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Screening and identification of DPP-IV inhibitory peptides from deer skin hydrolysates by an integrated approach of LC–MS/MS and *in silico* analysis

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ABSTRACT

An integrated approach combining liquid chromatography/tandem mass spectrometry (LC–MS/MS) with *in silico* analysis was used to screen dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from deer skin hydrolysates. A total of 203, 244 and 60 peptides were identified from deer skin hydrolysates prepared with pepsin, pepsin + trypsin and pepsin + Alcalase, respectively, by LC–MS/MS. The percentages of peptides in the above mentioned hydrolysates containing a Pro residue at the penultimate position were 5.9, 20.9 and 20.0%, respectively. Five peptides with a Pro residue at the penultimate position were synthesized and assessed, and these five synthetic peptides possessed DPP-IV inhibitory activity with IC_{50} values from 83.3 to 1638.3 μM. One of the evaluated peptides contained an oxidized methionine. The effects of peptide modification and length on the DPP-IV inhibitory activity of the peptides were assessed. The results suggest that the integrated approach was efficient in identifying novel bioactive peptides from hydrolysates.

1. Introduction

Dipeptidyl peptidase IV (DPP-IV) is a post-proline-cleaving enzyme that can specifically cleave X-proline or X-alanine from the N terminus of peptides (Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López, & Dávila-Ortiz, 2012; Lambeir, Durinx, Scharpe, & De Meester, 2003). Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones responsible for modulating insulin synthesis and secretion and for maintaining the blood glucose at normal levels (Juillerat-Jeanneret, 2014). In type-2 diabetes, GIP and GLP-1 are rapidly degraded and inactivated by the action of DPP-IV, and

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Chemical compounds: Diprotin A (PubChem CID: 3107); Gly-Pro-p-nitroanilide hydrochloride (PubChem CID: 16219380); L-hydroxyproline (PubChem CID: 5810); Methionine sulphoxide (PubChem CID: 158980); L-proline (PubChem CID: 145742); L-methionine (PubChem CID: 6137); Glycine (PubChem CID: 750).

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their blood glucose-modulation functions are inhibited. Therefore, DPP-IV inhibitors have been demonstrated to be an effective treatment option for type-2 diabetes (Drucker & Nauck, 2006; Juillerat-Jeanneret, 2014). DPP-IV inhibitors have also emerged as novel pharmacological agents for inflammatory diseases (Yazbeck, Howarth, & Abbott, 2009). In addition to synthetic compounds (Aschner et al, 2006), protein hydrolysates have been investigated as good sources of DPP-IV inhibitors (Power, Nongonierma, Jakeman, & FitzGerald, 2014). DPP-IV inhibitory peptides have been identified from milk proteins (Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013a, 2013b, 2014a; Tulipano, Sibilia, Caroli, & Cocchi, 2011), amaranth proteins (Velarde-Salcedo et al., 2013), gelatin (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012; Velarde-Salcedo et al., 2013), meat proteins (Lafarga, O'Connor, & Hayes, 2014) and cereal proteins (Cavazos & de Mejia, 2013).

Collagen is the main protein in bone, skin and cartilage. Peptides with various activities have been identified from hydrolysates of fish gelatin (Alemán, Giménez, Montero, & Gómez-Guillén, 2011; Alemán, Giménez, Pérez-Santin, Gómez-Guillen, & Montero, 2011; Byun & Kim, 2001; Li-Chan et al., 2012; Mendis, Rajapakse, & Kim, 2005; Uriarte-Montoya et al., 2011), bovine gelatin (Kim, Kim, & Leem, 2014), porcine gelatin (Hsu, Tung, Huang, & Jao, 2013; Li, Chen, Wang, Ji, & Wu, 2007), and chicken collagen (Saiga et al., 2008). It is well known that the dominant sequence of collagen is a continuous repeating sequence of Gly-X-Y triplets, where X is mostly proline and Y is mostly hydroxyproline (Asghar & Henrickson, 1982). DPP-IV is an enzyme that specifically acts on a proline or alanine in the second position of the N terminus of polypeptides. The presence of a Pro residue in a given peptide is a good indicator of its DPP-IV inhibitory properties (Nongonierma & FitzGerald, 2013c). It has been demonstrated that collagen hydrolysates are the richest sources of DPP-IV inhibitory peptides (Lacroix & Li-Chan, 2012b).

The deer constitutes an important animal in traditional Chinese medicine and most deer organs are used as medicines or nourishment in traditional Chinese medicine. The most valuable portion of the deer is the antler. With the development of deer breeding in northeast China, many other deer organs are now harvested after the antler is collected. Similar to pork and beef skin, deer skin mainly contains collagen and is a potential source of DPP-IV inhibitory peptides. The aim of this study was to investigate the DPP-IV inhibitory peptides in hydrolysates of deer skin to provide insight into the possible utilization of deer skin.

Empirical and predictive approaches are the two main methods for discovering bioactive peptides from protein hydrolysates (Carrasco-Castilla et al., 2012). The classical empirical approach involves difficult steps for obtaining enriched bioactive peptides from crude protein hydrolysates. The predictive approach, which is also known as a bioinformatics-driven approach, is based on computational methods and analyses using knowledge of the chemical's structure and activity (Li-Chan, 2015). Many DPP-IV inhibitory peptides have been identified through *in silico* approach (Lafarga et al., 2014; Nongonierma & FitzGerald, 2014a, 2014b). The theoretical prediction does not consider the effects of processing on the generation of bioactive peptides and cannot reflect the real hydrolytic conditions. Moreover, the requirements regarding the enzyme's characteristics have limited the application of predictive approaches to only several known proteases. An approach that integrates the empirical and predictive approaches was previously proposed in the literature (Udenigwe, 2014). Proteases that are able to liberate specific bioactive peptides were predicted through a bioinformatics-driven approach and the activity of hydrolysates was validated using a classical approach. Because the integrated approach is based on predictive approaches, it confronts the same disadvantages as predictive approaches.

Mass spectrometry has emerged as an indispensable technology for the analysis of peptide mixtures arising from proteolytic degradation (Panchaud, Affolter, & Kussmann, 2012). In addition to its application to native protein and peptide analyses, mass spectrometry has become the most powerful technique for analysing post-translational modified proteins (Huang, Wang, Ye, & Zou, 2014). Proteomics and peptidomics based on mass-spectrometry technology have been applied in the food and nutrition fields (Sanchez-Rivera, Martinez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014). In the present study, we developed an integrated approach to screen and identify DPP-IV inhibitory peptides from deer skin hydrolysates. The integrated approach is based on liquid chromatography and tandem mass spectrometry (LC–MS/MS) and *in silico* analysis. The effects of proline-containing peptides and amino acid modifications on DPP-IV inhibitory activity were also assessed in this study.

2. Materials and methods

2.1. Materials and reagents

DPP-IV (from porcine kidney), diprotin A (Ile-Pro-Ile), Gly-Pro-p-nitroanilide hydrochloride, pepsin (from porcine gastric mucosa, 600 U g^{-1}), Alcalase (from *Bacillus licheniformis*, 2.4 U g^{-1}), trifluoroacetic acid (TFA) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma Aldrich (St. Louis, MO, USA). Trypsin (>250 U g^{-1}) and Tris were obtained from Amresco (Solon, OH, USA). Acetonitrile (ACN, HPLC grade) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) was obtained from Fluka (Buchs, Germany). Magic C18AQ (5 μm, 12 nm pore) was purchased from Michrom BioResources (Auburn, CA, USA). HCl and other chemicals were obtained from Kemiou (Tianjin, China). Diatomite filters (porosity 85%) were purchased from Damao (Tianjin, China). Ninety-six well plates were from Corning (Corning, NY, USA). Fused silica capillaries with an inner diameter of 75 μm were purchased from Yongnian Optical Fiber Factory (Hebei, China). All of the water used in the experiments was purified using a Milli-Q system from Millipore Company (Bedford, MA, USA). Red deer skins were supplied by Jiujiu Deer Industry Co., Ltd. (Fushun, Liaoning Province, China).

2.2. Enzymatic hydrolysis

The thawed deer skins were gently washed with running tap water, drained and cut into pieces (approximately 3 cm^2). The skin pieces (10 g) were incubated with 150 mL of 0.01 M HCl

at 37 °C for 5 min prior to enzymatic hydrolysis. The hydrolysis reaction was initiated by the addition of 0.2 g of pepsin (at an enzyme/substrate ratio of 0.02 (w/w)) and was conducted for 4 h. The pH of the reaction was maintained at 3.5 by the manual dropwise addition of 6 M HCl. After hydrolysis, the hydrolysates were heated in a water bath to 80 °C for 20 min to inactivate the enzyme and then cooled to room temperature. A portion of the hydrolysate was filtered using a diatomite filter, and the filtrate was lyophilized before analysis. The remaining hydrolysate was used for further enzymatic hydrolysis.

The pepsin hydrolysate was further hydrolysed sequentially by trypsin or Alcalase. The hydrolysis conditions for trypsin were an enzyme/substrate ratio of 0.005 (w/w), a temperature of 37 °C and a pH of 8.0 for 3 h. The hydrolysis conditions for Alcalase were an enzyme/substrate ratio of 0.005 (w/w), a temperature of 50 °C and a pH of 8.0 for 3 h. The hydrolysates were heated to 80 °C for 20 min to inactivate the enzyme and then cooled to room temperature. The hydrolysates used for analysis were filtered using a diatomite filter and lyophilized before analysis.

