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Systematic analysis of a dipeptide library for inhibitor development using human dipeptidyl peptidase IV produced by a *Saccharomyces cerevisiae* expression system

Aya Hikida [a,1], Keisuke Ito [a,b,c,1,*], Takayasu Motoyama [c], Ryuji Kato [d], Yasuaki Kawarasaki [a,b]

[a] Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan
[b] School of Food and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan
[c] Food Science Research Institute, Research & Development HQ, Fujioil Co., Ltd., Kinunodai 4-3, Tsukubamirai, Ibaraki 300-2497, Japan
[d] Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Furocho, Chikusaku, Nagoya 464-8601, Japan

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ABSTRACT

The inhibition of human dipeptidyl peptidase IV/CD26 (hDPPIV) is an accepted treatment for type 2 diabetes. In this study, an extracellular production system of hDPPIV using *Saccharomyces cerevisiae* was established to facilitate the screening of hDPPIV inhibitors. As dipeptides that mimic the hDPPIV substrate are candidate inhibitors of this protein, X–Ala or X–Pro dipeptides (in which X represents any amino acid) were tested systematically. Based on the results obtained in the first screening, a second screening was performed for Trp–X dipeptides. To elucidate the manner via which the physicochemical features at the P_1 and P_2 positions contributed to the hDPPIV inhibitory effect, correlations between the inhibitory activity of dipeptides and 13 amino acid indices were analyzed. The most effective inhibitory dipeptide was Trp–Pro (K_i = 0.04 mM). The mode of inhibition of hDPPIV by dipeptides was explained well by some amino acid indices and by the structure of the substrate-binding site of hDPPIV. The information obtained from the systematic analysis of a dipeptide library provides important clues for the development of hDPPIV targeting drugs and functional foods for type 2 diabetes.

1. Introduction

Human dipeptidyl peptidase IV/CD26 (hDPPIV) is a transmembrane serine protease that is widely expressed in many tissues and cells, including the kidney, lung, adrenal gland, jejunum, liver, glandula parotis, spleen, and testis [1]. hDPPIV is responsible for the degradation of many endogenous peptide hormones, including the glucagon-like peptide 1 (GLP-1) [1]. The biological activities of GLP-1 include: (i) stimulation of insulin secretion, (ii) promotion of beta cell neogenesis, (iii) inhibition of beta cell apoptosis, and (iv) inhibition of glucagon secretion [2–4]. In patients with type 2 diabetes, the insulinotropic response to GLP-1 is typically intact, with deficient circulating levels of postprandial GLP-1. Thus, hDPPIV inhibitors provide a new strategy for the treatment of type 2 diabetes: sitagliptin, vildagliptin, saxagliptin, and alogliptin have been used as clinical treatments [5].

Although some specific studies have shown that certain compounds act as hDPPIV inhibitors, there are few reports on hDPPIV inhibitory peptides generated from a naturally occurring protein [6,7]. The hDPPIV inhibitory effect of oligopeptides leads to their possible use as functional food ingredients that can be used to prevent type 2 diabetes. To develop hDPPIV inhibitory peptides, a dipeptide library was screened in this study. Because hDPPIV generates a product dipeptide via the hydrolysis of a substrate peptide, oligopeptides, which mimic the substrate, are candidate inhibitors of this protein [8]. Moreover, the systematic analysis of a dipeptide library has another advantage: the biological function of dipeptides can be attributed easily to the physicochemical features of amino acid residues because of their simple structure. Various amino acid features are available for this type of analysis (Supplemental Fig. S1). Therefore, obtaining information from systematic analyses of a dipeptide library is useful for the development of drugs for type 2 diabetes.

Some hDPPIV expression systems have been developed to facilitate the screening of inhibitors of this protein [9,10]. However, a *Saccharomyces cerevisiae* expression system is not available cur-

Abbreviations: hDPPIV, human dipeptidyl peptidase IV; GPNT, glycyl-L-proline-p-nitroanilide p-tosylate.

* Corresponding author at: Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan. Fax: +81 54 264 5540.
E-mail address: sukeito@u-shizuoka-ken.ac.jp (K. Ito).

[1] The first two authors contributed equally to this work.

