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

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25

**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 peroxy 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

## 51 **Statement of Novelty**

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

61

## 62 **1. Introduction**

63         Pacific white shrimp and its products have become economically important for  
64 Thailand [1]. It accounts for 90% of the global aquaculture shrimp production [2,1]. By the  
65 year 2012, frozen Pacific white shrimp and products were manufactured and exported over  
66 500,000 tons, particularly to the USA and Japan. During shrimp processing, approximately  
67 40-50% of its total weight are generated as by-products. Those contained 71.4%  
68 cephalothorax and 28.6% shell, which are rich in amino acids, peptides, proteins and other  
69 components [1,3]. Those leftovers generally have been transformed to animal feed and  
70 aquaculture diets [4]. Shrimp cephalothorax is an essential source of protein (50-65%, dry  
71 weight basis) and also serves as a source of lipid (11% dry weight basis), chitin (11% dry  
72 weight basis), enzymes and other nutritive components [5,4]. The utilization of shrimp  
73 cephalothorax by conversion to protein hydrolysate can be a promising approach to eliminate  
74 harmful environmental aspects as well as earn the revenue for shrimp processing industry [6].

75 Protein hydrolysates have attracted increasing interest as potential ingredients for  
76 many health-promoting functional foods due to biologically active peptides [5,7]. The  
77 recovery of proteins or peptides from shrimp waste by hydrolysis has been widely  
78 investigated [8,5,3,9-11]. Endogenous proteases were reported in hepatopancreas [12].  
79 Under optimal condition, those proteases could autolysis [8]. Autolysis and enzymatic  
80 hydrolysis of cephalothorax depend on several factors including pH, temperature, time as  
81 well as enzyme/substrate ratio [6,1]. Cao et al. [8] reported that optimum autolysis condition  
82 for protein recovery of cephalothorax from *Penaens vannamei* included 50 °C, pH 7.85 and a  
83 substrate concentration at 23% (w/v), in which the highest degree of hydrolysis (45%) was  
84 obtained. The autolysis of shrimp head by gradual increase in temperature (40-60 °C) at 5  
85 °C/30 min resulted in the higher protein recovery (87.4%), compared with autolysis  
86 performed at different single temperature (40, 50, and 60 °C) (43.6-73.6%) [5]. Additionally,  
87 a number of commercial proteases have been used for the production of protein hydrolysis  
88 from seafood processing by-products. Dey, Dora [6] suggested that protein recovery of  
89 hydrolysate from shrimp waste using Alcalase was higher than those using Neutrase,  
90 Protamex and Flavourzyme. Sila et al. [11] also documented that the protein hydrolysate  
91 from shrimp waste of deep-water pink shrimp was produced by Alcalase hydrolysis.  
92 Hydrolysate contained 80.8% protein, 2.74% lipid, 14.4% ash, 1.13% chitin and 1.08 µg  
93 carotenoid/g sample.

94 To maximize the hydrolysis of proteins from cephalothorax, autolysis could be  
95 exploited. The liberated protein or peptides more likely served as the proteinaceous substrate  
96 for subsequent hydrolysis by commercial proteases. The hydrolysates containing varying  
97 peptides from different hydrolysis processes might possess different compositions as well as  
98 bioactivities, especially antioxidant activity. Nevertheless, a little information regarding the  
99 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-methylpropionamide) (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™ 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,

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

126

## 127 2.3 Production of hydrolysate from shrimp cephalothorax

### 128 2.3.1 Preparation of cephalothorax

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

132

### 133 2.3.2 Hydrolysis of cephalothorax using different processes

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

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

145 For two-step hydrolysis processes, the homogenate (pH 8.0), was firstly incubated at  
146 50 °C for 3 h for initial autolysis. After autolysis, the mixture was pre-incubated at 60 °C for  
147 15 min. Subsequently, Alcalase (0.5 or 1.0% w/w) was added into the mixture. Hydrolysis  
148 was conducted at 60 °C for 2 h. Enzymatic reaction was terminated as previously described.

