Effect of proteases and alcohols used for debittering on characteristics and antioxidative activity of protein hydrolysate from salmon frames

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Research highlights

- Protein hydrolysates were produced from salmon frames using Alcalase or Flavourzyme
- Hydrolysate debittered by 2-butanol had less bitterness and surface hydrophobicity
- Lower bitterness score was correlated with the decreased surface hydrophobicity
- Debittered hydrolysate from salmon frame could be used as a nutritive ingredient at high levels

Abstract

Protein hydrolysates were obtained from salmon frame using Alcalase or Flavourzyme at 3% (w/w protein) for 180 min. Protein hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) had DH and yield of 25.1-26.9 % and 28.5-32.3 g/100g sample, respectively. HF showed lower bitterness score (5.78) than that of HA (8.68) ($P<0.05$). When HA and HF were further subjected to debittering with 2-butanol or isopropanol, the recovery of 77.88-81.60 % was obtained ($P<0.05$). HF and HA debittered with 2-butanol possessed less bitterness score, 3.60 and 3.77, respectively ($P<0.05$). Surface hydrophobicity of 81.4 and 124.8 was attained when HF and HA were debittered with 2-butanol ($P<0.05$). Selected debittered hydrolysates, produced using flavourzyme, followed by fractionation using 2-butanol (HF-B) contained glutamic acid/glutamine (15.14 g/100g), aspartic acid/asparagine (10.07 g/100g) and glycine (9.30 g/100g) as the predominant amino acids. HF-B had the decreased ABTS radical scavenging activity and metal chelating activity. A_{280} of peptides separated by gel filtration was lowered to some extent and coincided with the lower bitterness score and surface hydrophobicity. Thus, debittered protein hydrolysate from salmon frame could serve as a nutritive ingredient at high levels in health promoting foods.

Keywords: Salmon frame, Protein hydrolysate, Alcalase, Flavourzyme, Debittering

1. Introduction

The global demand for foods from aquaculture surpassed 160 million tons in 2014 (FAO 2016). Salmon is one of popular species owing to its delicacy. Salmon (*Salmo salar*) has been imported to Thailand to serve for increasing demand for Thai consumers. Generally, salmon is sold as a fillet or whole fish (Idowu et al. 2018). During salmon filleting, by-products obtained from salmon processing include frames, trimmings (containing muscle, bone and skin), heads (containing the gills) and viscera (liver, kidney and roe) (See et al. 2011). Those leftovers contain significant amount of protein, which can be recovered as a source of food ingredients through enzymatic hydrolysis (Idowu et al. 2018). Fish processing by-products were subjected to enzymatic hydrolysis for recovery of valuable components (Nalinanon et al. 2011). Fish protein hydrolysates (FPH) have been reported to exhibit good functional and nutritional properties as well as bioactivities (Idowu et al. 2018). Nevertheless, a major drawback of protein hydrolysate is the sensation of bitter taste. The bitterness of protein hydrolysates inevitably limits their potential use as a nutritive food ingredient. Consequently, they are incorporated into foods at low level to avoid the bitterness (Hou et al. 2011). Bitterness of hydrolysate is influenced by type of proteases used for hydrolysis. Whey protein hydrolysates produced using Alcalase were more bitter than those produced with Prolyve 1000 or Corolase 7089 (Spellman et al. 2009). The bitterness of protein hydrolysate could be as a result of the specificity of enzyme used to produce the hydrolysate (Spellman et al. 2009). Seo *et al.* (2008) documented that the hydrolysate from soy protein isolate prepared using Flavourzyme showed the lowest bitterness, compared to those prepared using Alcalase, neutrase, protamex and papain. Flavourzyme is a mixture of enzymes containing both endo- and exo-peptidase. The exopeptidase is able to decrease the bitterness of a bitter peptide by removing terminal hydrophobic amino acids (Tavano 2013).

The bitter taste in protein hydrolysate is due to the formation of low molecular weight peptides made up of hydrophobic amino acids (Idowu et al. 2018). In general, most hydrophobic amino acids are positioned towards the interior of the globular proteins. However, peptides containing hydrophobic amino acids are exposed during enzymatic hydrolysis and interact with taste buds, resulting in a bitter taste (Idowu et al. 2018). Hydrophobic amino acid residues become more exposed with increasing degree of hydrolysis, thereby augmenting bitterness (Idowu et al. 2018). To conquer such a problem, debittering processes of protein hydrolysates have been developed, e.g. selective extraction with alcohols, absorption of bitter peptides on activated carbon and chromatographic removal using different matrices (FitzGerald and O'Cuinn 2006).

Protein hydrolysates from salmon frames have been recently produced. However, they had undesirable bitter taste (Idowu et al. 2018). Debittering using some selected alcohols such as 2-butanol and iso-propanol to remove hydrophobic peptides or free amino acids could be a means to lower bitterness of hydrolysate from salmon frame. However, debittering process might impact on the bioactivity, particularly antioxidant activity. A little information regarding the bitterness of protein hydrolysates from salmon frames using different enzymes subjected to subsequent fractionation using various alcohols exists. Therefore, this work aimed to examine the effect of debittering of protein hydrolysates from salmon frame prepared by Alcalase or Flavourzyme using some alcohols and to characterize and determine antioxidative activities of the resulting debittered hydrolysates.

