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Dipeptidyl Peptidase-IV Inhibitory Activity of Peptides in Porcine Skin Gelatin Hydrolysates

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1. Introduction

During a meal, two incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), are released from the small intestine into the vasculature and augment glucose-induced insulin secretion from the islet β -cells [1]. It has been estimated that 50-60% of the total insulin secreted during a meal results from the incretin response, mainly the combined effects of GIP and GLP-1 [2]. Previous studies have shown both GIP and GLP-1 stimulate β -cell proliferation, differentiation, and prevent apoptosis [3-6]. GLP-1 also has some actions such as inhibition of glucagon secretion and food intake, glucose homeostasis and slowing the gastric emptying [7-9]. However, GLP-1 has a short half-life of 1-2 min following secretion in response to the nutrients ingestion because of its inactivation by dipeptidyl peptidase-IV (DPP-IV) [10], resulting in loss of insulintropic activities.

DPP-IV (CD26; E.C. 3.4.14.5) is a 110-kDa plasma membrane glycoprotein ectopeptidase that belongs to the prolyl oligopeptidase family [11]. It acts as a cleaving enzyme with the specificity for removing X-Pro or X-Ala dipeptides from the N terminus of polypeptides and proteins. It has a strong preference for Pro > Ala > Ser as the penultimate amino acid residue [10-12]. This enzyme is also capable of cleavage of N-terminal dipeptides with hydroxyproline (Hyp), dehydroproline, Gly, Val, Thr or Leu [10-14]. GLP-1 has Ala as the N-terminal penultimate amino acid residue, and therefore it is the substrate of DPP-IV. This finding that over 95% of the degradation of GLP-1 is attributed to the action of DPP-IV led to an elevated interest in inhibition of this enzyme for the treatment of type 2 diabetes [15]. Some previous studies have shown that specific DPP-IV inhibition increased the half-life of total circulating GLP-1, decreased plasma glucose, and improved impaired glucose tolerance in animal and human experiments [16-18].

There are several chemical compounds used *in vitro* and in animal models to inhibit DPP-IV activity, such as Val-pyrrolidide [17], NVP-DPP728 [18], Lys[Z(NO₂)]-thiazolidide and

Lys[Z(NO₂)]-pyrrolidide [19]. However, such chemical compounds, which often have to be administered by injection, may result in side effects as chemical drugs often do. Thus, to develop safe and natural DPP-IV inhibitors as the therapeutic agents of type 2 diabetes is necessary.

Proteins are well known as precursors of bioactive peptides. In recent years, peptides have been identified to possess physiological functions, such as immunomodulatory [20], antimicrobial [21], antihypertensive [22], anticancer [23], antioxidative [24] and cholesterol-lowering activities [25]. These bioactive peptides are mostly derived from milk, wheat, soybean, egg and fish proteins by enzymatic hydrolysis or fermentation [26]. Food protein hydrolysates are well-used and natural food ingredients, and therefore they are believed to be safe for consumers when they are served as functional foods. Some studies have reported that bioactive peptides possessed DPP-IV inhibitory activity. Diprotins A and B, isolated from culture filtrates of *Bacillus cereus* BMF673-RF1, are bioactive peptides found to exhibit the DPP-IV inhibitory activity with IC₅₀ values of 1.1 and 5.5 µg/mL; they were elucidated to be Ile-Pro-Ile and Val-Pro-Leu, respectively [27]. Two bioactive peptides, Ile-Pro-Ala and Val-Ala-Gly-Thr-Trp-Tyr, derived from β-lactoglobulin hydrolysed by proteinase K and trypsin, showed IC₅₀ values of 49 and 174 µM, respectively, against DPP-IV *in vitro* [28,29]. Two patents, WO 2006/068480 and WO 2009/128713 have shown that peptides derived from casein and lysozyme hydrolysates, respectively display DPP-IV inhibitory activity, and the peptides show in particular the presence of at least one Pro within the sequence and mostly as the penultimate N-terminal residue [30,31].

