JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

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ABSTRACT: The dipeptidyl-peptidase IV (DPP-IV)-inhibitory activity of peptides derived from Atlantic salmon skin gelatin hydrolyzed by alcalase (ALA), bromelain (BRO), and Flavourzyme (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 an IC_{50} value of 1.35 mg/mL. The F-1 fraction further isolated by HPLC showed the IC_{50} 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 dose-dependent inhibition effects 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 foods or pharmaceuticals against type 2 diabetes.

KEYWORDS: *dipeptidyl-peptidase IV inhibitor, Atlantic salmon, gelatin, type 2 diabetes, bioactive peptide*

INTRODUCTION

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

Dipeptidyl-peptidase IV (dipeptidyl-peptide hydrolase, EC 3.4.14.5) is a postproline-cleaving enzyme with a specificity for removing X-proline or X-alanine dipeptides from the N-terminus of polypeptides.[9] The cleavage of N-terminal peptides with Pro in the second position is a rate-limiting step in the degradation of peptides. There are several chemical compounds used in vitro and in animal models to inhibit DPP-IV activity, such as valine-pyrrolidide,[7] NVP-DPP728,[8] and Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide.[10] However, such chemical compounds, which often have to be administered by injection, may result in side effects as chemical drugs. Diprotins A and B, isolated from culture filtrates of *Bacillus cereus* BMF673-RF1, were found to exhibit the inhibitory activity on DPP-IV with IC_{50} values of 1.1 and 5.5 μg/mL, respectively;[11] they were elucidated to be Ile-Pro-Ile and Val-Pro-Leu. There were also two peptides, Ile-Pro-Ala and Val-Ala-Gly-Thr-Trp-Tyr, prepared from β-lactoglobulin hydrolyzed by proteinase K and trypsin, that showed IC_{50} values of 49 and 174 μM against DPP-IV.[12,13] Patents WO 2006/068480 and WO 2009/128713 have demonstrated that the peptides derived from casein and lysozyme hydrolysates display DPP-IV-inhibiting activity, and the peptides show in particular the presence of at least one proline within the sequence and mostly in the second N-terminal residue.[14,15]

It is well-known that the dominant amino acid in gelatin is glycine, whereas the imino acids (proline and hydroxyproline) come second in abundance.[16] The amino acid composition is characterized by a repeating sequence of Gly-X-Y triplets, where X is mostly proline and Y is mostly hydroxyproline.[17] Furthermore, previous studies have reported that the DPP-IV-inhibitory peptides consisted of at least one proline and mostly as the penultimate N-terminal residue.[11−15] Therefore, the aim of this study was to examine the DPP-IV-inhibitory activity of peptides derived from Atlantic salmon skin gelatin. This is

Received: November 17, 2011
Revised: January 1, 2012
Accepted: January 4, 2012
Published: January 4, 2012

dx.doi.org/10.1021/jf204720q | J. Agric.Food Chem. 2012, 60, 973−978

expected to give insight into the possible utilization of Atlantic salmon skin as a potential source of DPP-IV inhibitors that may be used in the treatment of type 2 diabetes.

■ MATERIALS AND METHODS

Materials and Reagents. Atlantic salmon (*Salmo salar*) fish skins, processing byproducts recovered from fresh skin-off fillets, were supplied by Albion Fisheries Ltd. (Vancouver, BC, Canada). The fish skins were transferred on ice to our laboratory, vacuum packed, and stored at −25 °C until use. Three food-grade proteolytic enzymes were donated by Neova Technologies Inc. (Abbotsford, BC, Canada). Alcalase 2.4 L FG (from *Bacillus licheniformis*, 2.4 AU/g) and Flavourzyme 1000 L (from *Aspergillus oryzae*, 1000 LAPU/g) were products from Novo-zymes North America Inc. (Salem, NC, Canada), whereas bromelain (from pineapple stem, 2000 GDU/g) was manufactured by Ultra Bio-Logics Inc. (Montreal, QC, Canada). Dipeptidyl-peptidase IV (D7052, from porcine kidney), Gly-Pro-*p*-nitroanilide hydrochloride, trichloroacetic acid (TCA), L-leucine, and diprotin A were purchased from Sigma-Aldrich (St. Louis, MO). Trinitrobenzenesulfonic acid (TNBS) was from Fluka Biochemika (Oakville, ON, Canada). Other chemicals and reagents used were of analytical grade and commercially available.

