Title:

Dipeptidyl-Peptidase IV Inhibitory Activity of Peptides Derived from Tuna Cooking Juice Hydrolysates

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Running title:

Inhibition of Dipeptidyl-Peptidase IV

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Abstract

The *in vitro* DPP-IV inhibitory activity of isolated peptides from of tuna cooking juice hydrolyzed by Protease XXIII (PR) and orientase (OR) was determined. The results showed that the peptide fractions with the molecular weight over 1422 Da possessed the greatest DPP-IV inhibitory activity. The amino acid sequences of the three peptides isolated from PR and OR hydrolysates were identified by MALDI-TOF/TOF MS/MS, and they were Pro-Gly-Val-Gly-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu (1412.7 Da), Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg (1690.8 Da) and Pro-Ala-Cys-Gly-Gly-Phe-Try-Ile-Ser-Gly-Arg-Pro-Gly (1304.6 Da), while they showed the dose-dependent inhibition effect of DPP-IV with IC_{50} values of 116.1, 78.0 and 96.4 μM, respectively. The results suggest that the peptides derived from tuna cooking juice would be beneficial ingredients for functional food or pharmaceuticals against type 2 diabetes.

Key Words: Dipeptidyl-peptidase IV inhibitor; Tuna cooking juice; Type 2 diabetes; Bioactive peptide.

1. Introduction

Dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5), a serine protease, has a specificity for removing dipeptides from the N-terminus of substrate peptides and proteins by cleaving postproline or alanine residues [1]. It is present in body fluids and a variety of tissues, including liver, kidney and small intestine, and exists as a soluble circulating form [2,3]. DPP-IV has been shown to degrade chemokines, neuropeptides and many hormones, such as glucagon-like peptide 1 (GLP-1) [4].

GLP-1 is an incretin hormone that has potential to induce insulin secretion from the islet β-cells in a glucose-dependent manner. GLP-1 has multifaceted actions, including glucose-induced stimulation of insulin biosynthesis and secretion, inhibition of glucagon secretion, glucose homeostasis, β-cell proliferation and survival, inhibition of food intake, and slowing of gastric emptying [5-7]. However, GLP-1 has a short half-life of only 1 to 2 min following secretion in response to the nutrients ingestion because of its degradation by DPP-IV [8]. The finding that over 95% of GLP-1 is degraded by the action of DPP-IV led to an elevated interest in inhibition of this enzyme for the treatment of type 2 diabetes [9]. Experiments in animals and humans have showed that specific DPP-IV inhibition increased the half-life of total circulating GLP-1, decreased plasma glucose, and improved impaired glucose tolerance [10-12].

There are some reports describing bioactive peptides possess DPP-IV inhibitory activity. Diprotin A and B, isolated from culture filtrates of *Bacillus cereus* BMF673-RF1, were found to exhibit the inhibitory activity on DPP-IV [13] and they were elucidated to be Ile-Pro-Ile and Val-Pro-Leu, respectively. A bioactive peptide, Ile-Pro-Ala, prepared from β-lactoglobulin showed an IC_{50} value

of 49 μM for DPP-IV *in vitro*; in the meanwhile, Diprotin A showed the IC_{50} value of 7 μM *(14)*. Patent WO 2006/068480 and WO 2009/128713 have demonstrated that the peptides derived from milk and egg protein hydrolysates display DPP-IV inhibiting activity, and the peptides are in particular characterized by the presence of a proline within the sequence in the second N-terminal residue [5,15].

In our previous studies, we have successfully isolated 10 antioxidative peptides from tuna cooking juice hydrolyzed by Protease XXIII and orientase; meanwhile, nine of the peptides comprised at least one proline residue [16,17]. In our preliminary test, the major amino acid compositions of tuna cooking juice were Gly, His, Ala, Glu and Pro; and further, the proline content was 27.1 μmol/mL cooking juice (data not shown). Therefore, the objective of the present study was to determine the DPP-IV inhibitory activities of tuna cooking juice hydrolyzed by Protease XXIII and orientase. Then the sequences of the DPP-IV inhibitory peptides were identified.