It is well-known that the dominant amino acid in gelatin is Gly, while the imino acids (Pro and Hyp) come second in abundance [32]. The amino acid composition of gelatin is characterized by a repeating sequence of Gly-X-Y triplets, where X is mostly Pro and Y is mostly Hyp. Inside gelatin molecule, Gly constitutes approximately 27% of the total amino acid pool [33]. The total amount of the imino acids is higher in mammalian (20-24%) than in fish (16-20%). In our previous study, we successfully isolated two peptides, Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala from Atlantic salmon skin gelatin, that showed dose-dependent inhibitory effects on DPP-IV with IC₅₀ values of 49.6 and 41.9 µM, respectively [34]. According to the report of previous studies, DPP-IV inhibitory peptides consisted of at least one Pro and mostly as the penultimate N-terminal residue [32]. Therefore, the aim of this study was to examine the DPP-IV inhibitory activity of peptides derived from porcine skin gelatin, which constitutes higher content of imino acids than skin gelatin of Atlantic salmon, a kind of cold-water fish. This is expected to give insight into the possible utilization of porcine skin as a potential source of DPP-IV inhibitors that may be used in the treatment of type 2 diabetes to lower the risk of side effects.

2. Materials and methods

2.1. Materials and reagents

Porcine skin gelatin (G-2500) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alcalase® 2.4 L FG (from *Bacillus licheniformis*, 2.4 AU/g) (ALA) was the product from Novo-

zymes North America Inc. (Salem, NC, Canada). DPP-IV (D7052, from porcine kidney), Gly-Pro-*p*-nitroanilide hydrochloride, trichloroacetic acid (TCA), L-Leu and Diprotin A were purchased from Sigma-Aldrich. Trinitrobenzenesulfonic acid (TNBS) was from Fluka Biochemika (Oakville, ON, Canada). Other chemicals and reagents used were analytical grade and commercially available.

2.2. Enzymatic hydrolysis

One gram of the gelatin added with 50 mL ddH₂O was incubated at 50°C for 10 min prior to the enzymatic hydrolysis. ALA in liquid form were weighed 10, 30, 50 mg and mixed with 1 mL ddH₂O. The hydrolysis reaction was started by the addition of enzymes at various enzyme/substrate ratios (E/S: 1%, 3%, and 5%). The reaction with ALA was conducted at pH 8.0, respectively, and 50°C for up to 6 h. After hydrolysis, the hydrolysates were heated in boiling water for 15 min to inactivate enzymes and then cooled in cold water at room temperature for 20 min. Hydrolysates were adjusted their pH to 7.0 with 1 M NaOH and centrifuged (Du Pont Sorvall Centrifuge RC 5B, Mandel Scientific Co. Ltd, Guelph, ON, Canada) at 12,000g and room temperature for 15 min. The supernatant was lyophilized and stored at -25°C .

2.3. Measurement of degree of hydrolysis

Immediately prior to termination of hydrolysis, 4 mL of the hydrolysate were mixed with an equal volume of 24% TCA solution and centrifuged at 12200g 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 the mixture was added with 1.0 mL of 2.0 M NaH₂PO₄ containing 18 mM Na₂SO₃, and the absorbance was measured at 420 nm using a spectrophotometer (Cary 50 Bio UV-vis spectrophotometer, Varian, Inc., Santa Clara, CA, USA) [35,36]. Degree of hydrolysis (DH) was calculated as % $DH = (h/h_{tot}) \times 100$, where DH=percent ratio of the number of peptide bonds broken (*h*) to the total number bonds per unit weight (*h*_{tot}) and *h*_{tot}=11.1 mequiv/g of gelatin [35]. L-Leu was used for drawing a standard curve.

2.4. Determination of DPP-IV inhibitory activity

DPP-IV activity determination in this study was performed in 96-well microplates measuring the increase in absorbance at 405 nm using Gly-Pro-*p*-nitroanilide as DPP-IV substrate [37]. The lyophilized hydrolysates were dissolved in 100 mM Tris buffer (pH 8.0) to the concentration of 10 mg/mL and then serially diluted. The hydrolysates (25 µL) were added with 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.

2.5. Ultrafiltration

Hydrolysates were fractionated by ultrafiltration (UF; 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 collected fractions were lyophilized and stored in a desiccator until use.

2.6. High Performance Liquid Chromatography (HPLC)

The fractionated hydrolysates by ultrafiltration exhibiting DPP-IV inhibitory activity were further purified using high performance liquid chromatography (Model L-2130 HPLC, Hitachi Ltd., Katsuda, Japan). The lyophilized hydrolysate fraction (100 μ g) by gel filtration was dissolved in 1 mL of 0.1% trifluoroacetic acid (TFA) and 90 μ L of the mixture, was then injected into a column (ZORBAX Eclipse Plus C18, 4.6 \times 250 mm, Agilent Tech. Inc., CA, USA) using a linear gradient of acetonitrile (5 to 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 lyophilized and stored in a desiccator until use.