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

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

Enzymatic Hydrolysis. One gram of the freeze-dried gelatin with 50 mL of ddH_2O added was incubated at 50 °C for 10 min prior to the enzymatic hydrolysis. The enzymes in liquid form were weighed as 10, 20, 30, 60 mg and mixed with 1 mL of ddH_2O. The hydrolysis reaction was started by the addition of enzymes at various enzyme/substrate ratios (E/S: 1, 2, 3, and 6%). The reactions with alcalase (ALA), bromelain (BRO), and Flavourzyme (FLA) were conducted at pH 8.0, 7.0, and 7.0, respectively, and 50 °C for 4 h. After hydrolysis, the hydrolysates were heated in boiling water for 10 min to inactivate enzymes and then cooled in cold water at room temperature for 20 min. Hydrolysates were adjusted to pH 7.0 with 1 M NaOH and centrifuged (DuPont Sorvall Centrifuge RC 5B, Mandel Scientific Co. Ltd., Guelph, ON, Canada) at 12000*g* and room temperature for 15 min. The supernatant was lyophilized and stored at −25 °C.

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

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

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

High-Performance Liquid Chromatography (HPLC). The fractionated hydrolysates by ultrafiltration exhibiting DPP-IV-inhibitory activity were further purified using HPLC (model L-2130 HPLC, Hitachi Ltd., Katsuda, Japan). The lyophilized hydrolysate fraction (100 μg) by gel filtration was dissolved in 1 mL of 0.1% trifluoroacetic acid (TFA), and 90 μL of the mixture was then injected into a column (Zorbax Eclipse Plus C18, 4.6 × 250 mm, Agilent Techologies Inc., Santa Clara, CA) using a linear gradient of acetonitrile (5−15% in 20 min) in 0.1% TFA under a flow rate of 0.7 mL/min. The peptides were detected at 215 nm. Each collected fraction was then lyophilized and stored in a desiccator until use.

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

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

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

■ RESULTS AND DISCUSSION

Amino Acid Composition of Atlantic Salmon Skin Gelatin. The amino acid composition of Atlantic salmon skin gelatin is presented in Table 1. The glycine content of salmon skin gelatin was 223.63 mg/g sample, slightly higher than that of Nile tilapia skin gelatin (211.8 mg/g protein) and similar to that of porcine skin gelatin (224.5 mg/g protein).[23] The alanine content (7.06 mol/100 mol amino acids) of salmon skin gelatin in the present study was relatively lower than those (9.6−12.3 mol/100 mol amino acids) of skin gelatins from other fish species, such