2. Materials and Methods

2.1 Sample preparation

A canned tuna processor in Chiayi County (Taiwan) supplied the tuna cooking juice in which the protein content was 5.44%. The whole tuna fish (*Thunnus tonggol*) was cooked by steam (100-105℃) for 3-4 h, after which, the hot collected cooking juice was sealed in 400-mL polyethylene bags and then transferred to our laboratory immediately, and stored at 4℃ overnight. The cooking juice was filtrated through two layers of gauze to remove floating fats and solids, and the filtrate was collected and stored at -30℃ within 30 days until used.

98

99 *2.2 Chemicals and reagents*

100 Protease XXIII (specific activity of 3.8 units/mg solid), an endopeptidase
101 prepared from *Aspergillus melleus*, was obtained in dry powder form from
102 Sigma-Aldrich, Inc. (St. Louis, Mo, USA). Orientase 90N (specific activity of
103 70,000 units/mg solid), an endopeptidase prepared from *Bacillus subtilis* was
104 obtained in dry powder form from Hankyu Bioindustry Co. (Osaka, Japan).
105 Dipeptidyl peptidase IV (D7052, from porcine kidney), Gly-Pro-*p*-nitroanilide
106 hydrochloride, Bacitracin, L-tryptophan, L-leucine and Diprotin A were purchased
107 from Sigma-Aldrich (St. Louis, MO, USA). H-Phe-Phe-Phe-Phe-Phe-OH was
108 obtained from Bachem AG (Bubendorf, Switzerland). Trinitrobenzenesulfonic acid
109 (TNBS) was from Fluka Biochemika (Oakville, ON, Canada). Other chemicals and
110 reagents used were analytical grade and commercially available.

111

112 *2.3 Enzymatic hydrolysis*

113 Twenty-five milliliter of cooking juice was preincubated at 50℃ for 20 min
114 prior to enzymatic hydrolysis. The hydrolysis reaction was started by the addition
115 of 25 or 50 mg proteases (1 or 2 mg/mL of cooking juice; enzyme/protein substrate
116 ratio: 1.84 or 3.68%). The reactions with Protease XXIII (PR) and orientase (OR)
117 were conducted at pH 7.5, 37℃ and pH 7.0, 50℃, respectively, for up to 6 h. The
118 hydrolysates were heated in boiling water for 15 min to terminate hydrolysis and
119 then centrifuged (Centrifuge 05P-21, Hitachi Ltd., Katsuda, Japan) at 10,000×g for
120 10 min. A part of the supernatant was collected to determine its degree of
121 hydrolysis, and the other part was freeze-dried (FDU-540, EYELA freeze-dryer,

Tokyo, Japan) and stored in a desiccator for further purification.

2.4 Measurement of degree of hydrolysis

The degree of hydrolysis (DH) of the hydrolysates was determined as the ratio of the amount of α-amino acid released during hydrolysis to the maximum amount of α-amino acid in cooking juice [18]. Properly diluted samples (125 μL) were mixed with 2 mL of 0.2125 M sodium phosphate buffer (pH 8.2), followed by the addition of 1 mL of 0.01% TNBS. The mixtures were then incubated at 50℃ for 30 min in the dark, and added 2 mL of 0.1 M sodium sulfite to terminated the reaction. The mixtures were cooled down at ambient temperature for 20 min. The maximum amount of α-amino acid in cooking juice was obtained by acid hydrolysis with 6 N HCl at 110℃ for 24 h. The acid-hydrolyzed sample was then filtered through Whatman filter paper No. 1 to remove the unhydrolyzed debris. The supernatant was neutralized with 6 N NaOH before α-amino acid determination. The absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine. The DH was calculated as follows:

$$\text{DH (\%)} = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t; L_0 is the amount of α-amino acid in original cooking juice; L_{max} is the maximum amount of α-amino acid in cooking juice [19].

2.5 Determination of DPP-IV inhibitory activity (in vitro)

DPP-IV activity determination in this study was performed in 96-well microplates by measuring the increase in absorbance at 385 nm using

Gly-Pro-*p*-nitroanilide as DPP-IV substrate *(20)*. The lyophilized hydrolysates were dissolved in 100 mM Tris buffer (pH 8.0) at various concentrations. The hydrolysate solution (25 μL) was 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℃ for 20 min, followed by the addition of 50 μL of DPP-IV (diluted with the same Tris buffer to 0.01 units/ml). The reaction mixture was incubated at 37℃ for up to 60 min, and the reaction was stopped every 5 min by adding 100 μL of 1 M sodium acetate buffer (pH 4.0). The absorbance of the resulting solution was measured at 385 nm with an ELISA reader (Bio Tek μ QUANT; Bio Tek Instruments, Inc., Winooski, VT, USA). Recorded data were plotted versus time, and the DPP-IV activity was quantified from the linear part of the curve. The % DPP-IV inhibition was defined as the percentage of DPP-IV activity inhibited by a given concentration of hydrolysate (protein basis). The IC_{50} value corresponds to the concentration of sample needed to inhibit DPP-IV by 50%. 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.

