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Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran

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ABSTRACT

The insulinotropic hormone glucagon-like peptide-1 is metabolised extremely rapidly by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV). Therefore, human DPP-IV is a key regulator involved in the prevention and treatment of type 2 diabetes. To simplify the method of producing an inhibitory peptide against DPP-IV, we focused on rice bran (RB) as a source and subjected proteins from defatted RB to enzymatic proteolysis using 2 commercial enzymes. The RB peptides produced with Umamizyme G exhibited 10 times the inhibitory activity as those produced with Bioprase SP. The half-maximal inhibitory concentration (IC_{50}) value of the RB peptides was 2.3 ± 0.1 mg/ml. Leu-Pro and Ile-Pro were identified as the inhibitory peptides among the RB peptides produced with Umamizyme G. Ile-Pro was the strongest DPP-IV inhibitor among the 15 Xaa-Pro dipeptides and Pro-Ile tested. Ile-Pro competitively inhibited DPP-IV (K_i = 0.11 mM). Mass spectrometry indicated that the contents of Leu-Pro and Ile-Pro in the RB peptides were 2.91 ± 0.52 μg/mg.

1. Introduction

Rice is the main staple food of the Japanese. Rice bran (RB) is the pericarp and germ of *Oryza sativa* seeds and constitutes approximately 10% of rough rice grain by weight. Although RB is a good source of protein, fat, and antioxidants, it is currently underutilised despite its high potential as a raw material for the preparation of functional foods or nutraceuticals (Bandyopadhyay, Misra, & Ghosh, 2008; Parrado et al., 2006). In this study, RB was used as a source of bioactive peptides. Bioactive peptides are specific and small protein fragments that are inactive within the sequence of their parent protein; these peptides are 2–9 amino acids in size and typically possess specific amino acid sequences mainly comprised of hydrophobic groups in addition to proline, arginine, and lysine (Dizuba, Minkiewicz, & Nalcez, 1999; Kitts & Weiler, 2003; Korhonen & Pihlanto, 2003). Several bioactive peptides exhibit antioxidant, anti-obesity, anti-angiogenic, and anti-hypertensive activities (Algaron, Miranda, Le bars, & Monnet, 2004; Dizuba et al., 1999; Gauthier & Pouliot, 2003; Li & Zhang, 2001; Murakami et al., 2004; Parkash, Ng, & Tso, 2002; Quiros, Hernandez-Ledesma, Ramos, Amigo, & Recio, 2005).

The present paper focuses on the inhibitory activity of RB peptides against human dipeptidyl peptidase IV (EC 3.4.14.5, DPP-IV). DPP-IV is a serine protease that modulates the biological activity of specific circulating peptide hormones by specifically cleaving 2N-terminal amino acids: Xaa-Pro and Xaa-Ala (Bjelke et al., 2006). The insulinotropic hormone, glucagon-like peptide-1 (GLP-1), is metabolised extremely rapidly by the ubiquitous enzyme, DPP-IV. GLP-1 is a gut hormone released in response to nutrient ingestion. The enhancement of insulin secretion by gut-derived factors such as GLP-1 is called the "incretin effect." Recently, DPP-IV inhibitors that protect active GLP-1 from being cleaved by DPP-IV have been used as drugs to control postprandial glycemia in type 2 diabetes (Richard, 2008; Richter, Banderia-Echtler, Bergerhoff, & Lerch, 2008). Thus, DPP-IV inhibitors are effective in preventing and treating type 2 diabetes.

In this study, we used two commercial proteases to hydrolyse RB protein and evaluated the inhibitory activity of their hydrolysates against DPP-IV activity. Furthermore, the bioactive peptides from RB were identified using gel filtration, high-performance liquid chromatography (HPLC), protein sequencing, and HPLC–mass spectrometry (LC–MS).

