Peptides Derived from Atlantic Salmon Skin Gelatin as Dipeptidyl-Peptidase IV Inhibitors

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Inhibition of Dipeptidyl-Peptidase IV

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ABSTRACT The dipeptidyl-peptidase IV (DPP-IV) inhibitory activity of peptides derived from Atlantic salmon skin gelatin hydrolysed by alcalase (ALA), bromelain (BRO) and flavouzyme (FLA) was determined. The FLA hydrolysate with the enzyme/substrate ratio of 6% showed the greatest DPP-IV inhibitory activity. The hydrolysate was fractionated by ultrafiltration with 1 and 2.5-kDa cutoff membranes, and the < 1 kDa fraction had the highest DPP-IV inhibitory activity with the IC50 value of 1.35 mg/mL. The F-1 fraction further isolated by HPLC showed the IC50 value against DPP-IV of 57.3 μg/mL, and the peptide sequences were identified as Gly-Pro-Ala-Glu (372.4 Da) and Gly-Pro-Gly-Ala (300.4 Da). The synthetic peptides showed the dose-dependent inhibition effect on DPP-IV with IC_{50} values of 49.6 and 41.9 μM, respectively. The results suggest that the peptides derived from Atlantic salmon skin gelatin would be beneficial ingredients for functional food or pharmaceuticals against type 2 diabetes.

KEYWORDS Dipeptidyl-peptidase IV inhibitor; Atlantic salmon; Gelatin; Type 2 diabetes; Bioactive peptide.

35 **INTRODUCTION**

36 During a meal, two incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and 37 glucagon-like peptide-1 (GLP-1), are released from the small intestine into the vasculature and augment 38 glucose-induced insulin secretion from the islet β-cells *(1)*. It has been reported that approximately 50- 39 60% of the total insulin secreted during a meal results from the incretin response; mainly the effects of 40 GIP and GLP-1 *(2)*. However, GIP and GLP-1 had extremely short half-lives of about 1-2 min 41 following secretion due to the rapid degradation and inactivation by the enzyme dipeptidyl peptidase IV 42 (DPP-IV), resulting in loss of their insulinotropic activities *(3)*. It has been reported that most of the 43 degraded GLP-1 is attributed to the action of DPP-IV *(4)*, therefore, the use of DPP-IV inhibitors as a 44 new therapeutic approach for the management of type 2 diabetes was also developed *(5)*. Some studies 45 on the administration of DPP-IV inhibitors in animal and clinical experiments have shown to increase 46 half-life of total circulating GLP-1, decrease plasma glucose, and improve impaired glucose tolerance 47 *(6-8)*.

48 Dipeptidyl peptidase IV (dipeptidyl-peptide hydrolase, EC 3.4.14.5) is a postproline cleaving enzyme 49 with a specificity for removing X-proline or X-alanine dipeptides from the N-terminus of polypeptides 50 *(9)*. The cleavage of N-terminal peptides with Pro in the second position is a rate-limiting step in the 51 degradation of peptides. There are several chemical compounds used in vitro and in animal models to 52 inhibit DPP-IV activity, such as valine-pyrrolidide *(7)*, NVP-DPP728 *(8)*, Lys[Z(NO_2)]-thiazolidide and 53 Lys[Z(NO_2)]-pyrrolidide *(10)*. However, such chemical compounds, which often have to be 54 administered by injection, may result in side effects as chemical drugs. Diprotins A and B, isolated from 55 culture filtrates of *Bacillus cereus* BMF673-RF1, were found to exhibit the inhibitory activity on DPP- 56 IV with IC_{50} values of 1.1 and 5.5 μg/mL, respectively *(11)*; and they were elucidated to be Ile-Pro-Ile 57 and Val-Pro-Leu. There were also two peptides, Ile-Pro-Ala and Val-Ala-Gly-Thr-Trp-Tyr, prepared 58 from β-lactoglobulin hydrolyzed by proteinase K and trypsin showed the IC_{50} values of 49 and 174 μM 59 against DPP-IV *(12, 13)*. Patent WO 2006/068480 and WO 2009/128713 have demonstrated that the

60 peptides derived from casein and lysozyme hydrolysates display DPP-IV inhibiting activity, and the
61 peptides are in particular the presence of at least one proline within the sequence and mostly in the
62 second N-terminal residue *(14, 15)*.

63 It is well known that the dominant amino acid in gelatin is glycine, while the imino acids (proline and
64 hydroxyproline) come second in abundance *(16)*. The amino acid composition is characterized by a
65 repeating sequence of Gly-X-Y triplets, where X is mostly proline and Y is mostly hydroxyproline *(17)*.
66 Further, previous studies have reported that the DPP-IV inhibitory peptides consisted at least one
67 proline and mostly as the penultimate N-terminal residue (*11*-*15*). Therefore, the aim of this study was
68 to examine the DPP-IV inhibitory activity of peptides derived from Atlantic salmon skin gelatin. This is
69 expected to give insight into the possible utilization of Atlantic salmon skin as a potential source of
70 DPP-IV inhibitors that may be used in the treatment of type 2 diabetes.

