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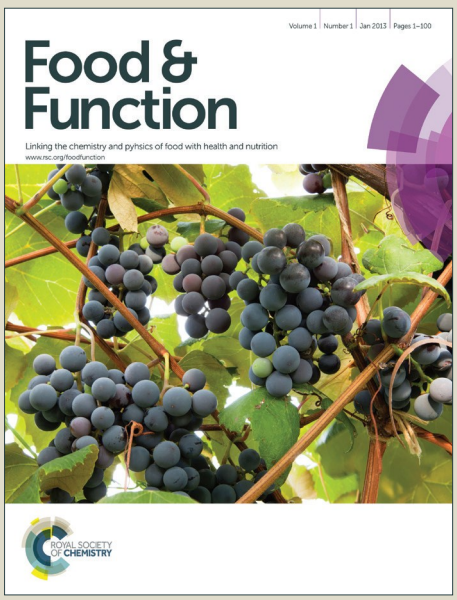

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In silico, *in vitro* and *in vivo* analyses of dipeptidyl peptidase IV inhibitory activity and antidiabetic effect of a sodium caseinate hydrolysate

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The frequency (*A*), 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 [1]. 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 [2]. DPP-IV is a serine protease that has a specificity for removing X-proline (Pro) or X-alanine (Ala) dipeptides from the N-terminus of polypeptides or proteins [3]. 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 [4]. DPP-IV inhibitors have shown to decrease blood glucose and improve impaired glucose tolerance, against the degradation by DPP-IV [5,6]. There have been several synthetic DPP-IV inhibitory drugs, e.g. sitagliptin, vildagliptin, saxagliptin and alogliptin, approved for the management of type 2 diabetes in the United States and European Union. Although these drugs generally have a high DPP-IV inhibitory potency, their side effects, e.g. asthenia, cardiac and vascular events are still the issues of concern [7]. A wild range of the hydrolysates from various dietary protein sources, e.g. milk [8,9], rice [10], amaranth, wheat, soybean [11], salmon [12], tuna cooking juice [13], has been reported to possess *in vitro* DPP-IV inhibitory activity.

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 [14,15]. 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 silico analysis, a computer-aided technique, is useful to predict the potential of proteins to act as precursors of DPP-IV inhibitory peptides [16,17]. 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 only considered that all DPP-IV inhibitory peptide sequences were pretend to be released from the relevant protein; however, that the peptide fragments obtained from the proteolytic hydrolysis of the protein determined by the specificity of the proteases was ignored. In addition, it is well documented that DPP-IV displays a preference for substrate having Pro or Ala as the second N-terminal residue [18], and the penultimate N-terminal residue of DPP-IV inhibitory peptides reported in the literature is mostly Pro or Ala [16]. In the present study, therefore, the peptide fragments released by a given protease from the relevant protein were taken into account, and the frequency of release of fragments with Pro and Ala as the penultimate N-terminal residues by a selected protease was also calculated [19].

Previous studies on the *in vitro* DPP-IV inhibitory activity of dietary proteins hydrolysed by various proteases were reviewed [11,16], 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 (**Table 1**). 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 great DPP-IV inhibitory activity, and then the results were compared to *in vitro* and *in vivo* analyses.

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Table 1 The hydrolysates obtained with the combination of protein sources and enzymes reported in the literature to present great DPP-IV inhibitory activity.

Protein source	Enzyme	E/S[a] (%)	pH	Temp. (℃)	Time (min)	Inhibitory activity (%)/ concentration (mg/mL)	Reference
Sodium caseinate	Bromelain	5	6.7	45	60	61/0.475	16
Sodium caseinate	Thermolysin	3	8	70	20	53/0.475	16
Wheat	Gastrointestinal digestion[b]	5/5-5	2/7.5	37/37	180/180	50/0.8	11
Soybean	Gastrointestinal digestion	5/5-5	2/7.5	37/37	180/180	40/1.4	11

[a] E/S: enzyme/substrate ratio.
[b] Gastrointestinal digestion: pepsin/trypsin-pancreatin.

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 pancrease), dipeptidyl peptidase-IV (DPP-IV; D7052, from porcine kidney), Gly-Pro-p-nitroanilide hydrochloride, and streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The characteristics and specificities of the commercial proteases were showed in **Table 2**. Other chemicals and reagents used were analytical grade and commercially available.

