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Original Article

Optimal condition to remove mercury in yellowfin tuna protein isolates and ACE-inhibitory property of peptide prepared using commercial proteases

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Abstract

Response surface methodology (RSM) was performed to maximize the mercury (Hg) reduction from yellowfin tuna (*Thunnus albacare*) by products protein isolates (YBPI). The optimal condition of Hg reduction (89.3%) was 10.5 mM $CaCl_2$ and a Water:YB of 12.9:1, while other variables were fixed at 5 mM citric acid, 60 min incubation, pH 11 and 8,000 x g for 15 min of centrifugation. At these conditions, the significant protein recovery (80.1%) was obtained. Hydrolysates sequentially hydrolyzed with G6 followed by GN exhibited the highest angiotensin I-converting enzyme (ACE) inhibitory activity than other enzyme preparations. Fractionated yellowfin tuna by products protein hydrolysate (YBPH) increased in ACE-inhibitory activity when peptide size decreased. *In-vitro* gastrointestinal (GI) digestion significantly increased bioactive property. ACE-inhibitory activity of YBPH with and without simulated GI digestion significantly increased after incubating against ACE, demonstrating *pro-drug type* peptides.

Keywords: response surface methodology, commercial enzymes, *pro-drug type* peptides, gastrointestinal digestion

1. Introduction

Yellowfin tuna (*Thunnus albacore*) is commercially the most important species to use in tuna frozen industry in Thailand and the production of this tuna species has increased continuously. In 2012, they accounted for 27% of total tuna catches (FAO, 2012). However, only tuna meat is used in a fish frozen process since solid wastes such as viscera, head, skin, bone, and some muscle tissue are generated as high as 50-70% of original material (Torres *et al.,* 2007). Over past 10 years, fish byproducts have seen an increased interest because of their high amounts of protein and a good pattern of essential amino acids (Guerard *et al.,* 2002). Tuna can be contaminated by heavy metals in their tissues (Oh and Lee, 1981). Additionally, heavy metal ions such as mercury, arsenic, cadmium, or lead can form strongly complex bonds with sulfhydryl-groups (SH group) of proteins (Kowalski *et al.,* 2002). Especially, inorganic mercury can be methylated by microorganism to form methyl mercury ($HgCH_3$). This organic compound is extremely toxic and moderately soluble in water. For removal of $HgCH_3$, a complete dissolution of the biological tissue should be achieved, assuring accomplished cleavage of the bonds between $HgCH_3$ and organic groups as well as reduction of

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organic matrix interferences. However, many researches revealed that the protein extraction by alkali-aided conditions could remove hazardous heavy metal (Hwang *et al.,* 2008). Andrew-Hong *et al.* (2000) indicated that the solubilization of sludge protein, change of pH of up to 12.5, was the most effective method for heavy metal removal and protein recovery. Chelators (ethylenediaminetetraacetate (EDTA) and citric acid) enhance the efficiency of solubilization method by extracting heavy metals from a heavy-metal-containing substance (Oh and Lee, 1981). Chloride ion can complex with Hg^{+} to form HgCl precipitate (Kowalski *et al.,* 2002). In addition, calcium ion fairly aggregates phospholipids through an anhydrous calcium-phospholipids complex, thus enhancing the reduction of phospholipids from protein recovery (Liang and Hultin, 2005). Therefore, isoelectric precipitation combined with calcium ion and citric acid can be used to prepare protein isolates from fish byproducts to reduce Hg and increase protein recovery. This would be an alternative way to sustain a management and add value of fishery byproducts.

Protein isolates can be subjected to hydrolytic treatments in order to produce a high quality of protein hydrolysates, which exhibited the inhibition of the angiotensin I-converting enzyme (Kitts and Weiler, 2003). Angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is physiologically significant in blood pressure regulation. This enzyme converts the inactive peptide angiotensin I to vasoconstrictive peptide angiotensin II (Li *et al.,* 2004) resulting in a raising blood pressure. Peptides can inhibit ACE activity via competitive inhibition (Sato *et al.,* 2002). This enzyme inhibition mechanism is competition of the peptides with ACE substrate for the enzyme catalytic sites (Kitts and Weiler, 2003).

This research primarily focused on optimization of the selected variables to obtain maximal Hg reduction and protein recovery in yellowfin tuna byproduct protein isolate production. Consequently, ACE-inhibitory peptides which prepared by various commercial proteases was evaluated. In addition, the effects of membrane fractionation using ultrafiltration on ACE-inhibitory activity of yellowfin tuna by products protein hydrolysates as well as their stabilities against simulated gastrointestinal digestion and ACE were investigated.