2.7. Identification 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, UK) coupled with an electrospray (ESI) source. The purified peptides were separately infused into the ESI 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, CA, USA).

2.8. 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, USA), and their purity was verified by analytical RP-HPLC-MS/MS.

2.9. Statistical analysis

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

3. Results and discussion

3.1. Degree of hydrolysis

The DH of porcine skin gelatin hydrolyzed with ALA increased dramatically during the initial 1 h, and then increased gradually thereafter (Fig. 1). Also the highest DH was obtained with the highest E/S ratio. The highest DH (%) for ALA was 16.7% and obtained at the E/S ratio of 5% and 6-h hydrolysis.

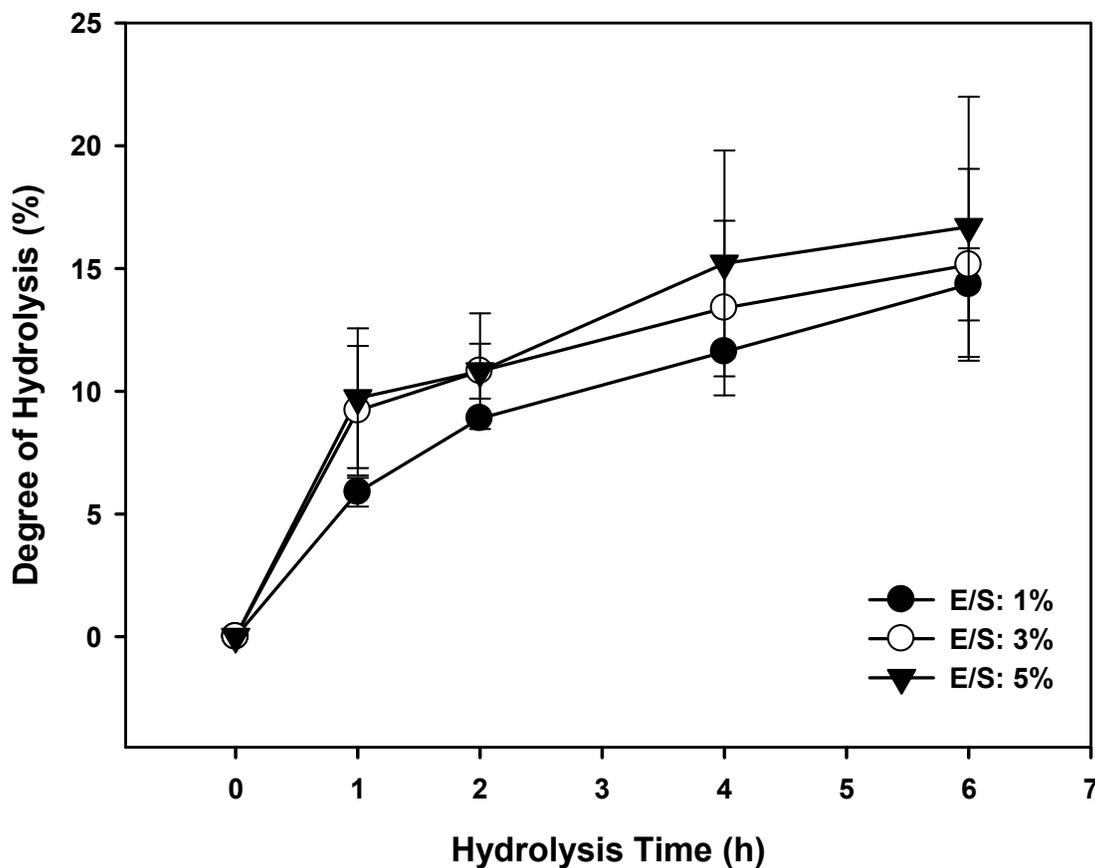


Figure 1. Degree of hydrolysis of porcine skin gelatin hydrolyzed with ALA at various E/S ratio.