149 All the obtained mixtures were cooled down to room temperature (25 °C) using a running  
150 water. The mixtures were filtered with two layers of cheesecloth to remove the undigested  
151 carapace and pereopods (solid phase). The resulting filtrates were centrifuged at 4000xg at 4  
152 °C using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA,  
153 USA) for 15 min. Then, the supernatants were freeze-dried using a freeze-dryer (CoolSafe  
154 55, ScanLaf A/S, Lyngby, Denmark) for 72 h. The hydrolysate from autolysis was named  
155 “AU”, while those from one-step hydrolysis process with the aid of 0.5 and 1.0% Alcalase  
156 were referred to as “0.5A” and “1.0A”, respectively. The hydrolysates attained from two-step  
157 hydrolysis process using autolysis, followed by hydrolysis with 0.5 and 1.0% Alcalase were  
158 termed “AU+0.5A” and “AU+1.0A”, respectively. All the hydrolysate samples were then  
159 subjected to analyses.

160

## 161 2.4 Analyses

### 162 2.4.1 Proximate analysis

163 Ground cephalothorax and hydrolysate samples were analyzed for moisture, protein,  
164 fat and ash contents using the analytical method no. of 950.46, 920.153, 960.39 and 928.08,  
165 respectively [14]. The conversion factor used for calculation of protein content was 6.25.  
166 Chitin content was determined according to the method of Senphan et al. [9] with a slight  
167 modification. Samples (2 g) were mixed with 30 ml of 1.25 M NaOH at 100 °C for 3 h. The  
168 mixture was filtered under vacuum using a Whatman No.1 filter paper. The residue was  
169 shaken with 30 ml of 1 M HCl for 30 min at 25 °C, filtered and washed with distilled water.  
170 The washed residue was then homogenized with cold acetone (-20 °C) at a speed of 13,000  
171 rpm using a homogenizer for 3 min to remove lipids and pigments. After washing with 3  
172 volumes of distilled water, 30 ml of 0.5% NaOCl were mixed with the sample and stirred for  
173 30 min at 25 °C. The mixture was then filtered and washed with distilled water. The residue



174 was dried at 60 °C 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 °C 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]:

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

192 where 0.2 is the  $A_{468}$  of 1  $\mu\text{g/ml}$  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:

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

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 °C 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 ( $\Delta E^*$ ) was calculated according to the following equation [18]:

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

220 where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  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  $\mu\text{mol Trolox (TE) equivalent/g sample}$ , except for metal chelating activity,  
257 which was reported as  $\mu\text{mol EDTA equivalent/g sample}$ .

258

#### 259 2.4.10 Measurement of inhibitory activity toward peroxy radical induced supercoiled 260 plasmid DNA strand scission

261 Peroxy 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) ( $0.025 \mu\text{g}/\mu\text{l}$ ;  $4 \mu\text{l}$ ) dissolved in 10 mM Tris-HCl  
264 containing 1 mM EDTA (pH 7.8) was mixed with  $2 \mu\text{l}$  of different hydrolysates to obtain the  
265 final concentrations of 1, 3 and 5 mg/ml. To initiate the oxidation reaction,  $4 \mu\text{l}$  of 10 mM  
266 AAPH were added. The mixture was incubated at  $37 \text{ }^\circ\text{C}$  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,  $2 \mu\text{l}$  of the loading dye (0.25% bromophenol blue, 50% glycerol) were added to  
269 the reaction mixture. Then, the mixture ( $6 \mu\text{l}$ ) 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<sup>®</sup> cell GT, Biorad, Hercules, CA,

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

275

## 276 2.5 Statistical analysis

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

281

## 282 **3. Results and discussion**

### 283 3.1 Yield and protein recovery

284 Yield and protein recovery of the hydrolysates from cephalothorax prepared using  
285 different hydrolysis processes are shown in Table 1. Generally, yield of hydrolysate samples  
286 correlated well with protein recovery. The highest yield (54.04%) and protein recovery  
287 (84.15%) were found for AU-1.0A sample, compared to other hydrolysates ( $P < 0.05$ ). Yield  
288 of hydrolysates prepared by one-step hydrolysis process was lower than that of hydrolysates  
289 prepared using two-step hydrolysis processes ( $P < 0.05$ ). However, no difference in the yield  
290 between 1.0A and AU+0.5A samples was observed ( $P > 0.05$ ). For hydrolysate prepared using  
291 single process, in which cephalothorax were hydrolyzed by Alcalase without prior autolysis,  
292 there were no differences in yield and protein recovery between 0.5A and 1.0A samples  
293 ( $P > 0.05$ ). The result indicated that proteins of cephalothorax could be hydrolyzed to higher  
294 degree when two-step process including autolysis, followed by hydrolysis with 1.0%  
295 Alcalase was conducted. Shrimp cephalothorax contained hepatopancreas, which was an  
296 excellent source of proteases, especially trypsin and chymotrypsin [12]. Proteases in