2. Materials and Methods

2.1 Preparation of pretreated fish mince

Yellowfin tuna byproducts (YB), including belly and trimmed meat obtained from Thai Ocean Venture Co., Ltd. frozen industry in Phuket Province, Thailand. The fish was kept in an ice box with a 2:1 fish to ice ratio and transferred to the laboratory in 6 hrs. YB was cut into smaller pieces (2x2 cm) immediately upon arrival, and then minced in a food grinder (Biro 8-22 E97, The BIRO MFG. Co., USA.) using two different grinding plates with 10 and 5 mm (diameter) perforations. The mince was vacuum-packed (Type SAE 10, Supervac Ltd., Germany) and kept frozen (SF-C695, Sanyo Co. Ltd., Thailand) at -30°C until used in the experiments. Each experiment replication of protein extraction by alkali-aided conditions assisted with additives was accomplished within 1 month of frozen storage.

2.2 Protein extraction by alkali-aided conditions assisted with additives

YB mince was thawed at 25-27°C for about 1 hr and then homogenized with various designed levels of cold $CaCl_2$ solution containing 5 mM citric acid (4-6°C) at various ratios of water to yellowfin tuna by products mince (W:YB) (w/w) using a homogenizer (Ace homogenizer, Nihonseiki Kaisha Ltd., Japan) at a speed of 8,000 rpm for 2 min (Montero and Gomez-Guillen, 1996). The concentration of calcium chloride and ratios of water to yellowfin tuna by products mince were shown in Table 1. The initial pH of the homogenate was in the range of 6.0-6.5 and then was adjusted to pH 11 (pH meter Lab 850, Schott Instruments, Germany) with 2.0 N NaOH solutions in an ice bath (>10°C). After centrifugation (AVANTI ™ J25, Beckman Ltd., USA.) at 8,000 x g, 4°C for 20 min, the middle liquid layer was collected for protein analysis compared to protein content in various homogenates. The protein solution phase was decanted and subsequently adjusted to the isoelectric pH 5.50±0.05 using 2 N HCl. At the pH 5.5 fish muscle proteins are precipitated due to the increase of protein-protein hydrophobic interactions and the decrease of protein-water interactions including the decrease

Table 1. Independent variables and levels of CCD applied to the alkali-aided extraction added with additives.

Independent variables		Levels				
Code	Real	-a	+1	0	+1	+a
x_1	$CaCl_2$ concentration (mM)	8.4	9.0	10.4	11.8	12.4
x_2	W:YB (w/w)	11.4	12.0	13.5	15.0	15.6

of protein-protein electrostatic repulsion (Jaczynski, 2008). The precipitate or yellowfin tuna by products protein isolate (YBPI) was collected using centrifugation (8,000 x g at 4°C for 20 min).

Variation effects in calcium chloride concentration ($CaCl_2$) and ratios of water to YB mince (W:YB) were analyzed using RSM. These variables were selected according to our previous study (Kokkaew *et al., 2015*). 2^2 central composite design (CCD) was used to determine the optimal condition for the extraction process (Guo-qing *et al.,* 2005), which maximizes the responses; Hg reduction (Y_1), and protein recovery (Y_2). Meanwhile, the other variables were fixed at pH 11, 5 mM citric acid, 60 min incubation time, and 8,000 x g centrifugation. Symbols and coded variable levels for the variables ($CaCl_2$; x_1) and W:YB; x_2) are given in Table 1 (response surface were obtained using $\pm|\alpha| = 1.41$). 11 experiments were generated (Table 2). The second-order model proposed for the response (Y) was:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

where Y is the response variable, x_1 and x_2, are the coded process variables and β_0, β_1, β_2, β_{11}, β_{22}, and β_{12} are the regression coefficients.

2.3 Enzymatic hydrolysis

To produce the various yellowfin tuna by products protein hydrolysates (YBPH), YBPI was digested by commercial enzymes including Protease G6 (G6), Alcalase 2.4 L FG (Alc), Protease GN (GN), and Flavourzyme® 500 MG (Fla). The biochemical characteristics of proteases and hydrolysis conditions used in the experiments were shown in Table 3 and 4, respectively. The enzymatic hydrolysis processes were performed in a stirred batch and thermostat reactor (1 L) (Fermenter Biostat B; B. Braun Biotech International, Germany). Briefly, a constant concentration of YBPI (10%, w/v, protein basic) was used in all hydrolysis processes (Megias *et al.,* 2009). G6 or Alc was used to be the first protease applied in the sequential hydrolysis and then the second protease (GN or Fla) was added into the process. YBPH was randomly taken every 60 min to analyze the degree of hydrolysis and ACE-inhibitory activity. To terminate the hydrolysis process, enzymes were inactivated by heating at 80°C for 20 min. The heated suspension was cooled and then centrifuged at 10,000 x g at 4°C for 20 min. YBPH solution was freeze-dried (Freezone 4.5; Lab Conco Freeze dry system Ltd., USA) and then kept at -30°C.