2.3. *DPP-IV inhibition assay*

The DPP-IV inhibition assay was performed in 96-well microplates as described previously (Hsu et al., 2013). The lyophilized hydrolysates or peptides were dissolved in 0.1 M Tris–HCl buffer (pH 8.0) at concentrations ranging from 0.25 to 10 mg mL^{-1} or 12.5 to 500 μg mL^{-1}, respectively. Diprotin A was used as a positive control. Twenty-five microlitres of the hydrolysates, peptide or buffer were added to 25 μL of 1.59 mM Gly-pro-p-nitroanilide (in 0.1 M Tris–HCl buffer, pH 8.0) and the mixture was incubated at 37 °C for 10 min. The reaction was started by the addition of 50 μL of 10 U μL^{-1} DPP-IV (in 0.1 M Tris–HCl buffer, pH 8.0). The reaction mixture was incubated at 37 °C for 60 min. A total of 100 μL of 1 M sodium acetate (pH 4.0) was added to stop the enzymatic reaction. The absorbance of the resulting solution was detected at 405 nm using a microplate reader (BioTek Synergy H1, Winooski, VT, USA). The per cent inhibition was calculated using the following equation:

$$\text{Inhibition activity} = (1 - As/Ac) \times 100$$

where A_S is the absorbance in the presence of the sample and A_C is the absorbance in the presence of buffer instead of the sample.

The IC_{50} value was defined as the sample concentration required to inhibit 50% of the DPP-IV activity under the experimental conditions.

Lineweaver–Burk plots were used to determine the mode of DPP-IV inhibition of synthetic peptides as described by Nongonierma and FitzGerald (2013a), with minor revisions. The basic conditions of the experiment were the same as those used for the DPP-IV inhibition assay. The enzymatic activity was measured at various substrate (Gly-pro-p-nitroanilide) concentrations (0.133, 0.172, 0.265, and 0.398 mM) in the absence and presence of an inhibitor at concentrations of 0.8 mg mL^{-1} and 0.4 mg mL^{-1}. K_m and V_{max} values were deducted from Lineweaver–Burk double reciprocal plots.

2.4. *Identification of the peptides by LC–MS/MS*

2.4.1. *Sample preparation*

The sample preparation for HPLC analysis was performed according to procedures previously reported by our group with minor modifications (Wu et al., 2009). A homemade C18 solid-phase extraction (SPE) column was activated by 1 mL of ACN and washed with a 0.1% TFA aqueous solution (v/v). The hydrolysate solution was adjusted to pH 2.7 with 10% TFA in water (v/v) and was then loaded onto the SPE column. Desalting was then performed by loading 500 μL of a 0.1% (v/v) TFA aqueous solution, and this desalting step was performed three times. The desalting was performed 3 times. Then, 1.2 mL of an aqueous solution containing 0.1% (v/v) TFA and 80% (v/v) ACN was used as an elution buffer (three times) to wash the peptides. The eluate was collected, lyophilized and stored at –80 °C until use.

2.4.2. *Mass spectrometric analysis*

The mass spectrometric and data analysis was performed using previously published procedure with minor revisions (Wang, Dong, Jiang, Ye, & Zou, 2007). The peptides were separated on an HPLC system consisting of a degasser and a quaternary Surveyor MS pump (Thermo Finnigan, San Francisco, CA, USA). The capillary separation column with an internal diameter of 75 μm was manually packed with C18 AQ particles (5 μm, 12 nm) to a length of 17 cm. The lyophilized hydrolysate was dissolved in a 0.1% (v/v) FA aqueous solution to 0.5 μg L^{-1} for LC–MS/MS analysis. Mobile phase A was 0.1% FA in water (v/v) and mobile phase B was 0.1% FA in ACN (v/v). The flow rate was set to approximately 60 μL min^{-1}. Separation of the peptides was performed using a gradient from 100% A to 10% A in 160 min and then 10% A to 100% A in 25 min.

The MS analysis was performed on a linear trap quadrupole (LTQ) mass spectrometer (Thermo Finnigan) with the following parameters: ion transfer capillary temperature, 200 °C; spray voltage, 1.8 kV; and full MS range, 400–2000 Da. The six most intense precursors were selected for subsequent fragmentation using a data-dependent acquisition mode. The normalized collision energy for fragmentation was set as 35.0%. The dynamic exclusion settings were the following: repeat count, 2, repeat duration, 30 s and exclusion duration, 90 s.

2.4.3. *Data analysis*

The collagen database was downloaded from http://www.uniprot.org/. The acquired MS/MS spectra were searched on the database using Turbo SEQUEST in the BioWorks 3.3.1 software suite (Thermo Finnigan) and processed by in-house Armone software (Jiang, Ye, Han, Dong, & Zou, 2010). Peptide identification was set to m/z at 400~2000. Cysteine residues were searched as a static modification of 57.0215 Da. To identify methionine sulphoxide (Met(O)) and hydroxyproline (Hyp), methionine residues and proline were searched as a variable modification of +15.9949 Da. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. The peptides were considered to be positively identified if the Xcorr (cross correlation value) was higher than 1.9 for a single-charged peptide, 2.2 for a double-charged peptide and 3.75 for a triple-charged peptide. The false positive rate of peptide identification was set to <1%.

The enzymes were set as pepsin for hydrolysates prepared with pepsin, pepsin and trypsin for those prepared with

pepsin + trypsin, and pepsin and Alcalase for those prepared with pepsin + Alcalase, respectively.

2.5. *Peptide synthesis*

The peptides were synthesized by the Chinese Peptide Company (Hangzhou, China) and their purities were verified by LC–MS/MS. The purities of the synthesized peptides were the following: 90.6% for Gly-Pro-Gly-Ser-Pro-Gly-Gly-Pro-Leu, 92.8% for Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys, 90.6% for Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys, 94.7% for Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys and 91.3% for Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Val-Hyp-Gly-Leu.

2.6. *Statistical analysis*

All of the tests were performed in triplicate. The results shown are the mean values ± standard deviation. All of the statistical comparisons were evaluated by Tukey's test. Difference with $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. *DPP-IV inhibitory activity of deer skin hydrolysates*

Insoluble native collagen must be pre-treated by heating, or using an acidic or alkaline agent before it can be converted into a form that is suitable for hydrolysis (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011). In this study, the deer skin was pre-treated through synchronized hydrolysis using pepsin. Therefore, pepsin was used for the preparation of all of the hydrolysates in some cases, was combined with trypsin and Alcalase. The DPP-IV inhibitory activities of deer skin hydrolysates prepared using pepsin in the absence or presence of proteases were evaluated and are shown in Fig. 1. The DPP-IV inhibitory activities of deer skin hydrolysed by pepsin in combination with trypsin or Alcalase were significantly improved compared with that of deer skin hydrolysed using pepsin alone ($P < 0.05$).

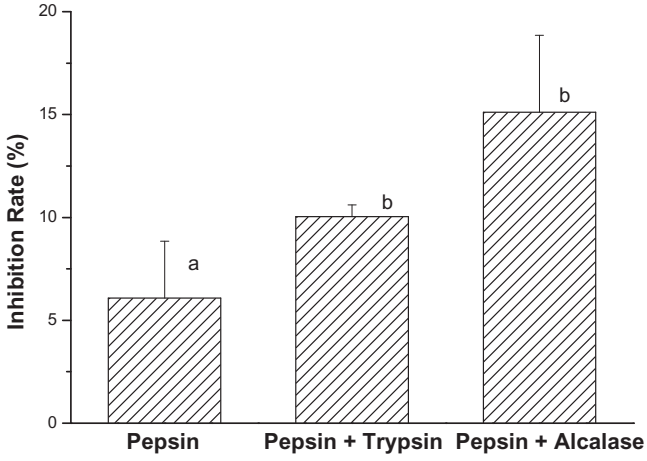

Fig. 1 – The DPP-IV inhibitory activity of deer skin hydrolysates prepared using pepsin, pepsin + trypsin or pepsin + Alcalase. The hydrolysate concentrations were set to 0.6 mg mL^{-1} for the DPP-IV inhibition assay. Each point is the mean of three determinations (n = 3) ± SD. Different letters indicate significant differences ($P < 0.05$).

3.2. *Analysis of peptides from deer skin hydrolysates*

The peptides identified in the deer skin hydrolysates are shown in Table 1. The number of amino acids in the identified peptides ranged from 7 to 22. The numbers of identified peptides from the deer skin hydrolysates prepared using pepsin, pepsin + trypsin and pepsin + Alcalase were 203, 244 and 60, respectively.

The amino acid residues in the identified peptides within each hydrolysate shown in Table 1 were determined. Within a hydrolysate, the percentage of the occurrence of each amino acid residue was obtained by dividing its number of occurrences by the total number of amino acid residues. As shown in Table 2, the result was normalized for 1000 residues. Gly, Pro, Ala and Hyp were the most frequently occurring amino acids identified within the three hydrolysates.

3.3. *Identification of DPP-IV inhibitory peptides with a Pro at the penultimate position*

It is well known that DPP-IV has strong specificity for a Pro residue at the P1 position. A previous study has demonstrated that many peptides with a Pro residue at the penultimate position behave as DPP-IV inhibitors (Nongonierma & FitzGerald, 2013c). The proportions of peptides from the deer skin hydrolysates prepared using pepsin, pepsin + trypsin and pepsin + Alcalase that presented a Pro residue at the penultimate position were 5.9, 20.9 and 20.0%, respectively.