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 peptides with Pro, Ala as the penultimate N-terminal residues and the total number of total peptide fragments released by proteases.

The value (A) of the frequency was used to quantify the potential of the proteins to serve as the DPP-IV inhibitory peptide precursors and calculated based on the following equation:

$$A = d/F$$

Where d is the number of the truncated peptides with Pro and Ala as the second N-terminal residues; F is the number of total peptide fragments released by the given proteases.

In vitro analysis

Each protein added with 25-fold volume (v/w) of ddH_2O and adjusted pH value by 1 N HCl or 1 N NaOH was incubated at the optimal temperature of the given proteases for 20 min prior to the enzymatic hydrolysis. The hydrolysis reaction was started by the addition of enzymes at various enzyme/substrate ratios (E/S; w/w). The reactions with bromelain (BRO) and thermolysin (THE) were conducted at pH 6.7, 8.0, temperature 45, 70℃, E/S 5, 3%, for 60, 20 min, respectively according to previous study [16]. The hydrolysis with gastrointestinal digestion (GAD) was done first by pepsin at pH 2.0, temperature 37℃, E/S 5% for 180 min, followed by the mixture of trypsin and pancreatin at pH 7.5, temperature 37℃, E/S 5 and 5%, respectively, for 180 min [11]. After hydrolysis, the hydrolysate solutions were heated in boiling water for 15 min to inactivate enzymes and then cooled in tap water at room temperature for 20 min. Hydrolysates were adjusted their pH to 7.0 with 2 N NaOH and centrifuged (Centrifuge 05P-21, Hitachi Ltd., Katsuda, Japan) at 10,000 *g* and 4℃ for 15 min. The supernatant was lyophilised and stored at -20℃.

DPP-IV activity determination in this study was performed in

Table 2 Characteristics and specificities of commercial proteases used in the present study [20].

Enzyme	Type	Opt. pH	Opt. Temp	Preferential Specificity[1]	Activity
Bromelain	Cysteine protease	5-8	45℃	Lys-, Arg-, Phe, Tyr-COOH	2000 GDU[a]
Thermolysin	Metalloprotease	7-9	70℃	Ala-, Ile-, Leu-, Val-, Phe-NH_2	50-100 units[b]/mg
Pepsin	Aspartic protease	1-4	37℃	Aromatic-COOH and –NH_2, Leu-, Asp-, Glu-COOH	250 units[c]/mg
Trypsin	Serine protease	7-9	37℃	Lys-, Arg-COOH	1000~2000 BAEE[d] units/mg
Pancreatin	Mixture of trypsin, chymotrypsin, elastase and carboxypeptidase A and B	7-9	37℃	Very broad specificity	4X USP[e] specifications

GDU[a]: gelatin digesting units.
Unit[b]: 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℃.
Unit[c]: one unit will produce a ΔA_{280} of 0.001 per min at pH 2.0 at 37℃, measured as TCA-soluble products using haemoglobin as substrate.
BAEE[d]: benzoyl L-arginine ethyl ester.
USP[e]: united states pharmacopeia units.

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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 [21]. 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℃ 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℃ 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, Inc., Winooski, VT, USA). Recorded data were plotted versus time, and the DPP-IV activity was quantified from the linear part of the curve. The % DPP-IV inhibition was defined as the percentage of DPP-IV activity inhibited by a given concentration of hydrolysate.

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 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 combination of high fat diet (HFD) and low-dose STZ referred to the method of Srinivasan et al. (2005) [22] 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 give 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 [23]. 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 CO_2. Blood samples were collected in chilled blood vases containing ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged (3,000 *g*, 15 min) and stored at -80℃. Plasma DPP-IV activity was measured using a DPPIV/CD26 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 was 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 [11,16]. 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

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.