71

72 **MATERIALS AND METHODS**

73 **Materials and reagents.** Atlantic salmon (*Salmo salar*) fish skins, the processing byproducts recovered
74 from fresh skin-off fillets, were supplied by Albion Fisheries Ltd. (Vancouver, BC, Canada). The fish
75 skins were transferred on ice to our laboratory, vacuum packed and stored at -25℃ until use. Three
76 food-grade proteolytic enzymes were donated by Neova Technologies Inc. (Abbotsford, BC, Canada).
77 Alcalase® 2.4 L FG (from *Bacillus licheniformis*, 2.4 AU/g) and Flavourzyme® 1000 L (from
78 *Aspergillus oryzae*, 1000 LAPU/g) were products from Novo-zymes North America Inc. (Salem, NC,
79 Canada) while bromelain (from pineapple stem, 2000 GDU/g) was manufactured by Ultra Bio-Logics
80 Inc. (Montreal, QC, Canada). Dipeptidyl peptidase IV (D7052, from porcine kidney), Gly-Pro-*p*-
81 nitroanilide hydrochloride, trichloroacetic acid (TCA), L-leucine and Diprotin A were purchased from
82 Sigma-Aldrich (St. Louis, MO, USA). Trinitrobenzenesulfonic acid (TNBS) was from Fluka

83 Biochemika (Oakville, ON, Canada). Other chemicals and reagents used were analytical grade and
84 commercially available.

85 **Extraction of gelatin.** The thawed skins were gently washed with running tap water, drained and cut
86 into pieces (about 5 × 10 cm). The fish skins were soaked in 0.2 M NaOH (1:10; w/v) and stirred in a
87 cold room at 4℃ for 30 min. This procedure was repeated three times to remove noncollagenous
88 proteins and pigments. The skins were washed with running tap water until pH was neutral. Afterwards,
89 the skins were soaked in 0.05 M acetic acid (1:10; w/v) and stirred at room temperature for 3 h and then
90 washed by running tap water until the pH was neutral. Almost all the scales could be removed. The
91 gelatin of the swollen skins was extracted in distilled, deionized water (ddH_2O; 1:2; w/v) at 70℃ for 3 h
92 *(18)*. The oil and aqueous layers of the extract were separated by separatory funnels, and the extract was
93 filtered through two layers of cheese-clothes, lyophilized and stored in a desiccator until use.

94 **Amino acid analysis.** The gelatin solutions were hydrolyzed in vacuum in 6 M HCl (1:1; v/v) at 110
95 ℃ for 24 h in the presence of 1% phenol (v/v), and the hydrolysates were analyzed using an amino acid
96 analyzer (Hitachi L-8900, Hitachi Ltd., Katsuda, Japan). The content of tryptophan was determined by
97 the colorimetric method at 550 nm after alkaline hydrolysis of gelatin at 105℃ for 24 h with 4 M NaOH
98 (*19*).

99 **Enzymatic hydrolysis.** One gram of the freeze-dried gelatin added with 50 mL ddH_2O was
100 incubated at 50℃ for 10 min prior to the enzymatic hydrolysis. The enzymes in liquid form were
101 weighed 10, 20, 30, 60 mg and mixed with 1 mL ddH_2O. The hydrolysis reaction was started by the
102 addition of enzymes at various enzyme/substrate ratios (E/S: 1%, 2%, 3% and 6%). The reactions with
103 alcalase (ALA), bromelain (BRO) and flavourzyme (FLA) were conducted at pH 8.0, 7.0 and 7.0,
104 respectively, and 50℃ for 4 h. After hydrolysis, the hydrolysates were heated in boiling water for 10
105 min to inactivate enzymes and then cooled in cold water at room temperature for 20 min. Hydrolysates
106 were adjusted their pH to 7.0 with 1 M NaOH and centrifuged (Du Pont Sorvall Centrifuge RC 5B,

107 Mandel Scientific Co. Ltd, Guelph, ON, Canada) at 12,000*g* and room temperature for 15 min. The
108 supernatant was lyophilized and stored at -25℃.

109 **Measurement of degree of hydrolysis.** Immediately prior to termination of hydrolysis, a 4 mL
110 aliquot of the hydrolysate was mixed with an equal volume of 24% TCA solution and centrifuged at
111 12200*g* for 5 min. The supernatant (0.2 mL) was added to 2.0 mL of 0.05 M sodium tetraborate buffer
112 (pH 9.2) and 1 mL of 4.0 mM TNBS and incubated at room temperature for 30 min in the dark. Then
113 the mixture was added with 1.0 mL of 2.0 M NaH_2PO_4 containing 18 mM Na_2SO_3, and the absorbance
114 was measured at 420 nm using a spectrophotometer (Cary 50 Bio UV-vis spectrophotometer, Varian,
115 Inc., Santa Clara, CA, USA) *(20, 21)*. DH was calculated as % DH=$(h/h_{tot}) \times 100$, where DH=percent
116 ratio of the number of peptide bonds broken (h) to the total number bonds per unit weight (h_{tot}) and
117 h_{tot}=11.1 mequiv/g of gelatin *(20)*. L-leucine was used for drawing a standard curve.