2.4 Ultrafiltration membrane fractionation

YBPH powder (800 mg) was suspended in 10 mL (80 mg/mL) distilled deionized water (ddH_2O) using a vortex mixture for 2 min and then fractionated via ultrafiltration membranes (The Kvick™ Start cassette, Healthcare Amersham Biosciences Corp., NJ, USA) with molecular weight cut-off of

Table 2. Response values for Y_1, and Y_2 with different combinations of x_1 and x_2 in CCD.

Experiment	Code level[1]		Response values[2, 3]	
	x_1 (mM)	x_2 (w/w)	Y_1 (%)	Y_2 (%)
1	-1 (9.0)	-1 (12.0)	88.2	69.8
2	+1 (11.8)	-1 (12.0)	88.3	66.8
3	-1 (9.0)	+1 (15.0)	87.0	80.8
4	+1 (11.8)	+1 (15.0)	86.5	74.4
5	-α (8.4)	0 (13.5)	87.7	74.5
6	+α (12.4)	0 (13.5)	88.0	73.0
7	0 (10.4)	-α (11.4)	88.4	72.5
8	0 (10.4)	+α (15.6)	86.4	77.9
9	0 (10.4)	0 (13.5)	89.4	78.8
10	0 (10.4)	0 (13.5)	89.3	84.8
11	0 (10.4)	0 (13.5)	88.7	82.7

[1] x_1: $CaCl_2$ concentration (mM), x_2: W:YB (w/w).
[2] Y_1: Hg reduction, Y_2: protein recovery.

Table 3. Biochemical characteristics of commercial enzymes as outlined by manufacturers.

Enzyme	Temp. range °C	pH Range	Source	Type of proteinase	Preferential specificity
Protease G6(G6)[1]	55-70	7.0-10.0	*Bacillus licheniformis*	Alkaline serine endopeptidase	Broad specificity, mainly hydrophobic-COOH
Alcalase 2.4 L FG (Alc)[2]	55-70	6.5-8.5	*Bacillus licheniformis*	Endoprotease	Broad specificity, mainly hydrophobic-COOH
Protease GN (GN)[1]	40-60	6.0-8.0	*Bacillus amyloliquefaciens*	Metallo neutral endopeptidase	Leu, Phe-NH_2 and others
Flavourzyme® 500 MG (Fla)[2]	50-55	5.5-7.5	*Aspergillus oryzae*	Endoprotease/ exopeptidase	Preferential cleavage: Arg, Lys-COOH

[1] Data from Genencor International, Inc., USA. [2] Data from Novozymes A/S, Denmark.

Table 4. Hydrolysis conditions of YBPI using commercial proteases.

Run	Hydrolysis conditions					
	Enzyme code	Enzyme conc. (%,w/w)	pH	Temp (°C)	Hydrolysis time (min)	YBPI/H_2O
1	G6	4	8	55	240	1:9
2	Alc	4	8	55	240	1:9
3	GN	4	8	55	240	1:9
4	Fla	4	8	55	240	1:9
5	G6/GN	3/1	8	55	60/180	1:9
6	G6/Fla	3/1	8	55	60/180	1:9
7	Alc/GN	3/1	8	55	60/180	1:9
8	Alc/Fla	3/1	8	55	60/180	1:9

10 and 5 kDa. When a 10 kDa membrane was used, more than 10 kDa retentate fraction (R10) and less than 10 kDa filtrate were obtained. Consequently, less than 10 kDa filtrate was again ultra-filtered as using a 5 kDa membrane filter to yield two fractions, a retentate (between 5 and 10 kDa, F5-10), and a permeate (less than 5 kDa, P5). All fractions were freeze-dried and stored at -30°C.