To verify the activity of the peptides with a Pro residue at the penultimate position, five peptides were selected from the hydrolysates for subsequent synthesis. Gly-Pro-Gly-Ser-Pro-Gly-Gly-Pro-Leu was selected from the hydrolysates prepared with pepsin. Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys and Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys were selected from the hydrolysate prepared with pepsin + trypsin. Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys and Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Val-Hyp-Gly-Leu were selected from the hydrolysates prepared with pepsin + Alcalase. These were the shortest sequences within the identified peptides. Fig. 2 shows the MS/MS spectrum of the five peptides detected within the hydrolysates. The IC_{50} values of the peptides are shown in Table 3. The most potent compound was Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys, with an IC_{50} value of 83.3 ± 3.2 μM, and the least potent was Gly-Pro-Gly-Ser-Pro-Gly-Gly-Pro-Leu, with an IC_{50} value of 1638.3 ± 233.8 μM. Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys is a peptide with an oxidized methionine and has an IC_{50} value of 226.9 ± 8.9 μM.

To compare the effects of amino acid modifications and peptide length on DPP-IV inhibitory activity, Gly-Pro-Met, Gly-Pro-Met(O) and Gly-Pro-Val were synthesized and their DPP-IV IC_{50} values were determined. As shown in Table 3, the IC_{50} values of Gly-Pro-Met(O), Gly-Pro-Met and Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys are of the same magnitude. The IC_{50} value of Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys was 226.90 ± 8.9 μM, which is 2.9- and ~2-fold lower than those of Gly-Pro-Met(O)

Table 1 – Peptides from deer skin hydrolysates identified by LC/MS/MS.

Protease	Pepsin	Pepsin + Trypsin	Pepsin + Alcalase
Peptides	AAESLPKIGDLQPQIVNL	AGEKGPSGEX[a]GTAGPXGTPGPQ	AGAQGPPGPAGPAGE
	AAQYDXGKGVGLGPGPMGL	AGEKGPSGEXGTAGPXGTXGPQ	AGAXGPRGLAIK
	ADGDKITFXLEDGTEL	AGPPGADGQPGAK	AGPAGPAGPAGPRGSXGE
	ADGLEIGDNLAGNAL	AGPPGADGQPGAKGEXGDAGAK	DTPVTPSTAPPTLATSAXY
	AEVIGMF	AGPPGADGQXGAKGEXGDAGAK	GATGXKGVMGPAGXPGL
	AGEDVQIEISGDEPL	AGPXGADGQPGAKGEXGDAGAK	GGPXGVAGPXGGSGPAGPXGPQ
	AGKEVDPDDLYIVEXL	AGPXGPPGAPGK	GIPGPTGSXGPK
	AGPAGMTGSPGPLGSPGL	AGPXGPXGAPGK	GLMGPRGPXGASGAPGPQ
	AGVPGIPGLXGLEGPMGPPGL	AGVM(O)[b] GPAGSR	GPAGPXGPXGTSGPPGLQ
	ALRGPAGPM(O)GL	AGVMGPAGSR	**GPAGPXGVXGL**[c]
	ALRGPAGPMGL	AGXKGDKGTSGLPGVPGK	GPPGIXGAPGAPGEVGLRGIE
	ANGEKVAQKEL	DFGFDGDF	GPPGPAGPA
	ANGLPVGKSLL	DGEAGAQGPPGPAGPAGER	GPPGPAGPAGERGE
	ATINXEVRLVSPL	DGEAGAQGPXGPAGPAGER	GPPGXAGPXGE
	AVVLGAPFTAIIGL	DGLNGLPGPXGPXGXR	GPRGXXGPNGAXGPQ
	CTGLNXGKPARFAVDADL	DGLNGLPGPXGXPGXR	GPRGXXGPPGAXGPQ
	CTQCIYTXGXXGLPGL	DGLNGLXGPIGXXGPR	GPSGPPGVL
	DAASLHSLSTXNATSSM(O)L	DGLNGLXGPXGPPGPR	GPSGPRGLXGPPGAXGPQ
	DASGKGTVTFIDGEQAGL	DGLNGLXGXPGPPGPR	GPSGPRGLXGPXGAXGPQ
	DKKSRPIPKVEL	DGNPGSDGLPGR	GPTGXPGKRGE
	DLXTSELXPVKL	DGNXGSDGLPGR	GPVGIPGRXGXPGPPGPK
	DRSGKLKTILNNTL	DGNXGSDGLXGR	GPXGPAGPA
	DXNECDVGTGAPGPXGPPGL	DGQNGKDGQDGAPGPPDPGL	GPXGPXGPXGPPGPPSGGY
	DXNECDVGTGAPGXPGPPGL	DGSPGEXGANGIXGAAGER	GQXGPPGVDGYPGPPGPPGXQ
	EGRPGRDGVXGXXGL	DGSXGEXGANGIXGAAGER	GSPGFXGXXGFPGQ
	EGVXGNTGKXGL	DGTSGHPGPIGPXGPR	GSPGXXGSPGLPGPK
	EMPXTDEKGATKAEL	DGTSGHXGPIGPXGPR	GTPGIXGLDGREGHPGL
	ESIKKEVKGDLENAF	DGVLGEIGSPGPNGPXGAK	GTPGLPGTPGPVGDRGFPGE
	EWIAGGTWTPSALKF	EGAPGAEGSPGRDGSXGPK	GTPGNEGSAGRDGAPGPK
	FGGXQDYDSGPPXPEF	ELVELPTXGANDGHRM(O)L	GXAGPRGLXGPPGSXGPQGF
	FNDATDVMDALGYVTRF	ETYFSGXPGPPGPXGPK	GXXGAPGLPGADGAPGQPGXK
	FSICRSGLVTGRGRM(O)L	GAAGIXGPK	IGPVGNPGPAGPAGPRGE
	FVNGTM(O)L	GAAGLXGPKGDRGDAGPK	IGPVGNXGPAGPAGPRGE
	GAIGVKGRXGIXGSF	GAAGLXGVAGAXGLXGPR	IGVPGPXGXXGTPGRSGF
	GAPGSXGVVGNPGQRGAPGL	GAAGPXGNSGPXGPPGPSGK	KGAPGADGPAGAPGTPGPQ
	GDPGVLGDPGVLGDXGM(O)L	GALGYXGXXGLPGFTGPR	KGAPGADGPAGAXGTPGPQ
	GEAATGEDGKAVFELEL	GANGAXGIAGAXGFPGAR	KGAPGADGPAGAXGTXGPQ
	GEKGEKGEXAIIEXGMFF	GANGAXGIAGAXGFXGAR	KGAXGADGPAGAPGTPGPQ
	GEKGEQGIPGVL	GAPGAXGIAGAXGFPGAR	KGPSGEXGTAGPXGTXGPQ
	GELGPXGRXGL	GAPGAXGIAGAXGFXGAR	KGSPGAQGPXGAXGPL
	GEXGAQGEXGVDGKDGAL	GAPGWXGPXGDPGXS	KVGPRGXAGPQ
	GGXAGPXGENGSAGQPGL	GAQGAXGATGFXGAAGR	PGXAGXPGPETTGXPGPPGK
	GIPGSXGEQGEXGIPGTPGL	GARGEXGPAGLXGPXGER	PPXSNLVISEVTXRSF
	GLPGAVGQKGEQGSPGL	GATGFXGAAGR	QGHAGAPGPSGANGAPGXK
	GLXGAIGQKGSLGEPGL	GATGQKGETGXAGPAGAK	RGEXGAXGSXGFQ
	GNRTNSPMMIMPF	GATGSXGIAGAXGFXGAR	RGLXGVAGSVGEXGPL
	GPAGPIGPSGFNGF	GAXGDRGELGPXGPAGF	RGPXGPM(O)GPPGLAGPXGE
	GPGSPGGPL	GAXGTAGPSGPSGLPGER	RGPXGPMGPPGLAGPXGE
	GPIGM(O)PGLDGLDGL	GDGGPPGATGFXGAAGR	RGPXGPMGPXGLAGPXGE
	GPKGDXGDXGLXGRQL	GDGGPXGATGFXGAAGR	RGPXGXMGPPGLAGPXGE
	GPLGPDGEXGKPGVPGL	GDIGATGPVGAXGPKGAK	RGPXGXMGPPGLSGPXGE
	GPPGKQGPQGDPGFIGL	GDKGNVGLDGPIGPPGXL	RGVPGPPGAVGPAGKDGEAGAQ
	GPPGPPGRPGL	GDPGQXGEKGEPGRVGPPGL	RGXPGPMGPXGLAGPXGE
	GPPGPTGPXGPM(O)GPPDF	GDQGDVGXLGPQGPK	TGPAGRXGEVGXPGPXGPAGE
	GPTGXQGPL	GDXGEAGXVGXKGEPGPR	TGPIGPXGPAGAXGDKGE
	GPXGPRGQPGL	GDXGLXGLPGKDGPPGLR	TGPIGPXGXGGAXGDKGE
	GPXGPTGEPGPAGXL	GDXGPAGPAGPR	VDLRITISFSAGPGDK
	GPXGXXGVAGPL	GEAGAXGIXGGK	VGPXGPXGPAGE
	GQPGQQGSPGLPGPL	GEAGAXGIXGGKGDSGAXGER	VGTPGIM(O)GPPGPPGPPGPPGXE
	GQPGXMGETGRPGLXGL	GEAGIQGPQGKAGKDGAXGK	WGTPGXHGQ
	GRSGQMGLPGPEGIVGIPGQ	GEAGPAGPAGPAGPR	
	GTNPLKSSGIENGAF	GEAGSXGIXGPK	
	GVXGKAGKKGDIGFQGF	GEIGPVGNPGPAGPAGPR	
	GXIGXXGLKGNPGLQGF	GEIGPVGNXGPAGPAGPR	
	GXPGDSGXAGLEGRQGPPGL	GENGIXGENGAXGPMGPR	