2. Materials and methods

2.1 Enzymes and chemicals

Alcalase (2.4 L enzyme) from *Bacillus licheniformis* and Flavourzyme (500L) were obtained from Novozyme (Bagsvaerd, Denmark). 2,4,6-trinitrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-triazine (TPTZ), 3-(2-

pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caffein was procured from Merck KGaA Co. (Darmstadt, Germany). SephadexTM G-25, blue dextran and gel filtration calibration kits (vitamin B12, flavin adeninedinucleotide and glycine-tryrosine) were procured from GE Healthcare (Uppsala, Sweden). All the chemicals used were of analytical grade.

2.2 Preparation of salmon frames

Frames of salmon (*Salmo salar*) of about 30-35 cm in length were obtained from Kingfisher Holding Ltd., Songkhla, Thailand. They were packed in a polyethylene bag, placed in a polystyrene box and embedded in ice with sample/ice ratio of 1:2 (w/w). The samples were delivered to Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, within approximately 2 h. The samples were stored at -20 ºC until used, but not more than 3 months.

2.3 Production of protein hydrolysates from salmon frames using different proteases

2.3.1 Hydrolysis of salmon frame

Protein hydrolysates were prepared from salmon frames as described by Idowu et al. (2018). Frozen salmon frames were firstly thawed overnight at 4 °C and the size was reduced to 4-5 cm in length with the aid of electric cutting machine (W210E, Union Kitchen & Service, Bangkok, Thailand). The salmon frame was mixed with distilled water at a ratio of 1:1 (w/v). Thereafter, the pH of the mixtures was adjusted to 8.0 using either 1.0 M NaOH or 1.0 M HCl. The mixture was pre-heated using a water bath (Model W350, Memmert, Schwabach, Germany) for 15 min at 55 ºC or 60 °C for Flavourzyme or Alcalase, respectively. Subsequently, Alcalase or Flavourzyme at a level of 3.0% (w/w) was added to

133 the mixture. Hydrolysis was conducted for 3 h. Thereafter, the mixtures were heated at 90 ºC
134 for 15 min to terminate the reaction. All the mixtures obtained were cooled down to room
135 temperature using running water and filtered with two layers of cheesecloth to remove the
136 undigested bones. Then filtrates were centrifuged at 4000x*g* at 4 ºC using an Avanti® J-E
137 refrigerated centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) for 15 min. The
138 supernatants were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge,
139 Denmark) for 72 h. The hydrolysates from salmon frames with the aid of Alcalase and
140 Flavourzyme were referred to as "HA" and "HF", respectively. Both hydrolysates were
141 subjected to analyses.

142 **2.3.2 Analyses**

143 **2.3.2.1 Determination of degree of hydrolysis (DH)**

144 DH of the hydrolysates was determined as tailored by Benjakul and Morrissey (1997).

145 **2.3.2.2 Yield**

146 The yield of hydrolysate was calculated based on the dry weight of initial salmon
147 frames after drying at 105 ºC for 12 h in a hot air oven.

148 $$\text{Yield (\%)} = \frac{\text{weight of dry hydrolysate (g)}}{\text{weight of dried ground frame}} \times 100$$

149

150 **2.3.2.3 Determination of bitterness**

151 Bitterness of hydrolysates was examined by five female and six male panelists aged
152 between 25 and 33 as detailed by Idowu et al. (2018).

153 **2.4 Debittering of protein hydrolysates from salmon frames using various alcohols**

154 **2.4.1 Debittering of protein hydrolysates**

155 Removal of bitter compounds using various alcohols was carried out following the
156 method of Lalasidis and Sjoberg (1978) with a slight modification. Both HA and HF were
157 solubilized in distilled water to obtain a concentration of 10% (w/v). The alcohols (2-butanol

158 and iso-propanol) were added and mixed with hydrolysate at a ratio of 1:4 (v/v). The
159 mixtures were stirred for 10 min at room temperature. Then the mixtures were centrifuged at
160 2250xg at 4 ºC using an Avanti® J-E refrigerated centrifuge (Beckman Je-avanti Fullerton,
161 CA, USA) for 30 min. The lower fraction (hydrolysate rich fraction) was collected and
162 subjected to debittering with the same manner for another time. Thereafter, hydrolysate rich
163 phase was collected and freeze-dried using a freeze-dryer. All the hydrolysates obtained were
164 then analyzed.

165 **2.4.2 Analyses**

166 **2.4.2.1 Determination of nitrogen recovery**

167 Nitrogen recovery in the debittered hydrolysates was calculated based on initial
168 nitrogen content of hydrolysate before debittering as follows:

170 $$\text{Nitrogen recovery (\%)} = \frac{\text{Total nitrogen in debittered hydrolysate (mg)}}{\text{Total nitrogen of initial hydrolysate sample (mg)}} \times 100$$

169

171 **2.4.2.2 Determination of surface hydrophobicity**

172 Surface hydrophobicity of the hydrolysates was determined as per the method of
173 Quan and Benjakul (2018) using ANS as a probe.