2.5 Stability of peptide to pepsin-pancreatin simulated gastrointestinal digestion

Crude YBPH (C-YBPH), R10, F5-10, and P5 were used to evaluate the stability of peptide against pepsin-pancreatin simulated gastrointestinal (GI) digestion according to the method of Cinq-Mars *et al.* (2008). YBPH powder (225 mg) was mixed with 15 mL ddH_2O, and adjusted pH to 2.0 with 5 N HCl. Pepsin was added (E/S 1:35 w/w), and the reaction mixture was incubated with continually shaking at 37°C for 1 hr. The pH was adjusted to 5.3 with a saturated $NaHCO_3$ solution and further adjusted up to 7.5 with 5 N NaOH. Pancreatin was added (E/S 1:25 w/w), and then the reaction mixture was incubated at 37°C and thoroughly shaking with speed 50 rpm for 2 hrs. To terminate the digestion, the solution was heated in boiling water at 100°C for 10 min. All peptides before and after GI digestion were analyzed for ACE-inhibitory activity.

2.6 Digestibility of peptides by ACE

C-YBPH and three fractions before and after simulated GI digestion were evaluated the ACE-inhibitory peptide types according to the method of Fujita *et al.* (2000). ACE-inhibitory peptides can be classified into three types depending on their interaction with ACE including *inhibitor type, substrate type*, and *pro-drug type* (Pihlanto-Leppala *et al.,* 2000). Briefly, YBPH powder was prepared at 5 mg/mL in 0.1 M Na-phosphate and 0.3 M NaCl (pH 8.3). The solution was then incubated with ACE (6 mU/mg of peptide) at 37°C for 3 hrs. ACE was inactivated by boiling for 10 min. IC_{50} values of ACE-inhibitory activity were compared before and after pre-incubation.

2.7 Analytical methods

2.7.1 Mercury content

Mercury content was assayed with inductively coupled plasma mass spectroscopy (ICP-MS) (model 7500C, Agilent, Japan) followed by the method of Eaton (2005). Sample and 1.25 mL nitric acid 65% were added in the test tube and thoroughly mixed for 1 min. Digestion was performed in water bath at 95°C for 2 hrs and left at room temperature (25°C). The reaction mixture was filtered through Whatman no. 1 filter paper. The filtrate was run by ICP-MS. The percentage of Hg reduction was calculated as: Hg reduction (%) = {[(Hg content in YB x wt. of YB) - (Hg content in protein extract x wt. of protein extract)] / (Hg content in YB x wt. of YB)} x 100%

2.7.2 Protein recovery

The adjusted homogenate (pH 11) and protein solution were analyzed for the total soluble protein by the modified Lowry method (Stoscheck, 1990). The protein recovery was determined based on the total soluble protein content of protein solution to the total protein content in adjusted homogenate. The percentage of protein recovery was calculated as: Protein recovery (%) = [(Protein content in supernatant x wt. of supernatant) / (Total protein in homogenate x wt. of homogenate)] x 100

2.7.3 Degree of hydrolysis (DH)

DH was analyzed using *o*-phthaldialdehyde (OPA) method described by Nielsen *et al.* (2001). OPA reagent was prepared as follows: A 7.620 g disodium tetraborate decahydrate and 200 mg sodium-dodecyl-sulfate (SDS) were thoroughly dissolved in 150 mL ddH_2O. OPA solution was prepared by 160 mg OPA 97% dissolved in 4 mL ethanol and

then transferred to the above solution. A 176 mg dithiothreitol 99% (DTT) was added to the solution and made up to 200 mL with ddH_2O. To assay DH, standard or sample (400 µL) was added to a test tube containing 3 mL OPA reagents and completely mixed for 5 s. The mixture was left for 2 min before measure the absorbance at 340 nm (Spectrophotometer; LAMBDA 25, Perkin Elmer, Inc., Germany). DH was calculated as: DH (%) = (h / h_{tot}) x 100, where h_{tot} of fish is 8.6. Meanwhile, h was calculated as: h = (serine-NH_2 - β) / α meqv/g protein, where α and β of fish are 1.0, and 0.4 respectively. Serine-NH_2 = [(OD_{sample} - OD_{blank}) / ($OD_{standard}$ - OD_{blank})] x [(0.9516 meqv/L) x 0.1 x (100/X) x P], where serine-NH_2 = meqv serine NH_2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L).