(*continued on next page*)

Table 1(*continued*)

Protease	Pepsin	Pepsin + Trypsin	Pepsin + Alcalase
	GXQGXQGILGAQGLXGL	GENGNGKPGPTGIRGPXGLK	
	GXYGPKGDKGSM(O)GVXGF	GEPGSXGENGAXGQMGPR	
	IHLQHNQL	GERGEAGSXGIXGPK	
	INNKISKISPGAF	GERGPXGESGAAGPAGPIGSR	
	INNKISKISPGAFAPL	GERGQAGXTGPQGPKGER	
	IRDVWGIEGPIDAAF	GETGLRGDVGSXGR	
	IXMGQSM(O)VDPTGNITL	GETGPAGPAGPIGPVGAR	
	KASMKGLGTDEDSL	GETGPAGPAGXVGPVGAR	
	KATDPVKDLLGNHAF	GETGPSGPAGPTGAR	
	KGALGKAGKXGEAGLPGL	GEVGPAGSXGSNGAPGQR	
	KGEIGGAGLXGQXGFPGVXGL	GEVGPAGSXGSNGAXGQR	
	KGEIGGAGLXGQXGFXGVPGL	GEXGAXGENGTPGQTGAR	
	KGEKGDPGSLGISPPGL	GEXGAXGENGTXGQTGAR	
	KGEPGFGVPGXQGPXGL	GEXGPAGAVGPAGAVGPR	
	KGERGEAGETGSPGLPGL	GEXGPAGLXGPXGER	
	KGESGPPGERGXTGXIGL	GEXGPTGIQGPPGPAGEEGK	
	KGSXGAXGLPGKDGLPGL	GEXGPTGIQGPXGPAGEEGK	
	KGXPGPM(O)GFTGRSGXL	GEXGPTGIQGXPGPAGEEGK	
	KSVMDPVIVEVXL	GEXGSPGENGAXGQMGPR	
	KTTSEPLPQDXDKL	GEXGSXGENGAXGQMGPR	
	LDGVQGEVGADGPPGFXGL	GFPGADGVAGPK	
	LGETGITGXXGKPGL	GFPGLXGXAGEPGK	
	LGFPGAIGPPGXKGVKGL	GFPGSXGNIGPAGK	
	LIGGGAEAEGPEDPEL	GFXGADGVAGPK	
	LKGEPGDCGLXGPPGL	GFXGADGVAGPKGPAGER	
	LKGEXGDCGLXGPPGL	GFXGERGVQGPXGPAGPR	
	LLDGSERIGEKNF	GFXGIXGSXGIDGK	
	LMGXPGXKGDCGLPGPPGL	GFXGLPGXAGEPGK	
	LSM(O)PRLKDL	GFXGLXGPSGEPGK	
	LTLKQKYEL	GFXGLXGXAGEPGK	
	LXGDQGDRGXXGNL	GFXGSXGNIGPAGK	
	M(O)GLPGIQGNPGIPGNXGNL	GFXGXPGAPGSPGQPGL	
	M(O)HAXRRRALIGLL	GFXGXXGAPGSPGQPGL	
	M(O)LRGXGPGLL	GGAAGALGXSGXLL	
	MGLXGIQGNPGIPGNXGNL	GGAGXXGATGFXGAAGR	
	NAIRDFLAKVIQRL	GGDPILRXETLPSGSNF	
	NAVNGXGQTLIRGGIL	GGKXGTSGTIGPXGAR	
	NDLNGNGKQDPNEPLL	GGPGGPGPQGPAGK	
	NGXGGXAGVGAGDIGIGGGL	GIPGPPGPQGPKGEXGISGL	
	NHFVPEAGSRL	GIVGEXGPAGSK	
	NHM(O)IKDQLASKYL	GIXGPVGAAGATGAR	
	NITNNATGIQVTKTGNTL	GKPGGAGSPGLPGK	
	NIVSXDLSGKGLVL	GLAGPXGMXGAR	
	NLKQSGVVPFIL	GLDGIDGEXGPXGPK	
	NLTDTQNLKPGQL	GLQGPPGPSGXQGER	
	NQMTKLPSGLPVSL	GLTGSPGSPGPDGK	
	NQXSGTLVSDNKL	GLTGSXGSPGPDGK	
	PCIIXGSYGXSGFXGTPGF	GLTGSXGSXGPDGK	
	PGAEGSPGEDGAXGF	GLTGXIGXPGPAGNXGDK	
	PGDPGPKGEEGERGLDGF	GLXGEFGLXGPAGAR	
	PGEDGAXGQKGEAGLXGL	GLXGPPGAXGPZGF	
	PGEKGMKGESGLXGL	GLXGPXGAXGPZGF	
	PGERGRPGAPGPAVSTPGPL	GLXGVAGSVGEXGPL	
	PGGSGPMGXPGLXGGXGL	GPAGANGLXGEK	
	PGLDGPAGPXGKDGLPGTKGL	GPAGANGLXGEKGPXGDR	
	PGLGTTGEKGEKGIXGL	GPAGESGKPGPK	
	PGPKGDAGVRGPPGLPGL	GPAGPQGPRGDKGETGEK	
	PGPKGDAGVRGXPGLPGL	GPAGPQGXRGDKGETGEK	
	PGPKGDRGIGGPPGL	GPKGERGETGPQGGQGPR	
	PGPKGDTGQPGPPGL	**GPM(O)GPXGVK**	
	PGPKGDTGQPGXPGL	GPNGDSGRXGEXGL	
	PGPPGPPGXXGKPGMF	GPNGDSGRXGEXGLMGPR	
	PGPXGPPGXQGPPGNSATAHGL	GPPGASGEXGAPGPXGK	
	PGQKGEAGLXGLXGSPGKF	GPPGNVGNPGVNGAXGEAGR	
	PGQPGIXGKXGPSGEPGL	GPPGNVGNXGVNGAXGEAGR	

(*continued on next page*)

Table 1(*continued*)

Protease	Pepsin	Pepsin + Trypsin	Pepsin + Alcalase
	PGSPGMKGLXGXXGFPGSPGL	GPPGPMGPPGLAGPXGESGR	
	PGSPGXDGXPGXLGPPGL	GPPGPMGPXGLAGPXGESGR	
	PGSRXXDPPL	GPPGXAGIXGXPGF	
	PGTSGASGQKGEXGLXGL	GPPGXQGPKGR	
	PGXAGASGTAGSSGAAGSPGL	GPSGLXGSPGVXGPK	
	PGXAGPKGMPGFGKPGL	GPSGPQGPGGXXGPK	
	PGXAGXPGPPGDKGNDGAPGL	GPSGPQGPSGPPGPK	
	PGXKGSDGLPGQXGXSGL	GPSGPQGPSGPPGXK	
	PGXSGPKGYSGAXGAPGL	GPSGPQGPSGPXGPK	
	PGXSGXAGNAGFNGAXGL	GPSGPQGXSGPPGXK	
	PGXXGLQGPMGLXGKHGL	GPSGPQGXSGPXGPK	
	PGXXGLSGRPGPPGXPGL	GPSGPSGAPGK	
	PKDGKETDIFVF	GPVGAXGPVGK	
	PSGEEDLEGSASVXL	GPVGPSGPPGK	
	QAISGXNNL	**GPVGPSGPXGK**	
	QGEPGLXGRM(O)GLXGQPGEL	**GPVGXAGPPGK**	
	QGIXGIPGAPGLTGXXGLL	GPXGAAGSPGPK	
	QGMPGPSGPAGDSGXAGXNGL	GPXGAGGPPGPR	
	QGNXGEPGEXGAAGPL	GPXGAGGPXGPR	
	QGTGVXGPRGGPGAPGL	GPXGAIGAXGAPGK	
	QGXLGXRGKTGSRGPVGL	GPXGATGFPGSAGR	
	QGXMGXMGPQGAXGL	GPXGATGFXGAAGR	
	QGXXGDAGGLIGIIXL	GPXGESGAAGPAGPIGSR	
	QGXXGTRGRXGDDGLHGL	GPXGNVGNPGVNGAPGEAGR	
	QIIKTDEEGKLL	GPXGNVGNPGVNGAXGEAGR	
	QLKGDKGDXGLAGL	GPXGNVGNXGVNGAXGEAGR	
	QNDYQXSTEAPGGGL	GPXGPM(O)GPPGLAGPXGESGR	
	QNXSQNNIDNLIVAL	GPXGPMGPPGLAGPXGESGR	
	QTGDGLISGSVTGXDGXL	GPXGPMGPPGLAGXPGESGR	
	RGEKGDTGFPGPPGLPGL	GPXGPMGPPGLAGXSGEPGR	
	RGEXGPAGLPGF	GPXGPMGPXGLAGPXGESGR	
	RGIPGENGLXGXKGEAGXAGL	GPXGPPGPXGVRGXVGPPGL	
	RGQXGLXGGKGDQGPXGL	GPXGPQGLXGLAGAAGEXGR	
	RGQXGLXGGKGDQGXPGL	GPXGPXGTNGAXGQR	
	RGRXGPL	GPXGQXGLXGPAGSR	
	RGSHGSQGPSGPPGXXGL	GPXGSAGTPGKDGL	
	RGSHGSQGPSGPXGXXGL	GQAGVM(O)GFPGPK	
	RKTDEEGNSLAGATF	GQAGVMGFPGPK	
	RRCLGPPAAHALSEEL	GQAGVMGFXGPK	
	RSLGGAIGVSALGAVL	GQXGQPGLPGLPGSPGLPGPK	
	RVITAASAIEASDPDNSL	GQXGVMGFXGPK	
	SGQXGLRGPPGXPGF	GRPGLPGSAGAR	
	STNAPIXM(O)MPL	GRVGGXGXTGAR	
	TEACIIVAGKIL	GSAGPPGATGFPGAAGR	
	TEXEDIYVATSLL	GSAGPPGATGFXGAAGR	
	TGAIPTSIADVVVLKPKL	GSAGPXGATGFPGAAGR	
	TGAQGXIGTTTATGXQGL	GSAGPXGATGFXGAAGR	
	TGPTGPLGDKGDKGPL	GSPGEAGRXGEAGLXGAK	
	TGTLLNVAKVKXEEL	GSTGAXGIAGPXGIPGPR	
	TITXLAGGTRXVSAHL	GSTGEIGPAGPPGPPGLR	
	VAQKGLESKVDAIL	GSTGEIGPAGPXGPPGLR	
	VDEFAPQDF	GSTGEIGPAGPXGPXGLR	
	VEVFSPPGSDRASF	GSXGEAGRXGEAGLPGAK	
	VGGQXQEYEFSLGDL	GSXGEAGRXGEAGLXGAK	
	VGLDKTSLQNDXAF	GSXGERGEVGPAGPNGF	
	VGLERVANL	GSXGGXGTAGFXGGR	
	VGSXGLRGL	GVAGEXGRDGLPGGPGLR	
	VKISLSPEYVF	GVAGEXGRDGLXGGPGLR	
	VRREXMVTLAVPESL	GVAGEXGRDGLXGGXGL	
	VVKFTXAGIAAMEXGATL	GVDGLXGMDAPSGAPGPR	
	VXGQAXISIGDFVF	GVKGXXGTVGXSGPAGPL	
	XDALSVGTFASRDGYXIL	GVPGPXGAVGPAGK	
	XDLAPGSYVVKTVL	GVQGPPGPAGPR	
	XGAPGYPGEXGAPGL	GVQGPXGPAGPR	
	XGESGSKGDRGFDGLPGL	GVVGLPGKR	