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rently. This expression system is one of the most well-characterized experimental systems in modern biology and exhibits advantages as a high-throughput analysis system [11]. We previously reported an advanced system that was useful for the expression of proteins bearing various mutations [12]. A taste-modifying protein, miraculin, was expressed successfully via the optimization of the secretion signal sequence, which is an application of the mutation method, and its structure–function relationship was analyzed [13]. In addition to high-speed performance, yeast cells have the ability to select folded proteins because the yeast secretory pathway is characterized by the presence of a quality control system that prevents the release of misfolded or incompletely-folded proteins [14]. These characteristics of the yeast expression system are suitable for the production of recombinant hDPPIV for inhibitor screening. In the present study, we report the systematic analysis of a dipeptide library using recombinant hDPPIV produced using a *S. cerevisiae* expression system.

2. Materials and methods

2.1. Materials and chemicals

Glycyl-L-proline-p-nitroanilide p-tosylate (GPNT) was purchased from Peptide Institute Inc. (Osaka, Japan). Dipeptides were purchased from Anaspec (California, USA). Trypton (casein peptides) was obtained from BD Bioscience (New Jersey, USA). Hinute-AM (soy peptides) was procured from Fujioil (Osaka, Japan). The hDPPIV cDNA (GenBank: AB590646) was purchased from Promega (Wisconsin, USA). The *S. cerevisiae* strain FGY217 (*MATa*, *ura3-52*, *lys2Δ201*, *pep4Δ*) was used as the host for the extracellular production of hDPPIV [12].

2.2. Construction of an hDPPIV expression plasmid

The 12 yeast signal sequences were selected according to the report by Sahara et al. [15]. DNA fragments of the signal sequences were synthesized. To obtain the PCR fragment of hDPPIV, the gene-specific primers *GGTGGTGGTGATTATAAAGATGATGATGATAAA* **GCTGACAGTCGCAAAACTTAC** and *AAATTGACCTTGAAAATATAAAT TTTCCCC***TCAAGGTAAAGAGAAACATTGTTTTATG** were used. Primers contained a gene-specific region (bold) and a homologous region (italic). The PCR fragment, the signal sequence, and the *SmaI*-linearized pRS426 GAL1 vector were cotransformed into *S. cerevisiae* strain FGY217. These fragments and vector were then linked at a specific gene region encoding a 3× glycine and FLAG tag (GGG DYKDDDDK) via homologous recombination [13]. After the cotransformation, cells were spread on a Ura$^-$ selection plate (2% agar, 0.2% yeast synthetic drop-out medium without uracil, 0.67% yeast nitrogen base without amino acids, and 2% glucose). The transformants were cultured at 30 °C for 48 h.

2.3. Extracellular production of hDPPIV

For the expression of hDPPIV, transformants were selected and grown in Ura$^-$ selection medium (0.2% yeast synthetic drop-out medium without uracil, 0.67% yeast nitrogen base without amino acids, and 2% glucose). After precultivation at 30 °C for 24 h, cells were harvested by centrifugation, resuspended in an equal volume