2.7.4 Angiotensin I-converting enzyme inhibitory activity

ACE-inhibitory activity assay was performed by high performance liquid chromatography method, which modified method of Li *et al.* (2005). For each assay, a sample solution was pre-incubated with Hippury-L-histidyl-L-Leucine (HHL) 0.2% in 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl at 37°C for 5 min. Reaction was initiated by addition of ACE solution (100 mU/mL), and the mixture was incubated at 37°C for 30 min. Reaction was stopped by adding 1 M HCl and HEPES buffer was then added to reaction mixture. Reaction mixture (20 mL) after stopping the reaction was analyzed on a Jupiter C18 (5 µm, 4.6 mm x 250 mm; Phenomenex, USA). UV absorbance of eluent was monitored at 228 nm to detect HHL. After injection in HPLC of control and assay with inhibitor, HHL peak areas obtained in two cases were measured and ACE inhibition (%) was calculated as: ACE inhibition (%) = [1 - ($A_{inhibitor}$/$A_{control}$)] x 100, where $A_{inhibitor}$ and $A_{control}$ are relative areas of HHL peak of assay with inhibitor and control sample without inhibitor, respectively. The IC_{50} was determined by regression analysis of ACE-inhibitory activity (%) versus log protein concentration (µg/mL). Protein content was analyzed by Lowry method (Lowry *et al.,* 1951).

2.8 Statistical analysis

Central composite design (CCD) was conducted using Design Expert software version 5.08 (Stat-Ease, Inc., Minneapolis, MN, USA.). Analysis of variance was performed by ANOVA. Significant differences between means were determined using Duncan's multiple range test (P<0.05). Statistical analysis was carried out using SPSS statistic program (Version 19) for Windows (SPSS Inc., Chicago, IL, USA). Means were accepted as significantly different at a 95% confidence interval (P<0.05) (Zhuang, 2009). All tests were conducted in duplicate and data were averaged.

3. Results and Discussion

3.1 Central composite design

Hg reduction (Y_1), and protein recovery (Y_2) in CCD varied from 86.5 to 89.4%, and 66.8 to 84.8%, respectively (Table 2). The regression models for these variables did not show any lack of fit ($P > 0.05$) and were statistically significant ($P < 0.05$) with R^2 = 0.9621 and 0.8535 respectively, indicating that the adjustment of the model to the experimental data were appropriate. The second-order models were presented in Equation 2 and 3.

$$Y_1 = 89.13 - 0.74x_2 - 0.67x_1^2 - 0.90x_2^2 \quad (2)$$

$$Y_2 = 82.13 + 3.28x_1 - 4.57x_1^2 - 3.58x_2^2 \quad (3)$$

The effect of $CaCl_2$ concentration (x_1) and W:YB (x_2) was illustrated in the response surface and contour graph (Figure 1a, b). Hg reduction increased as decreasing of these two variables. Maximum of Hg reduction (89.3%) was obtained at 10.5 mM $CaCl_2$ and 12.9:1 W:YB and then slightly decreased as continual increasing of $CaCl_2$ and W:YB (Figure 1a). Citric acid highly compete the basic amino acid residues of protein to bind Hg^+ (Oh and Lee, 1981) and then chloride ion can complex with Hg^+ to form HgCl precipitate (Kowalski *et al.,* 2002) and could be removed by filtration or centrifugation (Andrew-Hong *et al.,* 2000). Meanwhile, calcium ion enhance the phospholipids aggregation via an anhydrous calcium-phospholipids formation (Liang and Hultin, 2005), thus releasing phospholipids (PLs) from the system. As the results of Kokkaew *et al.* (2015), who reported that the dissociation of proteins during the alkaline extraction processing assisted with citric acid and $CaCl_2$ would reduce amount of MeHg as well as PLs in tilapia protein concentrate (Kokkaew *et al.,* 2015). Kawakami *et al.* (2010) reported that the total mercury concentrations in wild and farmed bluefin tuna with various fat contents in opposite proportion to the fat content. This might suggest that greater protein regions would be contained more mercury than high fat regions. Therefore, the results of our study confirmed that mercury could be removed through the alkali-aided protein recovery process assisted with citric acid and $CaCl_2$.

Protein recovery determination through independent variables x_1 and x_2 in terms of response surface and contour plot were shown in Figure 1b. Protein recovery decreased with a linear increase in $CaCl_2$ and then an exponential decrease in $CaCl_2$ and W:YB. The maximum of protein recovery (83.0%) was 10.1 mM $CaCl_2$ and 14.2:1 W:YB. However, Liang and Hultin (2005) indicated that the increase of phospholipids (PLs) removal from muscle homogenates