3.2. DPP-IV inhibitory activity of hydrolysates

The DPP-IV inhibitory activity of porcine skin gelatin hydrolysates at the concentration of 10 mg/mL was shown in Fig. 2. The gelatin sample without hydrolysis (0 h) showed 9.2% inhibitory rate on DPP-IV. The DPP-IV inhibitory activity of the gelatin hydrolysates increased with E/S ratio and hydrolysis time. The DPP-IV inhibition rates of the 1-h hydrolysates with the E/S ratio of 1, 3 and 5% were 27.2, 44.3 and 48.8%, respectively; while those of 6-h hydrolysates increased to 52.0, 59.7 and 60.0%. The hydrolysates with the E/S

ratio of 3% and 5%, and the hydrolysis time of 4 h and 6 h showed the highest DPP-IV inhibition rates between 57.4 to 60.0% among all the samples ($P<0.05$); in the meanwhile, the inhibition rates between the four samples were not significantly different ($P>0.05$). The results showed that the hydrolysates with the smaller size of peptides due to the higher DH possessed greater DPP-IV inhibitory activity. 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 hydrolysed peptide bonds of most preferably 20 to 40% [30]. As the economic concern for saving time and enzyme used, the hydrolysate with E/S ratio of 3% and 4-h hydrolysis was adopted for further purification and analysis.

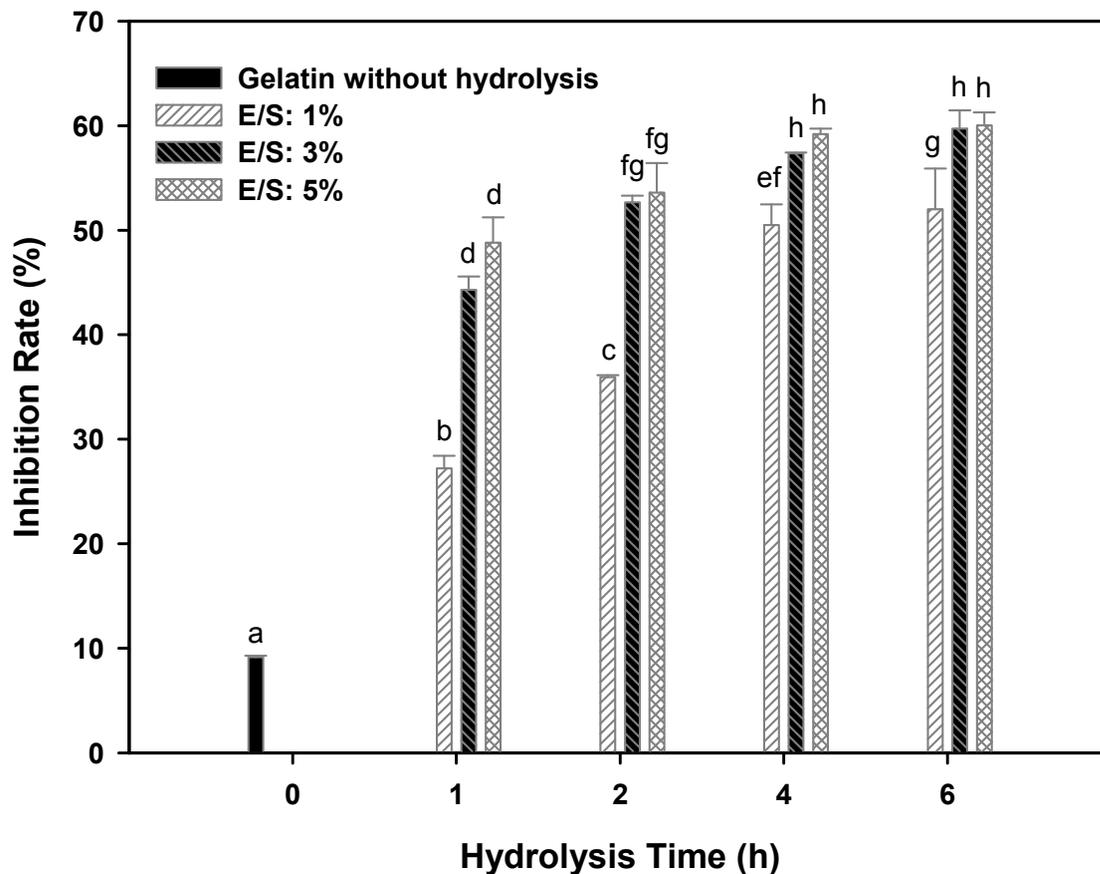


Figure 2. DPP-IV inhibitory rate of porcine skin gelatin hydrolysates. Different letters indicate the significant differences ($P<0.05$).

