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Title: Antiproliferative Activity of Peptides Prepared from Enzymatic Hydrolysates of Tuna Dark Muscle on Human Breast Cancer Cell Line MCF-7

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Abstract: To produce and identify antiproliferative peptides, two commercial enzymes, papain (PA) and Protease XXIII (PR) were used to hydrolyze tuna dark muscle byproduct, and the protein hydrolysates were purified and evaluated for antiproliferative activities against human breast cancer cell line MCF-7. The results showed that the peptide fractions with the molecular weight ranging from 390 to 1,400 Da possessed the greatest antiproliferative activity. The amino acid sequences of the two antiproliferative peptides isolated from PA and PR hydrolysates were Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1,206 Da) and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1,124 Da), while they show the dose-dependent inhibition effect of the MCF-7 cells with IC50 values of 8.1 and 8.8 μM, respectively. We thus conclude that antiproliferative hydrolysates from tuna dark muscle byproduct may be useful ingredients in food and nutraceutical applications.

Title: Antiproliferative Activity of Peptides Prepared from Enzymatic Hydrolysates of Tuna Dark Muscle on Human Breast Cancer Cell Line MCF-7

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Category: Bioactive constituents of foods

Significance:

Protein-rich byproducts from the seafood industry, especially dark muscles, have limited uses due to their dark color, accessibility to oxidation and off-flavor. They are mainly processed into low market-value products, such as fish waste meal and fertilizer. Therefore, to recover the seafood proteins for further utilization, such as an application of enzymatic technology, may produce highly valuable products. In Taiwan, canned tuna is the largest commercial canned fishery product, and the annual production quantity and value of canned tuna exceeds 1,619 tons and 169 million NT dollars in 2008. There have been some researches on anticancer peptides from food protein, such as fish sauce, soy protein, milk protein and beef protein, however, there has been no study used fish protein byproduct as the source for anticancer peptides. Further, there were only a few of studies identified the amino acid sequences of anticancer peptides. Therefore, we tried to use two commercial proteases, papain and Protease XXIII, to hydrolyze tuna dark muscle byproduct and then identify the antiproliferative activity of the hydrolysates against human breast cancer cell MCF-7. Moreover, the antiproliferative hydrolysates were isolated by gel filtration and high performance liquid chromatography (HPLC) in sequence, and amino acid sequences of the anticancer peptides were also determined.

According to the reviewers' comments, the manuscript has been revised for suitability to publish in "Food Chemistry". The revised comments are typed in "red

fonts” in the revised manuscript.

*Response to Reviewers

Dear Prof. Birch,

I have received the manuscript (FOODCHEM-D-10-02801) which was refereed for "Food Chemistry". Thank you and the referees for your patience to review this manuscript. According to the reviewers' comments, the manuscript has been revised for suitability to publish in this Journal.

The revision is shown as follows, and the revised comments are typed in "red fonts" in the revised manuscript.

Chia-Lin Jao Ph.D.
Oct. 19, 2010

To Reviewer 1:

1. Authors frequently use in the article the adjective "anticancer". However, the authors must carefully use that adjective, as "anticancer" may be used if the antiproliferative effect has been proved to be effective against cancer disease. You only prove the antiproliferative effect of the hydrolysates, not their "anticancer" effect.

Response: We have changed all "anticancer" to "antiproliferative" throughout the manuscript.

2. Line 51: Please correct "indentified"

Response: We have corrected "indentified" to "identified".

3. Line 53: Do you mean "antihypotensive", or "antihypertensive"?

Response: We have corrected "antihypotensive" to "antihypertensive".

4. Line 61: in vitro must be written in italics.

Response: We have changed "in vitro" to "*in vitro*".

5. Line 66: Please correct the name of the author "Macasieb".

Response: We have changed "Masasieb" to "Macasieb".

6. Line 81: Please change "showed" by "showing"

Response: We have changed "showed" by "showing".

7. Line 84-86: The phrase must be rewritten. It is not understandable as it was redacted.

Response: We have rewritten this phrase: "Although their analysis did not exclude the contribution of peculiar bioactive lipids and trace elements of minerals on inhibition of the two breast cancer cell lines, they demonstrated the antiproliferative activities of the hydrolysates were mainly attributed by peptides. However, they did not purify the antiproliferative peptides and determine their sequences".

8. Line 120: Please indicate (if it is known) the concentration of the enzyme inside the enzyme solution.

Response: We have changed the words in parenthesis to "63.25 mg/mL in deionized water"**.**

9. Line 160: That is true that the antiproliferative assays were performed with varying concentrations of the hydrolysates after fractionation, but the first assay was done at different times of hydrolysis, and I suppose the same concentration of raw material. It should be indicated in Materials and Methods.

Response: We have added the details in Materials and Methods. "The cells were seeded in a 96-well microtiter plate (1×10^4 cells/well) overnight, then treated with 1 mg/mL of hydrolysates and varying concentrations of hydrolysate fractions by following purified procedures, and incubated for 72 h".

10. Line 168: Please change "antioxidative" by "antiproliferative". The same in line 182.

Response: We have changed "antioxidative" by "antiproliferative".

11. Line 214: Please correct "Guimas".

Response: We have changed "Guimsa" to "Guimas".

