ABSTRACT

Inhibition of dipeptidyl peptidase-IV (DPP-IV) activity is a promising strategy for treatment of type 2 diabetes. In the current study, DPP-IV inhibitory peptides were identified from mare whey protein hydrolysates obtained by papain. The results showed that all the mare whey protein hydrolysates obtained at various hydrolysis durations possessed more potent DPP-IV inhibitory activity compared with intact whey protein. The 4-h hydrolysates showed the greatest DPP-IV inhibitory activity with half-maximal inhibitory concentration of 0.18 mg/mL. The 2 novel peptides from 4-h hydrolysate fractions separated by successive chromatographic steps were characterized by liquid chromatography–electrospray ionization tandem mass spectrometry. The novel peptides Asn-Leu-Glu-Ile-Ile-Leu-Arg and Thr-Gln-Met-Val-Asp-Gln-Glu-Ile-Met-Glu-Lys-Phe-Arg, which corresponded to β-lactoglobulin 1 f(71–77) and β-lactoglobulin 1 f(143–155), demonstrated DPP-IV inhibitory activity with half-maximal inhibitory concentrations of 86.34 and 69.84 μM, respectively. The DPP-IV inhibitory activity of the 2 peptides was retained or even improved after simulated gastrointestinal digestion in vitro. Our findings indicate that mare whey protein-derived peptides may possess potential as functional food ingredients in the management of type 2 diabetes.

Key words: dipeptidyl peptidase IV, mare milk, whey protein, bioactive peptide

INTRODUCTION

Type 2 diabetes mellitus is a worldwide metabolic disorder and is one of the leading public health problems, which is increasing at an explosive rate. Globally, an estimated 382 million people were affected by type 2 diabetes in 2013 and this number is predicted to reach 592 million by 2035 (International Diabetes Federation, 2013; Guariguata et al., 2014). Type 2 diabetes is characterized by insulin resistance and pancreatic β-cell dysfunction, which can result in severe complications such as nephropathy, retinopathy, cardiovascular disease, and peripheral vascular disease (Deshpande et al., 2008; Alonso-Magdalena et al., 2010). Therefore, effective interventions that may delay or prevent the onset of type 2 diabetes have attracted increasing attention.

Different therapies for type 2 diabetes have been proposed during the past decades. Among these treatments, a novel therapeutic strategy is targeting incretin hormones, which include glucagon-like peptide-1 and glucose-dependent insulinoergic peptide. These hormones are secreted from the small intestine within several minutes after food ingestion and subsequently promote glucose-dependent insulin secretion from pancreatic islet β-cells (Drucker, 2006; Tulipano et al., 2011). Moreover, they inhibit β-cell apoptosis and enhance β-cell proliferation (Khan et al., 2013). The increased levels of glucagon-like peptide-1 and glucose-dependent insulinoergic peptide improve glucose tolerance in normal and diabetic rats (Higuchi et al., 2013). However, incretin hormones are metabolized rapidly in vivo by endogenous protease dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5; Hatanaka et al., 2012; Zambrowicz et al., 2015). Therefore, additional supplementation of DPP-IV inhibitors can extend incretin action and thereby enhance insulin secretion, which has been regarded as one of the newest treatments for type 2 diabetes (Nauck et al., 2009). To inhibit DPP-IV, several synthetic inhibitors, such as vildagliptin and sitagliptin, have been developed for the treatment of type 2 diabetes over the past decade (Pratley et al., 2006; Karasik et al., 2008; Lacroix and Li-Chan, 2013). However, adverse side effects of these synthetic inhibitors are noted, such as upper respiratory tract infections, nasopharyngitis, and headaches (Reid, 2012). Thus,
there is an increased focus on identifying natural DPP-IV inhibitors for the management of type 2 diabetes.

Whey protein dietary supplements improve obesity-related blood pressure, vascular function, fatty liver disease, and type 2 diabetes (Pal and Ellis, 2010; Shertzler et al., 2011). Several peptides derived from bovine whey proteins have been shown to possess antihypertensive activity, antiinflammatory activity, and antioxidant activity (Wang et al., 2012; de Carvalho-Silva et al., 2014; Brandelli et al., 2015). Bovine whey protein-derived peptides inhibit DPP-IV activity in vitro (Lacroix and Li-Chan, 2013, 2014). Moreover, DPP-IV inhibitory peptides derived from bovine β-LG decrease blood glucose levels after an oral glucose tolerance test in C57BL/6 mice (Uchida et al., 2011). In addition, preservation of active incretin hormones by DPP-IV inhibitory peptides generated during gastrointestinal digestion of whey proteins is considered to be associated with the insulinogenic and glucose-lowering effects of whey protein in vivo (Jakubowicz and Froy, 2013).