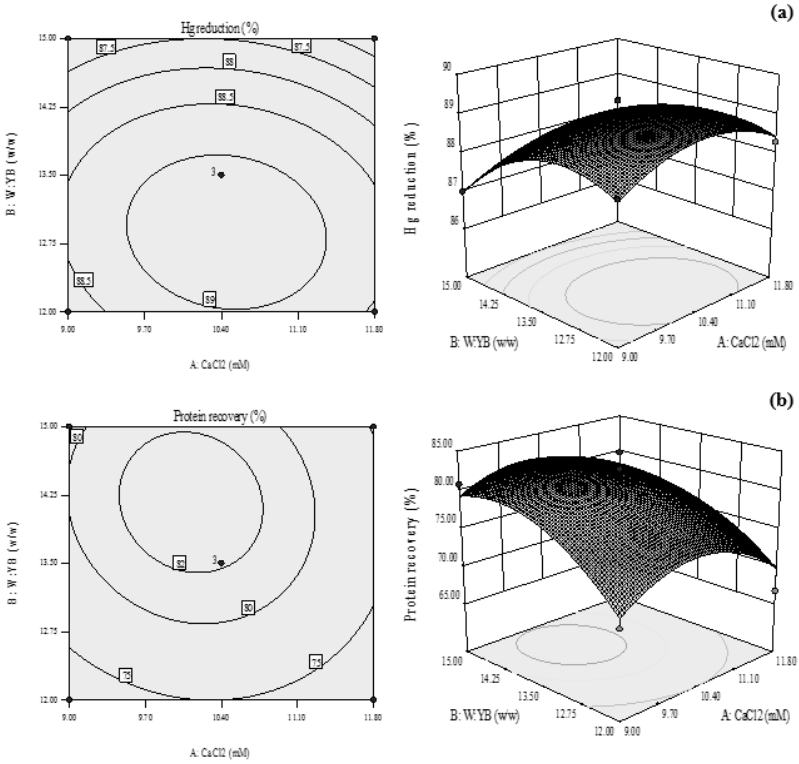

Figure 1. Contour plots and response surface curves showing effects of $CaCl_2$ concentration and W:YB of alkali-aided proteins isolated from YB on Hg reduction (a) and protein recovery (b).

effected to the high protein removal. As the results of previously our study (Kokkaew *et al.*, 2015) found that in the presence of 5 mM citric acid, an increase in Ca concentration continually increased PLs removal in minced tilapia frame homogenates solubilized at pH 11.0. This might cause the concurrent protein precipitation with PLs, which resulted in less protein content in the supernatant and a lower percentage of protein recovery.

To confirm the validity of the statistical model, three verification experiments were achieved (data not shown). All observation values were close to the predicted values using RSM showing % error between 0.30 and 4.42%. Therefore, the models were fit under these alkali-aided extractions with additives and could be used for reduction of mercury in YBPI.

3.2 Degree of hydrolysis and ACE-inhibitory activity of YBPH

DHs of all treatments were increased by extending hydrolysis time (Figure 2a). In particular, greater DHs were achieved when GN was applied in the sequential hydrolysis of G6 and Alc (G6/GN and Alc/GN), which resulted in DH of 43.8, and 41.7 %, respectively, at 240 min compared to those

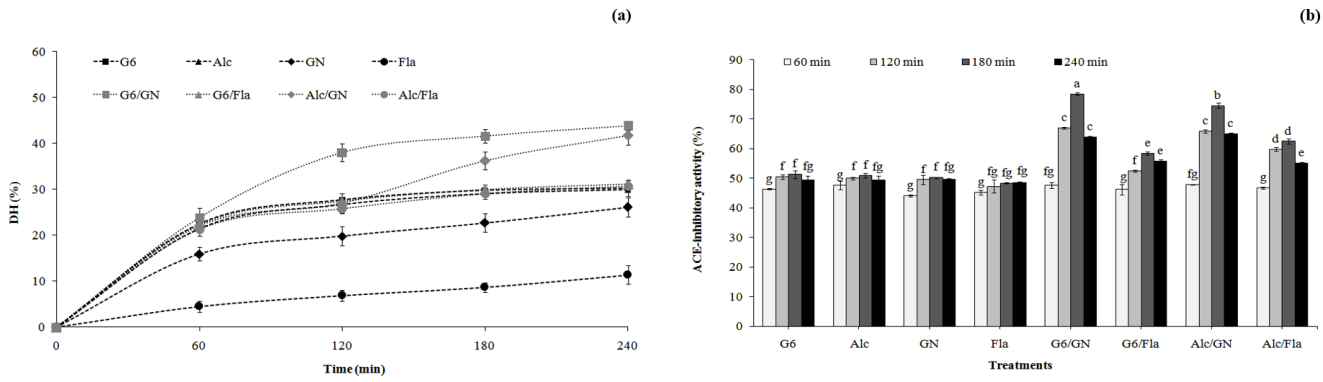

Figure 2. Changes in DH (a), and ACE-inhibitory activity (b) of YBPH. Abbreviations for treatments follow the enzyme codes and hydrolysis conditions in Table 5.