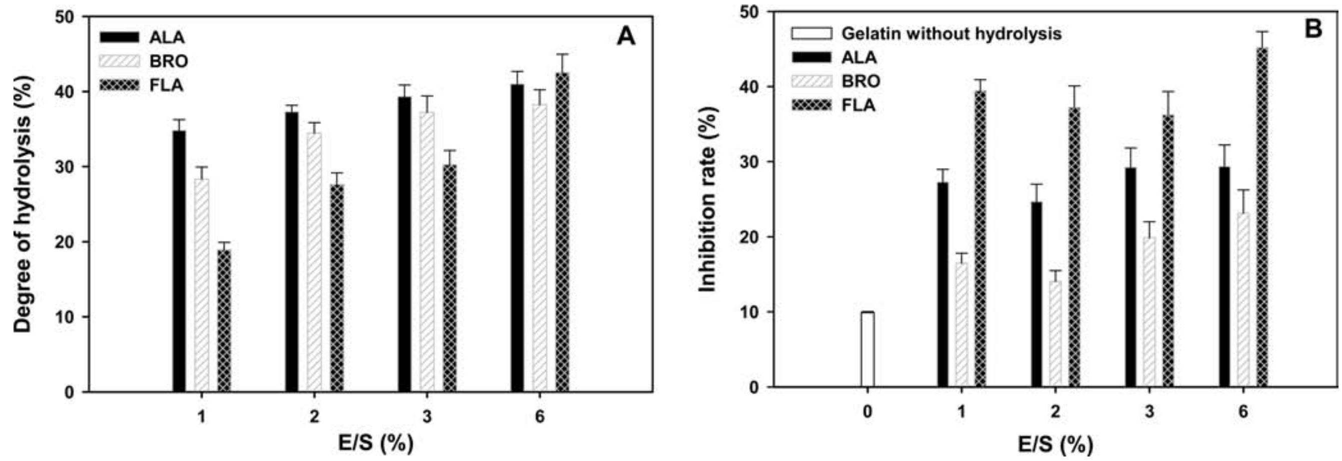

Figure 1. (A) DH and (B) DPP-IV inhibition rate of Atlantic salmon skin gelatin hydrolyzed with ALA, BRO, and FLA at various E/S ratios. The DPP-IV inhibition rate was determined with the hydrolysates at the concentration of 5 mg solid/mL. Bars represent standard deviations from triplicate determination.

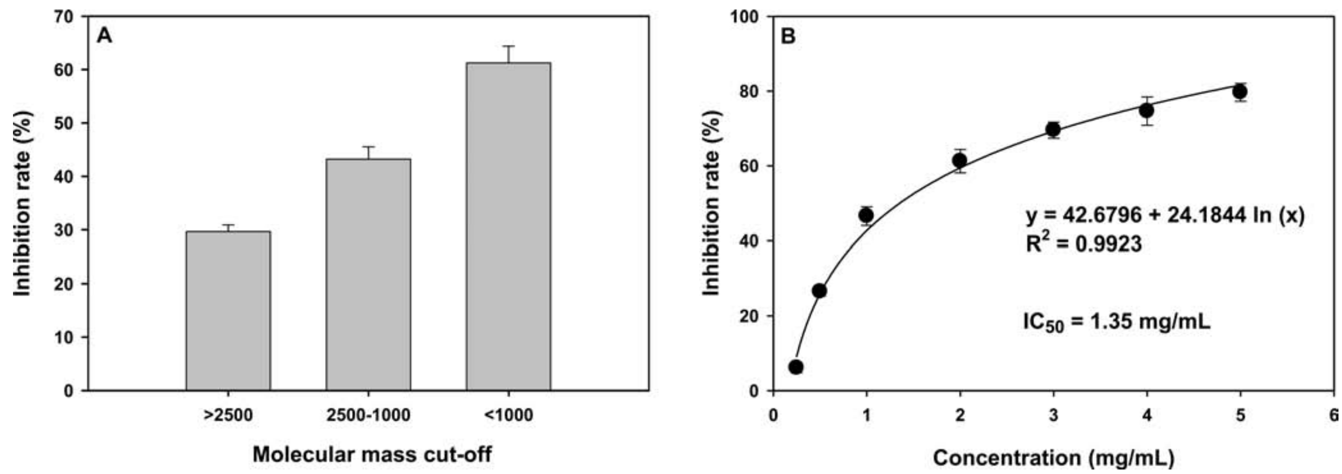

Figure 2. (A) DPP-IV inhibition rate of Atlantic salmon skin gelatin hydrolysates fractionated by UF at the concentration of 2 mg solid/mL. (B) DPP-IV inhibition rate of the <1 kDa UF fraction at various concentrations. Bars represent standard deviations from triplicate determinations.

