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In silico, in vitro and in vivo analyses of dipeptidyl peptidase IV inhibitory activity and antidiabetic effect of a sodium caseinate hydrolysate

Cheng-Hong Hsieh, Tzu-Yuan Wang, Chuan-Chuan Hung, Chia-Ling Jao, You-Liang Hsieh, Si-Xian Wu and Kuo-Chiang Hsu

The frequency, a novel in silico parameter, was developed by calculating the ratio of the number of truncated peptides with Xaa-proline and Xaa-alanine to all peptide fragments from a protein hydrolyzed with a specific protease. The highest in vitro DPP-IV inhibitory activity (72.7%) was observed in the hydrolysate of sodium caseinate by bromelain (Cas/BRO), and the constituent proteins of bovine casein also had relatively high A values (0.10-0.17) with BRO hydrolysis. 1CBR (the < 1 kDa fraction of Cas/BRO) showed the greatest in vitro DPP-IV inhibitory activity of 77.5% and was used for in vivo test by high-fat diet-fed and low-dose streptozotocin-induced diabetic rats. The daily administration of 1CBR for 6 weeks was effective to improve glycaemic control in diabetic rats. The results indicate that the novel in silico method has the potential as a screening tool to predict dietary proteins to generate DPP-IV inhibitory and antidiabetic peptides.

Introduction

Type 2 diabetes, a chronic metabolic syndrome, is estimated to affect 366 million people worldwide by 2030. Dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) inhibitors have been accepted to be a novel approach in the management of type 2 diabetes. DPP-IV is a serine protease that has a specificity for removing Xaa-proline and Xaa-alanine (Ala) dipeptides from the N-terminus of polypeptides or proteins. The ubiquitous enzyme can degrade and inactivate a number of incretin hormones with Ala as the second N-terminal residue, such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP), which contribute to stimulate pancreatic β-cell to boost glucose-dependent insulin secretion. DPP-IV inhibitors have shown to decrease blood glucose and improve impaired glucose tolerance, against the degradation by DPP-IV inhibitors have shown to decrease blood glucose and improve impaired glucose tolerance, against the degradation by DPP-IV inhibitors have shown to decrease blood glucose and improve impaired glucose tolerance.

In silico analysis, a computer-aided technique, is useful to predict the potential of proteins to act as precursors of DPP-IV inhibitory peptides. These in silico studies were based upon determination of the number of DPP-IV inhibitory peptides, which has been identified and reported in the literature, found within a dietary protein. The frequency of occurrence was calculated to express the potential of a protein as a precursor of DPP-IV inhibitory peptides. The frequency of occurrence was calculated to express the potential of a protein as a precursor of DPP-IV inhibitory peptides. The frequency of occurrence was calculated to express the potential of a protein as a precursor of DPP-IV inhibitory peptides. The frequency of occurrence was calculated to express the potential of a protein as a precursor of DPP-IV inhibitory peptides.

In this study, we used a novel in silico method to select the optimal protein source and protease, in order to predict hydrolysates with high Pro contents having the potential as the precursors of DPP-IV inhibitors. The specificity of the proteases we used for hydrolysis was not considered, hence, the DPP-IV inhibitory activity of the obtained hydrolysates was various and could not be expected.

Our research group has attempted a series of in vivo experiments which demonstrated that peptides from various protein sources were effective to lower blood glucose of diabetic rats by DPP-IV inhibition and/or GLP-1 stimulation. The rationale of these researches was to select protein sources with high Pro contents having the potential as the precursors of DPP-IV inhibitors. The specificity of the proteases we used for hydrolysis was not considered, hence, the DPP-IV inhibitory activity of the obtained hydrolysates was various and could not be expected.

In this study, we used a novel in silico method to select the optimal protein source and protease, in order to predict hydrolysates with high Pro contents having the potential as the precursors of DPP-IV inhibitors. The specificity of the proteases we used for hydrolysis was not considered, hence, the DPP-IV inhibitory activity of the obtained hydrolysates was various and could not be expected.

Previous studies on the in vitro DPP-IV inhibitory activity of dietary proteins hydrolysed by various proteases were reviewed, and the hydrolysates with relatively high DPP-IV inhibitory activity obtained from the combination of protein sources and hydrolysis conditions with various proteases were sorted. The aim of this study was to use a novel in silico method to select the optimal protein source and protease, in order to predict hydrolysates with high DPP-IV inhibitory activity, and then the results were compared to in vitro and in vivo analyses.