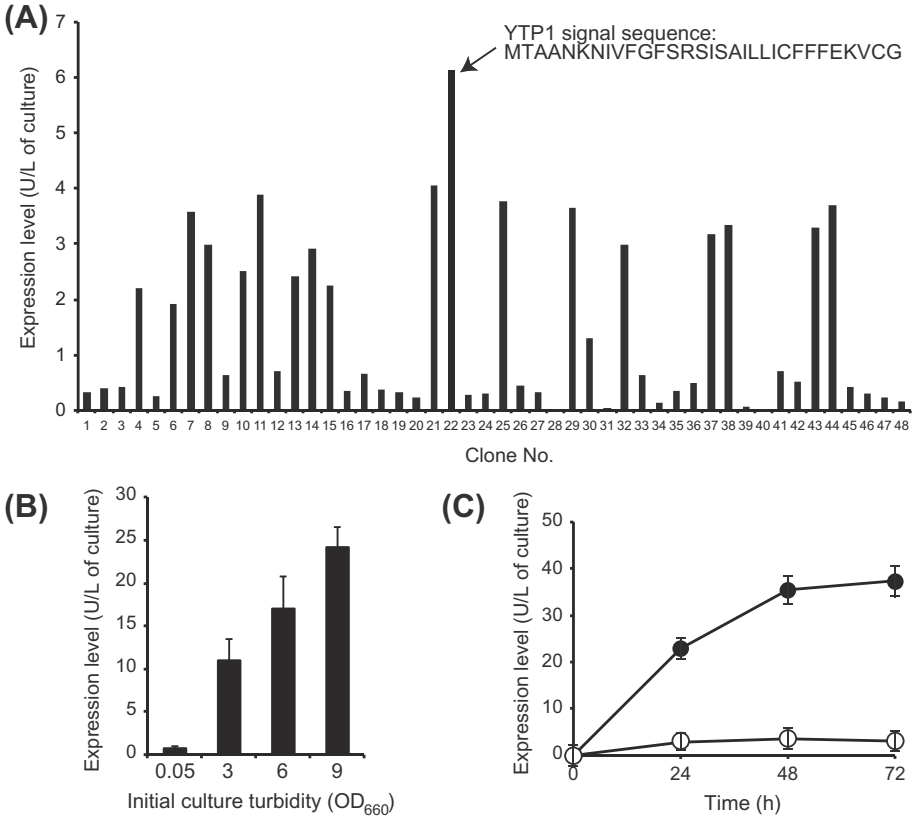

Fig. 1. Improvement of hDPPIV production level in the *S. cerevisiae* expression system. (A) Optimization of the secretion signal sequence for the extracellular production of hDPPIV. The optimization method is described in Section 2. (B) Effect of initial cell density. The cells resting in the post exponential growth phase in the preculture (OD_{660} = 3) were harvested and suspended in the induction medium, to give the depicted cell concentrations. The supernatants of cultures carried out at 20 °C for 24 h were subjected to the hDPPIV assay. (C) Time-course analysis of hDPPIV production at 20 °C. The hDPPIV activity from the cells suspended in the induction medium to give an OD_{660} = 9 was measured at the indicated time points. Black circle, hDPPIV expressing clone; white circle, parental strain (FGY217). The expression levels are presented as means ± SD (n = 3).

of induction medium [100 mM Na–phosphate buffer (pH 6.5), 0.2% yeast synthetic drop-out medium without uracil, 0.67% yeast nitrogen base without amino acids, and 2% galactose] at a final OD_{660} of 3, and grown at 20 °C for 24 h. The culture supernatant was collected for enzymatic analysis.

2.4. Enzymatic assay

Kinetic experiments were conducted at 37 °C in 100 mM Na–phosphate buffer, pH 8.0. hDPPIV-catalyzed GPNT hydrolysis was quantified by measuring the absorbance of p-nitroaniline at 405 nm [9]. One unit of hDPPIV activity was defined as the amount of enzyme that produces 1.0 mM of p-nitroaniline from GPNT/min. The k_m value was calculated using a Lineweaver–Burk plot. The K_i values of inhibitors were calculated using the Cheng–Prusoff equation from the k_m of GPNT and the IC_{50} of the inhibitor [16].

2.5. Dipeptide library screening

An assay system using 96-well plates was established for the high-throughput screening of hDPPIV inhibitors. Each well of the 96-well plate contained 100 mM Na–phosphate buffer (pH 8.0), 1 mU hDPPIV, 0.35 mM GPNT, and 1 mM test inhibitor (dipeptide). For the second screening, a 0.5 mM test inhibitor (dipeptide) was used. After incubation at 37 °C for 60 min, the hydrolysis of the substrate was measured using a SpectraMAX 190 apparatus (Molecular Devices, California, USA). The inhibition ratio (%) was calculated using the following equation: inhibition ratio (%) = $100 \times [(\Delta A_{405}$ of the negative control $- \Delta A_{405}$ of the test well)/ ΔA_{405} of the negative control].