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

311

### 312 3.2 Degree of hydrolysis (DH)

313 DH of protein hydrolysates obtained from various hydrolysis processes is presented in  
314 Table 1. All hydrolysate samples had DH in the range of 35.31-44.93%. AU-1.0A sample  
315 showed the highest DH, compared with other samples ( $P < 0.05$ ), while the lowest DH was  
316 found in AU sample (35.31%). Cao et al. (2008) documented that DH of hydrolysate from  
317 *Penaens vannamei* was 45.06% when autolysis was conducted at pH 7.85, 50 °C for 3 h. In  
318 the present study, autolysis process was carried out at 50 °C and pH 8.0 for 3 h. When  
319 autolysis proceeded, proteins were cleaved by endogenous proteases including trypsin,  
320 chymotrypsin and elastase. This resulted in the release of peptides from protein matrix during  
321 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 (*Penaeus 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  $\mu\text{g/g}$  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

347 contents in all hydrolysates were much lower than that of cephalothorax. This indicated the  
348 effective removal of lipids from the hydrolysates. Lipid contents of all the hydrolysate were  
349 lower than that of hydrolysates from *Penaeus monodon* (2.4-3.0%) [6] and deep-water pink  
350 shrimp (2.74%) [11]. The low amount of lipid in the hydrolysate samples might lead to the  
351 stability towards lipid oxidation [11]. AU+0.5A and AU+1.0A samples had the higher ash  
352 content, compared with others ( $P<0.05$ ). The higher ash content of hydrolysate samples was  
353 most likely caused by the release of mineral during the longer hydrolysis process. Cao et al.  
354 [5] reported that the most abundant mineral in protein hydrolysate was potassium, followed  
355 by calcium and magnesium. Although proteins could be effectively recovered from  
356 cephalothorax using two-step hydrolysis process involving autolysis with subsequent  
357 Alcalase hydrolysis, minerals were plausibly co-extracted.

358         Additionally, all hydrolysate samples had lower chitin content (0.67-1.08%) than  
359 shrimp cephalothorax (8.32%). It indicated that chitin was not liberated during hydrolysis by  
360 endogenous proteases or/and Alcalase. As a result, chitin was still retained in carapace or  
361 pereiopods. Carotenoid contents of protein hydrolysate samples were in the range of 3.37-  
362 6.11  $\mu\text{g/g}$  sample. Senphan et al. [9] reported that the carotenoid content in hydrolysate from  
363 shrimp shell increased with increasing enzyme levels. Carotenoproteins from Pacific white  
364 shrimp waste were more hydrolyzed with combination of protease and lipase, compared to  
365 those hydrolyzed by only protease [28]. When proteins associated with carotenoid, e.g.  
366 astaxanthin were hydrolyzed, carotenoids, which were lipid soluble, were more likely  
367 localized in lipid phase or pellet associated with lipids. As a consequence, lower carotenoid  
368 content was obtained in the resulting hydrolysates.

369

370

371



### 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

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

407

### 408 3.5 Color

409 The color has the direct influence on the appearance of a product, which has the  
410 impact on the overall acceptability of consumers. The color values of the hydrolysates from  
411 different hydrolysis processes expressed as  $L^*$ ,  $a^*$ ,  $b^*$  and  $\Delta E^*$  are shown in Table 1. AU  
412 sample had the higher lightness ( $L^*$ -value) with lower redness ( $a^*$ -value) and yellowness ( $b^*$ -  
413 value), compared with other hydrolysate samples ( $P < 0.05$ ). However, no differences in  $a^*$ -  
414 value were obtained between AU and 0.5A samples ( $P > 0.05$ ).  $L^*$ -values of hydrolysate  
415 samples decreased when Alcalase was used in hydrolysis processes ( $P < 0.05$ ). AU+1.0A  
416 sample had the lowest  $L^*$ -values but showed the highest  $a^*$  and  $b^*$ -values ( $P < 0.05$ ). Higher  $a^*$   
417 and  $b^*$ -values in hydrolysate were associated with the increase in carotenoid content during  
418 hydrolysis process as shown in Table 2. The difference in color was therefore governed by  
419 the different pigment contents [30]. Moreover, enzymatic browning reactions occurring  
420 during hydrolysis at temperature range of 50-60 °C plausibly contributed to the decrease in  
421 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 ( $\Delta E^*$ -  
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  $4.16 \pm 1.52$ ,  $3.04 \pm 1.63$ ,  $2.66 \pm 1.49$ ,  $2.14 \pm 1.55$  and  $2.12 \pm 1.14$   
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  $5.44 \pm 1.34$ . 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