Mare milk is a traditional foodstuff consumed in Mongolia and southern states of the former Soviet Union (Uniacke-Lowe et al., 2010). Also, in several European countries, such as Germany, Italy, and the Netherlands, the production and consumption of mare milk have gained importance, and approximately 1 million kilograms of mare milk is produced in Europe (Fox and Uniacke, 2010). It is reported that 30 million individuals regularly consume mare milk all over the world (Martuzzi and Vaccari Simonini, 2010). This increased interest in consuming mare milk is considered to be associated with special nutritional and therapeutically properties of mare milk. It has a positive influence on bowel flora, metabolic disorders, stiff joints, and cancer, which benefits the diets of the elderly, convalescent, or newborn (Uniacke-Lowe et al., 2010; Rad et al., 2013). The whey protein content in mare milk is 8.3 g/kg of milk, which is higher than those in bovine milk (5.7 g/kg of milk; Malacarne et al., 2002). Although mare α-LA is similar to bovine α-LA in terms of AA sequence, mare β-LG possesses only 58% identity with bovine β-LG (El-Salam and El-Shibiny, 2013). Based on the difference in the AA profiles between mare whey and bovine whey, bioactive peptides from 2 types of whey proteins are expected to be different. However, mare whey proteins as a source of bioactive peptides have not been studied.

In the current study, mare whey protein hydrolysates were prepared by incubating with papain for different durations, and their DPP-IV inhibitory activities were evaluated in vitro. The hydrolysates with the greatest DPP-IV inhibitory activity were fractioned by successive chromatographic steps, and peptides were further identified by liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI MS/MS).

MATERIALS AND METHODS

Materials and Reagents

Mare milk was sampled in a traditional pasture area, Inner Mongolia, China. Papain (EC 3.4.22.21, from papaya latex, 0.5–2.0 units/mg), pepsin (EC 3.4.23.1, from porcine gastric mucosa, 250 units per milligram), pancreatin (from porcine pancreas, 8 × UPS), Gly-Pro-p-nitroanilide (H-Gly-Pro-pNA-HCl), dipeptidyl peptidase-IV (EC 3.4.14.5, from porcine kidney, ≥10 units per milligram), and diprotin A (Ile-Pro-Ile) were purchased from Sigma-Aldrich (St. Louis, MO). The HPLC-grade acetonitrile, trifluoroacetic acid, and formic acid were from Fisher Scientific (Fair Lawn, NJ). All other chemicals used in the study were of analytical grade.

Isolation of Mare Whey Proteins

Mare milk was defatted by centrifugation at 5,000 × g at 10°C for 30 min. The mare casein was precipitated from skim mare milk at pH 4.6 with 10% (vol/vol) acetic acid by centrifuging at 5,000 × g for 30 min at 10°C. The supernatant containing whey proteins was dialyzed for 48 h and freeze-dried. The protein content in protein lyophilizates was determined by Kjeldahl method, as previously described (Finete et al., 2013). The protein content was 71.27 ± 0.59% (nitrogen-protein conversion factor, 6.38).

Preparation of Mare Whey Protein Hydrolysates

Lyophilized mare whey was dissolved in distilled water at a concentration of 5% (wt/vol). The solutions were adjusted to pH 6.0 and preincubated at 55°C for 10 min. The papain enzyme was added (5% enzyme/substrate ratio on a wt/wt protein basis) to hydrolyze the whey protein with a constant temperature and pH. Hydrolysates of mare whey proteins were collected at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 h. These hydrolysate samples were heated to 85°C and maintained for 15 min to inactivate protease, followed by centrifugation at 1,800 × g at 4°C for 20 min with TGL-20M high-speed centrifuge (Pingfan Instrument Co. Ltd., Changsha, China). The supernatants were collected and ultrafiltered using an Ultracel Amicon model 8400 ultrafiltration unit (Millipore Corporation, Bedford, MA) with membranes of molecular weight cutoffs of 10 kDa. The <10 kDa fraction was collected and freeze-
dried, and was defined as mare whey protein hydrolysates.