174 **2.4.2.3 Determination of solubility**

175 Hydrolysates were dissolved in deionized water to obtain a concentration of 1%
176 (w/v). The pH was adjusted to 7 with either 2 N NaOH or 2 N HCl. The samples were
177 centrifuged at 14000xg for 15 min at room temperature. Protein content in the supernatant
178 was measured using the Biuret method (Robinson and Hogden 1940). For total protein, the
179 samples were solubilized with 0.5 M NaOH. Solubility was reported as the percentage.

180 **2.4.2.4 Molecular weight distribution**

Molecular weight distribution of hydrolysate samples was analyzed using a Sephadex G-25 gel filtration column (2.5 x 50 cm) (17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden) as detailed by Idowu et al. (2018).

2.4.2.5 Determination of antioxidative activities

Antioxidative activities of hydrolysate samples were determined as follows: ABTS radical scavenging activity, DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) and metal chelating activity (Sae-leaw et al. 2016). The activities were expressed as µmol Trolox (TE) equivalent/g sample, except for metal chelating activity, which was reported as µmol EDTA equivalent/g sample.

2.4.2.6 Amino acid analysis

Hydrolysates without debittering (HA and HF) and those subjected to debittering using 2-butanol (HA-B and HF-B) showing the lowered bitterness were analyzed for amino acid composition as described by Benjakul *et al.* (2009).

2.5 Statistical analysis

All the experiments were carried out in triplicate. Analysis of variance (ANOVA) was done and mean comparisons were performed using the Duncan's multiple range test (Steel and Torrie 1980). Completely randomized design (CRD) was used for all studies, except randomized complete block design (RCBD) was used for analysis of bitterness. SPSS software (IBM software, New York, NY, USA) was used for analysis.

3. Results and Discussion

3.1 Impact of proteases on characteristics and bitterness of protein hydrolysate from salmon frame

3.1.1 Degree of hydrolysis (DH)

DHs of protein hydrolysate from salmon frame prepared using different proteases are shown in Table 1. DH was higher for HA (26.9 %) than that of HF (25.0 %) ($P<0.05$),

206 suggesting that Alcalase was more reactive in cleavage of polypeptide chain of proteins
207 localized in frame than Flavourzyme. Alcalase has been reported to exhibit higher efficacy in
208 hydrolysis of yellow stripe trevally muscle proteins and Persian sturgeon (*Acipenser*
209 *persicus*) viscera protein (Klompong et al. 2008; Ovissipour et al. 2012). The result
210 reconfirmed high hydrolytic activity of Alcalase toward proteins in salmon frame as indicated
211 by rapid hydrolysis of polypeptides chain within a short time (Idowu et al. 2018). In general,
212 continuous stirring, incubation temperature and hydrolysis time influenced the cleavage of
213 most compact protein when both enzymes were used (Benjakul and Morrissey 1997). Apart
214 from both proteases used, endogenous proteases such as cathepsins, etc. in raw fish muscle
215 have been reported to play a role in hydrolysis (Mackie 1982). This could assist the
216 breakdown of proteins into smaller peptides during hydrolysis. It can be inferred that the type
217 of proteases used affected the degree of hydrolysis of proteins from salmon frame.

218 **3.1.2 Yield**

219 Yields of HA and HF were 32.3 and 28.5 g/100g, respectively. Higher yield was
220 obtained for HA than HF ($P<0.05$). In general, state of substrate and surface area of
221 proteinaceous matter played an essential role in protein hydrolysis as well as the yield
222 obtained (Benjakul et al. 2009). Also, higher yield of hydrolysates was well related with
223 greater DH found in HA (Idowu et al. 2018). Idowu et al. (2018) reported that higher DH led
224 to higher yield. Smaller peptides are more water soluble and recovered in hydrolysate to a
225 higher extent. Overall, yield of hydrolysates varied with the type of proteases used, in which
226 Alcalase rendered the higher yield.

227 **3.1.3 Bitterness**

228 HA possessed higher bitterness score than HF ($P<0.05$). Bitterness is related with
229 peptides containing bulky hydrophobic groups toward their C-terminal. Bulky hydrophobic
230 group such as leucine, tryptophan, valine, tyrosine, isoleucine and phenylalanine at C-

terminal contributed to bitterness (Idowu et al. 2018). During hydrolysis, the hidden hydrophobic peptides are exposed or liberated. This resulted in the enhanced bitterness sensation. Concomitantly, the higher bitterness score of HA observed could be as a result of higher exposure of buried hydrophobic group of peptides in HA than HF ($P<0.05$). Also, Flavourzyme, containing exopeptidase, has been reported to remove bitter peptides by removing hydrophobic amino acids at the terminal (Tavano, 2013). Thus, the decrease in bitterness of HF observed could be as a result of removal of hydrophobic peptides or free amino acids by exopeptidase in Flavourzyme. Several factors have been documented to influence bitterness. Those include number of carbons in side chain, DH and concentration (Idowu et al. 2018). Thus, bitterness of hydrolysates from salmon frame was directly affected by the type of proteases used.