118 **Determination of DPP-IV inhibitory activity.** DPP-IV activity determination in this study was
119 performed in 96-well microplates and to measure the increase in absorbance at 405 nm using Gly-Pro-p-
120 nitroanilide as DPP-IV substrate *(22)*. The lyophilized hydrolysates were dissolved in 100 mM Tris
121 buffer (pH 8.0) to the concentration of 10 mg/mL and then serially diluted. The hydrolysates (25 μL)
122 were added with 25 μL of 1.59 mM Gly-Pro-p-nitroanilide (in 100 mM Tris buffer, pH 8.0). The
123 mixture was incubated at 37℃ for 10 min, followed by the addition of 50 μL of DPP-IV (diluted with
124 the same Tris buffer to 0.01 Unit/mL). The reaction mixture was incubated at 37℃ for 60 min, and the
125 reaction was stopped by adding 100 μL of 1 M sodium acetate buffer (pH 4.0). The absorbance of the
126 resulting solution was measured at 405 nm with a microplate reader (iEMS reader MF; Labsystems,
127 Helsinki, Finland). Under the conditions of the assay, IC_{50} values were determined by assaying
128 appropriately diluted samples and plotting the DPP-IV inhibition rate as a function of the hydrolysate
129 concentration.

130 **Ultrafiltration.** The DPP-IV inhibitory peptides of the hydrolysates were fractionated by
131 ultrafiltration (Model ABL085, Lian Sheng Tech. Co., Taichung, Taiwan) with spiral wound
132 membranes having molecular mass cutoffs of 2.5 and 1 kDa. The fractions were collected as follows:
133 >2.5 kDa, peptides retained without passing through 2.5 kDa membrane; 1-2.5 kDa, peptides
134 permeating through the 2.5 kDa membrane but not the 1 kDa membrane; <1 kDa, peptides permeating
135 through the 1 kDa membrane. All fractions collected were lyophilized and stored in a desiccator until
136 use.

137 **High performance liquid chromatography (HPLC).** The fractionated hydrolysates by
138 ultrafiltration exhibiting DPP-IV inhibitory activity were further purified using high performance liquid
139 chromatography (Model L-2130 HPLC, Hitachi Ltd., Katsuda, Japan). The lyophilized hydrolysate
140 fraction (100 μg) by gel filtration was dissolved in 1 mL of 0.1% trifluoroacetic acid (TFA), and 90 μL
141 of the mixture was then injected into a column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm, Agilent
142 Tech. Inc., CA, USA) using a linear gradient of acetonitrile (5 to 15% in 20 min) in 0.1% TFA under a
143 flow rate of 0.7 mL/min. The peptides were detected at 215 nm. Each collected fraction was then
144 lyophilized and stored in a desiccator until use.

145 **Determination of amino acid sequence.** An accurate molecular mass and amino acid sequence of
146 the purified peptides was determined using a Q-TOF mass spectrometer (Micromass, Altrincham, UK)
147 coupled with an electrospray ionization (ESI) source. The purified peptides were separately infused into
148 the electrospray source after being dissolved in methanol/water (1:1, v/v), and the molecular mass was
149 determined by the doubly charged $(M+2H)^{+2}$ state in the mass spectrum. Automated Edman sequencing
150 was performed by standard procedures using a 477-A protein sequencer chromatogram (Applied
151 Biosystems, Foster, CA, USA).

152 **Peptide synthesis**. Peptides were prepared by the conventional Fmoc solid-phase synthesis method
153 with an automatic peptide synthesizer (Model CS 136, CS Bio Co. San Carlos, CA, USA), and their
154 purity was verified by analytical RP-HPLC-MS/MS.

155 **Statistical analysis.** Each data point represents the mean of three samples was subjected to analysis
156 of variance (ANOVA) followed by Tukey's studentized range test, and the significance level of $P < 0.05$
157 was employed.

158

159 **RESULTS AND DISCUSSION**

160 **Amino acid composition of Atlantic salmon skin gelatin.** Amino acid composition of Atlantic
161 salmon skin gelatin are presented in **Table 1**. The glycine content of salmon skin gelatin was 223.63
162 mg/g sample, slightly higher than that of Nile tilapia skin gelatin (211.8 mg/g protein) and similar to
163 porcine skin gelatin (224.5 mg/g protein) *(23)*. The alanine content (7.06 mol/100 mol amino acids) of
164 salmon skin gelatin in the present study was relatively lower than those (9.6-12.3 mol/100 mol amino
165 acids) of skin gelatins from other fish species, such as cod, Alaska Pollock, hake and tilapia *(24)*.
166 Salmon skin gelatin contained a high content of imino acids (175.02 mg/g sample), including proline
167 (86.78 mg/g sample) and hydroxyproline (88.24 mg/g sample), which showed slightly lower contents
168 (186.29 and 187.42 mg/g sample) as compared to bigeye snapper skin gelatins *(25)*.