**Determination of DPP-IV Inhibition Activity**

The inhibitory activity of samples against DPP-IV was assayed as previously described with minor modifications (Van et al., 2009; Nongonierma and FitzGerald, 2014). Briefly, lyophilized samples were resuspended in Tris-HCl buffer (100 mM, pH 8.0). In a well of a 96-well microplate, 20 μL of sample solution and 100 μL of substrate solution (H-Gly-Pro-pNA-HCl, final concentration 0.5 mM) were added. The enzyme reaction was started by adding 30 μL of DPP-IV (final concentration 0.0075 U/mL). The absorbance at 405 nm was monitored after the microplate was incubated at 37°C for 0.5 h. The DPP-IV inhibitory activity (%) was calculated as follows:

\[
\text{DPP-IV inhibitory activity}(\%) = \left(1 - \frac{\text{Absorbance(test sample)}}{\text{Absorbance(sample control)}}\right) \times 100,
\]

where Absorbance(test sample) was the absorbance at 405 nm in the presence of Tris-HCl buffer, DPP-IV, H-Gly-Pro-pNA-HCl, and sample; Absorbance(sample control) was the absorbance at 405 nm when DPP-IV was replaced with Tris-HCl buffer; Absorbance(negative reaction) was the absorbance at 405 nm when sample was replaced with Tris-HCl buffer; and Absorbance(negative control) was the absorbance at 405 nm when DPP-IV and sample were replaced with Tris-HCl buffer.

The half-maximal inhibitory concentration (IC\textsubscript{50}) value was defined as the concentration of sample that inhibited 50% of DPP-IV activity and was calculated by the logarithmic regression of DPP-IV inhibitory activity against the concentration of the sample.

**Purification of DPP-IV Inhibitory Peptides**

**Separation by Gel Filtration Chromatography.** Mare whey protein hydrolysate with the highest DPP-IV inhibitory activity was separated using gel permeation chromatography, as previously described (Huang et al., 2012). Briefly, the lyophilized hydrolysate (20 mg) was dissolved in 1 mL of deionized water. Fractions of 3 mL were collected at the flow rate of 0.6 mL/min and the absorbance at 220 nm was monitored to separate peptide fractions. To obtain enough samples for further analysis, the separation was repeated 10 times. Each fraction was lyophilized and its DPP-IV inhibitory activity was determined.

**Separation by HPLC.** The fractionated peptide gel permeation chromatography exhibiting the highest DPP-IV inhibitory activity was purified by HPLC (model LC-10AT, Shimadzu, Japan), as previously described with minor modifications (Lacroix and Li-Chan, 2014). The lyophilized fraction was dissolved in ultra-pure water to a concentration of 2 mg/mL and injected into a column (Eclipse XDB-C18, 4.6 mm × 250 mm, 5 μm, Agilent Technologies Inc., Shanghai, China). The sample was eluted with a linear gradient of acetonitrile (10–90% (vol/vol) in 30 min) and 0.1% trifluoroacetic acid (vol/vol) in H\textsubscript{2}O under a flow rate of 1 mL/min at 30°C. The elution was monitored at 220 nm with a UV/visible detector. Fractions eluting from 6 to 15 min were collected every minute, and the separation was performed 10 times to obtain enough samples for analysis. After removing acetonitrile by evaporation, these fractions were freeze-dried and assayed for their DPP-IV inhibitory activities.