3.2 Effect of debittering process on characteristics and bitterness of protein hydrolysate from salmon frame

3.2.1 Nitrogen recovery (NR)

Nitrogen recovery (NR) of hydrolysates debittered using different alcohols (2-butanol and iso-propanol) is presented in Table 2. HA and HF treated with 2-butanol referred to as 'HA-B' and 'HF-B', respectively, had NR of 80.2 and 81.6%, respectively. When treated with iso-propanol, NR of 81.3 and 77.9 %, respectively, were obtained for HA and HF, named HA-I and HF-I, respectively. Removal of insoluble protein fractions from hydrolyzed soluble fraction is associated with nitrogen recoveries (Kristinsson and Rasco 2000). Removal of some nitrogenous components during debittering could have led to the lower NR. In general, some peptides could be precipitated in the presence of alcohols (Scanu and Edelstein, 1971). Also, alcohol could solubilize some small peptides or free amino acids in hydrolysates. Hence, the slightly lower NR of hydrolysate was observed after treatment with

255 both alcohols. It can be deduced that alcohol treatment for debittering decrease NR of HA
256 and HF from salmon frame to some extent.

257 **3.2.2 Bitterness**

258 Bitterness scores of both HA and HF without and with debittering are shown in Table
259 2. Flavourzyme is a mixture of endo- and exo-peptidases. As a result, exopeptidase could
260 remove some hydrophobic residues, particularly at N- or C-terminal, leading to the lower
261 bitterness of resulting hydrolysate (HF) (6.07), compared with HA (9.13). The result
262 reconfirmed the result presented in Table 1. When 2-butanol and iso-propanol were used for
263 debittering, the significant decreases in bitterness scores were noticeable ($P<0.05$). For the
264 same protein hydrolysate used, the lower bitterness score was found for those treated with 2-
265 butanol in comparison with iso-propanol ($P<0.05$). The result indicated that the former
266 exhibited higher efficiency in debittering both HA and HF than the latter. Alcohols are
267 hydrophobic (hydrocarbon chain) and hydrophilic (presence of hydroxyl group) in nature
268 (Wasswa et al. 2007). Continuous stirring followed by centrifugation employed during
269 debittering with alcohol could have assisted the interaction with bulky hydrophobic group,
270 especially small peptides or free amino acids, in hydrolysates. As a result, interaction existed
271 between the bulky hydrophobic group of peptides with the hydrophobic part of the alcohol to
272 form hydrophobic-hydrophobic interaction. Via this interaction, more hydrophobic groups of
273 the peptides were solubilized in the alcohol used during debittering process leading to the
274 lower bitterness observed in all hydrolysates treated with alcohols ($P<0.05$). Non-polar
275 (hydrophobic) domains of the alcohol interacted strongly with the hydrophobic peptides,
276 especially towards their C-termini. The bitter peptides, particularly those liberated by
277 exopeptidases in Flavourzyme, were leached out. Those peptides with bitterness could be
278 removed by 2-butanol (both HA-B and HF-B). Consequently, both proteases and type of
279 alcohol used for debittering played a role in lowering the bitterness of hydrolysates.

3.2.3 Surface hydrophobicity

Surface hydrophobicity of both HA and HF before and after debittering is shown in Table 2. Higher surface hydrophobicity denotes the presence of bulky hydrophobic/aromatic amino acids (Vandaburg et al. 1994) . The bulky hydrophobic/aromatic amino acid could be released from internal loop of the folded protein during hydrolysis (Aspevik et al. 2016). HA and HF (without debittering) showed high surface hydrophobicity of 208.6 and 155.4, respectively. During hydrolysis, the buried hydrophobic/aromatic amino acid were leached out from the interior domain of globular protein and more likely localized at the surface. It was noted that HA possessed higher surface hydrophobicity than HF ($P<0.05$). Higher surface hydrophobicity observed in HA could be as a result of high specificity of Alcalase to cleave buried hydrophobic peptides during hydrolysis, which led to their enhanced exposure. Nevertheless, hydrophobic residues at termini of peptides could be further cleaved by exopeptidase in Flavourzyme. This led to the lower surface hydrophobicity of resulting peptides in HF.

Treatment with alcohol was able to extract hydrophobic amino acids or small peptides via hydrophobic-hydrophobic interaction between alcohol and hydrophobic residues of the hydrolysates. HA-B and HF-B had surface hydrophobicity of 124.8 and 81.4, while HA-I and HF-I possessed surface hydrophobicity of 141.5 and 116.9, respectively. This result suggested that bulky hydrophobic amino acids or peptides of HF had higher affinity to alcohol than HA. Hence, surface hydrophobicity of HF was much decreased. Similarly, between the two alcohols used, 2-butanol showed higher efficiency to reduce the surface hydrophobicity of hydrolysates than iso-propanol. 2-Butanol possessed solvent polarity of 0.506 relative to water (Aspevik et al. 2016). Nevertheless, the apolar nature of 2-butanol could be assumed to be sufficient to attract or bind with hydrophobic amino acids or small peptides of hydrolysates. This possibly led to the decrease in surface hydrophobicity of

hydrolysates debittered using 2-butanol than those using iso-propanol. Idowu et al. (2018) reported the influence of hydrophobic/aromatic amino acids on bitterness of hydrolysate. In this study, surface hydrophobicity of hydrolysates correlated well with the bitterness of hydrolysates (Table 2). Type of alcohol used for debittering drastically affected the surface hydrophobicity of the resulting hydrolysates.