2.6 Purification of DPP-IV inhibitory peptides

2.6.1 Gel filtration chromatography

The DPP-IV inhibitory peptides of the hydrolysates were separated using column chromatography [21]. The lyophilized hydrolysates (50 mg) were dissolved with 1 mL of deionized water. The resulting solution was fractionated by gel filtration on a Sephadex G-25 column (2.5×50 cm, Pharmacia, Sweden), and eluted

with deionized water. Each 5-mL fraction was collected at a flow rate of 30 mL/h, and the absorbance at 220 nm was monitored to separate peptide fractions. Bacitracin (Mr 1422 Da), H-Phe-Phe-Phe-Phe-Phe-OH (Mr 753.9 Da) and L-Tryptophan (Mr 204.2 Da) were used as the comparable standards of molecular weight. Each fraction was lyophilized and stored in a desiccator until use. A part of each lyophilized fraction was redissolved in 100 mM Tris buffer (pH 8.0) at the concentration of 5 mg/mL to determine its DPP-IV inhibitory activity.

2.6.2. High performance liquid chromatography (HPLC)

The fractionated hydrolysates by gel filtration 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 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 Tech. Inc., CA, USA) using a linear gradient of acetonitrile (5 to 25% in 40 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. A part of each lyophilized fraction was redissolved in 100 mM Tris buffer (pH 8.0) at the concentration of 2 mg/mL to determine its DPP-IV inhibitory activity.

2.7 Identification of amino acid sequence by MALDI-TOF/TOF MS/MS

The purified peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), using a delayed

194 extraction source and a 335 nm pulsed nitrogen laser [22]. This analysis was
195 carried out using a MALDI-TOF/TOF (UltraFlexIII, Bruker Daltonics Inc.,
196 Billerica, MA, USA). Peptides solution (0.6 μL) was mixed with 0.6 μL of
197 saturated α-cyano-4-hydroxycinnamic acid, and a droplet of the resulting solution
198 was placed on the sample target mass spectrometer. The droplet was dried by
199 evaporation at room temperature and then loaded into the mass spectrometer for
200 analysis. The instrument was operated in positive ion reflection mode with the
201 source voltage set at 20 kV. All spectra were the results of signal averaging of 200
202 shots. Measurements were determined in the mass range m/z 200-4000 Da, while
203 the peptide sequencing was determined by MS/MS spectra processing, using
204 BioTools (Version 3.2; Bruker Daltonics Inc.,Billerica, MA, USA).
205
206 *2.8 Peptide synthesis*
207 Peptides were prepared by the conventional Fmoc solid-phase synthesis method
208 with an automatic peptide synthesizer (Model CS 136, CS Bio Co. San Carlos, CA,
209 USA), and their purity was verified by analytical RP-HPLC-MS/MS.
210
211 *2.9 Statistical analysis*
212 Each data point represents the mean of three samples was subjected to analysis of
213 variance (ANOVA) followed by Tukey's studentized range test, and the significance
214 level of $P < 0.05$ was employed.
215
216 **3. Results**
217 *3.1 Degree of hydrolysis during enzymatic hydrolysis*

The DH of tuna cooking juice hydrolyzed with PR and OR increased dramatically during the initial 2 h and 1 h, respectively, and then increased gradually thereafter (**Fig. 1**). The highest DHs (%) for PR and OR were 19.4 and 23.6%, respectively, both obtained at the enzyme concentration of 2 mg/mL cooking juice and 6-h hydrolysis.