169 **Degree of hydrolysis and DPP-IV inhibitory activity of hydrolysates.** The DHs and DPP-IV
170 inhibitory activities of Atlantic salmon skin gelatin hydrolysed with ALA, BRO and FLA at various E/S
171 ratios for 4 h are shown in **Figure 1**. The three proteases used in the present study were Alcalase (a
172 serine protease), bromelain (a cysteine protease) and Flavourzyme (an exo- and endopeptidase complex).
173 The result showed the DHs of the gelatin hydrolysates obtained by all the three proteases hydrolysis
174 increased with the increment of E/S ratio (**Figure 1A**). The DHs of ALA and BRO hydrolysates with
175 the E/S ratio of 1% were 34.8 and 28.3%, respectively, and those with 6% were 41 and 38.2%. The DHs
176 of 1, 2 and 3% FLA hydrolysates were lower than those of ALA and BRO hydrolysates, and the 6%
177 FLA hydrolysate showed the slightly higher DH of 42.5% than the other two protease hydrolysates. At
178 the concentration of 5 mg solid/mL, the extracted gelatin (without hydrolysis) showed the DPP-IV

179 inhibition rate of about 10%, and the hydrolysates possessed significantly higher DPP-IV inhibitory
180 activities ($P<0.05$) than gelatin (**Figure 1B**). The DPP-IV inhibition rates of ALA hydrolysates with all
181 the various enzyme concentrations were between 24-30% with insignificant differences ($P>0.05$), and
182 that of 6% BRO hydrolysate showed 23.1%, the highest among all BRO samples ($P<0.05$). The FLA
183 hydrolysates showed the greatest DPP-IV inhibition rates as compared to ALA and BRO hydrolysates
184 with the same E/S ratio, and that with 6% E/S ratio possessed the highest inhibition rate of 45.2% in this
185 study ($P<0.05$). Therefore, the FLA hydrolysate with the E/S ratio of 6% was used for further
186 purification. Patent WO 2006/068480 has demonstrated that the hydrolysates possessed great DPP-IV
187 inhibitory activities referred to a mixture of peptides derived from hydrolysis of proteins with the
188 percentage of hydrolysed peptide bonds of most preferably 20 to 40% *(14)*. All the hydrolysates except
189 of those with 1 and 6% E/S ratios of FLA obtained in this study showed their DHs between 27.6 to
190 40.9%, however, the DPP-IV inhibitory activities of the two exceptions were higher than those of the
191 other hydrolysates. We suggested that the DPP-IV inhibitory activity should be determined by the
192 peptide structures and sequences but not dependent upon DHs.

193 **DPP-IV inhibitory activity of hydrolysates fractionated by ultrafiltration**. **Figure 2A** shows the
194 DPP-IV inhibitory activities of 6% FLA hydrolysate fractions separated by ultrafiltration at the
195 concentration of 2 mg solid/mL. The result showed the peptides within the < 1 kDa UF fraction had the
196 greatest DPP-IV inhibition rate of 61.2% ($P<0.05$), while those within the > 2.5 kDa and 1-2.5 kDa
197 fractions displayed the inhibition rates of 29.6 and 43.2%, respectively. The IC_{50} value of the < 1 kDa
198 fraction was determined and found to be 1.35 mg/mL (**Figure 2B**). The result in this study is in
199 agreement with the former studies using various protein sources that reported the preferable DPP-IV
200 inhibitory peptides derived from food protein consisted of 2-8 amino acid residues *(14, 15)*, and their
201 molecular weights were supposed between 200 to 1000 Da.

202 **Purification of DPP-IV inhibitory peptides by HPLC**. **Figure 3A and B** show the elution profile
203 and DPP-IV inhibitory activities of the peptide fractions from the < 1 kDa UF fraction separated by

204 HPLC. To obtain a sufficient amount of purified peptide, chromatographic separations were performed
205 repeatedly. Five fractions (F-1 to F-5) were obtained upon HPLC separation of the < 1 kDa UF fraction
206 (**Figure 3A**), and they were lyophilised and then used to determine their DPP-IV inhibitory activities at
207 the concentration of 100 μg solid/mL. The result showed that the fraction F-1 had the highest DPP-IV
208 inhibition rate of 68.0% (P<0.05) (**Figure 3B**), and its IC_{50} value was also determined as 57.3 μg/mL
209 (**Figure 3C**). Therefore, the fraction F-1 was used to identify the amino acid sequences of the peptides.

210 **Amino acid sequence of DPP-IV inhibitory peptdies**. Two peptides were identified in fraction F-1,
211 and their amino acid sequences were Gly-Pro-Ala-Glu (372.4 Da) and Gly-Pro-Gly-Ala (300.4 Da)
212 (**Table 2**). Patent WO 2006/068480 has reported that 21 peptides which were capable of inhibiting DPP-
213 IV activity showed a hydrophobic character, had a length varying from 3-7 amino acid residues and in
214 particular the presence of Pro residue within the sequence *(14)*. The Pro residue was located as the first,
215 second, third or fourth N-terminal residue, but mostly as the second N-terminal residue. Besides, the Pro
216 residue was flanked by Leu, Val, Phe, Ala and Gly. In the present study, both peptides comprised Pro as
217 the second N-terminal residue, and the Pro residue was flanked by Ala and Gly. Moreover, the peptides
218 were composed of mostly hydrophobic amino acid residues, such as Ala, Gly and Pro, and one peptide
219 comprised a charged amino acid, Glu, as the C-terminal residue. The present results therefore are
220 consistent with the hypothesis demonstrated in the previous study *(14)*.