3.2.4 Solubility of hydrolysates

Solubility of hydrolysates from salmon frame with and without debittering treatment is shown in Table 2. Solubility ranged between 88.3 and 95.6 %. Solubility is regarded to as one of the vital requirement for physicochemical and functional properties of protein hydrolysates (Thiansilakul et al. 2007). HA and HF without debittering treatment possessed higher solubility than others ($P<0.05$), except HA-B which had solubility similar to those two samples ($P>0.05$). Solubility of hydrolysates could be influenced by reduction in molecular size, exposure of more polar and ionizable groups to the aqueous environment and hydrophobic character of proteins or peptides (Nalinanon et al. 2011). The balance of hydrophilic and hydrophobic forces of peptides is another crucial factor determining the solubility of protein hydrolysate (Klompong et al. 2007). During the debittering process, some soluble peptides might be aggregated as induced by alcohols, thus limiting the solubility of the debittered hydrolysates. When comparing alcohols used, hydrolysates debittered using iso-propanol showed less solubility than those debittered by 2-butanol, indicating the loss of more soluble peptides in the former. Overall, solubility of hydrolysates was altered with the debittering treatment used, especially type of alcohol.

3.3 Molecular weight distribution

Elution profiles of hydrolysates obtained from salmon frame without and with debittering treatments are depicted in Figure 1. A_{280} measured proteins or peptides, mainly

consisting of hydrophobic/aromatic amino acids, while A_{220} indicated peptide bonds (Karnjanapratum and Benjakul 2015). HA and HF had two major peaks at A_{280} and A_{220} with molecular weight (MW) of 13,828 and 1,615 Da. However, higher peak heights were observed in HA than in HF. For $A_{220,}$ peak of 644 Da was more pronounced in HF than in HA. At A_{280} and A_{220}, peptide with MW of 362 Da was observed for both HA and HF, but the latter possessed higher peak height. Peak of peptide with MW of 120 Da was higher in HF than in HA.

Elution profile of peptides in hydrolysates debittered using 2-butanol was slightly altered. For $A_{280,}$ the decline in peak height of 13,828 Da peptide was observed in HA-B and HF-B. This confirmed that some hydrophobic/aromatic amino acids were more likely removed during debittering process. For A_{280} and $A_{220,}$ peptides of 644, 362 and 120 Da became less pronounced after debittering in HA-B and HF-B. However, peak heights were higher in HF-B than HA-B. Aggregation of peptides in HA-B and HF-B might be related with the removal of some soluble peptides during debittering.

For A_{280}, there was reduction in peak heights of peak with MW of 13,828 Da in both HA-I and HF-I, suggesting the removal of hydrophobic peptides causing bitterness in hydrolysates. For A_{280} and A_{220}, similar peak height of peptide with MW of 362 Da was observed between HA-I and HF-I. Peak height of peptide with MW of 120 Da at A_{280} and A_{220} in HF-I was more pronounced than HA-I. This result correlated well with surface hydrophobicity and bitterness of hydrolysate (Table 2), which were lower in HA-I than HF-I. Thus, debittering treatment as well as type of alcohol used slightly impacted on size distribution of peptides as indicated by different profiles.

3.4 Antioxidative activities

3.4.1 ABTS radical scavenging activities

ABTS activities of hydrolysates without and with debittering are shown in Table 3. ABTS assay is used to determine the antioxidant activity toward lipid peroxyl radicals and hydrophilic radicals (Binsan et al. 2008). There was no difference in ABTS radical scavenging activity between HA and HF ($P>0.05$). Increased hydrophobic domains of peptides have been regarded as a factor that increased the radical scavenging activities of hydrolysates (Ajibola et al. 2011). Furthermore, hydrophobic peptides possessed high efficiency to interact with lipids. This might affect their antioxidative activity (Zhu et al. 2006). Thus, higher surface hydrophobicity of HA and HF (Table 2) could have enhanced their activities. For HA, both alcohols used for debittering had no impact on ABTS radical scavenging activity ($P>0.05$). After debittering, the decreases in activity of HF were observed ($P<0.05$). Loss of some hydrophobic peptides or free amino acids such as leucine and phenylalanine during debittering of HF could lower its activity. Thus, debittering using both alcohols decreased the activity of HF ($P<0.05$). However, debittering had no impact on activity of HA ($P>0.05$).

3.4.2 DPPH radical scavenging activities

DPPH radical scavenging activities of hydrolysates without and with debittering are shown in Table 3. This assay measured the antioxidative activity of compounds as a hydrogen donor or free radical scavenger (Klompong et al. 2007). Both HA and HF showed higher activity than those with debittering ($P<0.05$). Kim *et al*. (2007) reported that hydrophobic peptides function as antioxidants by promoting the solubility of peptides in non-polar solvent, thereby promoting better interaction with free radicals to terminate their activities. This correlated with the higher activities observed in HA and HF without debittering ($P<0.05$). Loss of soluble and hydrophobic peptides during debittering could have led to the decrease in DPPH radical scavenging activities of debittered hydrolysates (HA-B, HA-I, HF-B and HF-I) ($P<0.05$). Nevertheless, both alcohols used during debittering showed

379 no differences in activities of the resulting HA ($P>0.05$). For HF, slight decrease in activity
380 was observed when iso-propanol was used for debittering, compared to that found in HF
381 ($P<0.05$). It can be deduced that debittering affected DPPH radical scavenging activities of
382 both HA and HF.