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

479

480 3.8 Antioxidative activities of shrimp cephalothorax hydrolysate produced with different  
481 hydrolysis processes

482 3.8.1 ABTS radical scavenging activity

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

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

501

### 502 3.8.2 DPPH radical scavenging activity

503 Hydrolysate produced by autolysis process (AU sample) exhibited the highest DPPH  
504 radical scavenging activity ( $P < 0.05$ ) (Table 4). However, there was no difference in DPPH  
505 radical scavenging activity between 0.5A and 1.0A samples ( $P > 0.05$ ). Also, no differences in  
506 activity between AU+0.5A and AU+1.0A samples were found ( $P > 0.05$ ). Overall, the increase  
507 in DH was shown to decrease DPPH scavenging radical activity of the resulting hydrolysates.  
508 Senphan, Benjakul [1] documented that hydrolysate prepared using Alcalase with DH of 10,  
509 20 and 30% had no difference in DPPH radical scavenging activity. Sila et al. [11] reported  
510 that the hydrolysate from shrimp waste had the lower DPPH radical scavenging activity than  
511 BHA at the same concentration. The DPPH radical scavenging assay has been widely used to  
512 determine antioxidant properties of compounds. DPPH is a stable free radical, which can be  
513 reduced by a proton donating substrate such as an antioxidant, causing the decolorization of  
514 DPPH and reducing the absorbance at 517 nm in ethanol [11]. The decrease in DPPH radical  
515 scavenging activity of hydrolysate with higher DH (AU+0.5A and AU+1.0A) might be  
516 caused by the increased hydrophilicity of peptides. As a consequence, those peptides had  
517 lower ability to scavenge lipophilic DPPH radicals. DPPH radical scavenging assay is widely  
518 used for measurement of radical scavenging capacities of lipophilic antioxidant [35]. The  
519 result indicated that hydrolysates from cephalothorax of Pacific white shrimp contain  
520 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  $\mu\text{mol TE/g}$  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  $\alpha$ -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  $\mu\text{mol EDTA/g}$  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  $\text{Fe}^{2+}$  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  $\mu\text{mol TE/g}$  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



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

582

### 583 3.9 Inhibitory activity toward supercoiled plasmid DNA strand scission oxidation

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

596 In the presence of the selected hydrolysates (AU, 1.0A and AU+1.0A) at different  
597 levels (1, 3 and 5 mg/ml), the retention of supercoiled DNA strand increased with increasing  
598 amount of hydrolysates added. The highest retention of supercoiled DNA was obtained when  
599 hydrolysate at 5 mg/ml was incorporated. The result demonstrated that the hydrolysate from  
600 cephalothorax of Pacific white shrimp had a protective activity against DNA scission induced  
601 by peroxy radical. This might be associated with their ability to scavenge free radical and  
602 metal chelating activity as shown in Table 4. The result was in agreement with  
603 Kittiphattanabawon et al. [21] who documented that the ability to scavenge hydroxyl and  
604 peroxy radical and chelating activity of gelatin hydrolysate from blacktip shark skin  
605 contributed to the protective ability against DNA damage. Oxidative stress caused by reactive  
606 oxygen species (ROS), such as peroxy and hydroxyl radicals, led to damaged DNA, is  
607 involved in mutagenesis and carcinogenesis [40,41]. At the same concentration of  
608 hydrolysate incorporated, AU+1.0A had the higher inhibitory effect on DNA scission,  
609 compared to AU and 1.0A samples. The higher protective effect on DNA damage of  
610 AU+1.0A sample was related with higher ABTS radical scavenging activity and chelating  
611 activity (Table 4). Yarnpakdee et al. [39] reported that hydrolysate prepared by two-step  
612 processes from Nile tilapia showed higher protection effect against supercoiled DNA damage,  
613 compared to that prepared from the one step. Furthermore, Kittiphattanabawon et al. [21] also  
614 reported that the retention of supercoiled plasmid DNA induced by peroxy and hydroxyl  
615 radicals increased as DH of hydrolysate increased. Thus, the hydrolysates from cephalothorax  
616 of Pacific white shrimp could inhibit DNA oxidation induced by peroxy radical. It was  
617 suggested that hydrolysates could be used functional food ingredient to prevent oxidative  
618 stress.  
619  
620