12. Line 242: "the smallest molecular size."

Response: We have changed "small molecular size" to "smaller molecular size".

13. Line 271: "RP-C18".

Response: We have changed "RP-18" to "RP-C18".

14. Line 281: The meaning of I.C. should be explained in Materials and Methods.

Response: We have added the details in Materials and Methods. "The hydrolysate concentration which gives 50% growth inhibition is referred to as the IC_{50}".

15. Line 294: The first phrase must be rewritten, as it is understandable.

Response: We have deleted the first phrase to make the "Conclusion" section understandable.

Research highlights

→Peptides with MW between 390 to 1400 Da showed great antiproliferative activities.
→A peptide, LPHVLTPEAGAT (1206 Da), showed MCF-7 inhibition with IC_{50} value of 8.1 μM.

→A peptide, PTAEGGVYMVT (1124 Da), showed MCF-7 inhibition with IC50 value

of 8.8 μM.

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1 # Antiproliferative activity of peptides prepared from enzymatic hydrolysates of
2 # tuna dark muscle on human breast cancer cell line MCF-7

3

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14 **Running title:**

15 Antiproliferative Peptides from Tuna Dark Muscle Hydrolysate

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25

26 **ABSTRACT**

27 To produce and identify antiproliferative peptides, two commercial enzymes,
28 papain (PA) and Protease XXIII (PR) were used to hydrolyze tuna dark muscle
29 byproduct, and the protein hydrolysates were purified, before being evaluated for
30 antiproliferative activities against human breast cancer cell line MCF-7. The results
31 showed that the peptide fractions with the molecular weight ranging from 390 to 1,400
32 Da possessed the greatest antiproliferative activity. The amino acid sequences of the
33 two antiproliferative peptides isolated from PA and PR hydrolysates were
34 Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1,206 Da) and
35 Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1,124 Da), while they show the
36 dose-dependent inhibition effect of the MCF-7 cells with IC_{50} values of 8.1 and 8.8
37 μM, respectively. We thus conclude that antiproliferative hydrolysates from tuna dark
38 muscle byproduct may be useful ingredients in food and nutraceutical applications.

39

40 Keywords: Tuna dark muscle; Antiproliferative activity; Peptide; Protein hydrolysate.

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1. Introduction

In recent years, peptides have been identified to possess physiological functions, such as immunomodulatory (Gauthier, Pouliot & Saint-Sauveur, 2006), antimicrobial (Galvez, Abriouel, Lopez & Ben Omar, 2007), antihypertensive (Hsu, Cheng & Hwang, 2007), anticancer (Kim, Kim, Kim, Kang, Woo & Lee, 2000), antioxidative (Hsu, Lu & Jao, 2009) and cholesterol-lowering activities (Fukui et al., 2002). These bioactive peptides are mostly derived from milk, wheat, soybean, egg and fish proteins by enzymatic hydrolysis or fermentation (Wang & de Mejia, 2005).

Proteins, peptides and amino acids have been reported to show antitumor or antiproliferative activities. Bowman Birk protease inhibitor (BBI), a water-soluble protein isolated from legumes and some monocotyledonous seeds, has shown anticarcinogenic activity *in vitro* and in animal models (Armstrong, Kennedy, Wan, Atiba, McLaren & Meyskens, 2000). Soybean Kunitz trypsin inhibitor was reported to suppress ovarian cancer cell invasion by blocking urokinase upregulation (Kobayashi, Suzuki, Kanayama & Terao, 2004). Lunasin, a novel chemopreventive peptide from soybean, has been found to suppress chemical carcinogen and viral oncogene-induced transformation of mammalian cells and inhibit skin carcinogens in mice (Galvez, Chen, Macasieb & de Lumen, 2001; Jeong, Jeong, Kim & Lumen, 2007). Hydrophobic peptides from defatted soy protein hydrolyzed with thermoase showed *in vitro* cytotoxicity on mouse monocyte macrophage cell line, and their sequence was X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr (1,157 Da) (Kim et al., 2000). Lactoferrin and lactoferricin from bovine milk had an inhibition effect on lung metastasis and angiogenesis in mice transplanted with murine melanoma, lymphoma or colon carcinoma (Yoo, Watanabe, Watanabe, Hata, Shimazaki & Azuma, 1997).

74 Fish protein hydrolysates (FPH) have been reported to be a source of promising
75 health benefits components for nutritional or pharmaceutical applications (Wergedahl,
76 Liaset, Gudbrandsen, Lied, Espe & Muna, 2004; Qian, Je & Kim, 2007; Hsu et al.,
77 2009). However, the antiproliferative activity of peptides derived from FPH was rarely
78 studied. Vitilevuamide, a bicyclic marine peptide isolated from marine ascidians, was
79 cytotoxic in P388 lymphocytic leukemia (Edler, Fernandez, Lassota, Ireland & Barrows,
80 2002). Picot et al. (2006) found hydrolysates obtained from three blue whiting, three
81 cod, three plaice and one salmon showing significant inhibition on two human breast
82 cancer cell lines, MCF-7/6 and MDA-MB-231. The hydrolysates contained a complex
83 mixture of free amino acids, peptides with various sizes ranging up to 7 kDa and in a
84 lower proportion, lipids and sodium chloride. Although their analysis did not exclude
85 the contribution of peculiar bioactive lipids and trace elements of minerals on inhibition
86 of the two breast cancer cell lines, they demonstrated the antiproliferative activities of
87 the hydrolysates were mainly attributed by peptides. However, they did not purify the
88 antiproliferative peptides and determine their sequences. In this study, therefore, we
89 tried to use commercial proteases, papain and Protease XXIII, to hydrolyze tuna dark
90 muscle byproduct and then identify the antiproliferative activity on the human breast
91 cancer cell line, MCF-7. Furthermore, the antiproliferative hydrolysates were purified
92 by gel filtration and high performance liquid chromatography (HPLC) in sequence, and
93 the amino acid sequences of the antiproliferative peptides were also determined.
94
95 **2. Materials and methods**
96 *2.1. Sample preparation*
97 Hsing-Yi Frozen Foods Industry Ltd. (Chiayi County, Taiwan) supplied the tuna