Please do not adjust margins
Table 1. The hydrolysates obtained with the combination of protein sources and enzymes reported in the literature to present great DPP-IV inhibitory activity.

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Enzyme</th>
<th>E/S (%)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>Time (min)</th>
<th>Inhibitory activity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium caseinate</td>
<td>Bromelain</td>
<td>5</td>
<td>6.7</td>
<td>45</td>
<td>60</td>
<td>61/0.475</td>
<td>16</td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>Thermolysin</td>
<td>3</td>
<td>8</td>
<td>70</td>
<td>20</td>
<td>53/0.475</td>
<td>16</td>
</tr>
<tr>
<td>Wheat</td>
<td>Gastrointestinal digestion</td>
<td>5/5-5</td>
<td>2/7.5</td>
<td>37/37</td>
<td>180/180</td>
<td>50/0.8</td>
<td>11</td>
</tr>
<tr>
<td>Soybean</td>
<td>Gastrointestinal digestion</td>
<td>5/5-5</td>
<td>2/7.5</td>
<td>37/37</td>
<td>180/180</td>
<td>40/1.4</td>
<td>11</td>
</tr>
</tbody>
</table>

*E/S: enzyme/substrate ratio

Materials and methods

Materials and reagents

Sodium caseinate (Cas), soybean protein (Soy) and wheat protein (Whe) were purchased from Gemfont Inc. (Taipei, Taiwan). Bromelain (from pineapple stem) was purchased from ST BIO Inc. (Taipei, Taiwan). Thermolysin (T0331, from *Bacillus thermoproteolyticus*), pepsin (P7000, from porcine gastric mucosa), trypsin (T4799, from porcine pancreas), pancreatin (P1750, from porcine pancreas), dipeptidyl peptidase-IV (DPP-IV; D7052, from porcine kidney), GlyuProu (from Sigma-Aldrich (St. Louis, MO, USA). The amino acid sequences of the constituent proteins of casein, soybean protein and wheat were selected in the UniProt Knowledgebase of ExPASy Proteomics Server available at http://expasy.org/. The peptide fragments obtained from the constituent proteins by hydrolysis with a given protease were determined using the enzyme action tool in BIOPEP database, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep. A new parameter was introduced to the ratio of the number of the truncated peptides with Pro and Ala as the penultimate N-terminal residues and the total number of total peptide fragments released by the given proteases.

In silico analysis

The amino acid sequences of the constituent proteins of casein, soybean protein and wheat were selected in the UniProt Knowledgebase of ExPASy Proteomics Server available at http://expasy.org/. The peptide fragments obtained from the constituent proteins by hydrolysis with a given protease were determined using the enzyme action tool in BIOPEP database, available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep. A new parameter was introduced to the ratio of the number of the truncated peptides with Pro and Ala as the penultimate N-terminal residues and the total number of total peptide fragments released by the given proteases.

Table 2. Characteristics and specificities of commercial proteases used in the present study.

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromelain</td>
<td>Cysteine protease</td>
<td>5-8</td>
<td>45°C</td>
<td>Lys-, Arg, Phe, Tyr-COOH</td>
<td>2000 GDU*</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>Metalloprotease</td>
<td>7-9</td>
<td>70°C</td>
<td>Ala-, Ile-, Leu-, Val-, PheNH₂</td>
<td>50-100 units/mg</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Aspartic protease</td>
<td>1-4</td>
<td>37°C</td>
<td>Aromatic-COOH and –NH₂, Leu-, Asp-, Gli-COOH</td>
<td>250 units/mg</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Serine protease</td>
<td>7-9</td>
<td>37°C</td>
<td>Lys-, Arg-COOH</td>
<td>1000-2000 BAEE* units/mg</td>
</tr>
<tr>
<td>Pancreatin</td>
<td>Mixture of trypsin, chymotrypsin, elastase and carboxypeptidase A and B</td>
<td>7-9</td>
<td>37°C</td>
<td>Very broad specificity</td>
<td>4X USP* specifications</td>
</tr>
</tbody>
</table>

GDU*: gelatin digesting units.

Unit*: one unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per min at pH 7.5 at 37°C.

Unit*: one unit will produce a ΔA₂₈₀ of 0.001 per min at pH 2.0 at 37°C, measured as TCA-soluble products using haemoglobin as substrate.