#### 621 **4. Conclusion**

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

634

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639

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**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			
				$L^*$	$a^*$	$b^*$	$\Delta E^*$
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 °C for 3 h, 0.5A; 0.5% Alcalase for 2 h (55 °C, pH 8), 1.0A; 1.0% Alcalase for 2 h (55 °C, pH 8), AU+0.5A; autolysis for 3 h, followed by 0.5% Alcalase for 2 h (55 °C, pH 8), AU+1.0A; autolysis for 3 h, followed by 1.0% Alcalase for 2 h (55 °C, pH 8).

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

Amino acids (mg/g dry sample)	Samples					
	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 activities, 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 ( $\mu\text{mol TE/g sample}$ )	DPPH ( $\mu\text{mol TE/g sample}$ )	FRAP ( $\mu\text{mol TE/g sample}$ )	Chelating ( $\mu\text{mol EDTA/g sample}$ )	ORAC ( $\mu\text{mol TE/g sample}$ )
AU	443.87 $\pm$ 30.16e	4.84 $\pm$ 0.26a	7.51 $\pm$ 0.08c	6.50 $\pm$ 0.12e	349.67 $\pm$ 11.21d
0.5A	611.71 $\pm$ 6.92d	4.13 $\pm$ 0.23b	8.51 $\pm$ 0.33b	11.56 $\pm$ 0.39d	343.27 $\pm$ 17.40bc
1.0A	693.79 $\pm$ 31.01c	3.96 $\pm$ 0.22b	9.83 $\pm$ 0.54a	14.26 $\pm$ 0.25c	358.47 $\pm$ 25.04bc
AU+0.5A	737.34 $\pm$ 6.94b	3.07 $\pm$ 0.35c	5.38 $\pm$ 0.22d	15.24 $\pm$ 0.82b	371.63 $\pm$ 7.83ab
AU+1.0A	857.89 $\pm$ 26.51a	2.69 $\pm$ 0.19c	5.40 $\pm$ 0.50d	17.95 $\pm$ 0.28a	391.81 $\pm$ 8.15a