Table 1. Amino Acid Composition of Gelatin from Atlantic Salmon Skins

amino acid	content (n = 3)	
	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 (Pro)	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

Table 2. Amino Acid Sequences of Purified DPP-IV-Inhibitory Peptides Derived from Atlantic Salmon Skin Gelatin Hydrolyzed with FLA

sequence	molecular mass
Gly-Pro-Ala-Glu	372.4
Gly-Pro-Gly-Ala	300.4

as cod, Alaska pollock, hake, and tilapia.[24] Salmon skin gelatin contained a high content of imino acids (175.02 mg/g sample), including proline (86.78 mg/g sample) and hydroxyproline (88.24 mg/g sample), which showed slightly lower contents (186.29 and 187.42 mg/g sample) as compared to bigeye snapper skin gelatins.[25]

DH and DPP-IV-Inhibitory Activity of Hydrolysates. The DHs and DPP-IV-inhibitory activities of Atlantic salmon skin gelatin hydrolyzed with ALA, BRO, and FLA at various E/S ratios for 4 h are shown in Figure 1. The three proteases used in the present study were alcalase (a serine protease), bromelain (a cysteine protease), and Flavourzyme (an exo- and endopeptidase complex). The result showed the DHs of the gelatin hydrolysates obtained by all three proteases hydrolysis increased with the increment of E/S ratio (Figure 1A). The DHs of ALA and BRO hydrolysates with the E/S ratio of 1% were 34.8 and 28.3%, respectively, and those with 6% were