383 **3.4.3 Ferrous reducing antioxidant power (FRAP)**

384 FRAP measures the ability of a compound to donate electron to a free radical in order
385 to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex (Binsan et al. 2008). HA rendered
386 higher activity than those subjected to debittering ($P<0.05$). Ajibola *et al*. (2011) reported that
387 hydrolysate with more hydrophobic peptides could display high reducing activity. This
388 reaffirmed the higher reducing activity of HA without debittering than those with debittering.
389 Similar reducing activities were observed after debittering of HA with both alcohols ($P>0.05$).
390 HF, debittered with 2-butanol (HF-B), showed similar FRAP to those without debittering
391 (HF) ($P>0.05$). There was no difference in FRAP between HF debittered using 2-butanol and
392 iso-propanol ($P>0.05$). This suggested similar electron donating ability of hydrophobic
393 peptides in those hydrolysates to reduce Fe (III) to Fe (II) complex. In general, debittering
394 influenced the activities of HA and HF to some degree.

395 **3.4.4 Metal chelating activity**

396 Metal chelating activity is used to determine the ability of antioxidative peptides to
397 chelate prooxidative metals (Binsan et al. 2008). Concentration of carboxyl and amino groups
398 in the side chains of amino acids has been reported to influence chelating activities of
399 antioxidative peptides (Saiga et al. 2003). HF showed the highest chelating activities than
400 others ($P<0.05$). Available carboxyl and amino group of HF without debittering might play a
401 role in binding with Fe. However, after debittering, activity of both HA and HF decreased
402 ($P<0.05$). Activity was similar between HA-B and HA-I when both alcohols were used
403 ($P>0.05$). Nevertheless, HF debittered with iso-propanol (HF-I) showed the decrease in

activity ($P<0.05$). This could be due to loss of some binding groups or domains during debittering with iso-propanol. In summary, debittering process affected the chelating activity of HA and HF.

3.5 Amino acid composition

Hydrolysates (HA and HF) without and with debittering using 2-butanol (HA-B and HF-B) with less bitterness scores were selected for amino acid analysis (Table 4). The dominant amino acids in all samples were glutamic acid/glutamine, aspartic acid/asparagine and glycine. HA-B and HF-B possessed higher glutamic acid/glutamine than HA and HF. Removal of hydrophobic amino acids or peptides could increase the proportion of acidic or polar amino acids (Johnson et al. 2003). Glutamic acid is notable to contribute to umami taste (Naknaen et al. 2015). The cleavage of N or C- terminal hydrophobic amino acid by exopeptidase in Flavourzyme, followed by fractionation using 2-butanol in debittering of HF resulted in the decrease in total hydrophobic amino acid as shown in HF-B. Lower hydrophobic amino acids of 34.63 and 35.05 g/100 g were obtained for HF-B and HA-B, respectively, compared to those without debittering 35.18 (HF) and 37.69 g/100 g (HA), respectively. Thus, less hydrophobic amino acid were related with less bitterness score attained (Table 2). Hydroxyproline was observed in all samples, which indicated the presence of collagen derivatives in hydrolysates. This collagen, particularly from bone or connective tissue, could be solubilized by proteases at a temperature higher than Tmax of fish collagen during hydrolysis (Idowu et al. 2018).

Essential and non- essential amino acids in hydrolysates were in the range of 36.04-38.87 g/100g and 46.33-59.25 g/100g, respectively. Debittering of HF with 2-butanol (HF-B) slightly increased essential amino acids. Consequently, amino acid composition of HA and HF were altered slightly by debittering process.

4. Conclusion

Protein hydrolysate could be derived from salmon frame. Alcalase rendered higher degree of hydrolysis and yield than Flavourzyme. Nevertheless, hydrolysate obtained from Flavourzyme (HF) rendered less bitterness score than those from Alcalase (HA). Debittering of HF with 2-butanol (HF-B) lowered the bitterness and surface hydrophobicity than iso-propanol. In addition, 2-butanol decreased hydrophobic/aromatic peptides as indicated in the elution profile and amino acid composition and markedly decreased bitterness of hydrolysate. However, ABTS radical scavenging activity and metal chelating activity of HF-B were decreased. However, 2-butanol could be recommended to fractionate HF in order to yield hydrolysate with lowered bitterness, which can be used as a nutritive ingredient for food fortification.

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Conflicts of Interest

The authors at this moment declared no conflict of interest.