98 dark muscle by-product. The whole tuna fish (*Thunnus tonggol*) was cooked by steam

99 (100 - 105°C) for 3 - 4 h, after which the dark muscle by-product was vacuum-sealed in

100 400-ml polyethylene bags and then transferred on ice to our laboratory immediately,

101 before being stored at - 80 °C until used. The protein, lipid, ash and moisture contents

102 of the dark muscle by-product were 25.3, 2.30, 1.29 and 71.3%, respectively, as

103 reported in the previous study (Hsu, 2010).

104

105 *2.2. Chemicals and reagents*

106 Papain (specific activity of 3.8 units/mg solid), an endopeptidase prepared from

107 papaya latex; and Protease XXIII (specific activity of 4.6 units/mg solid), an

108 endopeptidase prepared from *A. melleus*, were both obtained in dry powder form from

109 Sigma-Aldrich, Inc. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-diphenyl

110 tetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO),

111 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-glutamine, Doxorubicin hydrochloride

112 (Dox), N-Formyl-Ala-Gly-Ser-Glu (Mr 390), Bradykinin (Mr 1,400) and

113 Biocytin-neuropeptide Y (Mr 4,500), trifluoroacetic acid (TFA), and acetonitrile were

114 purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sephadex G-25 was

115 produced by Pharmacia Biotech Co. (Uppsala, Sweden). Other chemicals and reagents

116 used were analytical grade and commercially available.

117

118 *2.3. Enzymatic hydrolysis*

119 Fifty grams of defatted mince was homogenized with 100 ml deionized water and

120 preincubated at either 25 or 37 °C for 20 min prior to enzymatic hydrolysis. The

121 hydrolysis reaction was started by the addition of 2 ml enzyme solution (63.25 mg/ml in
122 deionized water) at an enzyme/substrate ratio of 1:100 (w/w). The reaction with papain
123 (PA) and Protease XXIII (PR) were conducted at pH 6.2, 25℃ and pH 7.5, 37 ^{o}C,
124 respectively, for up to 6 h. The pH of the mixture was left constant by using 1 N NaOH
125 during hydrolysis. After hydrolysis, the hydrolysates were heated in a boiling water bath
126 for 20 min to inactivate the enzymes. Hydrolysates were centrifuged (Centrifuge himac
127 CR21, Hitachi Ltd., Katsuda, Japan) at 12,000 × g for 15 min. A part of the supernatant
128 was collected to determine the degree of hydrolysis, in addition to this the other part
129 was freeze-dried (FDU-2000, EYELA freeze-dryer, Tokyo, Japan) and stored in a
130 desiccator.
131
132 *2.4. Measurement of the degree of hydrolysis*
133 According to the method of Benjakul and Morrissey (1997) with some
134 modifications, the degree of hydrolysis (DH) of the hydrolysate was determined as the
135 ratio of the amount of α-amino acid released during hydrolysis to the maximum amount
136 of α-amino acid in dark muscle. Properly diluted samples (125 μl) were mixed with 2
137 ml of 0.2125 M sodium phosphate buffer (pH 8.2), followed by the addition of 1 ml of
138 0.01% TNBS. The mixtures were then incubated in a water bath at 50℃ for 20 min in
139 the dark. The reaction was terminated by the addition of 2 ml of 0.1 M sodium sulfite.
140 The mixtures were cooled down at ambient temperature for 20 min. The maximum
141 amount of α-amino acid in dark muscle, on the other hand, was obtained by acid
142 hydrolysis with 6 N HCl at 105℃ for 24 h. The acid-hydrolyzed sample was then
143 filtered through Whatman filter paper No. 1 to remove the unhydrolyzed debris. The

144 supernatant was neutralized with 6 N NaOH before α-amino acid determination. The
145 absorbance was measured at 420 nm and α-amino acid was expressed in terms of
146 L-leucine. The DH was calculated as follows:

147 $$DH(\%) = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

148 where L_t is the amount of α-amino acid released at time t; L_0 is the amount of α-amino
149 acid in original dark muscle; L_{max} is the maximum amount of α-amino acid in dark
150 muscle (Beak & Cadwallader, 1995).
151
152 *2.5. Cell culture*
153 Human breast cancer MCF-7 cells purchased from American Type Culture
154 Collection were cultured in a 37℃ humidified atmosphere with 5% CO_2 in DMEM
155 (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum
156 (FBS), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.
157
158 *2.6. MTT assay*
159 To avoid pH variation of the cell culture medium during sample solubilization, fish
160 hydrolysate stock solution was prepared in 0.1 M PBS (pH 7.4). The cells were seeded
161 in a 96-well microtiter plate (1×10^4 cells/well) overnight, then treated with 1 mg/ml of
162 hydrolysates and varying concentrations of hydrolysate fractions by following purified
163 procedures, then incubated for 72 h. The effect of hydrolysates on cell growth was
164 examined by the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide)
165 assay. 20 μl of MTT solution (5 mg/ml, Sigma Chemical Co., St. Louis, MO) were
166 briefly added to each well and incubated for 4 h at 37℃. The supernatant was aspirated

167 and the MTT-formazan crystals formed by metabolically viable cells were dissolved in
168 200 μl of DMSO. Finally, the absorbance was read at 595 nm with a microplate reader.
169 The hydrolysate concentration which gives 50% growth inhibition is referred to as the
170 IC_{50}.
171
172 *2.6. Purification of antiproliferative peptides*
173 *2.6.1. Gel filtration chromatography*
174 The antiproliferative peptides of the hydrolysates were separated using column
175 chromatography as described by Chen et al. (1995). The lyophilized hydrolysates (270
176 mg) were dissolved with 2 ml of deionized water. The resulting solution was
177 fractionated by gel filtration on a Sephadex G-25 column (2.5 × 50 cm), and eluted with
178 deionized water. Each 5 ml fraction was collected at a flow rate of 40 ml/h, and the
179 absorbance monitored at 220 nm to separate peptide fractions.
180 N-Formyl-Ala-Gly-Ser-Glu (Mr 390), Bradykinin (Mr 1,400) and
181 Biocytin-neuropeptide Y (Mr 4,500) were used as the comparable standards of
182 molecular weight. Each collected fraction was then lyophilized and stored in a
183 desiccator until use.
184
185 *2.6.2. High performance liquid chromatography (HPLC)*
186 The fractionated hydrolysates by gel filtration exhibiting antiproliferative activity
187 were further purified using high performance liquid chromatography (Model L-2130
188 HPLC, Hitachi Ltd., Katsuda, Japan). The lyophilized hydrolysate fraction (100 μg) by
189 gel filtration was dissolved in 1 ml of 0.1% TFA. 90 μl of the mixture was then injected
190 into a column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm, Agilent Tech. Inc., CA,

191 USA) using a linear gradient of acetonitrile (5 to 25% in 50 min) in 0.1% TFA under a
192 flow rate of 0.8 ml/min. The peptides were detected at 215 nm. Each collected fraction
193 was then lyophilized and stored in a desiccator until use.
194
195 *2.7. Determination of amino acid sequence*
196 An accurate molecular mass and amino acid sequence of the purified peptides was
197 determined using a Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled
198 with an electrospray ionization (ESI) source. The purified peptides were separately
199 infused into the electrospray source after being dissolved in methanol/water (1:1, v/v),
200 and the molecular mass was determined by the doubly charged $(M + 2H)^{+2}$ state in
201 the mass spectrum. Automated Edman sequencing was performed by standard
202 procedures using a 477-A protein sequencer chromatogram (Biosystems, Foster, CA,
203 USA).
204
205 *2.8. Statistical analysis*
206 Each data point represents the mean and standard deviation of three samples. The
207 data was subjected to analysis of variance (ANOVA) followed by Duncan's multiple
208 range post hoc test, and the significance level of $P < 0.05$ was employed. Statistical
209 significance of growth inhibition induced by hydrolysates was calculated following χ^2
210 test with $\alpha = 0.05$.
211
212 **3. Results and discussion**
213 *3.1. Degree of hydrolysis during enzymatic hydrolysis*

214 The DH of tuna dark muscle with PA and PR both increased dramatically
215 during the initial 60 min and then increased gradually thereafter (Fig. 1), which
216 indicated that maximum cleavage of peptides occurred within 60 min of hydrolysis.
217 The result was similar to that reported for enzymatic hydrolysis of different fish
218 proteins (Benjakul & Morrissey, 1997; Guerard, Guimas & Binet, 2002; Dong,
219 Zeng, Wang, Liu, Zhao & Yang, 2008). The highest DHs (%) for PA and PR were
220 20.4 and 30.2%, respectively, both obtained at 360-min hydrolysis. The trend and
221 curve shape of the hydrolysis are similar to those previously reported in our studies
222 (Jao & Ko, 2002; Hsu et al., 2009; Hsu, 2010) and in other studies for various
223 protein sources (Klompong, Benjakul, Kantachote & Shahidi, 2007; Dong et al.,
224 2008; Bougatef et al., 2010).
225
226 *3.2. Antiproliferative activity of hydrolysates*
227 Figure 2 shows the antiproliferative effect of the hydrolysates (concentration of 1
228 mg solid/ml) derived from tuna dark muscle byproduct on breast cancer cell, MCF-7,
229 during hydrolysis process. As depicted in Fig. 2, PA hydrolysates possessed significant
230 antiproliferative activity during 30 to 300 min hydrolysis, while PR hydrolysates also
231 showed antiproliferative activity during hydrolysis at 30 to 270 min except that at 60
232 and 240 min. The PA and PR hydrolysates with the strongest antiproliferative activity
233 during hydrolysis were obtained at 60 min and 120 min, respectively; therefore, they
234 were used for further purification to identify their amino acid sequences. No correlation
235 between degree of hydrolysis and antiproliferative activity was observed in this or
236 previous studies (Picot et al., 2006).
237