221 **DPP-IV inhibitory activity of the synthetic peptides.** **Figure 4** shows the DPP-IV inhibitory
222 activity of the two synthetic peptides and Diprotin A at various concentrations. The IC_{50} was calculated
223 for each of the peptides. Diprotin A is well known as the peptide with the greatest DPP-IV inhibitory
224 activity, and its IC_{50} value was found to be 24.7 μM in the present study (**Figure 4**). The IC_{50} values of
225 the two synthetic peptides, Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala, were 49.6 and 41.9 μM, respectively.
226 In the previous study, the IC_{50} values against DPP-IV of Diprotin A and Diprotin B isolated from
227 culture filtrates of *B. cereus* BMF673-RF1 were 3.2 and 16.8 μM, respectively *(11)*. Moreover, Ile-Pro-
228 Ala and Val-Ala-Gly-Thr-Trp-Tyr, both prepared from β-lactoglobulin showed IC_{50} values of 49 and

229 174 μM against DPP-IV, respectively *(12, 13)*. Patent WO 2006/068480 reported that Diprotin A
230 showed the IC_{50} value of about 5 μM against DPP-IV, and five peptides, HPIK, LPLP, LPVP, MPLW
231 and GPFP, comprised 4 amino acids with Pro as the penultimate N-terminal residue displayed their IC_{50}
232 values between 76 to 120 μM *(14)*. The results showed that the two peptides obtained in this study
233 showed lower DPP-IV inhibitory activity than only Diprotin A and B, which were composed with 3
234 amino acid residues. However, they had similar inhibition effect to Ile-Pro-Ala but greater than other
235 peptides comprised 4 or more amino acid residues. It is interesting that the ultimate N-terminal residues
236 of the peptides mentioned above are all hydrophobic amino acids, and Gly is smaller than the other
237 residues. Therefore, we assumed that DPP-IV inhibitory activity of bioactive peptides may be
238 determined by the amino acid length and the two N-terminal amino acid sequence of X-Pro, where X is
239 the hydrophobic amino acid and preferably smaller in size. In conclusion, we found two peptides, Gly-
240 Pro-Ala-Glu and Gly-Pro-Gly-Ala, isolated from Atlantic salmon skin gelatin hydrolysates having the
241 inhibitory activity against DPP-IV. The two peptdies may be useful for the therapy or prevention of type
242 2 diabetes.

243 **ABBREVIATIONS USED**

244 DPP-IV, dipeptidyl-peptidase IV; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-
245 like peptide 1; HPLC, high performance liquid chromatography.

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305 **FIGURE CAPTIONS**

306 **Figure 1.** (A) Degree of hydrolysis and (B) DPP-IV inhibition rate of Atlantic salmon skin gelatin
307 hydrolysed with ALA, BRO and FLA at various E/S ratios. The DPP-IV inhibition rate was
308 determined with the hydrolysates at the concentration of 5 mg solid/mL. Bars represent standard
309 deviations from triplicate determination.

310 **Figure 2.** (A) DPP-IV inhibition rate of Atlantic salmon skin gelatin hydrolysates fractionated by UF at
311 the concentration of 2 mg solid/mL. (B) DPP-IV inhibition rate of the < 1 kDa UF fraction at
312 various concentrations. Bars represent standard deviations from triplicate determinations.

313 **Figure 3.** (A) Elution profile and (B) DPP-IV inhibition rate of the peptide fractions from the < 1 kDa
314 UF fraction separated by HPLC. (C) DPP-IV inhibition rate of the fraction F-1 at various
315 concentrations. The DPP-IV inhibition rate was determined with each HPLC fraction at the
316 concentration of 100 μg solid/mL.

317 **Figure 4.** DPP-IV inhibition rate and the IC_{50} value of the synthetic peptides and Diprotin A.

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321 **Table 1.** Amino acid composition of gelatin from Atlantic salmon skins.

Amino acid	Content*	
	mg/g sample	mol/100 mol amino acids
Alanine	50.24	7.06
Arginine	83.51	6.00
Aspartic acid	78.15	7.35
Cysteine	2.32	0.12
Glutamic acid	89.84	7.65
Glycine	223.63	37.31
Histidine	3.18	0.26
Hydroxyproline (Hyp)	88.24	8.43
Isoleucine	9.61	0.92
Leucine	21.83	2.08
Lysine	33.18	2.84
Methionine	16.26	1.36
Phenylalanine	18.24	1.38
Proline	86.78	9.44
Serine	27.21	3.24
Threonine	26.08	2.74
Tryptophan	1.09	0.07
Tyrosine	6.54	0.45
Valine	11.95	1.28
Imino aicds (Hyp+Pro)	175.02	17.87

322 * n = 3

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325 **Table 2.** Amino acid sequences of purified DPP-IV inhibitory
326 peptides derived from Atlantic salmon skin gelatin hydrolyzed
327 with FLA.