(*continued on next page*)

Table 1(*continued*)

Protease	Pepsin	Pepsin + Trypsin	Pepsin + Alcalase
	XGEXGPPGPQGLIGLXGL	GVVGLXGKR	
	XGSSGSXGSXGAPGPL	GVXGPXGAVGPAGK	
	XGXM(O)GPVGPPGPPGPPGPGF	GXPGDRGGPGPAGPR	
	XSGGETHPESSTSGIDL	GXXGPIGAPGPK	
	YLDNNKISNIPDEYF	GXXGPPGAXGPQGF	
	YQTVSRKVALD	HGNRGEXGPAGAVGPAGAVGPR	
	YRDASSGAAKKRLLL	HGSRGEPGXVGAVGPAGAVGPR	
	YTGSALDFVRNNL	IGDPGLXGLDGMPGPPGPK	
		IGPXGVAGXL	
		IPVPGXPGXPGXPGPPGLSL	
		KGAXGYGAPGLXGLXGQK	
		KGXPGPPGPXGSSGLSDGGAY	
		LGDXGQQGQXGLPGAF	
		NGDRGESGXAGPAGAXGXAGAR	
		NGDRGETGPAGPAGAXGPAGSR	
		NGXPGEXGEXGASGPMGPR	
		NNGTLVNVLAGDTTY	
		QGLXGPAGTAGEAGKXGER	
		QGPPGEXGEXGASGPMGPR	
		QGPPGEXGEXGASGXMGPR	
		QGPPGEXGEXGQTGPAGAR	
		QGPXGEXGEPGSSGPMGPR	
		QGPXGEXGEXGASGPMGPR	
		QGPXGEXGEXGQTGPAGAR	
		RGSTGEIGPAGPXGPPGL	
		RGSTGEIGPAGPXGPXGLR	
		RGXPGPAGXNGK	
		RGXXGPPGPXGPPGEHLR	
		SGDRGETGPAGPAGPIGPVGAR	
		SGDXGAQGPPGXGGPAGER	
		SGLDGAKGDAGPAGPK	
		SGLDGSKGDAGPAGPK	
		SGPVGPXGNPGANGLXGAK	
		SGPVGPXGNXGANGLXGAK	
		SGPVGXPGNPGANGLXGAK	
		TGGXGPAGMGGPPGXSGHAGK	
		TGPXGPSGISGPXGPXGPAGK	
		TGQPGAVGPAGIR	
		TGXXGPSGISGPXGPPGPSGK	
		TIXSKIXGPXGPPGYPGK	
		VGAPGPAGAR	
		VGPXGPSGNAGPXGPPGPSGK	
		VGPXGPSGNAGPXGPXGPAGK	
		VGPXGPSGNAGXPGPXGPAGK	
		VGPXGSPGDXGPAGPLGAPGK	
		VGPXGXAGNAGXPGPPGPAGK	
		VGPXGXAGNSGPPGPXGPAGK	
		VGXXGPSGNAGPXGPPGPSGK	
		WQDADDKPPK	

[a] X: hydroxyproline.
[b] M(O): Met(O).
[c] The peptides shown in bold were synthesized for DPP-IV activity assessment.

and Gly-Pro-Met, respectively. The IC_{50} value of Gly-Pro-Met(O) was 1.9-fold higher than that of Gly-Pro-Met even though they are the same length. These results indicate that the Met modifications affected the DPP-IV activity of the peptide.

3.4. *Mode of DPP-IV inhibition of peptides from deer skin*

The DPP-IV inhibition patterns of the five synthetic peptides were analysed by using a Lineweaver–Burk plot. Lineweaver–Burk double reciprocal plots for Gly-Pro-Gly-Ser-Pro-Gly-Gly-Pro-Leu, Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys, Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys, Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys and Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Val-Hyp-Gly-Leu are illustrated in Fig. 3. The Lineweaver–Burk plots of the peptides converged at the same y-intercept as that of the plot obtained in the absence of inhibitor. No significant difference in V_{max} ($P \geq 0.05$) was obtained for all of the peptides without or with inhibitor. These results suggest that the five peptides identified from deer skin hydrolysates acted as competitive inhibitors.

Table 2 – Amino acid composition of hydrolysates from deer skin.

Amino acid	Number of residues/1000 residues		
	Pepsin	Pepsin + Trypsin	Pepsin + Alcalase
Gly	242	336	318
Pro	101	153	217
Ala	56	95	88
Hyp	72	99	101
Ser	45	36	28
Glu	42	38	31
Thr	34	19	29
Leu	103	30	27
Asp	43	23	15
Gln	31	19	24
Lys	44	33	21
Arg	24	40	31
Val	35	23	23
Ile	36	15	0
Asn	27	19	6
Met	10	6	7
Met(O)	6	1	2
Phe	22	12	8
Tyr	7	2	3
His	5	2	3
Cys	4	0	0
Trp	1	1	1

Table 3 – The DPP-IV IC_{50} values of synthetic peptides.

Peptide	IC_{50} (μM)	Hydrolysate prepared with
GPGSPGGPL	1638.3 ± 233.8[a]	Pepsin
GPVGX[b]AGPPGK	83.3 ± 3.2	Pepsin + Trypsin
GPM(O)[c]GPXGVK	226.9 ± 8.9	Pepsin + Trypsin
GPVGPSGPXGK	93.7 ± 0.9	Pepsin + Alcalase
GPAGPXGVXGL	318.1 ± 2.5	Pepsin + Alcalase
GPM	417.9 ± 4.0	
GPM(O)	790.1 ± 9.3	
GPV	794.8 ± 12.1	
IPI (diprotin A)	4.74 ± 0.32	

[a] The values represent the mean IC_{50} values ± SD (n = 3).
[b] X: hydroxyproline.
[c] M(O): Met(O).

4. Discussion

The importance of a Pro residue in DPP-IV inhibitory peptides has been previously reported. The first reported DPP-IV inhibitory peptides, diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu), are examples of peptides with a proline residue at the P1 position (Rahfeld, Schierhorn, Hartrodt, Neubert, & Heins, 1991). Xaa-Pro has been reported to be one of the most frequently occurring sequences in DPP-IV inhibitory peptides (Hatanaka et al., 2012; Nongonierma & FitzGerald, 2013c). In the present study, a total of 507 peptides were identified from three types of deer skin hydrolysates by LC–MS/MS. More than 20% of the identified peptides from deer skin hydrolysates prepared using pepsin in combination with trypsin or Alcalase had a Pro residue at the penultimate position and were predicted to be potent DPP-IV inhibitory compounds. The assay results of the synthetic peptides verified the DPP-IV inhibitory activity of the peptides with a Pro residue at the penultimate position and demonstrated that these peptides with a Pro residue at the penultimate position contribute to the DPP-IV inhibitory activity of the deer skin hydrolysates.