from G6, Alc, GN, Fla, G6/Fla, and Alc/Fla, which had DHs between 11.4 and 30.7%. G6 and Alc produced a high DH with a short time under moderate conditions (Benjakul and Morrisey, 1997). GN or Fla was added after the first protease to increase the DH. The results showed that GN could promote DH of the sequential hydrolysis greater than Fla. As the results of Megias *et al.* (2009), who demonstrated that the sequential use of these two enzymatic preparations was adopted in order to obtain higher DH than those obtained from using a single protease. Moreover, Gilmartin and Jervis, (2002) reported that endoproteases had limited activity against di- and aminopeptidase substrates. Therefore, exopeptidase supplementation would be used to improve and produce a various peptides in the low-molecular-weight range exhibiting biologically active peptides.

ACE-inhibitory activity markedly increased up to 3 hrs of hydrolysis and slightly decreased after that (Figure 2b). YBPH treatments of G6/GN and Alc/GN exhibited greater ACE-inhibitory activity (78.5 and 74.5%, respectively) ($P<0.05$) than those from G6, Alc, GN, Fla, G6/Fla, and Alc/Fla (51.3, 50.9, 50.2, 48.5, 58.4, and 62.5%, respectively). Therefore, sequential hydrolysis treatments could improve ACE-inhibitory potency greater than single enzymatic hydrolysis according to type of the second protease. Notably, ACE-inhibitory activities of hydrolysates were concomitant with their DH. Raghavan and Kristinsson (2009) indicated that tilapia protein hydrolysates prepared using various proteases showed a sharp increase in ACE-inhibitory activity as increasing DH. In addition, Wu *et al.* (2008) demonstrated that the smaller peptides exhibited better ACE inhibitors than the larger peptides. Klompong *et al.* (2007) reports that an endopeptidase hydrolyses proteins with broad specificity for peptide bonds and more cleave peptide bonds at different positions, resulting in various bioactive activities. Meanwhile, an exopeptidase enzyme can produce both amino acids and peptides, thus enhancing the activity of endopeptidase. In our study, the sequential treatment of G6 and GN prepared with a hydrolysis time of 60 and 120 min, respectively, exhibiting the highest ACE-inhibitory activity was selected for ultrafiltration process.

3.3 ACE-inhibitory activityof YBPH before and after ultrafiltration

ACE-inhibitory activity (IC_{50} values) of C-YBPH, R10, F5-10, and P5 were 18.8, 23.9, 20.2, and 16.7 mg protein/mL, respectively. A significant increase (significantly lower IC_{50} values) in ACE-inhibitory potency as MW of YBPH fractions decreased. Figure 3 demonstrated that a significant increase (significantly lower IC_{50} values) in ACE-inhibitory potency as MW of YBPH fractions decreased. However, P5 showed the lowest IC_{50}, indicating that most of the potent ACE-inhibitory peptides are short-chain peptides. As a similar report of quinoa protein hydrolysates, ACE-inhibitory activity of quinoa peptides increased in low MW fractions (Aluko and Muno, 2003). However, in our study, ACE-inhibitory activity of

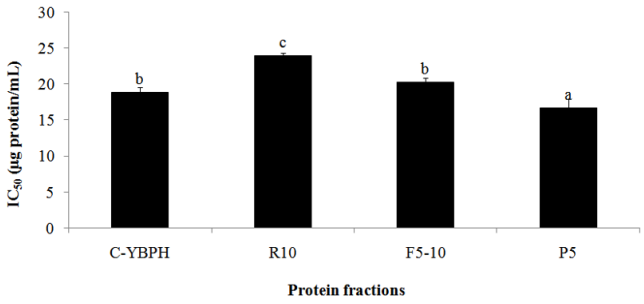

Figure 3. ACE-inhibitory activity of C-YBPH, retentate of 10 kDa (R10), fraction between 5 and 10 kDa (F5-10), and permeate of 5 kDa (P5). Each bar represents the mean ± standard deviation of triplicate. Bars labeled with different letters are significantly different ($P\leq0.05$).

C-YBPH and F5-10 were not significantly different ($P>0.05$). This might be due to C-YBPH mainly consisting of low MW peptides.