Sequence	Molecular mass
Gly-Pro-Ala-Glu	372.4
Gly-Pro-Gly-Ala	300.4

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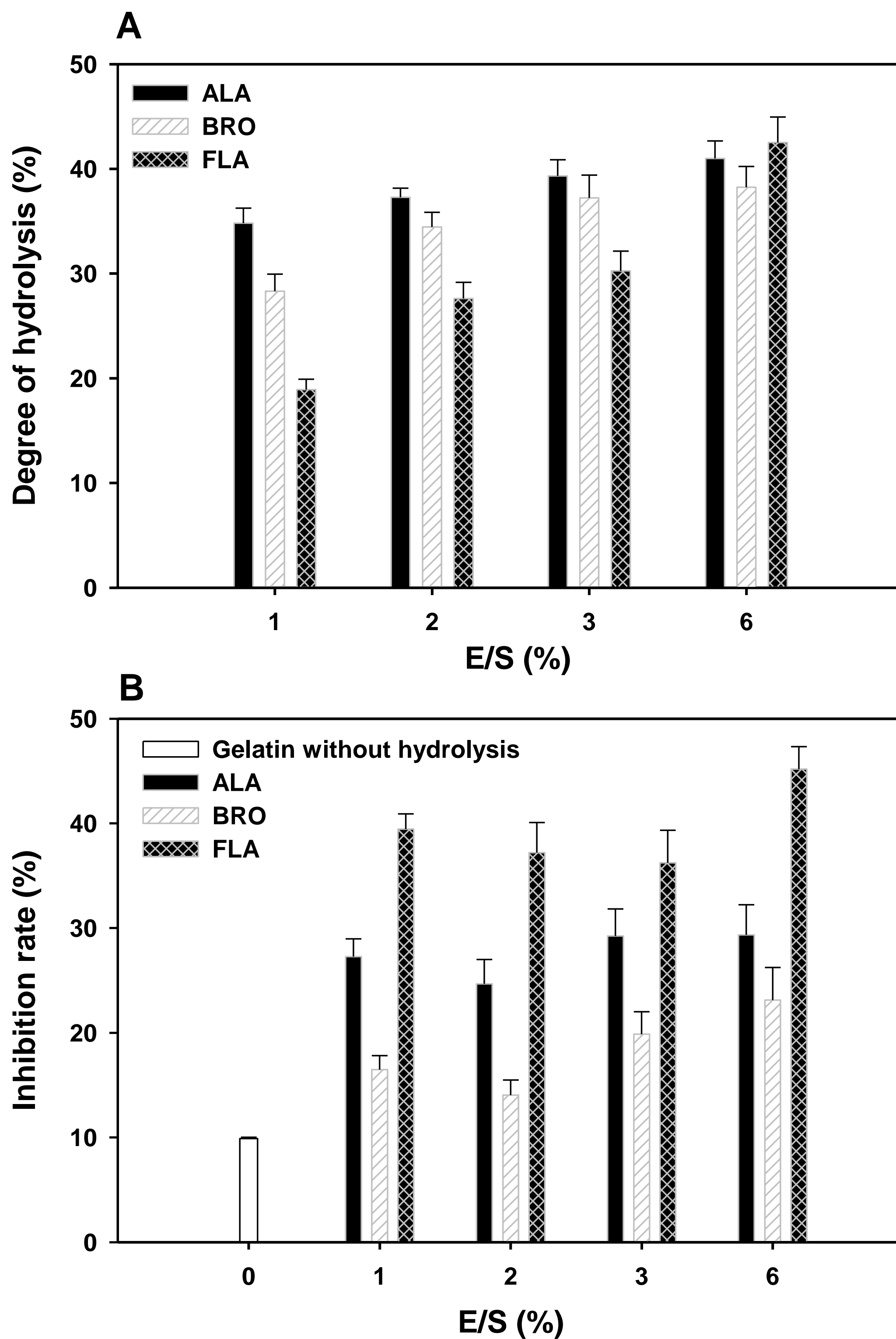

330

331 **Figure 1.** (A) Degree of hydrolysis and (B) DPP-IV inhibition rate of Atlantic salmon skin gelatin
332 hydrolysed with ALA, BRO and FLA at various E/S ratios. The DPP-IV inhibition rate was determined
333 with the hydrolysates at the concentration of 5 mg solid/mL. Bars represent standard deviations from
334 triplicate determination.