The five synthetic peptides analysed in this study acted as competitive inhibitors, as shown in Fig. 3, which indicates that the peptides directly interact with the active site of DPP-IV. The peptides with a Pro residue at position 2 from the N terminus may behave as substrate or prodrug DPP-IV inhibitors (Nongonierma & FitzGerald, 2014b). Diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) have been reported to be DPP-IV substrates (Rahfeld et al., 1991). Because DPP-IV specifically cleaves Xaa-Pro from the N terminus, Gly-Pro would be released from synthetic peptides when they were incubated with DPP-IV. Gly-Pro has been shown to not inhibit DPP-IV (Nongonierma, Mooney, Shields, & FitzGerald, 2014). Therefore, the synthetic peptides studied herein may act as substrate inhibitors.

In the present study, DPP-IV inhibitory peptides were identified through an integrated approach involving both LC–MS/MS and knowledge of the structure of DPP-IV inhibitory peptides. The peptides identified by LC–MS/MS were obtained from hydrolysates generated *in vitro*, and not from a hydrolysis simulated through an *in silico* approach. Compared with empirical approaches, the integrated approach omits the fractionation and purification steps. Therefore, the disadvantages of high labour intensity of the classical empirical approach and simulated hydrolysis of the bioinformatics-driven *in silico* digestion approach were overcome in this integrated approach. In our previous work, this approach was applied for the identification of angiotensin-converting enzyme (ACE) inhibitory peptides from deer plasma (Liu et al., 2011) and bee pollen (Guo, Yan, Guo, & Jin, 2014). This integrated approach is efficient for the discovery of novel bioactive peptides from peptide mixtures generated through proteolytic degradation.

Post-translational modifications are covalent protein processing events. More than 300 types of post-translational protein modifications are known to occur physiologically within living organisms, and these include phosphorylation, glycosylation, acetylation, methylation and oxidation/reduction. Oxidative stress caused by radical oxygen species (ROS) (Velarde-Salcedo et al., 2013) induced by extracellular stimuli may cause various forms of reversible or irreversible oxidative modifications to proteins (Bachi, Dalle-Donne, & Scaloni, 2013). Methionine (Met) is a target of oxidizing free radicals in peptides and proteins. Met residues of proteins are susceptible to ROS and are oxidized to methionine sulphoxide under mild oxidizing conditions (Bachi et al., 2013; Stadtman, Moskovitz, & Levine, 2003; Zhu, Heinemann, Schonherr, & Scriba, 2014). Methionine oxidation converts a hydrophobic residue (Met) into a hydrophilic one (Met(O)). The oxidation of a few surface methionine residues has little effect on polypeptide conformation and function, but an accumulation of methionine oxidation can cause significant structural changes in protein and polypeptides (Bachi et al., 2013). For short peptide sequences, the effects of methionine oxidation on the properties of the peptide are noticeable. In this study, 0.1~0.6% Met(O) was identified among all of the amino acids of the deer skin hydrolysates. Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys was a peptide with Met(O) identified from the

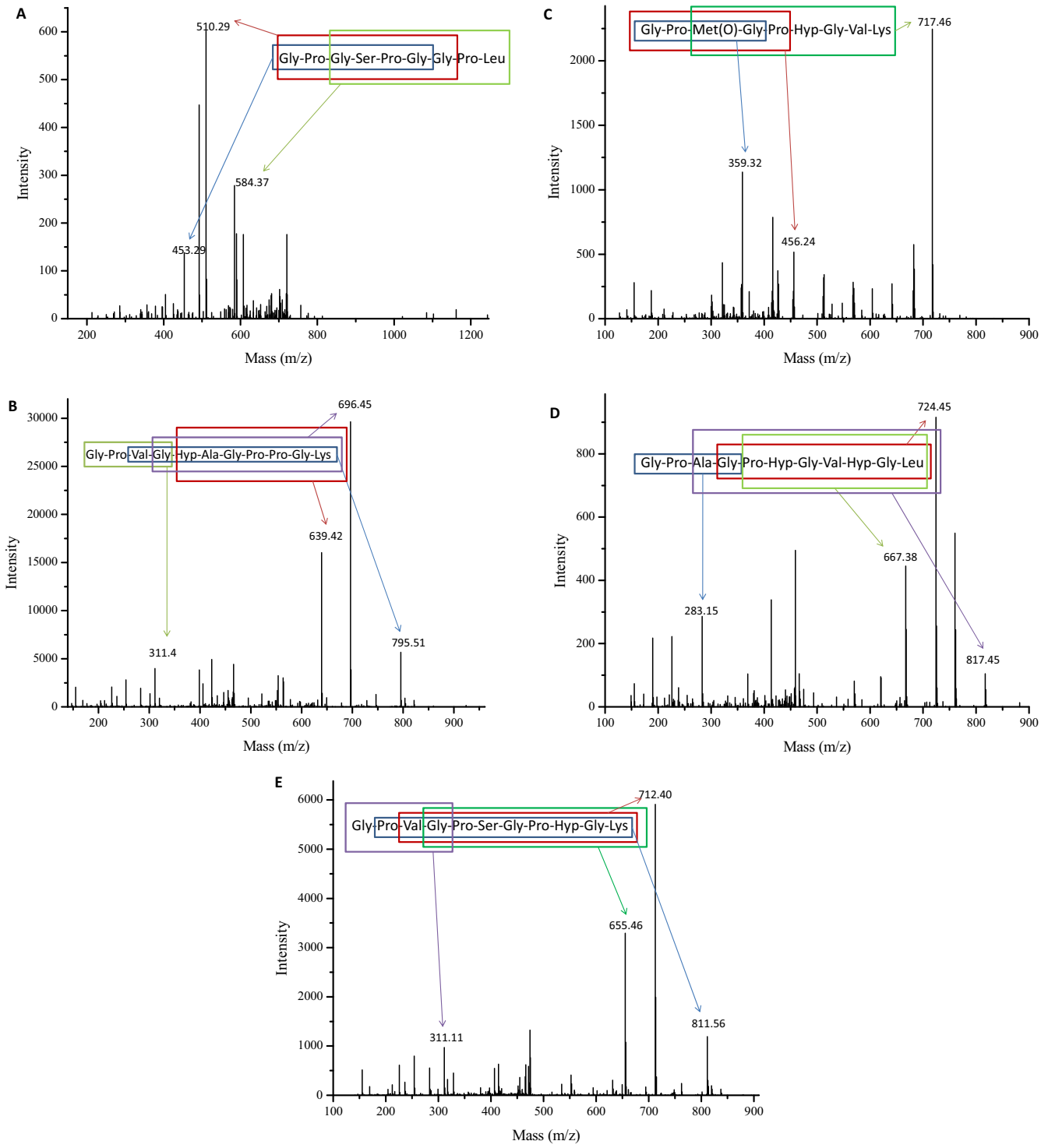

Fig. 2 – MS/MS spectrum of the precursor ions: A, m/z 739.37 for GPGSPGGPL; B, m/z 475.75 for GPVGXAGPPGK; C, m/z 436.71 for GPM(O)GPXGVK; D, m/z 483.75 for GPVGPSGPXGK; and E, m/z 476.24 for GPAGPXGVXGL.

deer skin hydrolysates prepared using pepsin and trypsin. This peptide displays DPP-IV inhibitory activity with an IC_{50} value of 226.9 ± 8.9 μM. The results obtained for Gly-Pro-Met(O)-Gly-Pro-Hyp-Gly-Val-Lys, Gly-Pro-Met and Gly-Pro-Met(O) indicated that the amino acid modifications affected the DPP-IV inhibitory activity of the peptides. The effect of peptide phosphorylation on DPP-IV activity was previously reported (Nongonierma & FitzGerald, 2013d). To the best of our knowledge, DPP-IV inhibitory peptides with amino acid oxidation modifications have not been investigated in a previous study. A systematic study of the mechanisms through which peptide modifications affect the inhibition of DPP-IV will be of benefit to those exploring novel DPP-IV inhibitory peptides from proteins.