Protein source	Constituent protein	ExPASy ID	No. (P+A)/ total a.a.[a]	Frequency (*A*)			*In vitro* DPP-IV inhibition rate (%)[b]		
				BRO	THE	GAD	BRO	THE	GAD
Sodium caseinate (bovine)	β-casein	P02666	44/224	0.16	0.21	0.09	72.7[a]	64.8[c]	64.1[c]
	κ-casein	P02668	37/190	0.15	0.09	0.11			
	α-S1-casein	P02662	29/214	0.17	0.15	0.06			
	α-S2-casein	P02663	21/222	0.10	0.06	0.05			
Soybean	Basic 7S globulin	P13917	55/427	0.14	0.08	0.05	61.6[d]	58.1[d]	50.9[e]
	Glycinin	P04347	58/516	0.14	0.06	0.06			
	β-conglycinin, β-chain	P25974	45/439	0.15	0.05	0.04			
	β-conglycinin, α-chain	P13916	68/605	0.13	0.08	0.04			
	β-conglycinin, α'-chain	P11827	63/639	0.09	0.06	0.04			
Wheat	α/β-gliadin	P02863	52/286	0.03	0.17	0.05	59.5[d]	69.9[b]	48.4[e]
	Glutenin, high molecular weight subunit 12	P08488	99/660	0.07	0.04	0.04			
	Glutenin, low molecular weight subunit 1D1	P10386	53/307	0.10	0.16	0.08			

[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.

hydrolysed with a specific protease. From the results shown in Table 3, the high frequency value (*A*) was observed in the hydrolysates of β-casein/THE (0.21), α-S1-casein/BRO (0.17) and α/β gliadin/THE (0.17). Based on *A* values from the constituent proteins, the hydrolysates of Cas/BRO, Soy/BRO and Whe/THE were supposed to possess great *in vitro* DPP-IV inhibitory activity. Each constituent protein accounting for the proportion of the protein source was different due to the various species and hence difficult to identify.

The highest *in vitro* DPP-IV inhibitory activity of the hydrolysate (sample concentration of 1 mg/mL) from each protein source was discovered in Cas/BRO (72.7%), Soy/BRO (61.6%) and Whe/THE (69.9%), respectively (Table 3). In the present study, the hydrolysate of sodium caseinate hydrolysed by BRO exhibited higher DPP-IV activity than that by THE (64.8%), which was consistent with the result in the previous study [16]. 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) [11], 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.

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.

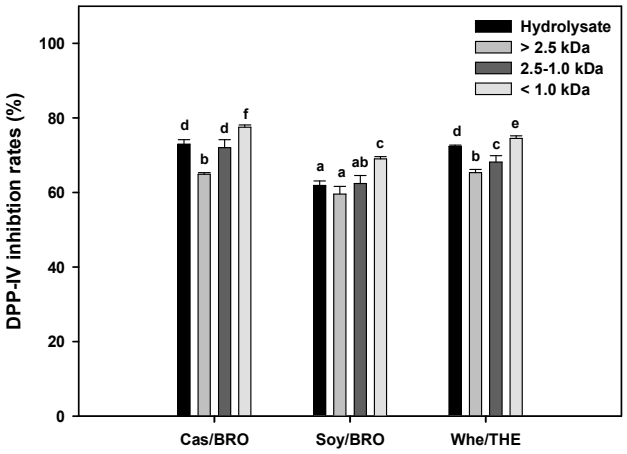

Fig. 1 DPP-IV inhibition rate of protein hydrolysates fractionated by UF at the concentration of 1 mg/mL. Bars with different letters are significantly different at P<0.05.

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 [15]. 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 antidiabetic agents for the treatment of type 2 diabetes [22]. The fasted blood glucose levels of this model rats 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 ofSTZ-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.