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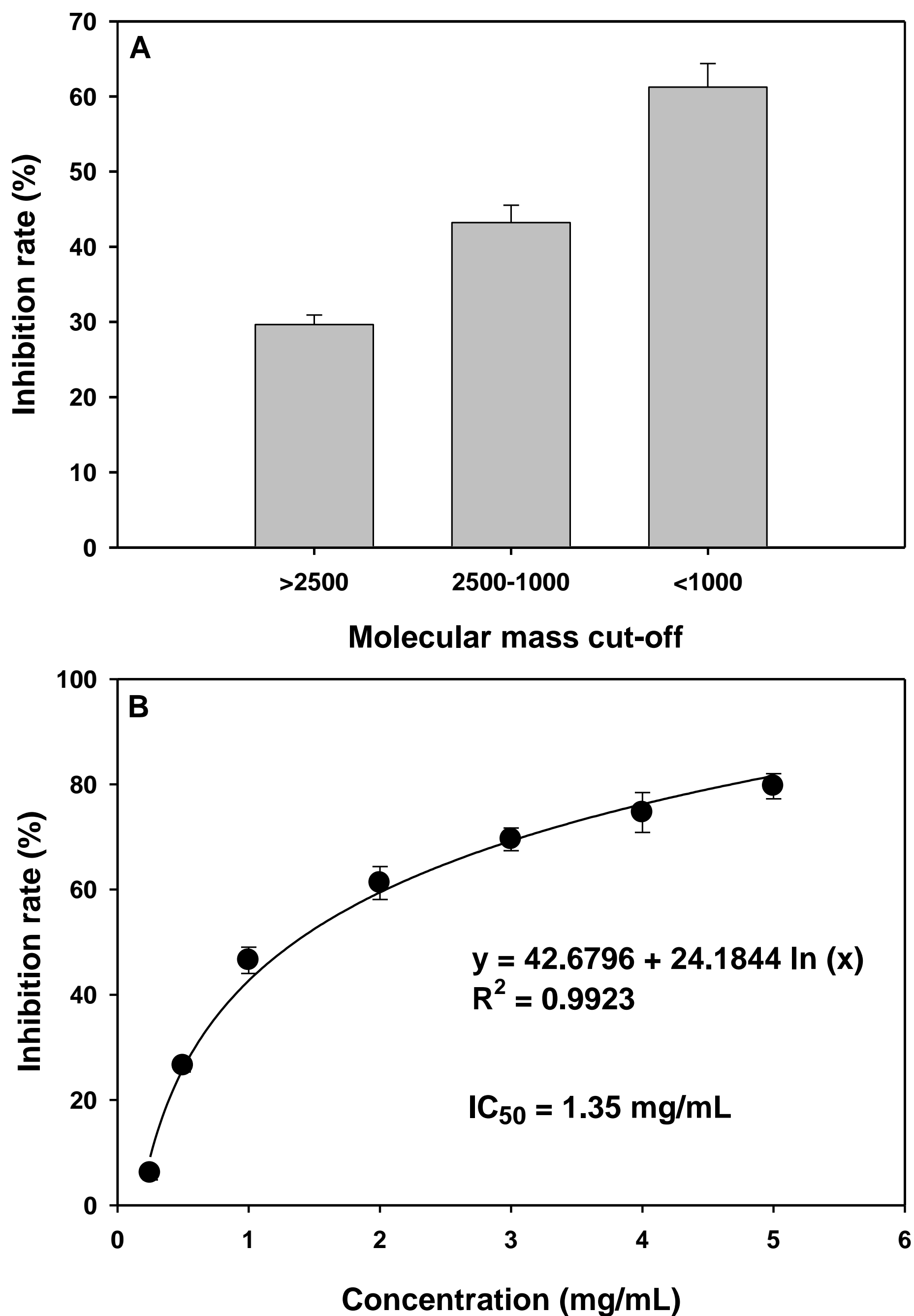
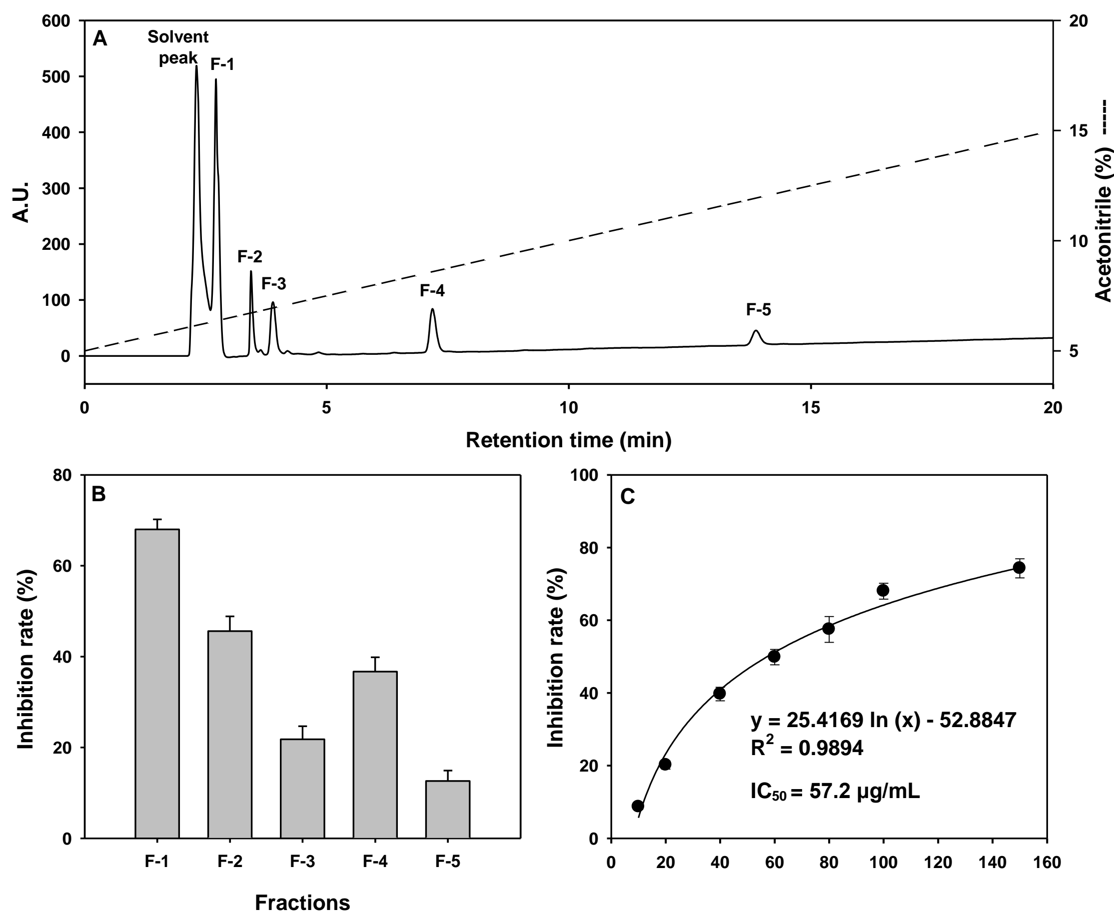