3.3. DPP-IV inhibitory activity of hydrolysates fractionated by UF

The DPP-IV inhibitory activity of hydrolysates with the E/S ratio of 3% and 4-h hydrolysis at the concentration of 1 mg/mL fractionated by UF was shown in Fig. 3A. The result showed the UF fractions of 1-2.5 kDa and < 1 kDa had insignificantly different ($P>0.05$) and higher

DPP-IV inhibition rates of 30.6% and 30.7%, respectively ($P < 0.05$), than that within the > 2.5 kDa fraction displaying an inhibition rate of 28.2%. < 1 -kDa fraction was selected for further analysis on the basis of the small size peptides may pass through the digestive tract without degradation. The IC_{50} value of the < 1 kDa fraction was determined and found as 1.50 mg/mL (Fig. 3B). The result in this study is in agreement with the former studies using various protein sources that reported the preferable DPP-IV inhibitory peptides derived from food protein consisted of 2-8 amino acid residues [30,31], and their molecular weights were supposed between 200 to 1000 Da.

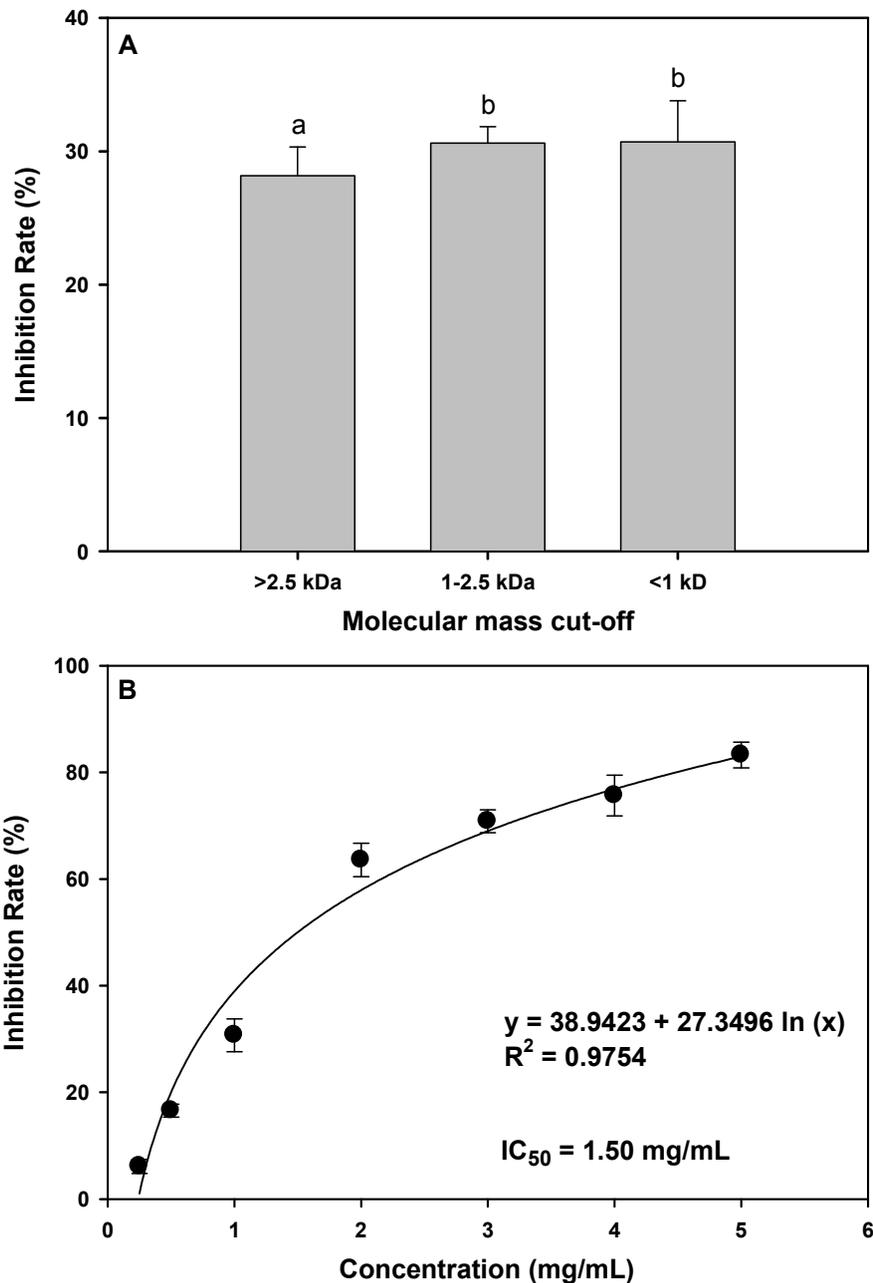


Figure 3. (A) DPP-IV inhibition rate of porcine skin gelatin hydrolysate fractionated by UF at the concentration of 1 mg/mL. (B) DPP-IV inhibition rate of the < 1 kDa UF fraction at various concentrations. Different letters indicate the significant differences ($P < 0.05$).

3.4. Purification of DPP-IV-inhibitory peptides by HPLC

The elution profile and DPP-IV inhibitory activity of the peptide fractions from the < 1 kDa UF fraction separated by HPLC were shown in Fig. 4A and B. To obtain a sufficient amount of purified peptide, chromatographic separations were performed repeatedly. Five fractions (F-1 to F-5) were obtained upon HPLC separation of the < 1 kDa UF fraction (Fig. 4A), and they were lyophilized and then used to determine their DPP-IV inhibitory activities at the concentration of 100 $\mu\text{g}/\text{mL}$. The result showed that the fraction F-3 had the highest DPP-IV inhibition rate of 64.6% ($P < 0.05$), as compared to the others which showed the inhibition rates between 26.7 to 45.6%, and its IC_{50} value was also determined as 62.9 $\mu\text{g}/\text{mL}$ (Fig. 4C). Therefore, the fraction F-3 was used to identify the amino acid sequences of the peptides.