3.2 DPP-IV inhibitory activity of hydrolysates

Fig. 2 shows the DPP-IV inhibition rate of the hydrolysates (concentration of 10 mg solid/mL) derived from tuna cooking juice during hydrolysis. As shown in **Fig. 2**, cooking juice without hydrolysis showed DPP-IV inhibition rate of 9.8%; while after hydrolysis, DPP-IV inhibition rate increased to 36-45%. PR hydrolysates showed 36 to 40% of inhibition rate with insignificant differences in all conditions in the present study, except of the 1-h hydrolysate with the enzyme concentration of 1 mg/mL. OR hydrolysates showed the highest inhibition rate of 45.2% at the enzyme concentration of 2 mg/mL and 1-h hydrolysis. Therefore, PR and OR hydrolysates with the enzyme concentration of 2 mg/mL and 1-h hydrolysis were used for further purification to identify their amino acid sequences. Furthermore, no correlation between degree of hydrolysis and DPP-IV inhibition rate was observed in this study.

3.3 DPP-IV inhibitory activity of hydrolysates fractionated by gel filtration chromatography

Fig. 3 shows the elution profiles and DPP-IV inhibitory activities of PR and OR enzymatic hydrolysates separated by gel filtration chromatography. Five

fractions were collected from either PA and PR hydrolysates, respectively, separated by Sephadex G-25 gel (**Fig. 3**). The molecular weight (MW) distributions of fraction PR-1 (fraction no. 10-14), PR-2 (fraction no. 15-22), OR-1 (fraction no. 10-14) and OR-2 (fraction no. 15-21) were all over 1422 Da; while fraction PR-3 (fraction no. 23-28), PR-4 (fraction no. 29-33), OR-3 (fraction no. 22-29) and OR-4 (fraction no. 30-35) showed the molecular size between 1422 and 753.9 Da. In addition, those of fraction PR-5 (fraction no. 34-57) and OR-5 (fraction no. 36-57) were between 753.9 and 204.2 Da. The fractions with the highest DPP-IV inhibitory activity ($P < 0.05$) obtained from each enzymatic hydrolysates were PR-2 and OR-2, and they showed 39.5 and 38.8% of inhibition rate, respectively, at the concentration of 5 mg/mL. Therefore, the two fractions, PR-2 and OR-2, were used for further purification by HPLC.

3.4 Purification of DPP-IV inhibitory peptides by HPLC

Fig. 4 shows the elution profiles and DPP-IV inhibitory activities of PR-2 and OR-2 separated by HPLC. To obtain a sufficient amount of purified peptide, chromatographic separations were performed repeatedly. The fraction PR-2 and OR-2 obtained from gel filtration chromatography were lyophilized and then subjected to HPLC on a RP-C18 column (4.6 × 250 mm) with a linear gradient of acetonitrile (5-25%) containing 0.1% TFA. According to the elution profiles of both PR-2 and OR-2, only one major peak (the other one is the solution peak) was obtained at the retention time of about 4.8 min, and a lot of minor peaks were observed thereafter. Hence, we collected a fraction from the major peak and 4 fractions from the minor peaks every 7 minutes for both PR-2 and OR-2 (**Fig. 4**).

All the collected HPLC fractions were lyophilized and then used to determine their DPP-IV inhibitory activities at the concentration of 2 mg/mL. Fraction PR-2c and OR-2c showed the greatest DPP-IV inhibition rates of 60.8 and 63.4% ($P < 0.05$), respectively; therefore, they were used to identify their amino acid sequences by MALDI-TOF/TOF MS/MS.

3.5 Amino acid sequence of DPP-IV inhibitory peptides

The molecular mass of the DPP-IV inhibitory peptides from PR-2c were 1412.733 Da and 1690.879 Da, and that from OR-2c was 1304.644 Da (**Fig. 5**). After the determination by MS/MS spectra processing with BioTools database, the amino acid sequences of the three peptides were Pro-Gly-Val-Gly-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu (1412.7 Da), Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg (1690.8 Da) and Pro-Ala-Cys-Gly-Gly-Phe-Try-Ile-Ser-Gly-Arg-Pro-Gly (1304.6 Da), respectively.

3.6 DPP-IV inhibitory activity of the synthetic peptides

Fig. 6 shows the DPP-IV inhibitory activity of the three synthetic peptides and Diprotin A. The IC_{50} was also calculated for each of the peptides. Diprotin A (Ile-Pro-Ile) is well known as the peptide with the greatest DPP-IV inhibitory activity, and its IC_{50} value is 24.7 μM (**Fig. 6**). The IC_{50} values against DPP-IV of the three synthetic peptides, PGVGGPLGPIGPCYE, CAYQWQRPVDRIR and PACGGFWISGRPG, were determined to be 116.1, 78.0 and 96.4 μM, respectively.