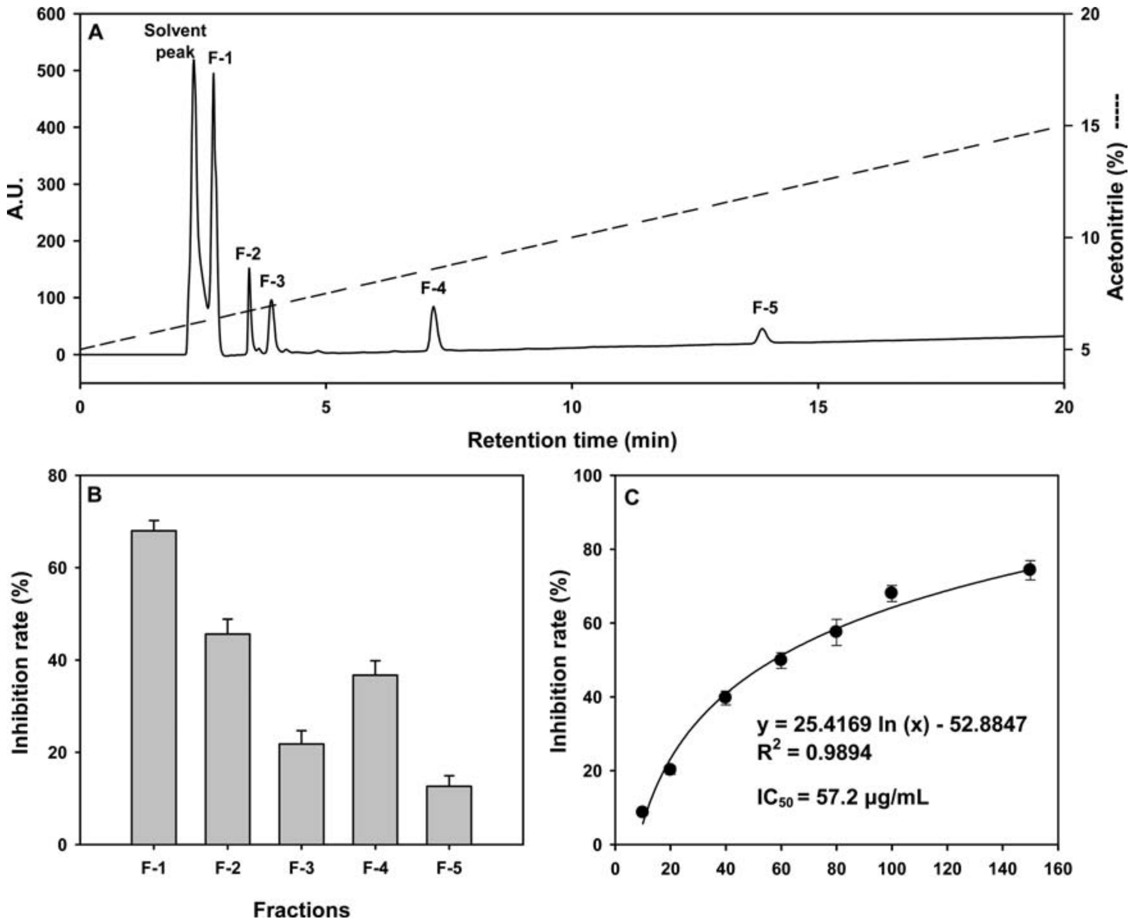

Figure 3. (A) Elution profile and (B) DPP-IV inhibition rate of the peptide fractions from the <1 kDa UF fraction separated by HPLC. (C) DPP-IV inhibition rate of fraction F-1 at various concentrations. The DPP-IV inhibition rate was determined with each HPLC fraction at the concentration of 100 μg solid/mL.

41 and 38.2%. The DHs of 1, 2, and 3% FLA hydrolysates were lower than those of ALA and BRO hydrolysates, and the 6% FLA hydrolysate showed the slightly higher DH of 42.5% than the other two protease hydrolysates. At the concentration of 5 mg solid/mL, the extracted gelatin (without hydrolysis) showed the DPP-IV inhibition rate of about 10%, and the hydrolysates possessed significantly higher DPP-IV-inhibitory activities ($P < 0.05$) than gelatin (Figure 1B). The DPP-IV inhibition rates of ALA hydrolysates with all of the various enzyme concentrations were between 24 and 30% with insignificant differences ($P > 0.05$), and that of 6% BRO hydrolysate showed 23.1%, the highest among all BRO samples ($P < 0.05$). The FLA hydrolysates showed the greatest DPP-IV inhibition rates as compared to ALA and BRO hydrolysates with the same E/S ratio, and that with 6% E/S ratio possessed the highest inhibition rate of 45.2% in this study ($P < 0.05$). Therefore, the FLA hydrolysate with the E/S ratio of 6% was used for further purification. Patent WO 2006/068480 has demonstrated that the hydrolysates possessed great DPP-IV-inhibitory activities referred to a mixture of peptides derived from hydrolysis of proteins with the percentage of hydrolyzed peptide bonds of most preferably 20−40%.[14] All of the hydrolysates except of those with 1 and 6% E/S ratios of FLA obtained in this study showed DHs between 27.6 and 40.9%, however, the DPP-IV-inhibitory activities of the two exceptions were higher than those of the other hydrolysates. We suggested that the DPP-IV-inhibitory activity should be determined by the peptide structures and sequences but not dependent upon DHs.

DPP-IV-Inhibitory Activity of Hydrolysates Fractionated by Ultrafiltration. Figure 2A shows the DPP-IV-inhibitory activities of 6% FLA hydrolysate fractions separated by ultrafiltration at the concentration of 2 mg solid/mL. The result showed the peptides within the <1 kDa UF fraction had the greatest DPP-IV inhibition rate of 61.2% ($P < 0.05$), whereas those within the >2.5 and 1−2.5 kDa fractions displayed inhibition rates of 29.6 and 43.2%, respectively. The IC_{50} value of the <1 kDa fraction was determined and found to be 1.35 mg/mL (Figure 2B). The result in this study is in agreement with former studies using various protein sources that reported the preferable DPP-IV-inhibitory peptides derived from food protein consisted of two to eight amino acid residues,[14,15] and their molecular weights were presumed to be between 200 and 1000 Da.