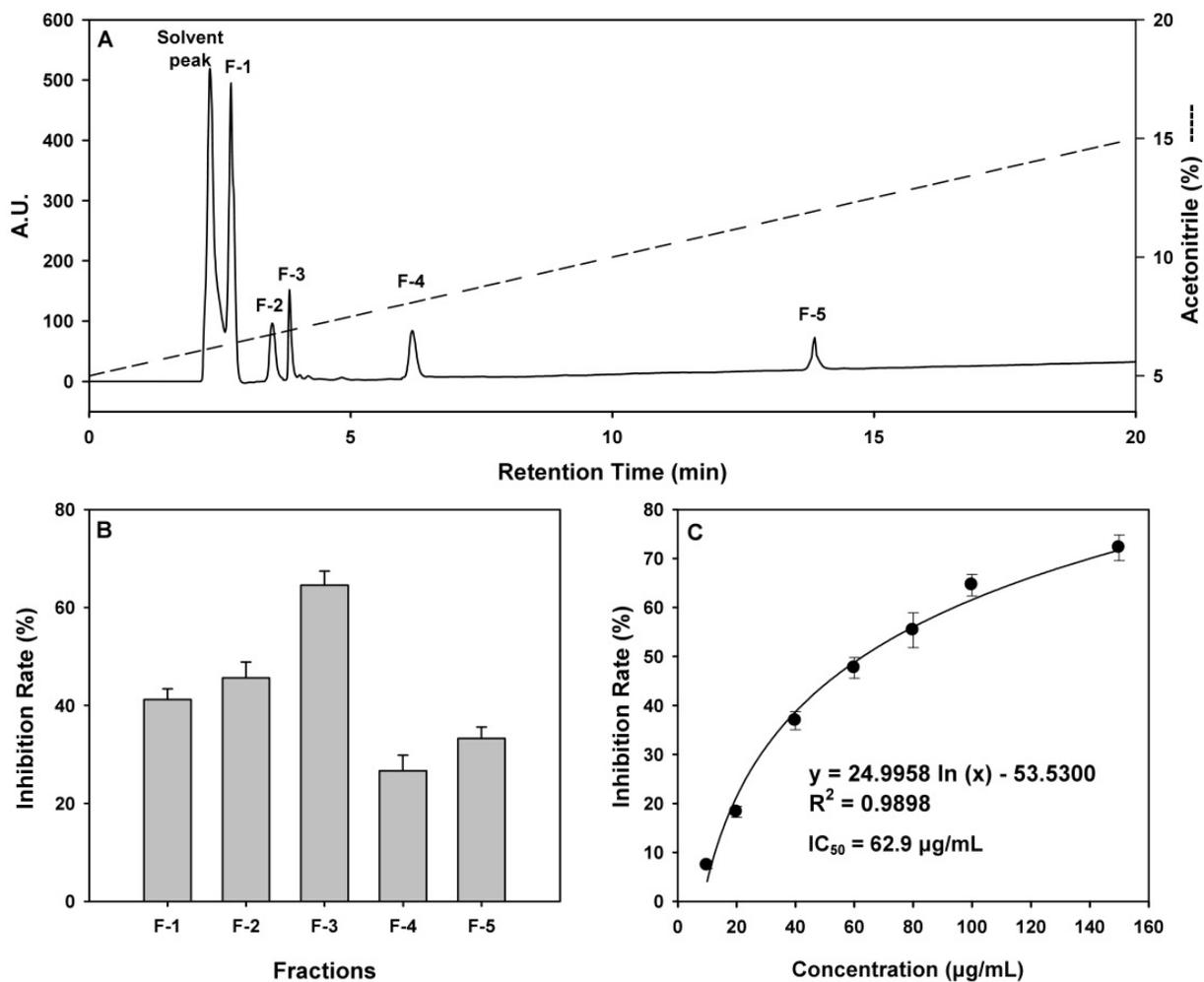


Figure 4. (A) Elution profile and (B) DPP-IV inhibition rate of the peptide fractions from the < 1 kDa UF fraction separated by HPLC. (C) DPP-IV inhibition rate of fraction F-3 at various concentrations. The DPP-IV inhibition rate was determined with each HPLC fraction at the concentration of 100 $\mu\text{g}/\text{mL}$.

3.5. Amino acid sequence of DPP-IV inhibitory peptides

Two peptides were identified in fraction F-3, and their amino acid sequences were Gly-Pro-Hyp (285.3 Da) and Gly-Pro-Ala-Gly (300.4 Da). Patent WO 2006/068480 has reported that 21 peptides which were capable of inhibiting DPP-IV activity showed a hydrophobic character, had a length varying from 3-7 amino acid residues and in particular the presence of Pro residue within the sequence [30]. 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 [30].

3.6. DPP-IV-inhibitory activity of the synthetic peptides

The DPP-IV inhibitory activity of the two synthetic peptides and Diprotin A at various concentrations was determined (Fig. 5). 