BAEE*: benzoyl L-arginine ethyl ester.

USP*: United States Pharmacopeia units.
96-well microplates and to measure the increase in absorbance at 385 nm using Gly-Pro-p-nitroanilide as DPP-IV substrate according to the method described previously. The lyophilized hydrolysates were dissolved in 100 mM Tris buffer (pH 8.0), and an aliquot of 40 µL was added with 40 µL of 1.59 mM Gly-Pro-p-nitroanilide (in 100 mM Tris buffer, pH 8.0). The mixture was incubated at 37°C for 10 min, followed by the addition of 80 µL of DPP-IV (diluted with the same Tris buffer to 0.01 Unit/mL). The reaction mixture was incubated at 37°C for up to 60 min, and the reaction was stopped every 5 min by adding 150 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, 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.

In vivo analysis

The DPP-IV inhibitory peptides of the hydrolysates were fractionated by ultrafiltration (Model ABL085, Lian Sheng Tech. Co., Taichung, Taiwan) with spiral wound membranes having molecular mass cutoffs of 2.5 and 1.0 kDa. The fractions were collected as follows: >2.5 kDa, peptides retained without passing through 2.5 kDa membrane; 1.0-2.5 kDa, peptides permeating through the 2.5 kDa membrane but not the 1.0 kDa membrane; <1.0 kDa, peptides permeating through the 1.0 kDa membrane. All fractions collected were lyophilized and stored in a desiccator until use. The fraction with the highest DPP-IV inhibitory activity was used as the sample for animal experiment. For animal experiment, the hydrolysates were stored in a desiccator until use. The fraction with the highest DPP-IV inhibitory activity was used as the sample for animal experiment. For animal experiment, the hydrolysates were prepared on large scale based on the same condition with in vitro analysis. The high-fat diet (HFD)-fed and low-dose STZ-induced diabetic rats were used as the animal model in this study. Male Sprague-Dawley (SD) rats (LASCO, Taipei, Taiwan), aged 7 weeks and weighing between 230-250 g were used. The rats were randomly divided into normal and diabetic groups. The rats in normal groups were fed a regular diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) consisting of a total kcal value of 4.07 kcal/g (14% fat, 57% carbohydrate, 29% protein). The induction of type 2 diabetic rats by the high-fat diet (HFD) and low-dose STZ referred to the method of Srinivasan et al. (2005) with some modifications. The rats were fed HFD with a total kcal value of 8.18 kcal/g (34% fat, 44% carbohydrate, 22% protein) for the initial period of 6 weeks. The rats were intraperitoneally injected with a buffer (0.01 M citrate, pH 4.5) solution of STZ at a low dosage of 30 mg/kg body weight while the respective control rats were given vehicle citrate buffer in a dose volume of 1 mL/kg. At 1 week after the injection of STZ, animals were considered to be diabetic if their plasma glucose levels over 200 mg/dL. All rats care and procedures were approved by the Institutional Animal Care and Use Committee of China Medical University.

Animals were divided into 6 groups of 12 rats each, and the rats in experimental groups were administrated samples by oral gavage. The experimental period was 6 weeks. Group A: normal control rats administered drinking water daily; group B: normal rats administrated 1CBR (500 mg/kg/day); group C: diabetic control rats administered drinking water daily; group D: diabetic rats administered low-dose of 1CBR (250 mg/kg/day); group E: diabetic rats administered high-dose of 1CBR (500 mg/kg/day); group F: diabetic rats administered sitagliptin (120 mg/kg/day; positive control).

At the end of the experiment, an oral glucose tolerance test (OGTT) test was performed in overnight fasted rats from all groups, and the plasma glucose levels were determined using a blood glucose meter (TD-4207, Taidoc, New Taipei, Taiwan). After 30 min of the administration of samples, glucose (2 g/kg) was fed to each rat. Blood was withdrawn from tail vein at 0, 30, 60, 90, 120 and 180 min for the assay of glucose levels.

On the morning after final administration on day 42, the animals were sacrificed by over dose of CO₂. Blood samples were collected in chilled blood vases containing ethylendiaminetetraacetic acid (EDTA). Samples were centrifuged (3,000 g, 15 min) and stored at -80°C. Plasma DPP-IV activity was measured using a DPPIV/C26 assay kit (Enzo Inc., Farmingdale, NY, USA). Plasma active GLP-1 concentration was measured using a glucagon like peptide-1 ELISA kit (Millipore Corp., Billerica, MA, USA). Plasma insulin concentration was measured using a Mercodia rat insulin kit (Mercodia Inc., Uppsala, Sweden).