**Identification of AA Sequence by LC-ESI MS/MS**

The purified peptide fraction displaying the greatest DPP-IV inhibitory activity was analyzed by LC-ESI MS/MS with a nanoAcquity nano HPLC system (Waters, Milford, MA), which was directly interfaced with a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA), as previously described (Zhang et al., 2015). Briefly, the sample was injected into a trap column (100 μm × 20 mm, Polymicro Technologies, Phoenix, AZ) packed with Aqua C18 column (5 μm particle size, 125 Å, Phenomenex, Torrance, CA) and separated on a micro analytical column (50 μm × 10 cm, Polymicro Technologies) packed with Aqua C18 column (3 μm particle size, 125 Å, Phenomenex). The eluting program was performed using a linear gradient of acetonitrile [1–40% (vol/vol) in 40 min] and 0.1% (vol/vol) formic acid in water at a flow rate of 200 nL/min. The eluate was directly injected into the MS system. The MS analysis was carried out in positive ion mode and full scans were performed for Auto MS/MS between 100 and 2,500 m/z. The acquired MS/MS data were preprocessed with Mascot Distiller 2.4 (MatrixScience, London, UK). The AA sequences of peptides were identified by comparison with identified peptide sequences from *Equus caballus* whey proteins (UniProt database, http://www.uniprot.org/).
Peptide Synthesis

Peptides identified by LC-ESI MS/MS were prepared by the conventional Fmoc solid-phase synthesis method, which were performed by Nanjing Leon Biological Technology Co. Ltd. (Nanjing, China). The purity of the peptides was ≥95%.

Simulated Gastrointestinal Digestion of Synthetic Peptides

The synthetic peptides were subjected to simulated gastrointestinal digestion, as described previously (Walsh et al., 2004). Peptides were dissolved in deionized water, and the pH value of solution was adjusted to 2.0 with 1 \(M\) HCl. Pepsin (enzyme to substrate mass ratio of 1:40) was added to hydrolyze the peptides. The hydrolysis reaction was performed at 37°C in a shaking incubator. After 1.5 h, the pH value of solution was adjusted to 7.5 with 1 \(M\) NaOH. Then, pancreatin at an enzyme/substrate mass ratio of 1/100 was added to the solution, which was incubated at 37°C for 2.5 h with shaking. Afterward, the solution was heated at 80°C for 20 min to terminate digestion. The digested peptides were lyophilized and then resuspended in Tris-HCl buffer (100 mM, pH 8.0) at a final concentration of 0.5 mg/mL to determine their DPP-IV inhibitory activity.

Statistical Analysis

All assays in the present study were performed at least in triplicate and data were expressed as means ± standard deviations. Significant differences (\(P < 0.05\)) between means were identified by Tukey’s procedures using SPSS software (version 20.0, IBM Inc., Chicago, IL).

RESULTS

DPP-IV Inhibitory Activity of Mare Whey Hydrolysates

As shown in Figure 1A, compared with intact mare whey (5.26 ± 0.21% inhibition), the obtained mare whey hydrolysates showed more potent DPP-IV inhibitory activity at a concentration of 1.0 mg/mL (53.25 ± 1.70% to 77.25 ± 1.60% inhibition), which varied depending on the hydrolysis durations. The 4-h hydrolysate displayed the greatest DPP-IV inhibitory activity (77.25 ± 1.60% inhibition), and its inhibitory activity increased dose-dependently from 0.025 to 1.0 mg/mL (Figure 1B). The IC\(_{50}\) value of the 4-h hydrolysate was 0.18 mg/mL. In addition, Ile-Pro-Ile was used as a reference inhibitor in our study. The IC\(_{50}\) value of the Ile-Pro-Ile was 10.45 \(\mu\)M.

DPP-IV Inhibitory Activity of Mare Whey Hydrolysates Fractionated by Gel Filtration Chromatography and HPLC

The 4-h hydrolysates were fractionated using a Sephadex G-25 gel filtration column, and 2 fractions, F1 and F2, were collected, which corresponded to fractions from 60 to 125 min and fractions from 130 to 210 min, respectively (Figure 2A). Compared with unseparated 4-h hydrolysates, the obtained 2 fractions showed more potent DPP-IV inhibitory activity, with inhibitory rates of 49.16 ± 3.56% and 67.46 ± 0.59% at a concentration of 0.1 mg/mL, respectively (Figure 2B). Therefore, the F2 fraction with higher DPP-IV inhibitory activity was lyophilized and used for further purification by HPLC. Several minor peaks were observed between the retention time from 6 to 15 min, as shown in Figure 3A. Thus, 9 fractions from the minor peaks every 1 min (F2-1 to F2-9) were collected, lyophilized, and used to determine their DPP-IV inhibitory activity. Fraction F2-7 showed the greatest DPP-IV inhibition rate of 38.33 ± 0.79% and was selected to identify peptide sequences (Figure 3B).