238 *3.3. Antiproliferative activity of hydrolysates fractionated by gel filtration*
239 *chromatography*

240 Figure 3 shows the elution profiles and antiproliferative activities of PA and
241 PR enzymatic hydrolysates separated by gel filtration chromatography. Three and
242 four major fractions were obtained from PA and PR hydrolysates, respectively,
243 separated by Sephadex G-25 gel (Fig. 3). The molecular weight (MW) distributions
244 of fraction PAA and PAB, with relative high absorbance at 220 nm, were between
245 4,500 and 1,400 Da, 1,400 and 390 Da, respectively, and fraction PAC showed the
246 smallest molecular size < 390 Da. In addition, that of fraction PRA and PRB was
247 between 4,500 and 1,400 Da, 1,400 and 390 Da, respectively. Fraction PRC and
248 PRD were less than 390 Da in molecular size. The fractions with highest
249 antiproliferative activity ($P < 0.05$) obtained from each enzymatic hydrolysates
250 were PAB and PRB, and they showed 59.7 and 58.7% of cytotoxicity against
251 MCF-7 cell lines, respectively, at the concentration of 200 μg/ml. Therefore, the
252 two fractions, PAB and PRB, were used for further purification by HPLC.

253 The results also showed the protein hydrolysates with MW, ranging from 390
254 to 1,400 Da, possessed the highest antiproliferative against MCF-7 cell lines. To
255 our knowledge, the only antiproliferative peptide derived from a fish source was
256 reported as a 440.9 Da hydrophobic peptide, which was able to induce apoptosis in
257 human U937 lymphoma cells through the increase of caspase-3 and caspase-8
258 activity, isolated from anchovy sauce (Lee et al., 2003; 2004). The MWs of the
259 antiproliferative peptides obtained from soy protein and algae protein waste were
260 1,157 and 1,309 Da, respectively (Kim et al., 2000; Sheih, Fang, Wu & Lin, 2010).

261 However, some studies reported the MW of the antiproliferative peptides derived
262 from various sources was greater than 1,400 Da. An antifungal peptide with MW of
263 approximately 3.9 kD isolated from buckwheat seeds possessed antiproliferative
264 activities toward leukemia (L1210), breast (MCF-7), liver embryonic (WRL68) and
265 liver (HepG2) cancer cells (Leung & Ng, 2007); and Lunasin, a cancer-preventive
266 peptide with MW of about 5.45 kDa, isolated and characterized in soy and barley
267 has been demonstrated to be effective against chemical carcinogens and oncogenes
268 in mammalian cells and in a skin cancer mouse model (de Lumen, 2005). It seems
269 there is no correlation between antiproliferative activity and MW of peptides.
270

271 *3.4. Amino acid sequences of antiproliferative peptides on MCF-7*

272 To obtain a sufficient amount of purified peptide, chromatographic separations
273 were performed repeatedly. The fraction PAB and PRB, obtained from gel filtration
274 chromatography, were lyophilized and then subjected to HPLC on a RP-C18(e)
275 column (4.6 × 250 mm) with a linear gradient of acetonitrile (5-25%) containing
276 0.1% TFA. Three fractions for PAB (PAB1, PAB2 and PAB3) and two fractions for
277 PRB (PRB1 and PRB2) were observed (Fig. 4). Fraction PAB2 and PRB2 showed
278 the greatest ($P < 0.05$) antiproliferative activities, which showed 71 and 70%
279 cytotoxicity, respectively, at the concentration of 20 μg/ml. The PAB2 and PRB2
280 fractions were then used to determine their amino acid sequences. Table 1 shows
281 the amino acid sequences of the peptides derived from PAB2 and PRB2,
282 Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1,206 Da) and
283 Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1,124 Da); while they show the
284 dose-dependent inhibition effect of the MCF-7 cells with IC_{50} values of 8.1 and 8.8

285 μM, respectively. As compared to the antiproliferative peptides reported in
286 previous studies (Leung & Ng, 2007; Sheih et al., 2010), the two peptides obtained
287 in this study showed lower IC_{50} values against cancer cell lines. The peptide,
288 Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe (1,309 Da), derived from algae
289 protein waste, showed antiproliferative effect on human gastric cancer cell lines
290 AGS with an IC50 value of 256.4 μM (Sheih et al., 2010). The antifungal peptide
291 (approximately 3.9 kDa), isolated from buckwheat seeds, inhibited proliferation of
292 Hep G2, L1210, MCF-7 and WRL 68 cells with an IC_{50} of 33, 4, 25 and 37 μM,
293 respectively (Leung & Ng, 2007). The results showed that tuna dark muscle
294 byproduct would be a good source to produce antiproliferative peptides.
295
296 **4. Conclusions**
297 This study has clearly demonstrated tuna dark muscle byproduct has the
298 potential to be used as a material to produce peptides with the antiproliferative
299 effect on human breast cancer cells. The purified peptides,
300 Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1,206 Da) and
301 Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1,124 Da), showed lower IC_{50}
302 values against MCF-7 than those reported in previous studies against various
303 cancer cell lines. The effects of the antiproliferative peptides on cell cycle or
304 apoptosis of MCF-7 need to be further investigated.
305
306 **Acknowledgement**
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309

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408

409 **Figure Legends**

410 **Fig. 1.** Degree of hydrolysis (DH) of tuna dark muscle byproduct during
411 hydrolysis with PA and PR at 1:100 (w/w) enzyme/protein substrate. Bars represent
412 means and standard deviations from triplicate determinations.