336

337 **Figure 2.** (A) DPP-IV inhibition rate of Atlantic salmon skin gelatin hydrolysates fractionated by UF at
338 the concentration of 2 mg solid/mL. (B) DPP-IV inhibition rate of the < 1 kDa UF fraction at various
339 concentrations. Bars represent standard deviations from triplicate determinations.

340



341

342 **Figure 3.** (A) Elution profile and (B) DPP-IV inhibition rate of the peptide fractions from the < 1 kDa
343 UF fraction separated by HPLC. (C) DPP-IV inhibition rate of the fraction F-1 at various concentrations.
344 The DPP-IV inhibition rate was determined with each HPLC fraction at the concentration of 100 μg
345 solid/mL.

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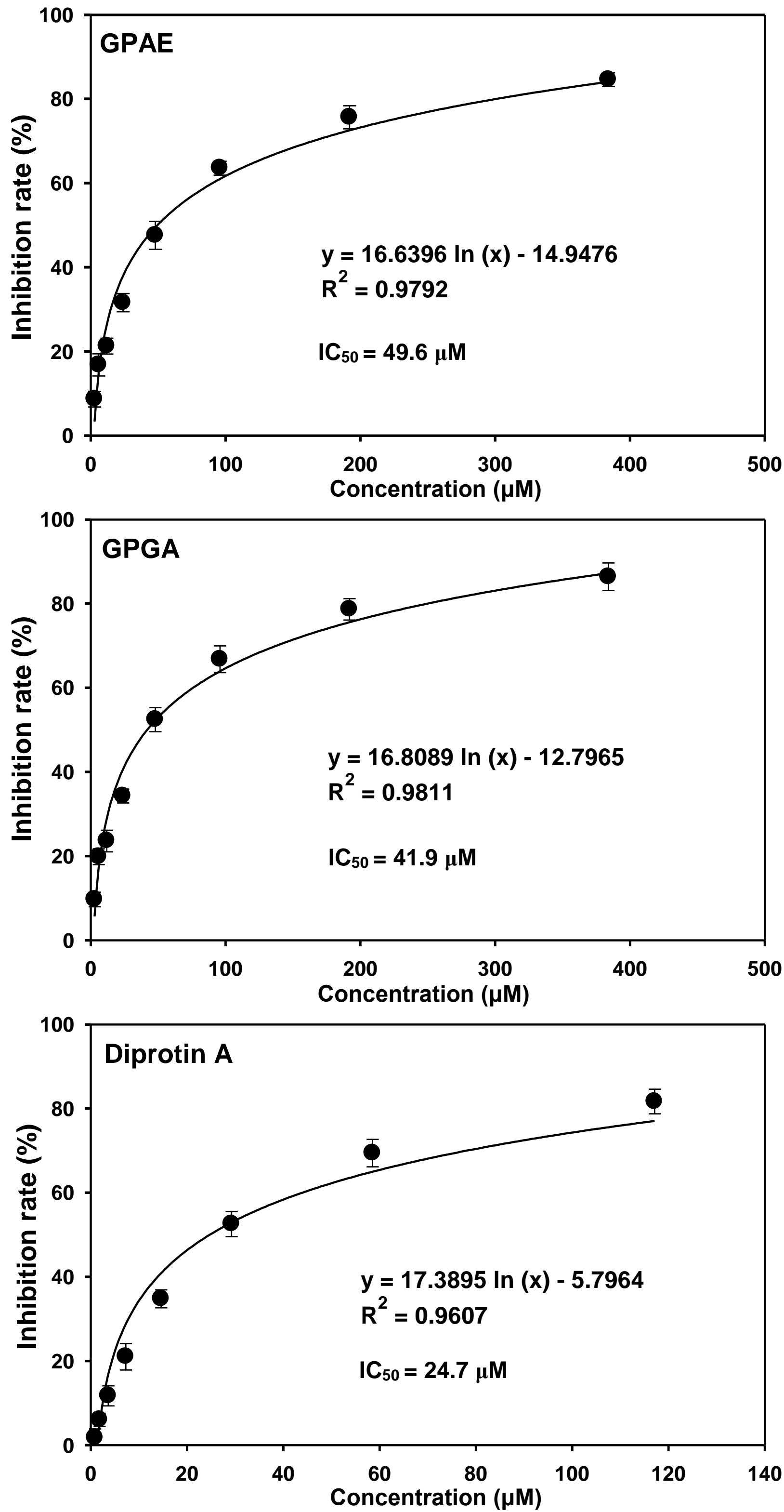

348 **Figure 4.** DPP-IV inhibition rate and the IC_{50} value of the synthetic peptides and Diprotin A.