Identification of DPP-IV Inhibitory Peptides

To identify the AA sequence of the peptides with potent DPP-IV inhibitory activity, the F2-7 fraction with the greatest DPP-IV inhibitory activity was analyzed by LC-ESI MS/MS. As an example, the LC-ESI MS/MS spectrum of a double-charged ion with \(m/z\) at 435.77 was shown in Figure 4. Following sequence interpretation and database searching, the LC-ESI MS/ MS spectrum of ion with \(m/z\) 870.54 was matched to sequence Asn-Leu-Glu-Ile-Ile-Leu-Arg (NLEIILR). A total of 5 peptides with lengths ranging from 6 to 13 AA were identified from the F2-7 fraction (Table 1). Two of them originated from β-LG and 3 peptides were from α-LA (Figure 5).

DPP-IV Inhibitory Activity of Selected Synthetic Peptides

It is reported that DPP-IV inhibitory peptides are generally characterized by a hydrophobic character (Boots, 2012). Moreover, the presence of positively charged Lys residue at the carboxyl-terminal end of peptides reduces the hydrophobic characteristics of peptides and thereby decreases the inhibition effect against DPP-IV (Yin et al., 2012; Silveira et al., 2013). Based on these structural features of DPP-IV inhibitory peptides, 2 of the peptides identified by LC-ESI MS/MS were selected for chemical synthesis and IC\(_{50}\) values against DPP-IV of the 2 synthetic peptides
were determined. Table 2 shows that the IC_{50} values of Asn-Leu-Glu-Ile-Leu-Arg (NLEIILR) and Thr-Gln-Met-Val-Asp-Glu-Glu-Ile-Met-Glu-Lys-Phe-Arg (TQMVDEIMEKFR) against DPP-IV were 86.34 and 69.84 μM, respectively. The tri-peptide diprotin A (Ile-Pro-Ile) was used as a reference inhibitor, and its IC_{50} value was 10.45 μM in the current study.

**Simulated Gastrointestinal Stability of Selected Synthetic Peptides**

The DPP-IV inhibitory activity of 2 synthetic peptides and diprotin A before and after in vitro simulated gastrointestinal digestion was determined. As shown in Table 3, before simulated gastrointestinal digestion, the
DPP-IV inhibition of NLEIILR, TQMVDEEIMEKFR, and diprotin A was 54.86 ± 2.71%, 63.14 ± 0.55%, and 38.77 ± 0.90% at a concentration of 0.5 mg/mL, 0.5 mg/mL, and 2 μg/mL, respectively. The DPP-IV inhibitory activity of NLEIILR and diprotin A non-significantly changed before and after simulated gastrointestinal digestion. However, the inhibition effect of TQMVDEEIMEKFR against DPP-IV increased by 21.00% after simulated gastrointestinal digestion.

**DISCUSSION**

Incretins are gut-derived enteroendocrine hormones released into the bloodstream in response to ingested food. They increase glucose-dependent insulin secretion from pancreatic β-cells. About two-thirds of the insulin secreted after an oral glucose load is elicited by incretin hormones (Holst and Gromada, 2004). Increased blood levels of incretins and insulin effectively lower blood glucose levels in type 2 diabetic mice (Zhang et al., 2013). However, the cleavage and inactivation of incretins happen within several minutes due to the presence of DPP-IV. Inhibition of incretin degradation by DPP-IV inhibitor enhances insulin secretion and improves glycemic control in diabetic rats (Hsieh et al., 2015). Thus, DPP-IV inhibitors may possess a potential for the treatment of type 2 diabetes. In the present study, 4-h mare whey protein hydrolysates produced by papain possessed potent DPP-IV inhibitory activity in...
vitro with IC$_{50}$ value of 0.18 mg/mL, which was lower than that of bovine whey protein hydrolysates generated with a foodgrade pancreatic enzyme preparation (1.34 mg/mL; Nongonierma and FitzGerald, 2013). This difference is mainly attributed to different peptide profiles of the hydrolysates, which is associated with the difference of the used proteases and protein sources (Zhang et al., 2016). To the best of our knowledge, this is the first time that mare whey protein hydrolysates are reported for their DPP-IV inhibitory properties.