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546 **Figure Legend**

547 **Figure 1** Elution profile by Sephadex G-25 size exclusion chromatography of
548 hydrolysates from salmon frame using Alcalase and Flavourzyme. HA; HF:
549 Hydrolysate obtained from Alcalase and Flavourzyme, respectively. HA-B;
550 HF-B: Hydrolysate from Alcalase and Flavourzyme subjected to debittering
551 using 2-Butanol, respectively. HA-I; HF-I: Hydrolysate from Alcalase and
552 Flavourzyme subjected to debittering using Iso-propanol, respectively.

553

554

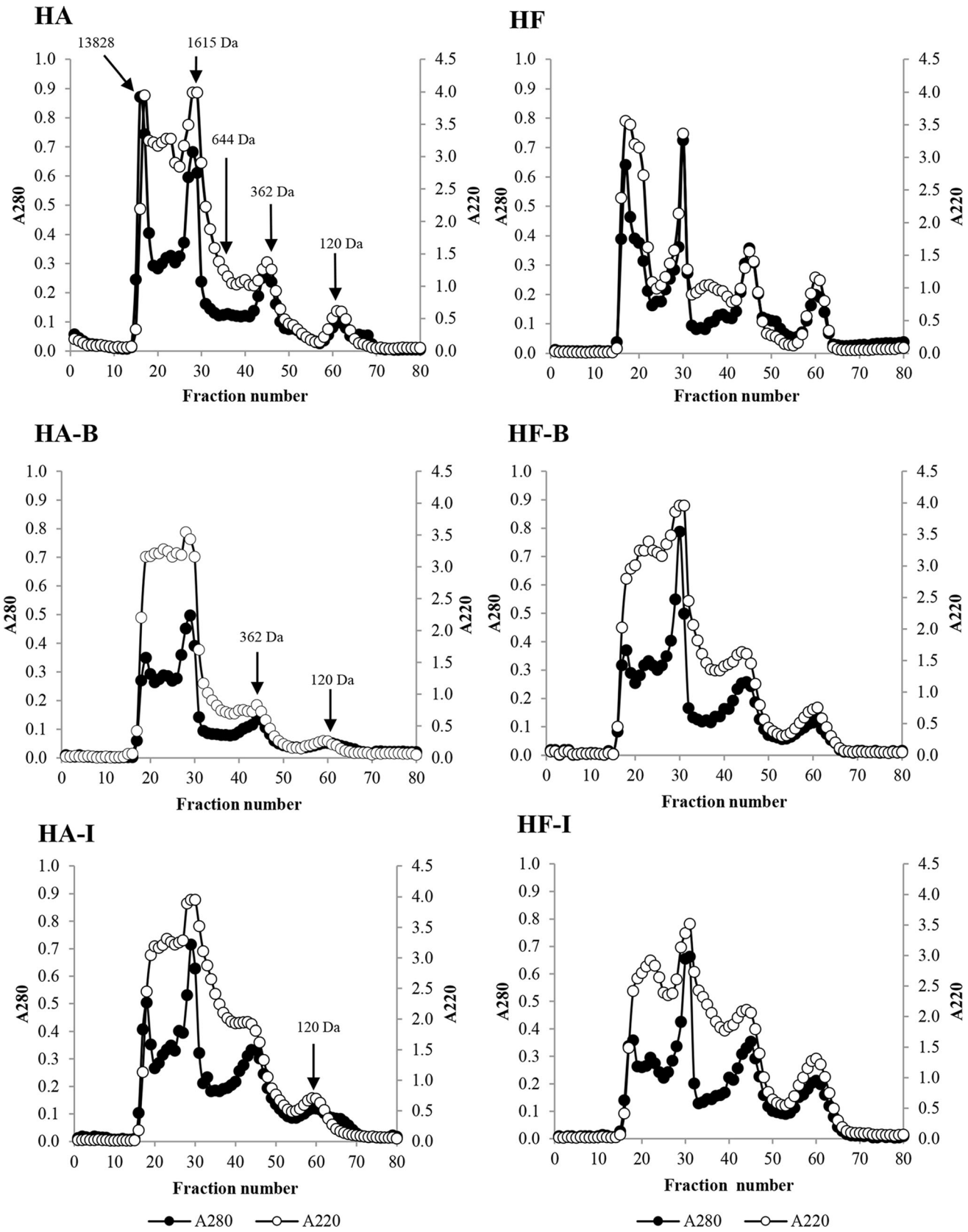
HA
13828
1615 Da
644 Da
362 Da
120 Da
A280
A220
Fraction number
HF
HA-B
HF-B
HA-I
HF-I
A280
A220

Table 1 Degree of hydrolysis (DH), yield and bitterness score of the hydrolysates from salmon frame prepared using different enzymes

Samples	DH (%)	Yield (g/100 g sample)	Bitterness score
HA	26.88±1.45a	32.27±1.68a	8.68±1.19a
HF	25.02±1.11b	28.51±0.96b	5.78±1.58b

Values are presented as mean ± SD (n = 3) (dry weight basis)
Different lowercase letters within the same column indicate significant differences ($P<0.05$).
HA and HF denote hydrolysates produced using Alcalase and Flavourzyme, respectively

Table 2 Characteristics of protein hydrolysates from salmon frame without and with different debittering processes

Samples	Alcohol	Nitrogen recovery (%)	Bitterness score	Surface hydrophobicity	Solubility (%)
HA	-	-	9.13±1.77aA	208.57±2.64aA	95.61±0.72aA
HA-B	2-Butanol	80.21±0.01	3.77±1.33cC	124.79±3.50cD	92.70±2.10bAB
HA-I	Iso-propanol	81.28±0.01	5.47±1.46bB	141.54±6.61bC	89.36±1.05cBC
HF	-	-	6.01±2.34aB	155.40±3.17aB	93.81±2.01aAB
HF-B	2-Butanol	81.60±0.01	3.60±1.28bC	81.414.88cE	90.88±1.35abB
HF-I	Iso-propanol	77.88±0.03	4.66±1.89abBC	116.98±4.82bD	88.31±3.56abC

Values are presented as mean ± SD (n = 3).