Abbreviations: DPP-IV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; HPLC, high-performance liquid chromatography; IC_{50}, half-maximal inhibitory concentration; LAP, leucine aminopeptidase; LC–MS, HPLC–mass spectrometry; pNA, *p*-nitroanilide; RB, rice bran; SIM, selected ion-monitoring mode; X-PDAP, X-proryl dipeptidyl aminopeptidase.

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2. Materials and methods

2.1. Materials

Defatted RB was a gift from SATAKE Co. Ltd. (Higshi-Hiroshima, Japan). Dipeptides were purchased from Bachem AG (Bubendorf, Switzerland). Asp-Pro, Asn-Pro, Glu-Pro, Gln-Pro, and Cys-Pro were not supplied by manufacturers. Diprotin A (Ile-Pro-Ile) and Ala-Pro-*p*-nitroanilide (Ala-Pro-pNA) were also obtained from Bachem AG. A protein assay kit was obtained from Bio-Rad Laboratories Inc. (Berkeley, CA).

2.2. Cloning and extracellular production of human DPP-IV protein

Recombinant DPP-IV was prepared as described previously (Metzler et al., 2008). The human *dpp-IV* gene was prepared using nested PCR as follows. Human adult kidney cDNA (PCR Ready First Strand cDNA; Bio Chain, Hayward, CA) was used as a template. We performed a primary PCR with 0.2 μM primers (sense and antisense primers: 5′-ATGAAGACACCGTGGAAGGTTCTTCTGGGA-3′ and 5′-CTAAGGTAAAGAGAAACATTGTTTTATGAA-3′) and with the DNA polymerase Prime STAR HS obtained from Takara Holdings Inc. (Kyoto, Japan) using the following protocol: 30 cycles of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 2 min 30 s. Next, the *dpp-IV* gene was amplified using a secondary PCR similar to the primary PCR protocol, and using the primary PCR products as a template with 0.2 μM sense (5′-GAATTCCGCAAAACTTACACTCTAACTGAT-3′) and antisense (5′-GCGGCCGCTAGAGGTATTCCTGTAGAAAGTGC-3′) primers (the underlined areas represent the *Eco*RI and *Not*I sites, respectively). The 2.2-kbp PCR product was cloned into pCR–Blunt II-TOPO purchased from Invitrogen Corp. (Carlsbad, California) and sequenced. The *dpp-IV* gene was subcloned into pPICZαA (Invitrogen Corp.) at the *Eco*RI and *Not*I sites. The recombinant extracellular expression vector pPICzαA/DPP-IV was linearised using *Pme*I. The DNA was purified and transformed to *Pichia pastoris* KM71H (Invitrogen Corp.).

Transformants were screened in the presence of 50 μg/ml Zeocin (Invitrogen Corp.). Afterwards, 20 clones were obtained using the selection; one clone, A-5, was chosen for enzyme preparation. A colony of A-5 was inoculated into a 500-ml baffled flask containing 25 ml of BMGY with 25 μg/ml Zeocin and shaken at 250 rpm at 30 °C for 2 d. This inoculum was used to inoculate eight 500-ml baffled flasks, each containing 100 ml of BMGY with 25 μg/ml Zeocin. After being shaken for 2 d at 250 rpm at 30 °C, the cells were harvested by centrifugation at 2000*g* for 10 min, and the supernatant was removed. The cells were resuspended in 80 ml of BMMY with 1% methanol and 25 μg/ml Zeocin, placed into two new 500-ml baffled flasks, and shaken again at 250 rpm for 3 d with methanol added to 1% each day. Cells were then harvested by centrifugation at 2000*g* for 30 min. The supernatant was dialysed twice against 4.5 l distilled water. The dialysate was used as the enzyme preparation. Then, BMGY and BMMY were produced according to the manufacturer's instructions (http://www.tools.invitrogen.com/content/sfs/manuals/easyselect_man.pdf#search='Pichia protocol invitorogen').