Previous studies have reported that bioactive peptides contribute to DPP-IV inhibitory activity of protein hydrolysates. The hexapeptide Val-Ala-Gly-Thr-Trp-Tyr (IC$_{50}$ value, 174 μM) is found to be responsible for the DPP-IV inhibitory activity of β-LG hydrolysates (Uchida et al., 2011). Moreover, Atlantic salmon skin gelatin-derived peptides with an IC$_{50}$ value of 1.35 mg/mL have been identified as Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala, and possess a superior antidiabetic effect in streptozocin-induced diabetic rats at a dose of 300 mg per day (Li-Chan et al., 2012; Hsieh et al., 2015).

In our study, 2 peptides, Asn-Leu-Glu-Ile-Ile-Leu-Arg and Thr-Gln-Met-Val-Asp-Glu-Glu-Ile-Met-Glu-Lys-Phe-Arg, were identified in 4-h mare whey protein hydrolysates. The IC$_{50}$ values against DPP-IV of them were 86.34 and 69.84 μM, respectively, suggesting that these 2 peptides may be responsible for the DPP-IV inhibitory activity of mare whey hydrolysates. Moreover, to our knowledge, the 2 identified peptides were first experimentally demonstrated to possess DPP-IV inhibitory activity, and the AA sequence of the 2 identified peptides only existed in mare whey protein, not in bovine whey protein. In addition, we found no significant difference in DPP-IV inhibitory activity of

**Table 1.** Liquid chromatography-electrospray ionization tandem MS identification of dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides in fraction F2-7 obtained by HPLC

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Calculated mass (Da)</th>
<th>Observed molecular ion, m/z (charge)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLEIILR</td>
<td>870.05</td>
<td>435.77 (2)/870.54 (1)</td>
<td>β-LG f (71–77)</td>
</tr>
<tr>
<td>TQMVDEEMEKFIR</td>
<td>1,655.90</td>
<td>828.39 (2)/1,655.78 (1)</td>
<td>β-LG f (143–155)</td>
</tr>
<tr>
<td>SMDGYK</td>
<td>699.77</td>
<td>350.65 (2)/700.29 (1)</td>
<td>α-LA f (14–19)</td>
</tr>
<tr>
<td>NNGKTEYG</td>
<td>881.90</td>
<td>441.70 (2)/882.39 (1)</td>
<td>α-LA f (44–51)</td>
</tr>
<tr>
<td>YDQTIVIK</td>
<td>967.09</td>
<td>484.26 (2)/967.51 (1)</td>
<td>α-LA f (36–43)</td>
</tr>
</tbody>
</table>

**Figure 4.** The liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) spectrum of double-charged ion with m/z 435.77. Following sequence interpretation and database searching, the LC-ESI MS/MS spectrum of ion with m/z 870.54 was matched to peptide NLEIILR. The peptide sequences are shown with the fragment (b, y) ions observed in the spectra. Color version available online.
peptide Asn-Leu-Glu-Ile-Ile-Leu-Arg after simulated gastrointestinal digestion, which suggested that the DPP-IV inhibitory activity of the peptide could be retained under simulated gastrointestinal conditions. However, the DPP-IV inhibitory activity of Thr-Gln-Met-Val-Asp-Glu-Ile-Met-Glu-Lys-Phe-Arg was improved by gastrointestinal enzyme treatment. This effect may be associated with the formation of new and smaller DPP-IV inhibitory peptides induced by

Table 2. The half-maximal inhibitory concentration (IC$_{50}$) values against dipeptidyl peptidase-IV (DPP-IV) of synthesized peptides NLEIILR, TQMVDEEIMEKFR, and diprotin A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLEIILR</td>
<td>86.34</td>
</tr>
<tr>
<td>TQMVDEEIMEKFR</td>
<td>69.84</td>
</tr>
<tr>
<td>Diprotin A</td>
<td>10.45</td>
</tr>
</tbody>
</table>

Table 3. Simulated gastrointestinal stability of synthetic dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides NLEIILR, TQMVDEEIMEKFR, and diprotin A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPP-IV inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before SG</td>
<td>After SG</td>
</tr>
<tr>
<td>NLEIILR</td>
<td>54.86 ± 2.71$^a$</td>
</tr>
<tr>
<td>TQMVDEEIMEKFR</td>
<td>63.14 ± 0.55$^b$</td>
</tr>
<tr>
<td>Diprotin A</td>
<td>38.77 ± 0.90$^a$</td>
</tr>
</tbody>
</table>

$^a$Values in a row bearing different letters are significantly different ($P < 0.05$).