2.6. Elucidation of contributing factors to the inhibitory effect of dipeptides

Independent amino acid indices for the elucidation of inhibitory effect of dipeptides were selected by correlation analysis of all candidate indices. Variables for conversion of peptide sequences were obtained from the AAindex1 reported in Genome Net Japan, organized by Kyoto University (http://www.genome.jp/dbget-bin/www_bfind?aaindex1) [17]. In the database (version 9.1, as of Aug 2006), 544 amino acid indices registered were found to consist of 21 clusters that have high correlation by average linkage hierarchical clustering using Cluster 3.0, distributed from University of Tokyo, Human Genome Center (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm). From all clusters, 13 independent clusters covering the majority of indices were selected as independent amino acid descriptors that provide characteristic physicochemical properties of residues. Other clusters that had small members with uninterpredictable indices were eliminated. From each 13 cluster, single index was selected as a representative index for the elucidation of dipeptide interaction [18–27].

3. Results and discussion

3.1. Functional expression of hDPPIV

S. cerevisiae has the disadvantage of yielding relatively low expression levels of recombinant protein compared with other hosts, with less extracellular production of endogenous proteins. To overcome this weakness, we focused on the secretion signal sequence as the first step in the construction of the system. hDPPIV is naturally expressed as a type II transmembrane protein. Hu et al. removed the transmembrane domain located at the N terminus by amplifying the coding region between amino acid residues 37 and 766 [9]. Instead, here, the yeast secretion signal sequence was added to the N terminus of hDPPIV using a method described previously [13]. To improve the expression level of hDPPIV, a signal sequence that maximizes the heterologous production of hDPPIV was screened. Approximately 5×10^2 colonies per experiment were obtained by cotransformation of the plasmid backbone with two gene fragments: a mixture of a signal-sequence repertoire and the hDPPIV fragment. Forty-eight clones were selected randomly from the plate and the hDPPIV expression levels were analyzed (Fig. 1A). Nineteen clones showed relatively higher hDPPIV activity (<1 U/L of culture). The signal sequences of these clones were confirmed by DNA sequencing. The best signal sequence was that from YTP1, a type III integral membrane protein of unknown function [28]. Under expression screening conditions, the use of the YTP1 signal sequence increased the expression level of hDPPIV to 6.13 U/L of culture.

To improve the expression levels of hDPPIV, we performed a subsequent optimization of the culture parameters, including cell density, induction time, and temperature. According to our previ-

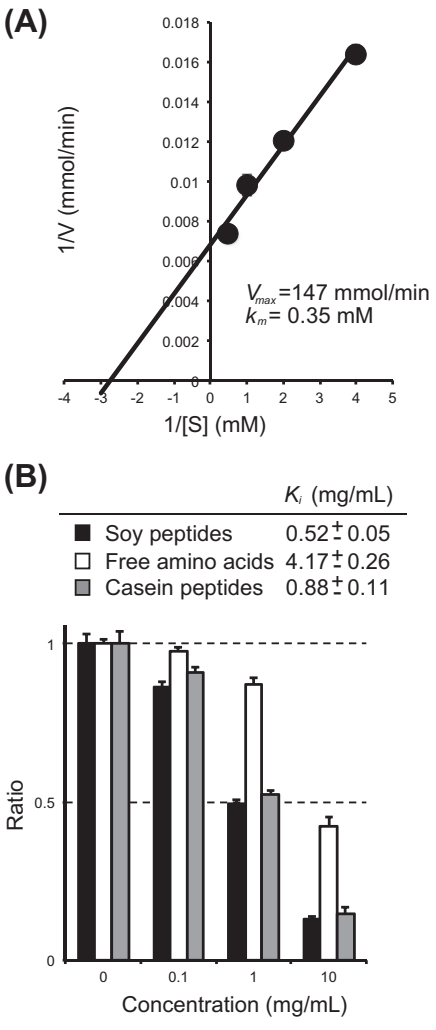

Fig. 2. Kinetic analysis of recombinant hDPPIV. A kinetic analysis of the enzymatic properties of recombinant hDPPIV was conducted using GPNT as a substrate. (A) Lineweaver–Burk plot of GPNT hydrolysis. The line shown was determined by linear regression of the reciprocal data. (B) Inhibitory effect of food peptides on hDPPIV. The degree of inhibition of hDPPIV activity by each sample is shown as a ratio of the control. The K_i values were calculated using the Cheng–Prusoff equation [16]. Black columns, soy peptides; white columns, free amino acids that were composed by reagent-grade free amino acids similar to the soy peptides; gray columns, casein peptides. Data are presented as means ± SD (n = 3).

