In type 2 diabetic mellitus, glucose-dependent insulino-}
notropic polypeptide (GIP) secretion is increased, but its
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result of reduced GIP receptor expression or reduced
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demonstrated a progressive decline in postprandial glu-
cagon-like peptide-1 (GLP-1) secretion, where individu-
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ment in GLP-1 secretion (3, 4). This difference has been
called the incretin effect. Although GLP-1 has been used
as a therapeutic drug, the clinical utility of native GLP-1
is limited by its short half-life (within 2 min) due to its
rapid degradation to inactive metabolites by the enzyme
dipeptidyl peptidase-4 (DPP-4) (5 – 8). Therefore, DPP-
4 inhibitor is also useful target for the therapy of type 2
diabetic mellitus. We already tested the effects of alpha-lac-
toalbumin and trypsin-treated alpha-lactoalbumin on
DPP-4 activity and found that these proteins did not show
any inhibitory effect on DPP-4. However, trypsin-treated
β-lactoglobulin showed a concentration-dependent inhibi-
tion for dipeptidyl peptidase-4, with an IC50 value of 210 μM, although non-treated β-lactoglobulin showed no significant effect in the in vitro assay. The active peptide was isolated from trypsin-treated β-lactoglobulin and identified as the hexapeptide Val-Ala-Gly-Thr-Trp-Tyr (β-lactoglobulin f15-20). This hexapeptide also exhibited a concentration-dependent inhibitory effect and IC50 value was 174 μM, suggesting that this hexa-
peptide is almost totally responsible for the DPP-4 inhibitory activity of trypsin-treated β-lacto-
globulin.

**Keywords:** DPP-4 inhibitor, β-lactoglobulin, Val-Ala-Gly-Thr-Trp-Tyr

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β-lactoglobulin showed inhibitory effect on it. Then, we
tested the hypoglycemic efficacy of trypsin-treated
β-lactoglobulin in the oral glucose tolerance test using
mice. Next we isolated the hypoglycemic peptide having
DPP-4 inhibiting activity and identified the active amino
acid sequence.

Test samples were prepared as follows: β-Lactoglobulin
(Davisco Foods International Inc., Eden Prairie, MN,
USA) dissolved in 0.01 M Tris-HCl buffer at a concentration of 1 g/20 ml was digested by adding 10 mg of trypsin (Wako Pure Chemical Industries, Ltd., Tokyo) for 24 h at 37°C. Digestion was stopped by heating for 5 min at 90°C. The reaction mixture was lyophilized.

The following animal studies were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Meiji Co., Ltd. C57BL/6 mice weighing about 17 g (7-week-old) were purchased from Japan SLC, Inc. (Shizuoka) and fasted for one night before the experiment. Trypsin-treated β-lactoglobulin was dissolved in 0.01 M Tris-HCl buffer and orally administered 30 min before the oral glucose tolerance test at a dose of 300 mg/kg (0.1 ml/10 g body weight). Sitagliptin phosphate hydrate (Januvia; MSD K.K., Tokyo) was used as a positive control, and it was suspended in 0.01 M Tris-HCl buffer and orally administered 1 h before oral glucose tolerance test at a dose of 3 mg/kg (0.05 ml/10 g body weight). Glucose was dissolved in distilled water and administered orally at a dose of 10 g/kg (0.1 ml/10 g body weight). Blood glucose levels were measured with Glucometer DEX (Bayer Health Care, Tarrytown, NY, USA) before and at 30, 60, 90, and 120 min after glucose administration. In the control mice, 0.01 M Tris-HCl buffer was administered instead of sample. Area under the curve of glucose (AUC120min) values (mg/dL·min) was calculated from the values of blood glucose level.

The DPP-4 inhibitory assay was performed as follows: To determine the potential of direct inhibitory activity against DPP-4, human standard serum (Liquid control serum I Wako, Wako Pure Chemical Industries, Ltd.) was used as a DPP-4 enzymatic source. Gly-Pro-p-nitroaniline (Gly-Pro-pNA) and p-nitroaniline were used as the substrate and standard, respectively. Gly-Pro-pNA and p-nitroaniline were dissolved in Tris-HCl buffer (pH 8.0). After mixing 50 μl of plasma with 50 μl of test sample on the microplate, 100 μl of 1 mM Gly-Pro-pNA solution was added to the mixture and incubated at 37°C. After 30 min, absorbance was measured at 405 nm with a microplate spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA, USA). In the control, 50 μl of Tris-HCl buffer (pH 8.0) was added instead of test sample. Inhibitory percentage was calculated from the ratio of the

![Graph A](image)

**Fig. 1.** Effects of trypsin-treated β-lactoglobulin and sitagliptin phosphate hydrate on the blood glucose level (mg/dL, A) and AUC120min values (mg/dL·min, B) after oral glucose administration in mice. Values represent the mean ± S.E.M. of 10 mice. **: Significant difference from the control group ($P < 0.01$).
absorbance of test sample against the control absorbance.