Statistical analysis

Each data point represents the mean of three replicates subjected to analysis of variance (one-way ANOVA) using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). A test of comparison of two means was analysed by Duncan’s test, and the significance level of P<0.05 was employed.

Results and discussion

In silico and in vitro analyses

According to the previous studies reported that the hydrolysates of sodium caseinate, wheat and soybean by various proteases possessed great DPP-IV inhibitory activity, we used these protein sources and proteases for in silico and in vitro analyses. Table 3 shows the frequency of the constituent proteins and in vitro DPP-IV inhibitory activity of the hydrolysates from the protein sources by various proteases. In most previous studies, researchers used the occurrence frequency of the DPP-IV inhibitory peptides, which have been reported in the literature, to present in the protein sequences of various food commodities to quantify the potential of the proteins as bioactive peptide precursors. In the present study, however, the frequency (A) was developed to display the potential of the combination of proteins and proteases to be DPP-IV inhibitors by calculating the ratio of the number of truncated peptides with Xaa-proline and Xaa-alanine to all peptide fragments from a protein
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protein accounting for the proportion of the protein source was
preliminary screening tool. Therefore, a further study will be
probably released by pepsin and pancreatin but rarely contributed by
positive correlation, which may reflect the novel
differences might be attributed to the poor digestive activity of
result in the previous study
activity than that by THE (64.8%), which was consistent with the
of sodium caseinate hydrolysed by BRO exhibited higher DPP-IV
inhibitory activity (69.9%), respectively (Table 3). In the present study, the hydrolysate
discovered in Cas/BRO (72.7%), Soy/BRO (61.6%) and Whe/THE
3, the high frequency value (40% at 1.4 mg/mL)
that the wheat protein hydrolysed via gastrointestinal digestion
showed greater DPP-IV activity (50% at 0.8 mg/mL) than soybean
(0.17). Based on
values from the constituent proteins, the hydrolysates of Cas/BRO, Soy/BRO and Whe/THE were supposed
to possess great
hydrolysates of Cas/BRO, Soy/BRO and Whe/THE were supposed
in silico
in vitro
analyses for DPP-IV inhibitory activity seemed consistent and
oral glucose tolerance test (OGTT)

DPP-IV inhibitory activity of UF fractions

The DPP-IV inhibitory activity of the UF fractions (sample concentration of 1 mg/mL) of the protein hydrolysates was
shown in Fig. 1. For each protein source, the UF fraction with smaller molecular weight showed greater DPP-IV inhibitory activity. The highest inhibition value (77.5%) in this study was observed in < 1 kDa fraction of Cas/BRO (P<0.05), while < 1 kDa fraction of Soy/BRO and Whe/THE showed 69.0 and 74.5% of DPP-IV inhibitory activity, respectively. The result in this study is in agreement with former studies using various protein sources that reported the preferable DPP-IV inhibitory peptides consisted of 2 to 8 amino acid residues, and their molecular weights were presumed between 200 and 1,000 Da
25,26. Then, the < 1 kDa fraction of Cas/BRO (1CBR) was used for in vivo experiment.

Table 3 In silico analysis of frequency (A) of the constituent proteins and in vitro DPP-IV inhibition rates of the hydrolysates obtained from the combinations of various protein sources and proteases.

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Constituent protein</th>
<th>ExPASy ID</th>
<th>No. (P+A)/total a.a.</th>
<th>Frequency (A)</th>
<th>In vitro DPP-IV inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium caseinate (bovine)</td>
<td>β-casein</td>
<td>P02666</td>
<td>44/224</td>
<td>0.16</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>κ-casein</td>
<td>P02668</td>
<td>37/190</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>α-S1-casein</td>
<td>P02662</td>
<td>29/214</td>
<td>0.17</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>α-S2-casein</td>
<td>P02663</td>
<td>21/222</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Basic 7S globulin</td>
<td>P13917</td>
<td>55/427</td>
<td>0.14</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Glycinin</td>
<td>P04347</td>
<td>58/516</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>Soybean</td>
<td>β-conglycinin, α-chain</td>
<td>P25974</td>
<td>45/439</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>β-conglycinin, β-chain</td>
<td>P13916</td>
<td>68/605</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>β-conglycinin, γ-chain</td>
<td>P11827</td>
<td>63/639</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>α/β-gliadin</td>
<td>P02863</td>
<td>52/286</td>
<td>0.03</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Glutelin, high molecular weight subunit 12</td>
<td>P08488</td>
<td>99/660</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Glutelin, low molecular weight subunit 1D1</td>
<td>P10386</td>
<td>53/307</td>
<td>0.10</td>
<td>0.16</td>
</tr>
</tbody>
</table>