413

414 **Fig. 2.** Effect of protein hydrolysates derived from tuna dark muscle byproduct
415 on cell proliferation of MCF-7 cells cultured for 72 h in cell culture medium
416 containing 1 mg/ml of hydrolysates. Each value is expressed as the mean ±
417 standard deviation (n=3). Statistical significance (*) of cell proliferation was
418 calculated following χ^2 test with $\alpha = 0.05$.

419

420 **Fig. 3.** Elution profile and cell proliferation of PA and PR hydrolysates separated
421 with gel filtration chromatography on Sephadex G-25.

422

423 **Fig. 4.** Purification of antiproliferative peptide fractions from gel filtration
424 chromatography on Sephadex G-25 by HPLC. The fractions PAB and PRB shown
425 in Fig. 3 were applied to a RP-18 column (4.6 × 250 mm), equilibrated with 0.1%
426 TFA in H_2O and eluted with a linear gradient of acetonitrile (5-25%) in 0.1% TFA
427 under a flow rate of 0.7 ml/min. Each fraction, at various concentrations, was used to
428 determine the cell proliferation.

429

430

431 **Table Captions**

432

433 **Table 1** Amino acid sequences and IC_{50} against MCF-7 cells of purified

434 antiproliferative peptides derived from tuna dark muscle byproduct hydrolyzed

435 with PA and PR.

Table1

Table 1 Amino acid sequences and IC_{50} against MCF-7 cells of purified antiproliferative peptides derived from tuna dark muscle byproduct hydrolyzed with PA and PR.

Fractions	Sequence (molecular weight)	IC_{50} (μM)
PAB2	Lue-Pro-His-Val-Lue-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1,206 Da)	8.1 ± 0.23
PRB2	Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1,124 Da)	8.8 ± 0.41