Purification of DPP-IV-Inhibitory Peptides by HPLC. Panels A and B of Figure 3 show the elution profile and DPP-IV-inhibitory activities of the peptide fractions from the <1 kDa UF fraction separated by HPLC. To obtain a sufficient amount of purified peptide, chromatographic separations were performed repeatedly. Five fractions (F-1−F-5) were obtained upon HPLC separation of the <1 kDa UF fraction (Figure 3A), and they were lyophilized and then used to determine their DPP-IV-inhibitory activities at the concentration of 100 μg solid/mL. The result showed that fraction F-1 had the highest

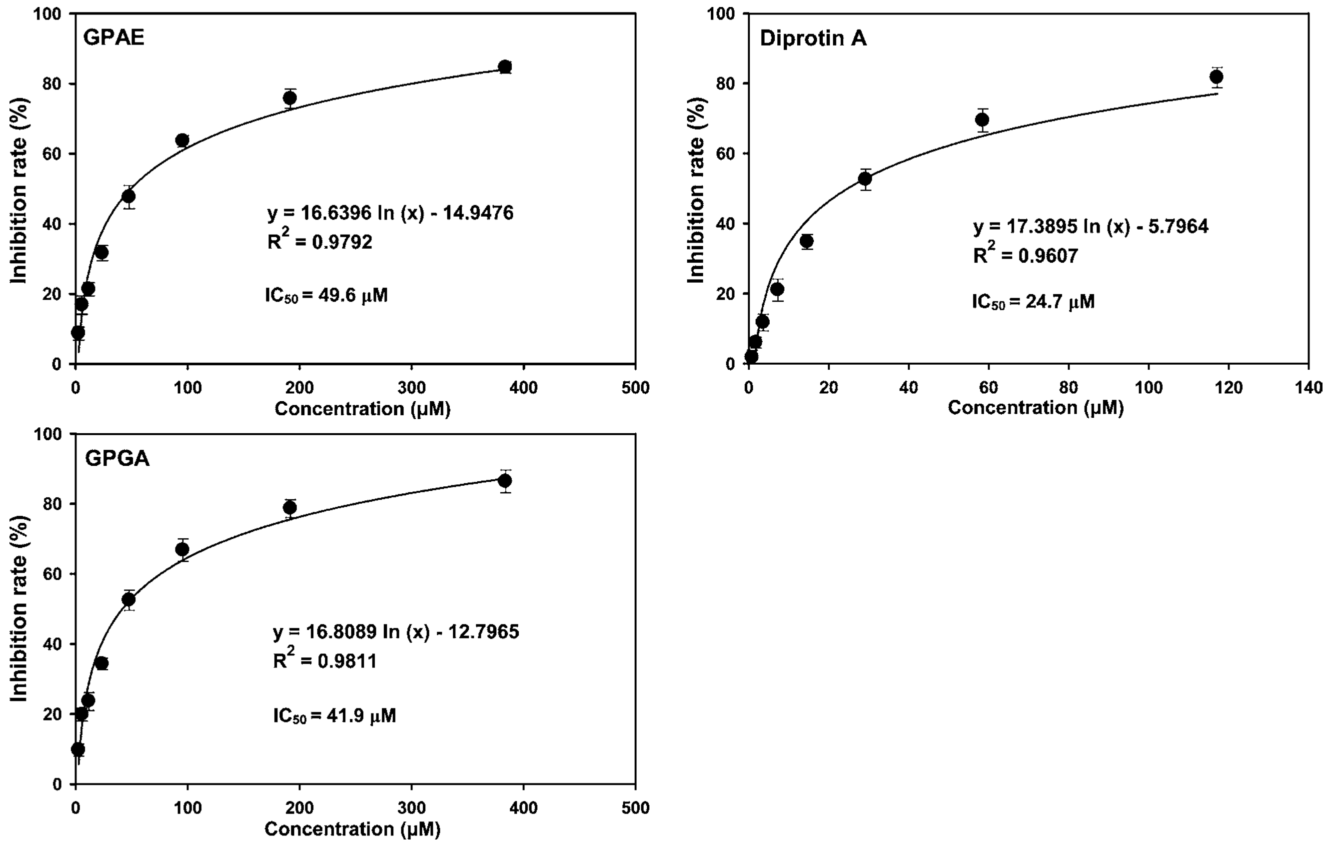

Figure 4. DPP-IV inhibition rates and IC_{50} values of the synthetic peptides and diprotin A.