The active substance was isolated as follows: Trypsin-treated β-lactoglobulin was dissolved in distilled water. The solution was then subjected to 5-μm BEH130 PREP C18 column chromatography (150 mm × 10 mm i.d.; Waters Corporation, Milford, MA, USA) initially equilibrated with 0.1% trifluoroacetic acid using a 600E HPLC separation module (Waters). Elution was performed by a linear gradient system. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was 90% acetonitrile containing 0.1% trifluoroacetic acid. A linear gradient from 5% to 50% B in 30 min then to 100% B in 15 min was used. The column was eluted at a flow rate of 4.0 ml/min. Elution of peptide was monitored at 215 nm. The active fraction whose retention time was from 16 to 22 min was further purified on a 150 mm × 10 mm i.d., 5 μm BEH 130 PREP C18 column using a 600E HPLC separation module. Elution was performed by a linear gradient system. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was 90% acetonitrile containing 0.1% trifluoroacetic acid. A linear gradient from 15% to 30% B in 30 min then to 100% B in 5 min was used. Flow rate was 4 ml/min.

Results were presented as the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using a Dunnett’s multiple-comparison test and P values < 0.05 were considered statistically significant.

In the control mice, blood glucose level was 90.3 ± 2.4 mg/dL at the initial time, increased at 30 min, and decreased thereafter (Fig. 1). Trypsin-treated β-lactoglobulin and sitagliptin phosphate hydrate significantly decreased the blood glucose level as compared with the control at 30 min (P < 0.01, Fig. 1A), but β-lactoglobulin did not show any significant effect (data not shown). Trypsin-treated β-lactoglobulin and sitagliptin phosphate hydrate also significantly lowered AUC120min values as compared with the control whose value was 22201 ± 852 mg/dL·min (P < 0.01, Fig. 1B).

The reversed phase liquid chromatography elution profile of β-lactoglobulin digested by trypsin showed several peaks, which demonstrate that β-lactoglobulin digested by trypsin was relatively complex. Then, we separated five fractions from the retention time. The active fraction for inhibiting DPP-4 activity was obtained at 16 – 22 min of retention time. The active fraction was fractionated again using reverse-phase HPLC. The active large peak was found at 16 – 18 min of retention time. This peak was analyzed by mass spectrometry, and its molecular weight was found to be 696.6. From the sequence of β-lactoglobulin, this peak was found to be Val-Ala-Gly-Thr-Trp-Tyr. Authentic Val-Ala-Gly-Thr-Trp-Tyr showed the same retention time.

Trypsin-treated β-lactoglobulin concentration-dependently inhibited the DPP-4 activity as shown in Fig. 2A and IC50 value was 210 μM, but β-lactoglobulin did not. Authentic Val-Ala-Gly-Thr-Trp-Tyr also concentration-dependently inhibited DPP-4 activity. The IC50 value of this peptide was 174 μM as shown in Fig. 2A. Sitagliptin phosphate hydrate showed concentration-dependent inhibition on DPP-4 activity, and its IC50 value was 19.6 nM, being extremely stronger than the hexapeptide (Fig. 2B).

Gannon et al. (13) reported that milk was a particularly potent insulin secretagogue; the observed insulin response was approximately 5-fold greater than would be anticipated from the glucose response. Östman et al. (11) also observed the same finding that milk products appear more insulinotropic than that expected from the corresponding glycemic indexes, and Nilsson et al. (12) that the whey fraction contains the predominating insulin secretagogue.

In this study, trypsin-treated β-lactoglobulin significantly lowered the blood glucose level in the oral glucose tolerance test using mice. This datum may support the report that milk proteins, in particular the whey fraction,
have a stimulating effect on insulin secretion in healthy subjects (9, 14). Trypsin-treated β-lactoglobulin showed a concentration-dependent inhibition of DPP-4 and the IC₅₀ value was 210 μM, suggesting that trypsin-treated β-lactoglobulin has a DPP-4 inhibitory activity. Then, we tried to identify the active peptide and found that it was Val-Ala-Gly-Thr-Trp-Tyr whose amino acid sequence was β-lactoglobulin f15-19. The IC₅₀ value of this peptide was 174 μM. These findings show that DPP-4 inhibiting activity of Val-Ala-Gly-Thr-Trp-Tyr could essentially account the action of trypsin-treated β-lactoglobulin.

Human native GLP-1 has the six N-terminal amino acid sequence of His-Ala-Glu-Gly-Thr-Phe. In this study we obtained the hexapeptide of Val-Ala-Gly-Thr-Trp-Tyr from β-lactoglobulin treated with trypsin. The second amino acid of each was the same, Ala. Furthermore, the Gly-Thr sequence was also contained in both 4 – 5 of native human GLP-1 and 3 – 4 of Val-Ala-Gly-Thr-Trp-Tyr. This structural resemblance is critically interesting. Indeed, the next amino acids of dipeptide (Gly-Thr) are also both aromatic amino acids.

In conclusion, we found a hexapeptide having the inhibitory activity against DPP-4 from β-lactoglobulin, which is a major protein of cow milk. These trypsin-treated β-lactoglobulin and hexapeptide may be useful functional materials for the therapy or prevention of type 2 diabetic mellitus.

References