$^b$Values are expressed as means ± SD of 3 independent determinations.

The DPP-IV inhibition activity of NLEIILR, TQMVDEEIMEKFR, and diprotin A was performed using 0.5 mg/mL, 0.5 mg/mL, and 2 μg/mL (final assay concentration). SG: simulated gastrointestinal digestion.
enzymatic-triggered cleavage of peptide bond cleavage. In agreement with our study, peptides derived from tuna cooking juice hydrolysates exhibits higher DPP-IV inhibitory activity upon simulated gastrointestinal digestion (Huang et al., 2012). Taken together, these results indicated that 2 DPP-IV inhibitory peptides, Asn-Leu-Glu-Ile-Leu-Leu-Arg and Thr-Gln-Met-Val-Asp-Glu-Glu-Ile-Met-Glu-Lys-Phe-Arg, isolated from mare whey may possess a potential effect in regulation of blood glucose and amelioration of type 2 diabetes.

Various studies have highlighted the role of the length of peptides, compositions, and sequences of AA in peptides on their DPP-IV inhibitory activities. Most dietary protein-derived DPP-IV inhibitory peptides described in the literature have a length of 2 to 8 AA (Lacroix and Li-Chan, 2012); however, peptides larger than 13 residues have been reported as novel DPP-IV inhibitor (Huang et al., 2012). Our study showed that heptapeptide NLEIILR and tridecapeptide TQMVDEEIMEKFR possessed DPP-IV inhibitory activity, which suggested that DPP-IV inhibitory activity of peptides may be associated with the composition and sequence of AA rather than their length. The presence of hydrophobic AA and a Pro residue, especially a Pro in the second N-terminal residue within the sequence of peptides, is a typical characteristic of DPP-IV inhibitory peptides (Li-Chan et al., 2012). The Pro residue is present within the sequences of bovine whey protein-derived DPP-IV inhibitory peptides as the second or third N-terminal residue (Silveira et al., 2013; Lacroix and Li-Chan, 2014). Peptides identified from goat milk casein display DPP-IV inhibitory activity, characterized by the presence of Pro residue and large amounts of hydrophobic AA, such as Phe, Tyr, Leu, Ile, Trp, Val, and Met (Zhang et al., 2015). In the present study, the 2 DPP-IV inhibitory peptides from mare whey protein were composed of many hydrophobic AA residues, such as Leu, Ile, Met, Phe, and Val. However, the AA sequences of DPP-IV inhibitory peptides generated from dietary proteins depend, to a very great extent, on hydrolysis conditions such as the types of proteases. Thus, whether DPP-IV inhibitory peptides containing Pro in the second N-terminal residue exist in the mare whey protein hydrolysates produced by other proteases need to be further investigated. In addition, it is reported that the peptides with acidic isoelectric point are capable of blocking the formation of DPP-IV dimers by locating at the dimerization interface, inhibiting DPP-IV activity (Velarde-Salcedo et al., 2013). In the present study, the heptapeptide NLEIILR and tridecapeptide TQMVDEEIMEKFR with a predicted acidic isoelectric point (pI 6.00 and 4.41, respectively, http://web.expasy.org/compute_pi/) showed potent DPP-IV inhibitory activity. However, the binding modes of these peptides with the DPP-IV enzyme need to be investigated via molecular docking simulations in our further study.

CONCLUSIONS

Peptides with DPP-IV inhibitory activity were successfully prepared from mare whey proteins through papain hydrolysis. Two novel peptides Asn-Leu-Glu-Ile-Leu-Leu-Arg and Thr-Gln-Met-Val-Asp-Glu-Glu-Ile-Met-Glu-Lys-Phe-Arg were identified from mare whey protein hydrolysates, and showed good DPP-IV inhibitory activity in vitro. The DPP-IV inhibitory activity of the 2 peptides was retained or even improved after simulated gastrointestinal enzyme treatment. These results suggested that peptides derived from mare whey may possess potential in the management of type 2 diabetes. Further studies using Caco-2 cells/islet β-cells and animal model systems are also necessary to evaluate DPP-IV inhibitory activity of the peptides derived from mare whey.

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