ous report on the efficient production of *Lentinula edodes* laccase using *S. cerevisiae*, the effect of initial cell density on hDPPIV production was evaluated based on activity (Fig. 1B) [29]. The precultured cells were suspended in the expression medium at various concentrations and allowed to produce hDPPIV for 24 h at 20 °C. The efficient production of hDPPIV was observed only when the cells were inoculated at high cell density (initial $OD_{660} \geqslant 3$). The hDPPIV production per cell [hDPPIV expressed (U/L of culture) per initial OD_{660}] was also high in the high-cell density cultures, suggesting that the individual cells in the dense culture showed a high performance regarding hDPPIV expression. Induction using an initial $OD_{660} = 9$ was used as the optimized condition in subsequent culture. The induction temperature and period were also investigated (Fig. 1C). The activity of hDPPIV was not detected in the supernatant at an induction temperature of 30 °C. The optimized culture condition was 20 °C for 48 h. In this condition, hDPPIV was successfully expressed in culture medium with 35 U/L of culture and the GPNT hydrolysis signal by the parental strain FGY217 was negligible (data not shown).

3.2. Enzymatic analysis of the recombinant hDPPIV

Enzymatic analysis of the recombinant hDPPIV was performed using GPNT as a substrate. The hydrolysis reaction was dependent on substrate concentration and its kinetic parameters were estimated (Fig. 2A). The k_m and V_{max} of GPNT hydrolysis by hDPPIV were 0.35 and 147 mmol/min, respectively.

The inhibitory effects of soy protein and milk protein hydrolysates on hDPPIV activity were also analyzed (Fig. 2B). The soy peptides, of which 65% of the weight consisted of di- or tripeptides from soy protein, showed a significant hDPPIV inhibitory effect in a concentration-dependent manner ($K_i = 0.52$ mg/mL), whereas the free amino acid mixture with the same amino acid composition as the soy peptides had an inhibitory effect that was 0.12 times lower ($K_i = 4.17$ mg/mL). Casein peptides also exhibited an hDPPIV inhibitory effect, with a K_i value that was 0.59 times higher than that of the soy peptides. These results suggest that the di- or tripeptides from soy protein have a potent inhibitory effect on hDPPIV.

3.3. Systematic analysis of a dipeptide library for the development of hDPPIV inhibitors

hDPPIV recognizes N-terminal X–Ala or X–Pro (in which X represents any amino acid) residues in the substrate polypeptide and liberates the N-terminal dipeptide [1]. Although the catalytic activity of hDPPIV depends strongly on the amino acids Pro, Ala, Ser, or Gly at the P_1 position, the amino acid residue at the P_2 position affects its enzymatic activity to a lesser extent [1,8]. Because substrate-mimicking oligopeptides are candidate hDPPIV inhibitors, X–Ala or X–Pro dipeptides were tested to identify specific dipeptides that exhibit the highest inhibitory effect on hDPPIV activity (Fig. 3A). We found that the dipeptides Ile–Ala, Leu–Ala, Val–Ala, Trp–Ala, Ile–Pro, Lys–Pro, Gln–Pro, Val–Pro, Trp–Pro, and Tyr–Pro showed hDPPIV inhibitory effects (inhibition ratio, >50%). In particular, Trp–Ala, Ile–Pro, Val–Pro, and Trp–Pro exhibited a relatively higher level of inhibition compared with the remaining dipeptides (inhibition ratio, >75%). Subsequently, the inhibitory modes of these dipeptides were analyzed in detail. The K_i values of these dipeptides were: Trp–Ala, 0.05 mM; Ile–Pro, 0.30 mM; Val–Pro, 0.35 mM; and Trp–Pro, 0.04 mM (Fig. 3B). The Lineweaver–Burk plots for these dipeptide inhibitors showed that all dipeptides increased the apparent k_m for GPNT hydrolysis, but had no effect