Figure1

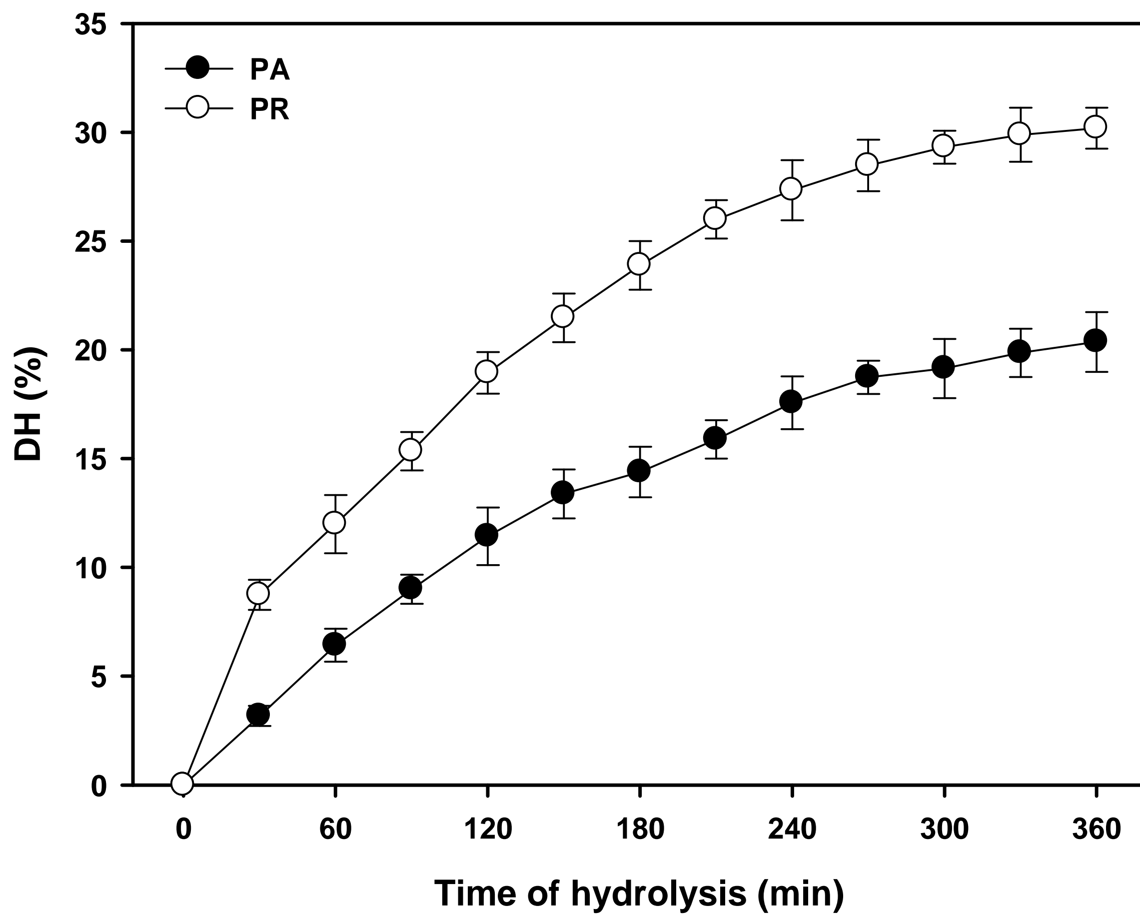

Figure 1

Figure2

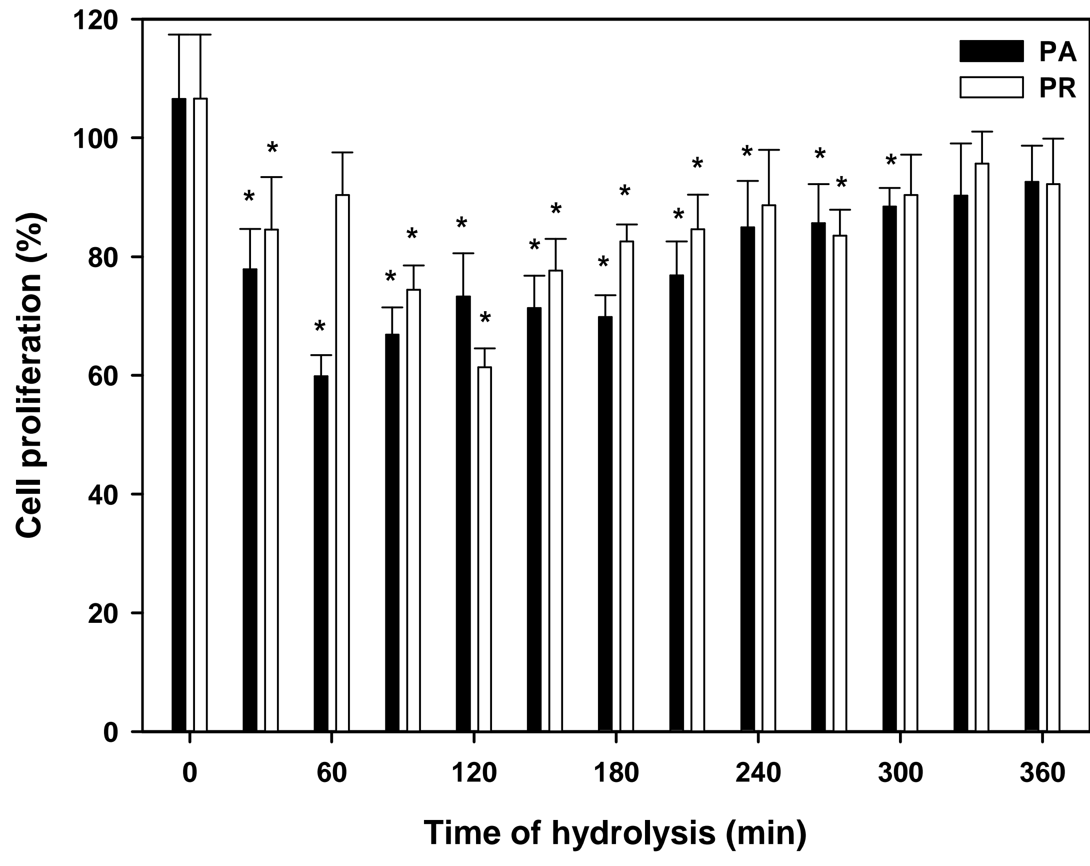

Figure 2