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 as 24.7 μ M in the present study (Fig. 4C). The IC_{50} values of the two synthetic peptides, Gly-Pro-Hyp and Gly-Pro-Ala-Gly, were 49.6 and 41.9 μ M, respectively (Fig. 4A, B). In the previous study, the IC_{50} values against DPP-IV of Diprotin A and Diprotin B isolated from culture filtrates of *B. cereus* BMF673-RF1 were 3.2 and 16.8 μ M, respectively [27]. Moreover, Ile-Pro-Ala and Val-Ala-Gly-Thr-Trp-Tyr, both prepared from β -lactoglobulin showed IC_{50} values of 49 and 174 μ M against DPP-IV, respectively [28,29]. Patent WO 2006/068480 reported that Diprotin A showed the IC_{50} value of about 5 μ M against DPP-IV, and five peptides, His-Pro-Ile-Lys, Leu-Pro-Leu-Pro, Leu-Pro-Val-Pro, Met-Pro-Leu-Trp and Gly-Pro-Phe-Pro, comprised 4 amino acids with Pro as the penultimate N-terminal residue displayed their IC_{50} values between 76 to 120 μ M [30]. 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 with 3 amino acid residues. However, they had similar inhibition effect to Ile-Pro-Ala but greater than other peptides comprised 4 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 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-Hyp and Gly-Pro-Ala-Gly, isolated from porcine skin gelatin hydrolysates having the inhibitory activity against DPP-IV.