a the number of Pro and Ala / the number of total amino acids in a constituent protein.
b the inhibition rate expressed by each hydrolysate at concentration of 1 mg/mL.

d DPP-IV inhibitory activity of the UF fractions (sample concentration of 1 mg/mL) from each protein source was
preliminary screening tool. Therefore, a further study will be
probably released by pepsin and pancreatin but rarely contributed by
positive correlation, which may reflect the novel
differences might be attributed to the poor digestive activity of
result in the previous study
activity than that by THE (64.8%), which was consistent with the result in the previous study
Another previous study demonstrated that the wheat protein hydrolysed via gastrointestinal digestion showed greater DPP-IV activity (50% at 0.8 mg/mL) than soybean (40% at 1.4 mg/mL) 21, however, the results in this study appeared that GAD hydrolysates of soybean and wheat possessed similar (P>0.05) but relatively low DPP-IV inhibitory activity. The differences might be attributed to the poor digestive activity of trypsin which is activated by calcium ions 24. In the present study, the peptides of GAD hydrolysates from various protein sources were probably released by pepsin and pancreatin but rarely contributed by trypsin due to calcium ions free.

From the results shown in Table 3, in silico and in vitro analyses for DPP-IV inhibitory activity seemed consistent and positive correlation, which may reflect the novel in silico method having the chance as a great screening tool to predict the potential of a protein source hydrolysed by a given protease as a DPP-IV inhibitor. However, the sample size in this study is small so we can only propose this in silico method to be a preliminary screening tool. Therefore, a further study will be done to determine the correlation between the in silico and in vitro analyses for DPP-IV inhibitory activity of hydrolysates from various protein sources and proteases for evaluation the feasibility of the novel in silico method.

Oral glucose tolerance test (OGTT)
At the end of the experiment, OGTT blood glucose responses of the rats in all groups were shown in Fig. 2A, and the area under curve (AUC) was shown in Fig. 2B. The blood glucose levels during OGTT test of DM group rats were significantly higher than the rats in the other groups \( (P<0.05) \). 1CBR did not result in hypoglycaemia in normal rats. 1CBR and sitagliptin was potent to lower the blood glucose levels of diabetic rats, but the levels were still higher than 200 mg/dL. As the results of the plasma glucose AUC, 1CBR significantly improved the blood glucose levels of diabetic rats after 6-week administration \( (P<0.05) \); meanwhile, glucose AUC of the rats administrated with high-dose of 1CBR was significantly lower than that with low-dose of 1CBR \( (P<0.05) \). The result was consistent with the previous study, which reported that the porcine skin gelatin hydrolysates (300 mg/day) showed hypoglycaemic effect on the STZ-induced rat model \(^1\). Our previous studies have reported that the oral administration of Atlantic salmon (300 mg/day) and tilapia (750 mg/kg/day) skin gelatin hydrolysates for 5 and 4 weeks, respectively, significantly lowered blood glucose of diabetic rats to the similar level to normal control rats \(^14,27\).

The HFD-fed and low-dose STZ-induced diabetic rat model was first adopted in our study for type 2 diabetes simulating the human syndrome that is also suitable for testing anti-diabetic agents for the treatment of type 2 diabetes \(^22\). The fasted blood glucose levels of this model rat were over 200 mg/dL, and the levels after 15-90 min during OGTT reached beyond 500 mg/dL \(^28\). The blood glucose levels of HFD-fed and low-dose STZ-treated rats during OGTT were much higher than those of STZ-nicotinamide (NA)-induced diabetic rats \(^14,15\). The plasma glucose AUC of STZ-NA-induced diabetic rats treated with porcine and Atlantic salmon skin gelatin was reduced by 10 and 33%, respectively, \(^14,15\); meanwhile, that of HFD-fed and low-dose STZ-induced diabetic rats treated with 1CBR decreased 37% \( (Fig. 2) \). The result represents that the administration of 1CBR for 6 weeks is effective for glycaemic control of diabetic rats, although the rats are still identified to be diabetic due to their blood glucose levels over 200 mg/dL.