Figure3

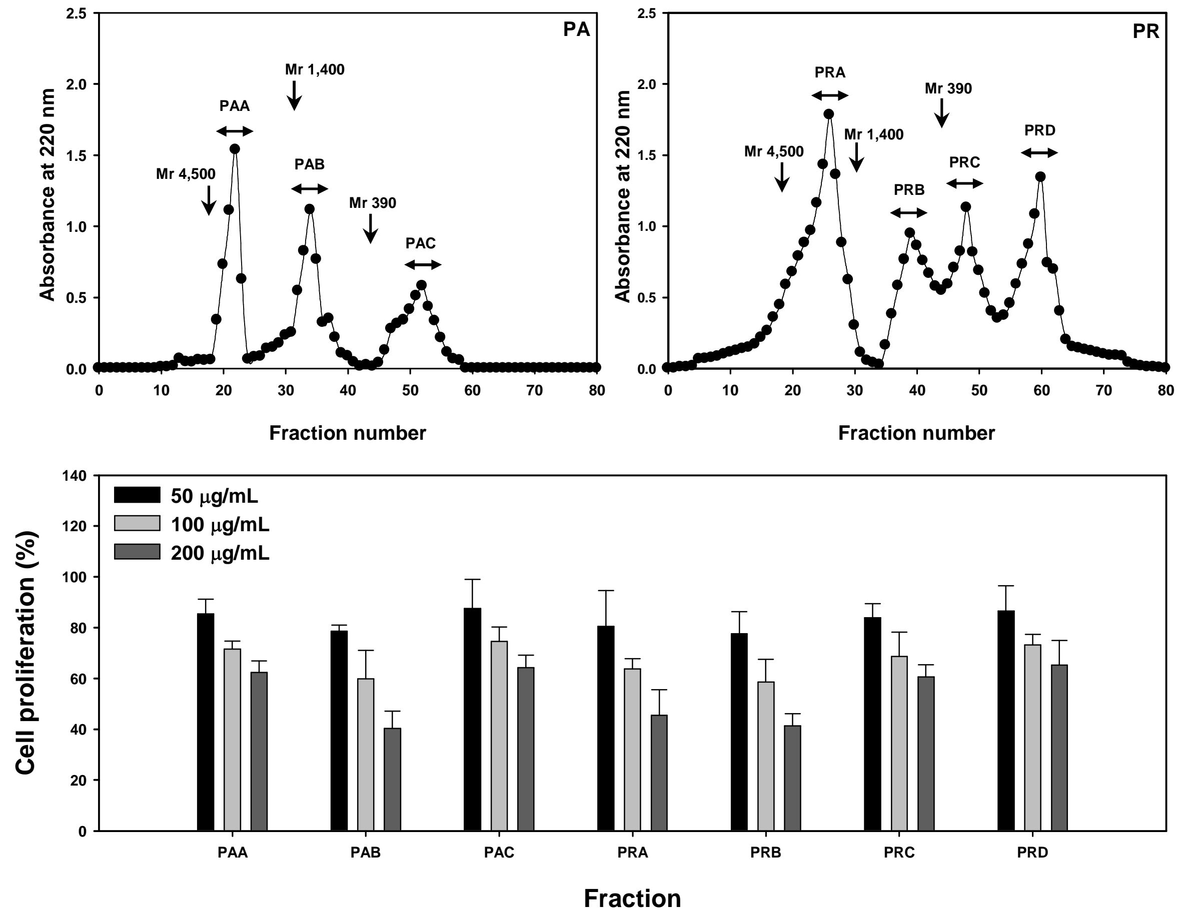

Figure 3

Figure4

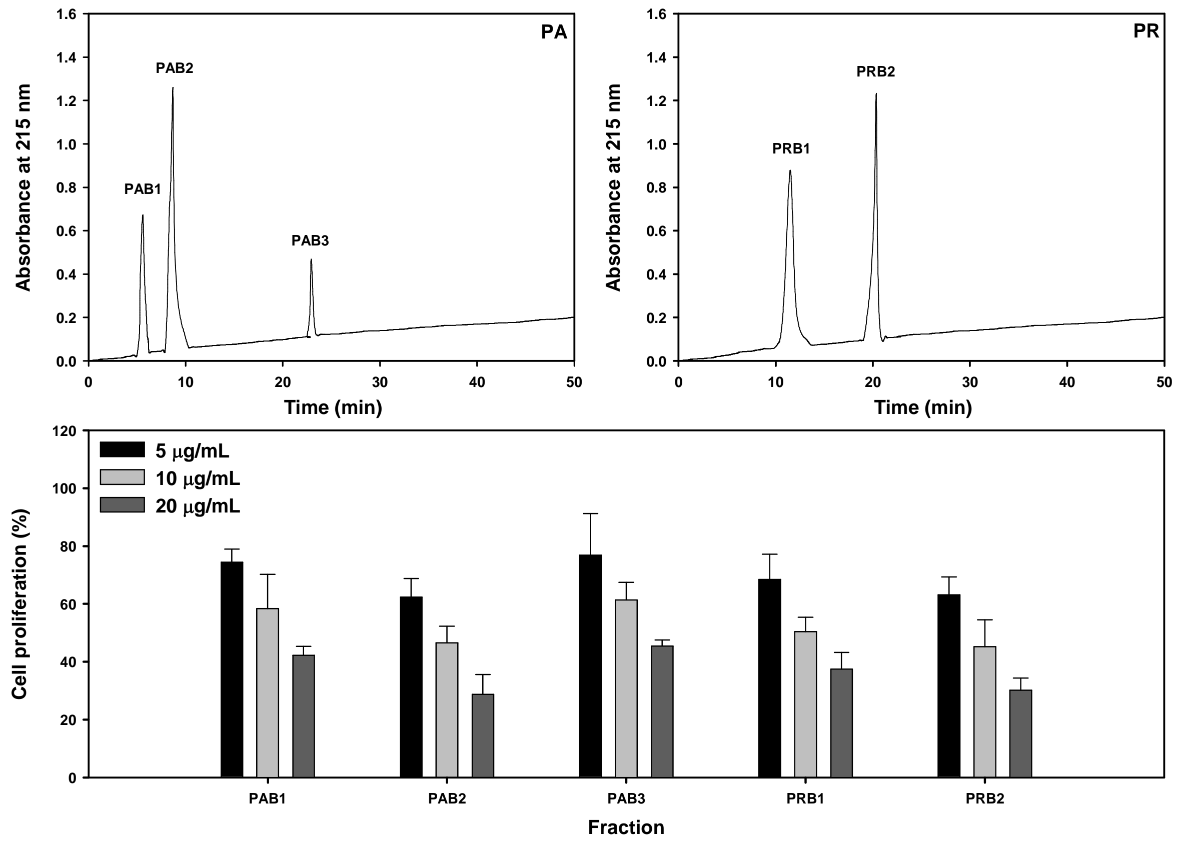

Figure 4