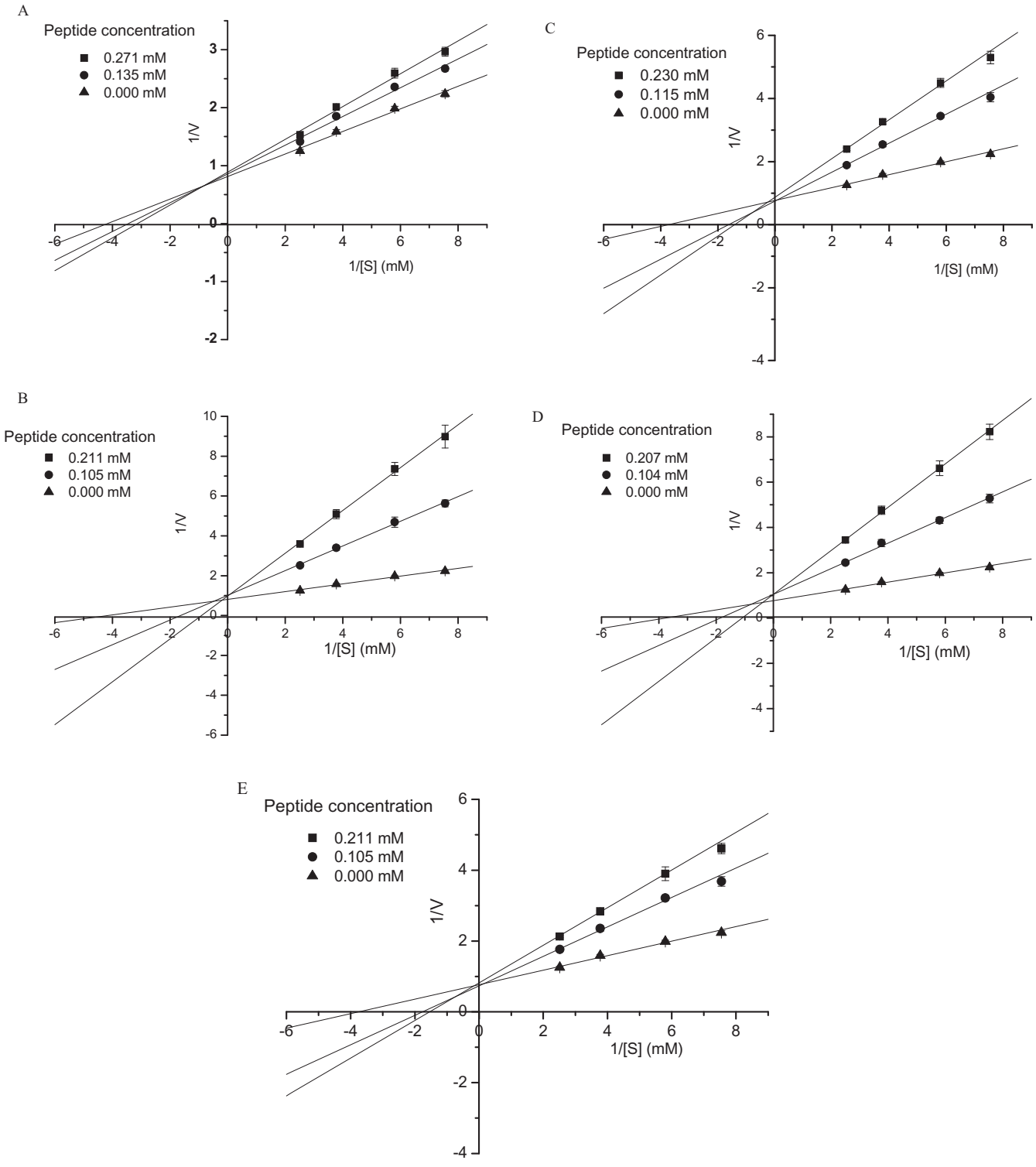

Fig. 3 – Lineweaver–Burk plots of DPP-IV inhibition by peptides from deer skin hydrolysates. The DPP-IV activity was measured in the presence or absence of synthetic peptides. Each point is the mean of three determinations (n = 3) ± SD. A, GPGSPGGPL; B, GPVGXAGPPGK; C, GPM(O)GPXGVK; D, GPVGPSGPXGK; and E, GPAGPXGVXGL.

Previous studies regarding DPP-IV inhibitory peptides focused on short peptides with two to seven amino acids using empirical approaches (Hatanaka, Kawakami, & Uraji, 2014; Hsu et al., 2013; Lafarga et al., 2014) or *in silico* approaches (Lacroix & Li-Chan, 2012b). Although larger peptides with 13 amino acids have been reported to possess DPP-IV inhibitory activity (Velarde-Salcedo et al., 2013), the effect of the peptide length on DPP-IV inhibition has rarely been discussed. The DPP-IV inhibitory peptides identified in the present study contain nine to eleven amino acids. To investigate the effect of peptide length on DPP-IV inhibitory

activity, Gly-Pro-Val was synthesized and investigated. Two peptides, Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys and Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys, have a Gly-Pro-Val sequence at their N terminus. Only three amino acids are different in the above two peptides. The IC_{50} values of the two peptides of the same length are very close and are almost 10% that of the IC_{50} value of Gly-Pro-Val. In this example, the peptide length improved the DPP-IV inhibitory activity of the peptides. Gly-Pro and Gly-Pro-Ala possess a high IC_{50} value and are thought to not inhibit DPP-IV (Bauvois, 1988; Nongonierma et al., 2014; Yoshimoto, Fischl, Orlowski, & Walter, 1978). Gly-Pro-Ala-Glu released from Atlantic salmon skin is a potent DPP-IV inhibitor with an IC_{50} value of 49.6 μM (Li-Chan et al., 2012). In the present study, Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Val-Hyp-Gly-Leu, which starts with Gly-Pro-Ala, has 11 amino acids and displayed DPP-IV inhibitory activity with an IC_{50} value of 318.1 ± 2.5 μM. The effect of peptide length on the DPP-IV inhibitory activity of peptides was the same as in the aforementioned example. These two examples both indicate that a greater peptide sequence length increases the DPP-IV inhibitory activity. Therefore, medium length peptides are also a good resource for DPP-IV inhibitory peptides.

Porcine skin gelatin hydrolysates (Huang, Hung, Jao, Tung, & Hsu, 2014) and salmon skin gelatin hydrolysates (Hsieh, Wang, Hung, Chen, & Hsu, 2015) have been proven to inhibit plasma DPP-IV in rats. Hyp-containing peptides were detected in human plasma after the ingestion of a collagen hydrolysate and exerted beneficial effects on human health (Shigemura, Kubomura, Sato, & Sato, 2014). This result indicates that Hyp-containing DPP-IV inhibitory peptides may resist gastrointestinal proteases/peptidases and exert effects *in vivo* in human plasma. The results described herein demonstrate that peptides with a Pro residue at the penultimate position from deer skin hydrolysate contribute to the DPP-IV inhibition activity of deer collagen hydrolysates. Hyp is the third- or fourth-most-frequent of the amino acids in the peptides identified from deer skin hydrolysates. Four of the five peptides contain a Hyp residue. Therefore, it may be deduced that some of the DPP-IV inhibitory peptides in deer skin hydrolysates are Hyp-containing peptides. The *in vivo* DPP-IV inhibitory activity of the deer skin hydrolysates is expected. Of the deer skin hydrolysates assessed in this study, 5.9–20.9% contain a Pro residue at the penultimate position. Five synthetic peptides were verified to act as competitive inhibitors and may behave as substrate-type DPP-IV inhibitors. It is possible that most of this type of peptides are competitive and substrate-type DPP-IV inhibitors. Certain competitive and substrate-type DPP-IV inhibitory peptides have been proven to exert a synergistic effect with antidiabetic drugs (gliptins) *in vitro* (Nongonierma & FitzGerald, 2015). The activity, stability, and synergistic effect with antidiabetic drugs of deer skin hydrolysates in humans needs to be studied further.

5. Conclusion

The proposed approach integrating LC–MS/MS with *in silico* analysis is an efficient method for the discovery of novel bioactive peptides from peptide mixtures arising through proteolytic degradation. Both modifications to and the length of the peptides were found to affect their DPP-IV inhibitory activity. These results may help promote the discovery of more potent bioactive peptides. The mechanisms through which peptide modifications and peptide length influence activity need to be investigated further.

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REFERENCES

Alemán, A., Giménez, B., Montero, P., & Gómez-Guillén, M. C. (2011). Antioxidant activity of several marine skin gelatins. *LWT – Food Science and Technology*, *44*(2), 407–413.

Alemán, A., Giménez, B., Pérez-Santin, E., Gómez-Guillén, M. C., & Montero, P. (2011). Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. *Food Chemistry*, *125*(2), 334–341.

Aschner, P., Kipnes, M. S., Lunceford, J. K., Sanchez, M., Mickel, C., Williams-Herman, D. E., & Sitagliptin Study 021 Group (2006). Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with type 2 diabetes. *Diabetes Care*, *29*(12), 2632–2637.

Asghar, A., & Henrickson, R. L. (1982). Chemical, biochemical, functional, and nutritional characteristics of collagen in food systems. *Advances in Food Research*, *28*, 231–372.

Bachi, A., Dalle-Donne, I., & Scaloni, A. (2013). Redox proteomics: Chemical principles, methodological approaches and biological/biomedical promises. *Chemical Reviews*, *113*(1), 596–698.

Bauvois, B. (1988). A collagen-binding glycoprotein on the surface of mouse fibroblasts is identified as dipeptidyl peptidase-IV. *Biochemical Journal*, *252*(3), 723–731.

Byun, H. G., & Kim, S. K. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (Theragrachal cogramma) skin. *Process Biochemistry*, *36*(12), 1155–1162.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Gutiérrez-López, G. F., & Dávila-Ortiz, G. (2012). Use of proteomics and peptidomics methods in food bioactive peptide science and engineering. *Food Engineering Reviews*, *4*(4), 224–243.

Cavazos, A., & de Mejia, E. G. (2013). Identification of bioactive peptides from cereal storage proteins and their potential role in prevention of chronic diseases. *Comprehensive Reviews in Food Science and Food Safety*, *12*(4), 364–380.

Drucker, D. J., & Nauck, M. A. (2006). The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*, *368*(9548), 1696–1705.

Gomez-Guillen, M. C., Gimenez, B., Lopez-Caballero, M. E., & Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids*, *25*(8), 1813–1827.