4. Discussion

290 We reported three bioactive peptides derived from tuna cooking juice
291 hydrolyzed by Protease XXIII and orientase which possess DPP-IV inhibitory
292 activities. In the present study, the maximum cleavage of peptides occurred within
293 2 h and 1 h of PR and OR hydrolysis, respectively. The result was similar to our
294 previous studies with tuna cooking juice and dark muscles (17,23). The DH values
295 showed lower than those in our previous studies also with tuna cooking juice
296 hydrolyzed by PR and OR (17,23) probably attributed to the addition of lower
297 enzyme concentration in the present study.
298 According to the reports in several patents (Patent WO 2006/068480 and WO
299 2009/128713), the DPP-IV inhibitory peptides have a length varying from 3-7
300 amino acids, and are generally characterized by a hydrophobic nature and in
301 particular the presence of a proline residue within the peptide sequence. The
302 proline residue is as the first or second N-terminal residue, and is also found as
303 C-terminal or penultimate C-terminal residue. In the present study, all the three
304 peptides comprised at least one proline residue in the sequence, and two peptides
305 had the proline as the first N-terminal residue. Moreover, all the three peptides
306 were composed of many hydrophobic amino acid residues, such as Val, Leu, Ile,
307 Trp, Phe and Cys. Two peptides had Ala as the penultimate N-terminal residue as
308 well as GLP-1, the substrate of DPP-IV *in vivo* [15,24]. However, the three
309 peptides obtained in this study comprised 13-15 amino acid residues which were
310 much longer than the preferable DPP-IV inhibitory peptides. Some peptides,
311 Ile-Pro-Ala, Val-Ala-Gly-Thr-Trp-Tyr, Gly-Pro-Phe-Pro and Pro-Leu-Leu-Gln,
312 comprised 3-7 amino acid residues as the preferable DPP-IV inhibitors showed
313 diverse IC_{50} values against DPP-IV ranging from 49 to 174 μM [14,15,25]. The

result demonstrates that the DPP-IV inhibitory activity of bioactive peptides is determined by the composition and sequence of amino acids but not the length. Therefore, tuna cooking juice can be a good source of bioactive peptides possess DPP-IV inhibitory activity.

5. Conclusions

This study has clearly demonstrated that tuna cooking juice could be a good and cost-free protein source to produce DPP-IV inhibitory peptides by hydrolysis with two commercial proteases, Protease XXIII and orientase, for 1 h. It is economically feasible to use the materials and hydrolysis process for medical or food applications.

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403

Figure Legends

Fig. 1. Degree of hydrolysis (DH) of tuna cooking juice during hydrolysis by PR and OR with the enzyme concentration of 1 mg/mL cooking juice (●) and 2 mg/mL cooking juice (○). Bars represent standard deviations from triplicate determination.

Fig. 2. DPP-IV inhibition rate of tuna cooking juice hydrolysates at the concentration of 10 mg/mL by PR and OR with the enzyme concentration of 1 mg/mL cooking juice (black) and 2 mg/mL cooking juice (grey). Bars represent standard deviations from triplicate determinations. Different letters indicate the significant differences ($P < 0.05$).

Fig. 3. Elution profile and DPP-IV inhibition rate of PR and OR hydrolysates separated with gel filtration chromatography on Sephadex G-25. Each fraction, at the concentration of 5 mg/mL, was used to determine the DPP-IV inhibition rate.

Fig. 4. Purification of DPP-IV inhibitory peptide fractions from gel filtration chromatography on Sephadex G-25 by HPLC. Fractions PR-2 and OR-2 shown in Fig. 3 were applied to a RP-C18 column (4.6 × 250 mm), equilibrated with 0.1% TFA in H_2O and eluted with a linear gradient of acetonitrile (5-25%) in 0.1% TFA under a flow rate of 0.7 mL/min. Each fraction, at the concentration of 2 mg/mL, was used to determine the DPP-IV inhibition rate.

Fig. 5. Identification of the DPP-IV inhibitory peptides. (a) MALDI-TOF/TOF MS

and (b) MALDI-TOF/TOF MS/MS spectrum of the peptides from PR-2c and OR-2c.

Fig. 6. DPP-IV inhibition rate and the IC_{50} value of the synthetic peptides identified in **Fig. 5** and Diprotin A.