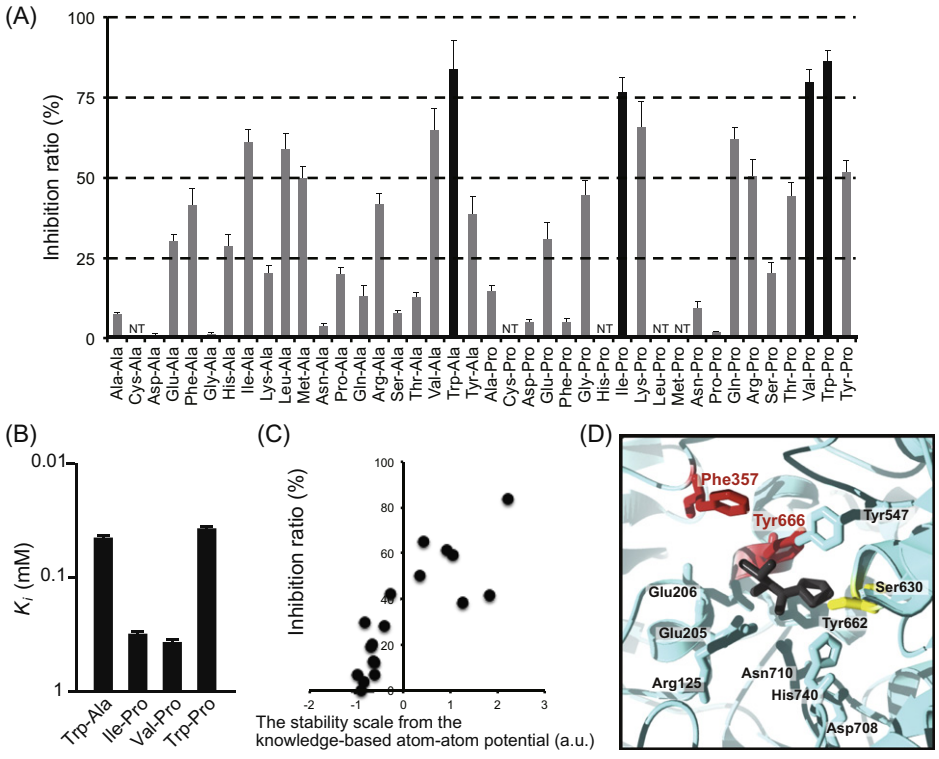

Fig. 3. First screening of the dipeptide library for hDPPIV inhibitors. (A) X–Ala and X–Pro dipeptide library screening. The degree of inhibition of hDPPIV activity by each sample is shown as a ratio (%) of the control. (B) K_i values of inhibitory dipeptides for hDPPIV. NT, not tested. Data are presented as means ± SD ($n = 3$). (C) Correlation between the inhibition ratio and the amino acid index "stability scale from the knowledge-based atom–atom potential" at the P_2 position of X–Ala. The data resulting from the correlation analysis with the other indices are shown in Supplemental Figs. S2 and S3. (D) Structure of the substrate-binding site of the hDPPIV–ligand complex (PDB: 1N1M) [10]. The competitive inhibitor Val–Pyr, the putative residues that interact with the P_2 position of inhibitory dipeptides, and the catalytic residue Ser are shown in black, red, and yellow, respectively.

