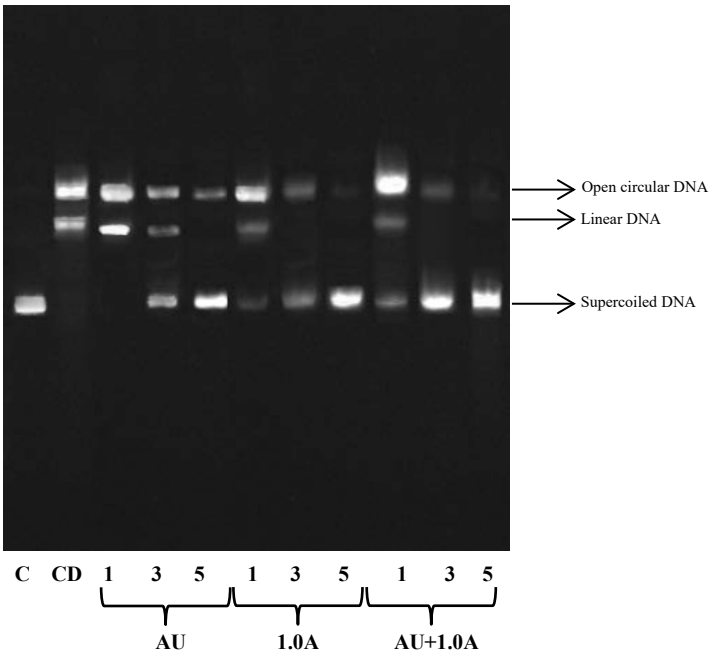
Open circular DNA
Linear DNA
Supercoiled DNA
C
CD
1
3
5
1
3
5
1
3
5
AU
1.0A
AU+1.0A

Figure 2