Guo, J., Yan, J. Z., Guo, M., & Jin, Y. (2014). Application of reverse-phase liquid chromatography-tandem mass spectrometry in the identification of protein and bioactivity

peptides from rape bee pollen. *Chinese Journal of Chromatography*, *32*, 284–289.
Hatanaka, T., Inoue, Y., Arima, J., Kumagai, Y., Usuki, H., Kawakami, K., Kimura, M., & Mukaihara, T. (2012). Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran. *Food Chemistry*, *134*(2), 797–802.
Hatanaka, T., Kawakami, K., & Uraji, M. (2014). Inhibitory effect of collagen-derived tripeptides on dipeptidyl peptidase-IV activity. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *29*(6), 823–828.
Hsieh, C., Wang, T., Hung, C., Chen, M., & Hsu, K. (2015). Improvement of glycemic control in streptozotocin-induced diabetic rats by Atlantic salmon skin gelatin hydrolysate as the dipeptidyl-peptidase IV inhibitor. *Food & Function*, *6*(6), 1887–1892.
Hsu, K.-C., Tung, Y.-S., Huang, S.-L., & Jao, C.-L. (2013). Dipeptidyl peptidase-IV inhibitory activity of peptides in porcine skin gelatin hydrolysates. In B. Hernandez-Ledesma & C.-C. H. Hsieh (Eds.), *Bioactive food peptides in health and disease* (pp. 205–218). Croatia: InTech.
Huang, J. F., Wang, F. J., Ye, M. L., & Zou, H. F. (2014). Enrichment and separation techniques for large-scale proteomics analysis of the protein post-translational modifications. *Journal of Chromatography. A*, *1372C*, 1–17.
Huang, S.-L., Hung, C.-C., Jao, C.-L., Tung, Y.-S., & Hsu, K.-C. (2014). Porcine skin gelatin hydrolysate as a dipeptidyl peptidase IV inhibitor improves glycemic control in streptozotocin-induced diabetic rats. *Journal of Functional Foods*, *11*, 235–242.
Jiang, X. N., Ye, M. L., Han, G. H., Dong, X. L., & Zou, H. F. (2010). Classification filtering strategy to improve the coverage and sensitivity of phosphoproteome analysis. *Analytical Chemistry*, *82*(14), 6168–6175.
Juillerat-Jeanneret, L. (2014). Dipeptidyl peptidase IV and its inhibitors: Therapeutics for type 2 diabetes and what else? *Journal of Medicinal Chemistry*, *57*(6), 2197–2212.
Kim, H. K., Kim, M. G., & Leem, K. H. (2014). Collagen hydrolysates increased osteogenic gene expressions via a MAPK signaling pathway in MG-63 human osteoblasts. *Food & Function*, *5*(3), 573–578.
Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012a). Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal*, *25*(2), 97–102.
Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012b). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an in silico approach. *Journal of Functional Foods*, *4*(2), 403–422.
Lafarga, T., O'Connor, P., & Hayes, M. (2014). Identification of novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides from meat proteins using in silico analysis. *Peptides*, *59*, 53–62.
Lambeir, A. M., Durinx, C., Scharpe, S., & De Meester, I. (2003). Dipeptidyl-peptidase IV from bench to bedside: An update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical Reviews in Clinical Laboratory Sciences*, *40*(3), 209–294.
Li, B., Chen, F., Wang, X., Ji, B. P., & Wu, Y. N. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry*, *102*(4), 1135–1143.
Li-Chan, E. C. Y. (2015). Bioactive peptides and protein hydrolysates: Research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science*, *1*, 28–37.
Li-Chan, E. C. Y., Hunag, S. L., Jao, C. L., Ho, K. P., & Hsu, K. C. (2012). Peptides derived from Atlantic salmon skin gelatin as dipeptidyl-peptidase IV inhibitors. *Journal of Agricultural and Food Chemistry*, *60*(4), 973–978.
Liu, X. Y., Song, C. X., Chen, R., Jiang, X. N., Jin, Y., & Zou, H. F. (2011). Identification of angiotensin I-converting enzyme inhibitors in peptides mixture of hydrolyzed red deer plasma with proteomic approach. *Chinese Journal of Chemistry*, *28*, 1665–1673.
Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry*, *53*(3), 581–587.
Nongonierma, A. B., & FitzGerald, R. J. (2013a). Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides*, *39*, 157–163.
Nongonierma, A. B., & FitzGerald, R. J. (2013b). Dipeptidyl peptidase IV inhibitory properties of a whey protein hydrolysate: Influence of fractionation, stability to simulated gastrointestinal digestion and food-drug interaction. *International Dairy Journal*, *32*(1), 33–39.
Nongonierma, A. B., & FitzGerald, R. J. (2013c). Inhibition of dipeptidyl peptidase IV (DPP-IV) by proline containing casein-derived peptides. *Journal of Functional Foods*, *5*(4), 1909–1917.
Nongonierma, A. B., & FitzGerald, R. J. (2013d). Inhibition of dipeptidyl peptidase IV (DPP-IV) by tryptophan containing dipeptides. *Food & Function*, *4*(12), 1843–1849.
Nongonierma, A. B., & FitzGerald, R. J. (2014a). An in silico model to predict the potential of dietary proteins as sources of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Chemistry*, *165*, 489–498.
Nongonierma, A. B., & FitzGerald, R. J. (2014b). Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis. *Food Chemistry*, *145*, 845–852.
Nongonierma, A. B., & FitzGerald, R. J. (2015). Utilisation of the isobole methodology to study dietary peptide-drug and peptide-peptide interactive effects on dipeptidyl peptidase IV (DPP-IV) inhibition. *Food & Function*, *6*(1), 313–320.
Nongonierma, A. B., Mooney, C., Shields, D. C., & FitzGerald, R. J. (2014). In silico approaches to predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV) inhibitors. *Peptides*, *57*, 43–51.
Panchaud, A., Affolter, M., & Kussmann, M. (2012). Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects. *Journal of Proteomics*, *75*(12), 3546–3559.
Power, O., Nongonierma, A. B., Jakeman, P., & FitzGerald, R. J. (2014). Food protein hydrolysates as a source of dipeptidyl peptidase IV inhibitory peptides for the management of type 2 diabetes. *Proceedings of the Nutrition Society*, *73*(1), 34–46.
Rahfeld, J., Schierhorn, M., Hartrodt, B., Neubert, K., & Heins, J. (1991). Are diprotin-A (Ile-Pro-Ile) and diprotin-B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase-IV? *Biochimica et Biophysica Acta*, *1076*(2), 314–316.
Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., & Morimatsu, F. (2008). Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. *Journal of Agricultural and Food Chemistry*, *56*(20), 9586–9591.
Sanchez-Rivera, L., Martinez-Maqueda, D., Cruz-Huerta, E., Miralles, B., & Recio, I. (2014). Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food Research International*, *63*, 170–181.
Shigemura, Y., Kubomura, D., Sato, Y., & Sato, K. (2014). Dose-dependent changes in the levels of free and peptide forms of hydroxyproline in human plasma after collagen hydrolysate ingestion. *Food Chemistry*, *159*, 328–332.
Stadtman, E. R., Moskovitz, J., & Levine, R. L. (2003). Oxidation of methionine residues of proteins: Biological consequences. *Antioxidants & Redox Signaling*, *5*(5), 577–582.

Tulipano, G., Sibilia, V., Caroli, A. M., & Cocchi, D. (2011). Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides*, *32*(4), 835–838.

Udenigwe, C. C. (2014). Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science & Technology*, *36*(2), 137–143.

Uriarte-Montoya, M. H., Santacruz-Ortega, H., Cinco-Moroyoqui, F. J., Rouzaud-Sandez, O., Plascencia-Jatomea, M., & Ezquerra-Brauer, J. M. (2011). Giant squid skin gelatin: Chemical composition and biophysical characterization. *Food Research International*, *44*(10), 3243–3249.

Velarde-Salcedo, A. J., Barrera-Pacheco, A., Lara-Gonzalez, S., Montero-Moran, G. M., Diaz-Gois, A., Gonzalez de Mejia, E., Barba de la Rosa, A. P. (2013). In vitro inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of amaranth (Amaranthus hypochondriacus L.) proteins. *Food Chemistry*, *136*(2), 758–764.

Wang, F. J., Dong, J., Jiang, X. N., Ye, M. L., & Zou, H. F. (2007). Capillary trap column with strong cation-exchange monolith for automated shotgun proteome analysis. *Analytical Chemistry*, *79*(17), 6599–6606.

Wu, M. H., Wu, R. A., Wang, F. J., Ren, L. B., Dong, J., Liu, Z., & Zou, H. (2009). "One-Pot" process for fabrication of organic-silica hybrid monolithic capillary columns using organic monomer and alkoxysilane. *Analytical Chemistry*, *81*(9), 3529–3536.

Yazbeck, R., Howarth, G. S., & Abbott, C. A. (2009). Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? *Trends in Pharmacological Sciences*, *30*(11), 600–607.

Yoshimoto, T., Fischl, M., Orlowski, R. C., & Walter, R. (1978). Post-proline cleaving enzyme and post-proline dipeptidyl aminopeptidase: Comparison of two peptidases with high specificity for proline residues. *The Journal of Biological Chemistry*, *253*, 3708–3716.

Zhu, Q. F., Heinemann, S. H., Schonherr, R., & Scriba, G. K. E. (2014). Capillary electrophoresis separation of peptide diastereomers that contain methionine sulfoxide by dual cyclodextrin-crown ether systems. *Journal of Separation Science*, *37*(23), 3548–3554.