**Plasma DPP-IV activity, active GLP-1 and insulin levels**

The effect of administration of 1CBR after 6 weeks on the plasma DPP-IV activity, active GLP-1 and insulin levels of diabetic rats was shown in Fig. 3. The plasma DPP-IV activity of diabetic control rats \( (Group \, C) \) was 204.4% and significantly higher than those of the other rat groups \( (P<0.05) \) \( (Fig. 3A) \). The DPP-IV activities of diabetic rats administrated with low- and high-dose of 1CBR were reduced by 21.4 and 22.8%, respectively, which showed higher levels than normal rats \( (135.0\%) \) \( (P<0.05) \). The plasma active GLP-1 levels of diabetic rats in Group D, E, and F were significantly higher than those of diabetic control rats \( (Group \, C) \) and normal rats \( (Group \, A \, and \, B) \) \( (P<0.05) \) \( (Fig. 3B) \). Moreover, the GLP-1 level of Group C rats was similar to those of Group A and B rats with insignificant differences \( (P>0.05) \). The active GLP-1 of Group D, E and F was proposed to be prevented from degradation by DPP-IV. The insulin levels of normal rats \( (Group \, A \, and \, B) \) were 2.15-2.21 µg/L and significantly higher than the other group rats \( (Group \, C \, F) \) \( (P<0.05) \) \( (Fig. 3C) \). The Group E rats showed their plasma insulin level of 0.99 µg/L and significantly higher than Group D and C rats \( (P<0.05) \). The results showed that the long-term administration of DPP-IV inhibitory peptides, 1CBR, was potent to improve the insulin secretion in diabetic rats due to DPP-IV inhibition and active GLP-1 level elevation.

The HFD-fed and low-dose STZ-induced diabetic rat model has been widely used for evaluation the potency of antidiabetic agents for the treatment of type 2 diabetes \(^24,30\); however, this is the first study to adopt this model for testing the antidiabetic effect of bioactive peptides. Sitagliptin, a DPP-IV inhibitor approved for use in the European Union, USA and Japan, administered to diabetic mice and rats has shown hypoglycaemic effect by DPP-IV inhibition, active GLP-1 level elevation and insulin secretion enhancement \(^31,32\). Our previous studies have also demonstrated that bioactive peptides from food protein sources as DPP-IV inhibitors improved glycaemic control by inhibition of DPP-IV, elevation of plasma GLP-1 and insulin levels, reduction of glucagon levels of STZ-NA-induced diabetic rats \(^14,15\). From the results in this study, high-dose of 1CBR (500 mg/kg/day) showed similar antidiabetic effect as sitagliptin (120 mg/kg/day) with the same mechanism as other bioactive peptides and therefore had the potential as a functional food for the treatment of type 2 diabetes mellitus.

**Conclusions**

1CBR from sodium caseinate, selected by a novel in silico method and proved by the in vitro analysis, had a superior antidiabetic effect in HFD-fed and low-dose STZ-induced diabetic rats, including the improvement of glucose tolerance, inhibition of plasma DPP-IV activity, elevation of active GLP-1 levels, resulting in the enhancement of insulin secretion. This study indicates the novel in silico analysis having the chance as a screening tool to successfully
predict the potential of protein sources to be precursors of DPP-IV inhibitors by given proteases.

Fig. 3 Effect of daily administration of 1CBR for 6 weeks on (A) plasma DPP-IV activity, (B) active GLP-1 and (C) insulin of diabetic rats. Bars with different letters are significantly different at P<0.05. The group description is the same as for Fig. 2.

Acknowledgements

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Notes and references

18. A. Davy, K. K. Thomsen, M. A. Juliano, L. C. Alves, I. Svendsen and D. J. Simpson. Purification and


Bovine sodium caseinate

Soy protein

Wheat protein

In silico analysis

In vitro analysis

Hydrolysate

DM rats

Pancrease

↑ Insulin
↓ DPP-IV activity
↑ Active GLP-1
↑ Glycemic control

Active GLP-1

Inactive GLP-1

DPP-IV