DPP-IV inhibition rate of 68.0% ($P < 0.05$) (Figure 3B), and its IC_{50} value was also determined as 57.3 μg/mL (Figure 3C). Therefore, fraction F-1 was used to identify the amino acid sequences of the peptides.

Amino Acid Sequence of DPP-IV-Inhibitory Peptides. Two peptides were identified in fraction F-1, and their amino acid sequences were Gly-Pro-Ala-Glu (372.4 Da) and Gly-Pro-Gly-Ala (300.4 Da) (Table 2). Patent WO 2006/068480 has reported that 21 peptides that were capable of inhibiting DPP-IV activity showed a hydrophobic character, had a length varying from three to seven amino acid residues, and in particular showed the presence of a Pro residue within the sequence.[14] The Pro residue was located as the first, second, third, or fourth N-terminal residue, but mostly as the second N-terminal residue. Besides, the Pro residue was flanked by Leu, Val, Phe, Ala, and Gly. In the present study, both peptides comprised Pro as the second N-terminal residue, and the Pro residue was flanked by Ala and Gly. Moreover, the peptides were composed of mostly hydrophobic amino acid residues, such as Ala, Gly, and Pro, and one peptide comprised a charged amino acid, Glu, as the C-terminal residue. The present results therefore are consistent with the hypothesis demonstrated in the previous study.[14]

DPP-IV-Inhibitory Activity of the Synthetic Peptides. Figure 4 shows the DPP-IV-inhibitory activity of the two synthetic peptides and diprotin A at various concentrations. The IC_{50} was calculated for each of the peptides. Diprotin A is well-known as the peptide with the greatest DPP-IV-inhibitory activity, and its IC_{50} value was found to be 24.7 μM in the present study (Figure 4). The IC_{50} values of the two synthetic peptides, Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala, were 49.6 and 41.9 μM, respectively. In the previous study, the IC_{50} values against DPP-IV of diprotins A and B isolated from culture filtrates of *B. cereus* BMF673-RF1 were 3.2 and 16.8 μM, respectively.[11] Moreover, Ile-Pro-Ala and Val-Ala-Gly-Thr-Trp-Tyr, both prepared from β-lactoglobulin, showed IC_{50} values of 49 and 174 μM against DPP-IV, respectively.[12,13] Patent WO 2006/068480 reported that diprotin A showed the IC_{50} value of about 5 μM against DPP-IV, and five peptides, HPIK, LPLP, LPVP, MPLW, and GPFP, comprised four amino acids with Pro as the penultimate N-terminal residue displayed their IC_{50} values between 76 and 120 μM.[14] The results showed that the two peptides obtained in this study showed lower DPP-IV-inhibitory activity than only diprotin A and B, which were composed of three amino acid residues. However, they had inhibition effects similar to that of Ile-Pro-Ala but greater than that of other peptides comprising four or more amino acid residues. It is interesting that the ultimate N-terminal residues of the peptides mentioned above are all hydrophobic amino acids, and Gly is smaller than the other residues. Therefore, we assumed that DPP-IV-inhibitory activity of bioactive peptides may be determined by the amino acid length and the two N-terminal amino acid sequence of X-Pro, where X is the hydrophobic amino acid and preferably smaller in size. In conclusion, we found two peptides, Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala, isolated from Atlantic salmon skin gelatin hydrolysates having inhibitory activity against DPP-IV. The two peptides may be useful for the therapy or prevention of type 2 diabetes.

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Funding

This study was financially supported by China Medical University (Project CMU 99-S-37).

■ ABBREVIATIONS USED

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

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