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

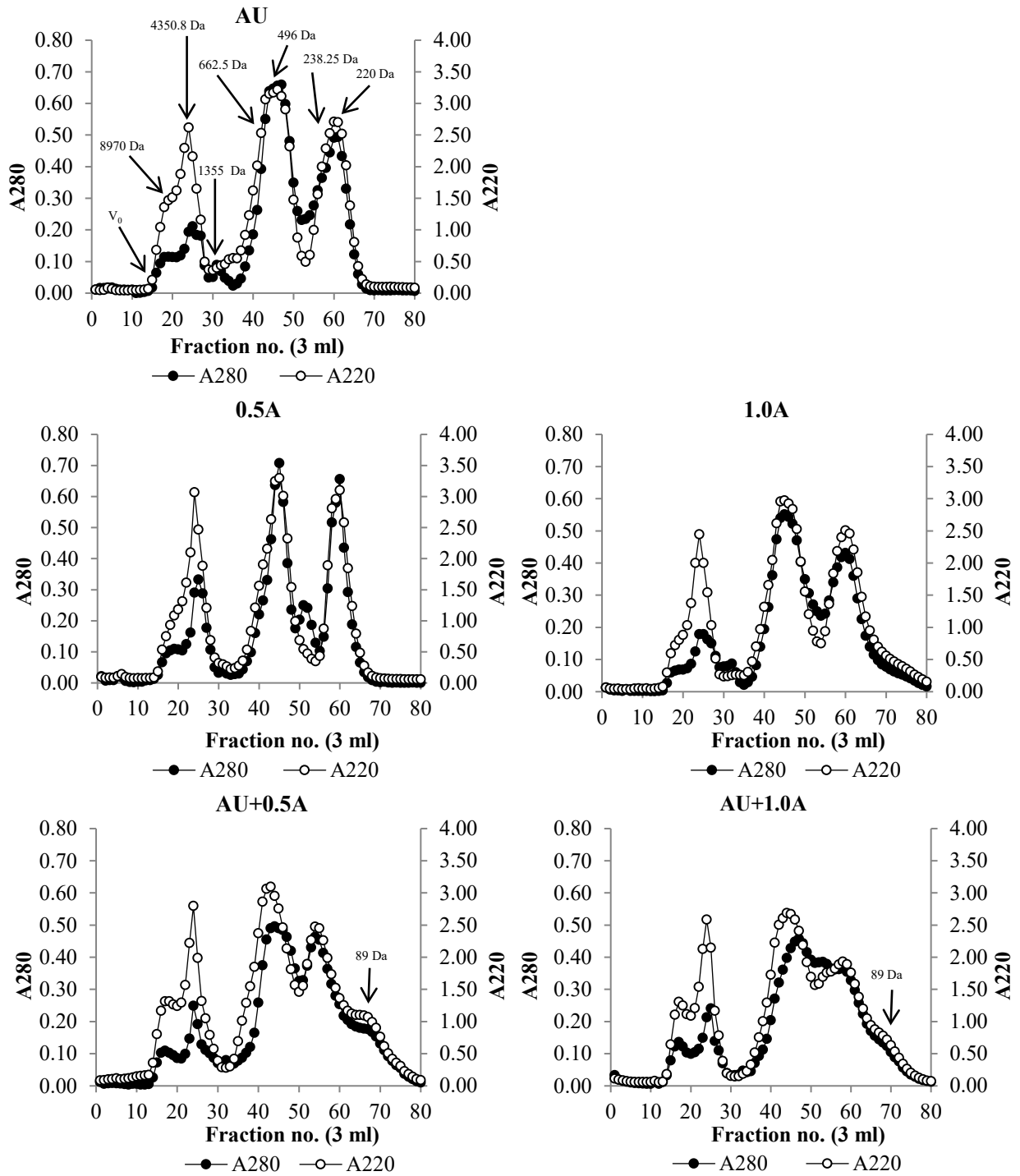
Different lowercase letters within the same column indicate significant difference (P<0.05).

Caption: see Table 1.

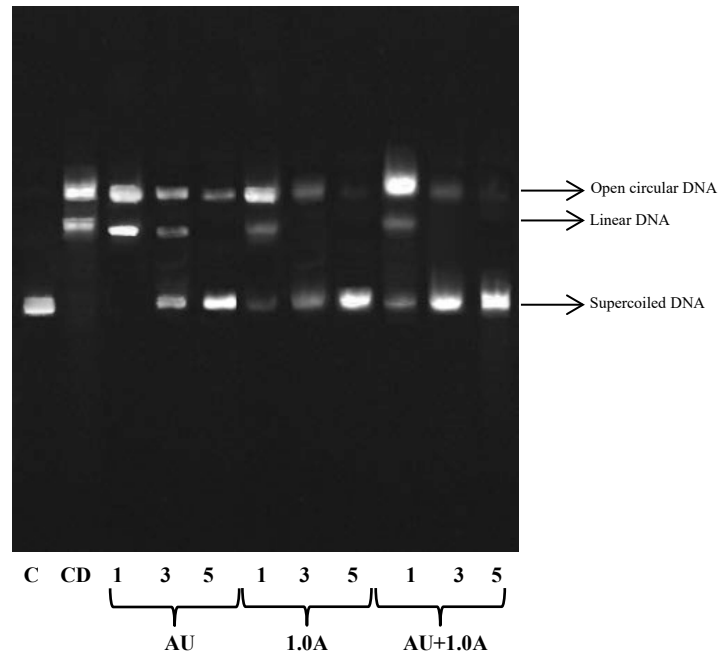
## **Figure legend**

**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 °C 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 peroxy 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.



**Figure 1**



**Figure 2**