2.3. DPP-IV activity

Among Xaa-Pro derivatives, Ala-Pro and Pro-Pro derivatives are reportedly suitable substrates for human DPP-IV (Edosada et al., 2006). Therefore, we chose Ala-Pro-pNA as a substrate for DPP-IV in this study. DPP-IV activity was determined using a final concentration 1.6 mM Ala-Pro-pNA in 0.1 M Tris–HCl (pH 7.5) at 37 °C. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol *p*-nitroaniline per min under the assay conditions. The assay was performed continuously at 405 nm (molecular extinction coefficient of pNA: $\varepsilon_{mM} = 10.6$). A kinetic study was conducted using 0.06–1.8 mM Ala-Pro-pNA in 0.1 M Tris–HCl (pH 7.5) at 37 °C. The activity and protein concentration of the enzyme preparation were determined as 0.29 u/ml and 0.12 mg/ml, respectively, and the K_m value toward Ala-Pro-pNA was estimated at 0.23 ± 0.02 mM.

2.4. Preparation of RB proteins

Defatted RB (25 g) was dispersed in 250 ml of distilled water adjusted to pH 12.5 with 1 g NaOH. The preparation was stirred at 45 °C for 2 h. The slurry was centrifuged at 2000*g* for 15 min. The pH of the supernatant liquid was further adjusted to 4.0 with 1 N HCl and it was centrifuged again at 2000*g* for 15 min. The solid residue (RB proteins) was collected and dried in a vacuum oven overnight at 40 °C. The protein content of the resultant RB proteins was ~75%.

2.5. Preparation of RB peptides

Two commercial proteases, Umamizyme G and Bioprase SP, were used. Umamizyme G (70 u/g) from *Aspergillus oryzae* was obtained from AMANO Enzyme Co. Ltd. (Nagoya, Japan). Bioprase SP (100,000 proteolytic units/g) from *Bacillus* sp. was a gift from NAGASE ChemteX Co. Ltd. (Osaka, Japan). RB proteins (2 g, 75% protein content) were dispersed in 40 ml of distilled water, adjusted to pH with 5 N NaOH, and incubated at 45–50 °C for 17 h with shaking. Each preparation was hydrolysed with a 1% (w/w) solution of each enzyme. The resultant hydrolysates were heated at 80 °C for 30 min and centrifuged at 2000*g* for 30 min to inactivate the proteases. The supernatants were freeze-dried and stored at 4 °C for further studies.

2.6. Inhibition assay

The inhibition assay was performed using a microplate reader (SH-8000Lab; Corona Electric Co. Ltd., Hitachi-Naka, Japan) and 96-well microtiter plates. Dried samples of the hydrolysates were dissolved and diluted to different extents with distilled water for the assay. A sample (10 μg) was added to 80 μl of 2 mM Ala-Pro-pNA in 0.1 M Tris–HCl (pH 7.5) in each well. Under the assay conditions, the samples were diluted to a final concentration between 0.31 and 5 mg/ml. Then, the mixture was pre-incubated at 37 °C for 5 min. The enzymatic reaction was initiated by adding 10 μl of DPP-IV stock solution. This mixture was incubated at 37 °C for 5 min as increased absorbance at 405 nm was measured every 10 s; the velocity of the enzymatic reaction was estimated according to the increase in absorption. The percentage of inhibition was determined relative to the velocity without dipeptides. The half-maximal inhibitory concentration (IC_{50}) values were calculated by plotting the logarithm of the concentration of the sample (mg/ml) against the inhibitory activity (%).

Using the synthetic peptides dissolved in distilled water at concentrations of 6.25–100 mM, the inhibition constants (K_i) and the type of inhibition were determined from Lineweaver–Burk and Dixon plots, respectively.

2.7. Gel filtration analysis

The molecular weight distributions of the RB peptides were determined by gel filtration analysis. The peptides were dissolved in 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl at 10 mg/ml. A sample (200 μg) was injected into a Superdex peptide column (GE Healthcare Japan, Tokyo, Japan) with the same buffer