3.4 Stability of peptide to pepsin-pancreatin simulated GI digestion

The IC_{50} of C-YBPH, R10, F5-10, and P5 were determined to investigate whether GI digestion leads to the release of ACE-inhibitory peptides. A higher IC_{50} value demonstrates a lower ACE-inhibitory activity. The IC_{50} of C-YBPH, R10, F5-10, and P5 significantly ($P<0.05$) decreased from 18.8, 23.9, 20.2, and 16.7 to 15.3, 18.5, 14.9, and 14.1 mg protein/mL, respectively, after GI digestion (Figure 4). These results indicated that *in-vitro* gastrointestinal (GI) digestion significantly increased ACE-inhibitory potency. Hura *et al.* (2011) reported that simulated GI digestion could break down large MW peptides into small peptide fractions, thus improving more effective in bioactive ability. However, C-YBPH, F5-10, and P5 showed similarly increase of ACE-inhibitory activity, after GI digestion. Therefore, ultrafiltration process for peptides production from YB in a commercial scale may not be necessary.

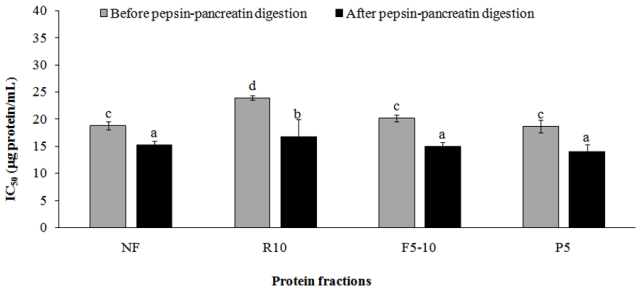

Figure 4. ACE-inhibitory activity of YBPH fractions (Abbreviations are as in Figure 3) before and after *in vitro* GI (pepsin-pancreatin) digestion. Each bar represents the mean ± standard deviation of triplicate. Bars labeled with different letters are significantly different ($P\leq0.05$).

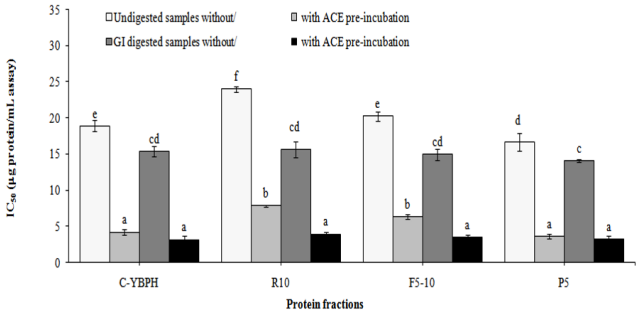

Figure 5. Effect of pre-incubation with ACE on the ACE-inhibitory activity of YBPH fractions (Abbreviations are as in Figure 3) digested with and without *in vitro* GI digestion. Each bar represents the mean ± standard deviation of duplicate. Bars labeled with different letters are significantly different ($P \leq 0.05$).

3.5 Digestibility of peptides by ACE

The IC_{50} of ACE-inhibitory activity of undigested (before GI digestion) C-YBPH, R10, F5-10, and P5 significantly ($P<0.05$) decreased from 18.8, 23.9, 20.2, and 13.7 to 4.1, 7.8, 6.3, and 3.6 g protein/mL, respectively, after pre-incubation with ACE, resembling the results of ACE-inhibitory activity of GI digested C-YBPH, R10, F5-10 and P5 (Figure 5). These results confirmed that ACE-inhibitory potency of all samples, both undigested and pre-digested with simulated GI enzymes, considerably increased. Therefore, these peptides demonstrated *pro-drug type* peptides. These *pro-drug type* peptides are also substrates for ACE, but they are converted by this enzyme to true inhibitors. Only *inhibitor* and *pro-drug type* peptides have the ability to lower blood pressure (Li *et al.,* 2004). Fujita *et al.* (2000) reported that the peptides, whose activities are increased with ACE pre-incubation, have been shown to demonstrate a milder but more prolonged antihypertensive effect *in vivo*.

4. Conclusions

The maximal Hg reduction of YBPI (89.3%) was 10.5 mM $CaCl_2$ and a W:YB of 12.9:1, while other variables were fixed at 5 mM citric acid, 60 min incubation, pH 11 and 8,000 x g for 15 min of centrifugation. At these conditions, the significant protein recovery (80.1%) was obtained. The sequential hydrolysis with G6 and GN could enhance the ACE-inhibitory activity of YBPH. In addition, YBPH demonstrated *pro-drug type* peptides, thus can be applied as potential nutraceuticals or functional ingredients in food industry.

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