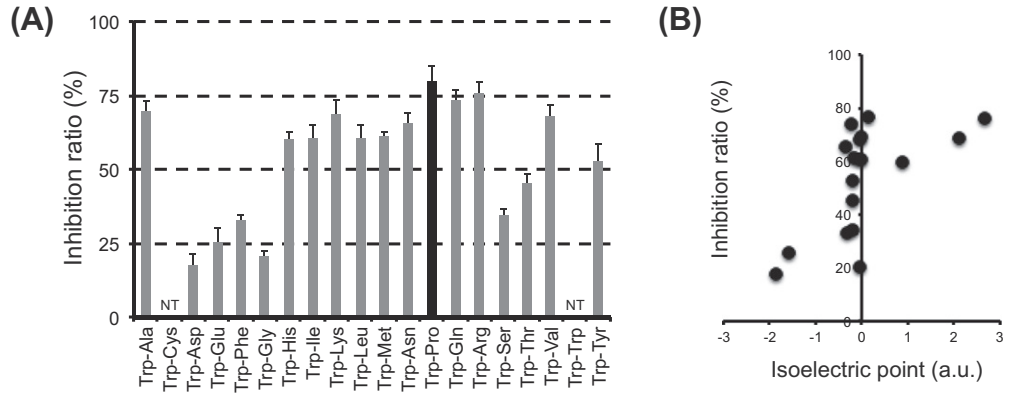

Fig. 4. Second screening of dipeptide library for hDPPIV inhibitors. (A) Trp–X dipeptide library screening. The concentration of inhibitory dipeptide used in this screening was 0.5 mM, which is half that used in the X–Ala and X–Pro dipeptide library screening. (B) Correlation between the inhibition ratio and the amino acid index "isoelectric point" at the P_1 position of Trp–X. The data resulting from the correlation analysis with the other indices are shown in Supplemental Fig. S4.

on the apparent V_{max}, indicating that they were competitive inhibitors. To elucidate the manner via which physicochemical features at the N-terminal P_2 position of dipeptides contributed to the hDPPIV inhibitory effect, correlations between inhibition ratios and 13 amino acid indices were analyzed (Supplemental Figs. S2 and S3). Two indices, "side-chain contribution to protein stability" and "stability scale from the knowledge-based atom–atom potential," were positively correlated with the inhibition ratios of the X–Ala and X–Pro dipeptides (Fig. 3C, Supplemental Figs. S2 and S3). This result indicates that the aromatic ring located at the P_2 position is a major contributor to the inhibitory effect of the molecule (Supplemental Fig. S1). Typically, the K_i of the Trp–Pro dipeptide was the lowest value among the X–Pro group (Fig. 3A and B). The crystal structure of the hDPPIV–ligand complex shows that the Val moiety located at the P_2 position of the competitive inhibitor Val–Pyr is located with the two aromatic amino acid residues of hDPPIV, Phe357 and Tyr666 (Fig. 3D) [10]. These amino acid residues of hDPPIV also contributed to the binding of Tat-driven nonapeptides with hDPPIV inhibitory effect [30]. Thus, it is plausible that the interaction between these aromatic rings and the side chain at the P_2 position amino acid is important for the competitive binding of dipeptides.

Based on the results obtained in the first screening, the amino acid residue located at the C-terminal P_1 position was changed in the second screening, whereas the Trp residue located at the N-terminal P_2 position was fixed (Fig. 4A). The dipeptides Trp–Ala, Trp–His, Trp–Ile, Trp–Lys, Trp–Met, Trp–Asn, Trp–Pro, Trp–Gln, Trp–Arg, Trp–Val, and Trp–Try exhibited an inhibitory effect on hDPPIV (inhibition ratio, >50%). In particular, Trp–Pro and Trp–Arg yielded higher inhibition compared with the remaining dipeptides (inhibition ratio, >75%). The most effective inhibitory dipeptide that was optimized at both the P_1 and P_2 positions was Trp–Pro (K_i = 0.04 - mM). This suggests that the interaction at the P_1 position of the dipeptide inhibitor is similar to that of the substrate. Although the pyrrolidide moiety of Val–Pyr is buried in a hydrophobic pocket (Fig. 3D) [10], a correlation between this dipeptide and any indices including "hydropathy index" and inhibitory ratio, was not observed (Supplemental Fig. S4). Conversely, the hDPPIV inhibitory activity was significantly affected by the charged amino acid residue located at the P_1 position (Fig. 4B). Two negatively charged residues, Glu205 and Glu206, are located at the ligand-binding site of hDPPIV (Fig. 3D), and a single mutation of either of them abolishes the enzymatic activity of the protein [31]. The side chains of the positively charged cluster extend into the P_1 position, suggesting a role for charged residues located at the P_1 position in the hDPPIV inhibitory effect. This information provides a new insight into the development of peptide-type inhibitors. Oligopeptides that have an Arg at the P_1 position likely act as undigestable inhibitors. The hDPPIV inhibitory dipeptides identified in this study can be used as functional food ingredients to prevent type 2 diabetes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.073.

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