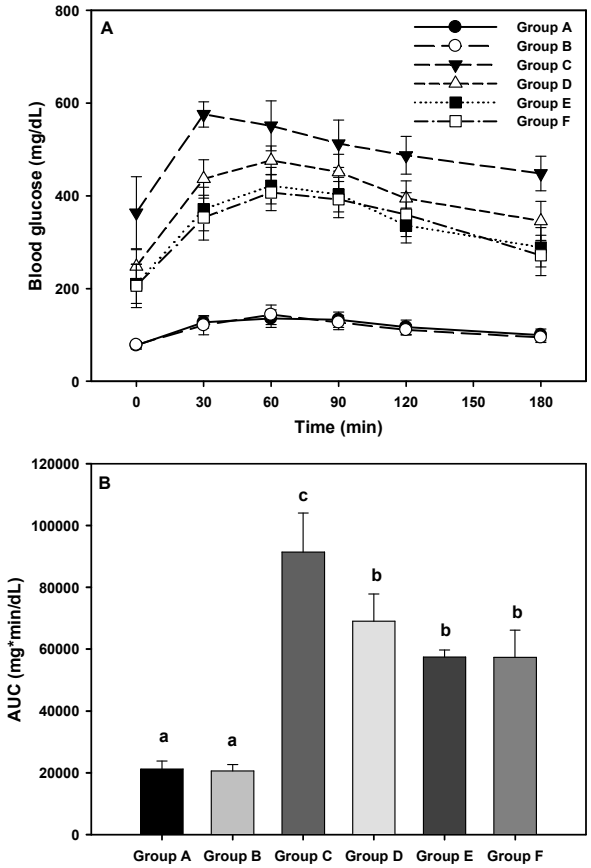

Fig. 2 (A) The blood glucose levels and (B) AUC_{0-180} values during oral glucose tolerance test of diabetic rats treated with 1CBR after 6-week experiment. Bars with different letters are significantly different at P<0.05. Group A: normal control rats, group B: normal rats + 1CBR (500 mg/kg/day), group C: diabetic control rats, group D: diabetic rats + 1CBR (250 mg/kg/day), group E: diabetic rats + 1CBR (500 mg/kg/day), and group F: diabetic rats + sitagliptin (positive control) (n = 12/group).

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 [29,30], 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.

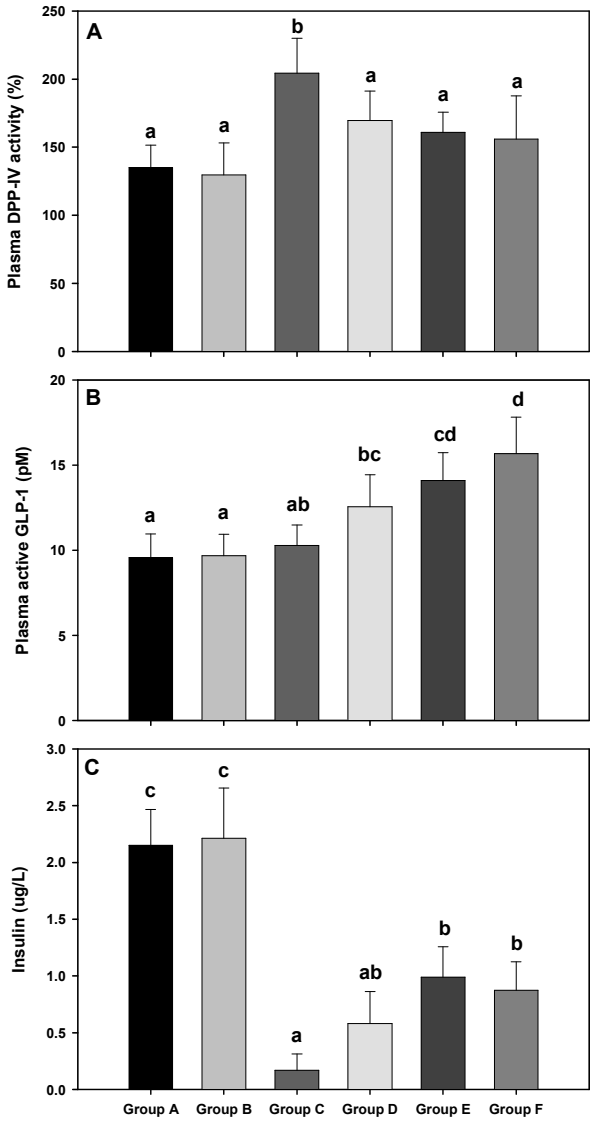

Fig. 3 Effect of daily administration of 1CBR for 6 weeks on (A) plasma DPP-IV activity, (B) active GLP-1 and (C) insulin of diabetic rats. Bars with different letters are significantly different at $P<0.05$. The group description is the same as for Fig. 2.

Acknowledgements

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Bovine sodium caseinate

Soy protein

Wheat protein

In silico analysis

In vitro analysis

Hydrolysate

Active GLP-1

DPP-IV

Inactive GLP-1

DM rats

Pancrease

↑ Insulin

↓ DPP-IV activity

↑ Active GLP-1

↑ Glycemic control