Different lowercase letters within the same hydrolysate sample in the same column indicate significant differences ($P<0.05$). Different uppercase letters in the same column indicate significant differences ($P<0.05$).

HA; HF denoted hydrolysates produced using Alcalase and Flavourzyme. HA-B;HF-B: Hydrolysate obtained from Alcalase and Flavourzyme, debittered using 2-butanol, respectively. HA-I;HF-I: Hydrolysate obtained from Alcalase and Flavourzyme, debittered using isopropanol respectively.

Table 3 ABTS and DPPH radical scavenging activities, ferric reducing antioxidant power (FRAP) and metal chelating activity of protein hydrolysates from salmon frame without and with different debittering processes

Samples	Alcohols	ABTS (μmol TE/g smple)	DPPH (μmol TE/g smple)	FRAP (μmol TE/g smple)	Metal chelating (μmol EDTA/g smple)
HA	-	211.87±9.56aAB	28.52±3.41aA	26.01±3.07aA	0.52±0.08aB
HA-B	2-Butanol	201.54±0.97aBC	18.99±0.43bB	23.26±1.62bB	0.25±0.10bC
HA-I	Iso-propanol	205.47±9.54aABC	15.49±1.38bB	21.12±0.39bBC	0.20±0.03bC
HF	-	216.32±5.71aA	25.53±3.38aA	23.11±2.60aB	0.91±0.40aA
HF-B	2-Butanol	196.30±5.12bC	19.20±3.54abB	22.98±2.68aBC	0.43±0.08bB
HF-I	Iso-propanol	200.74±9.54bBC	16.35±5.82bB	20.25±1.22abC	0.26±0.05cC

Values are presented as mean ± SD (n = 3). Different lowercase letters within the same hydrolysate sample in the same column indicate significant differences ($P<0.05$). Different uppercase letters in the same column indicate significant differences ($P<0.05$). Caption: see Table 2.

Table 4 Amino acid profile of selected protein hydrolysates without and with debittering

Amino acids (g/100 g)	HA	HF	HA-B	HF-B
Alanine	6.75±0.04	7.19±0.02	6.84±0.03	6.82±0.01
Arginine	6.52±0.02	6.56±0.04	6.87±0.03	6.91±0.04
Aspartic acid/asparagine	9.59±0.00	9.22±0.01	10.10±0.00	10.07±0.01
Cysteine	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00
Glutamic acid/glutamine	14.13±0.02	14.65±0.01	14.98±0.01	15.14±0.02
Glycine	9.03±0.01	10.93±0.00	9.40±0.00	9.30±0.00
Histidine	3.40±0.02	3.58±0.03	3.23±0.01	3.47±0.03
Isoleucine	3.76±0.01	3.33±0.01	3.56±0.01	3.37±0.01
Leucine	7.06±0.03	6.39±0.02	5.75±0.04	5.83±0.03
Lysine	8.23±0.01	8.15±0.02	8.67±0.02	8.80±0.02
Hydroxylysine	0.37±0.02	0.47±0.02	0.41±0.00	0.40±0.00
Methionine	3.14±0.04	2.83±0.03	2.80±0.04	2.79±0.03
Phenylalanine	3.55±0.01	2.99±0.02	2.80±0.02	2.85±0.01
Hydroxyproline	1.88±0.00	2.51±0.00	1.91±0.00	2.01±0.01
Proline	5.14±0.05	5.53±0.06	5.34±0.06	5.33±0.04
Serine	4.61±0.01	4.62±0.01	4.75±0.00	4.68±0.00
Threonine	4.56±0.02	4.13±0.02	4.63±0.01	4.59±0.00
Tryptophan	0.69±0.04	0.48±0.03	0.52±0.04	0.49±0.04
Tyrosine	3.11±0.03	2.27±0.02	2.90±0.03	2.81±0.02
Valine	4.48±0.01	4.17±0.02	4.54±0.01	4.33±0.01
Hydrophobic amino acids	37.69±0.22	35.18±0.21	35.05±0.24	34.63±0.23
Essential amino acids	38.87±0.18	36.04±0.12	36.50±0.14	36.53±0.16
Non essential amino acids	59.25±0.15	46.80±0.17	46.62±0.14	46.33±0.18

HA; HF: Hydrolysates produced using Alcalase and Flavourzyme, respectively. HA-B; HF-B: Hydrolysate produced using Alcalase and Flavourzyme and debittered with 2-butanol, respectively.