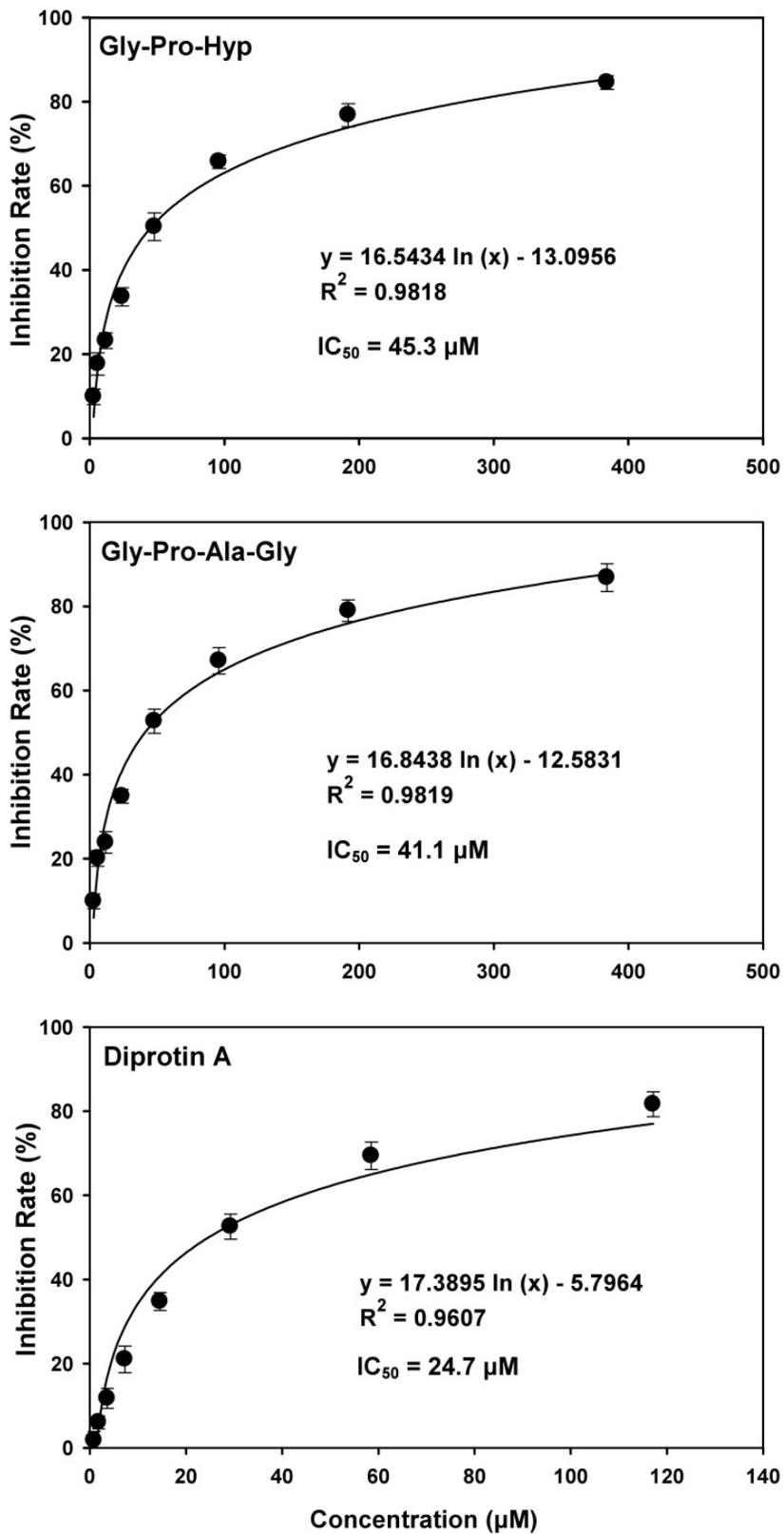


Figure 5. DPP-IV inhibition rates and IC₅₀ values of the synthetic peptides and diprotin A.

4. Conclusion

This study has clearly demonstrated that porcine skin gelatin could be a good protein source to produce DPP-IV inhibitory peptides by hydrolysis with ALA. The two peptides identified in this study may have the potential for the therapy or prevention of type 2 diabetes. Further studies using *in vitro* simulated gastrointestinal digestion and Caco-2 cell permeate analysis and *in vivo* animal model systems are therefore necessary to ascertain the required standards of evidence for DPP-IV inhibitory potential of bioactive peptides derived from porcine skin gelatin.

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