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Novel Tripeptides with α-Glucosidase Inhibitory Activity Isolated from Silk Cocoon Hydrolysate

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ABSTRACT: Active compounds with antidiabetic potential were isolated from silk peptide E5K6 by consecutive ultrafiltration and gel filtration using Biogel P-2 and RS-HPLC using a YMC-Pack Pro C18 column. The highest α-glucosidase inhibitory activity of silk peptide E5K6 resulted from fractions with MW <1 kDa. The activities of gel-filtered fractions from silk peptide E5K6 of <1 kDa were assayed in vitro, demonstrating that the fourth peak (F4) had the highest α-glucosidase inhibitory activity (IC_{50} = 37.1 mg/mL). F4 of silk peptide E5K6 was separated by HPLC into two peaks. Moreover, the purified compounds were identified as Gly-Glu-Tyr (GEY, MW = 367 Da) and Gly-Tyr-Gly (GYG, MW = 295 Da) according to amino acid sequences, and their α-glucosidase inhibitory activities (IC_{50}) were 2.7 and 1.5 mg/mL, respectively.

KEYWORDS: cocoon, silk peptide E5K6, diabetes, α-glucosidase

INTRODUCTION

The silkworm (*Bombyx mori*) spins silk threads to make a cocoon shell, where some pigments coexist and accumulate in the layers of the cocoon. Until recently, silk processing from cocoons to finished clothing has consisted of a series of steps. Since the 1970s, many bioactive peptides derived from food protein hydrolysates have been studied as potential nutraceuticals. On this basis, attention has recently increased to the application of silk peptides in the biotechnological and biomedical fields.[1,2]

Silk peptide, which is the hydrolysate of silk protein derived from cocoons, has been employed as a biomedical suture material and is believed to be safe for humans. Silk peptides consist of different peptide sizes, having peptides that are 18−20 amino acids in length, while enzymatic degradation results in specific sizes or compositions of peptides that exert diverse bioactivities, such as hypocholesterolemic, antioxidant, immunoregulatory, antitumor, antiviral, and antibacterial effects.[3−5] Many earlier investigations demonstrated that silk peptide influences blood glucose and insulin,[6,7] even though the mechanism underlying this antidiabetic activity has not been explored completely. In our previous study, we tested the effects of silk peptide on the insulin sensitivity of 3T3-L1 adipocytes and found that it increased glucose uptake and metabolism in these cells, suggesting that the hypoglycemic effects of silk peptide in *ob*/*ob* mice are a result of increased extrahepatic glucose uptake.[7] In this regard, silk peptide may be a potent natural supplement for lowering blood glucose. Several antidiabetic compounds such as acarbose, catechins, and plant extracts, which can decrease glucose levels in blood, not only influenced increased glucose uptake and secretion of insulin, but also α-glucosidase inhibitory activity.[8−10] Hu et al.[11] showed that the elastase hydrolysate of silk fibroin has α-glucosidase inhibitory activity and that the hydrolyzed fragments with molecular weights under 6 kDa exerted prominent inhibiting effects on mouse α-glucosidase.

Our research team reported on the antidiabetic activity of silk peptide from cocoons but did not identify the potential antidiabetic compounds. These compounds must be identified to explain the mechanism of the peptide. Therefore, in the present study, antidiabetic compounds in silk peptide were purified and identified using α-glucosidase inhibitory activity. This assay is an important tool for studying the mechanisms of action of compounds and examining prospective therapeutic components for certain degenerative diseases such as diabetes.

MATERIALS AND METHODS

Preparation of Silk Peptide E5K6. Silk peptide E5K6 was prepared from the cocoons of *B. mori*. Briefly, a 90 g portion of cocoons was cut into small pieces and boiled in 1000 mL of 50% (w/v) Ca_2Cl_2 for 6 h at 111 ± 5 °C and then filtered through filter paper. The solution was clarified by an ion exchange membrane electrodialyzer (Asahi Kasei Co., Kawasaki, Japan). A desalted solution was used to carry out the proteolytic reaction using protease N (Amano Enzyme Inc., Nagoya, Japan) at 53−55 °C for 3 h. The hydrolysates were immediately heated in a boiling water bath for 10 min and finally lyophilized.[12]

***α*-Glucosidase Inhibitory Activity.** α-Glucosidase was measured using a slightly modified method of Ohta et al.[13] A 0.1 mL amount of the silk peptide E5K6 fraction, 1 mL of 0.1 M potassium phosphate buffer (pH 6.8), 0.2 mL of *p*-nitrophenyl-α-D-glucopyranoside (NPG; 50 mM in 0.1 M potassium phosphate buffer), and 0.2 mL of α-glucosidase solution (0.15 U/mL of the buffer) were combined, and the mixture was incubated for 60 min at 37 °C. The reaction was terminated by 1.5 mL of 0.25 M sodium carbonate. The supernatants were transferred to a microplate, and absorbance (OD) was read at 405 nm.

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The α-glucosidase inhibitory activity of the silk peptide E5K6 fraction was expressed as the median effective concentration for α-glucosidase inhibitory activity (IC_{50}), that is, the amount of tested extract required for a 50% decrease in absorbance of α-glucosidase inhibitory activity.

$$\text{Inhibition (\%)} = [1 - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

A_{sample} is the absorbance of the test sample, A_{blank} is the absorbance of the sample (without the substrate), and $A_{control}$ is the absorbance of the control (without sample).

Isolation Procedures for α-Glucosidase Inhibitor as Potential Antidiabetic Compounds. A 40% silk peptide E5K6 solution was first passed through a 10 kDa molecular weight cutoff membrane (Satocon cassette, Sartorius, Germany). Next, a portion of the solution was immediately removed, and the filtrate was pumped through 3 and 1 kDa molecular weight cutoff membranes. The respective permeates were passed through each membrane, and fractions with >10, 10−3, 3−1, and <1 kDa molecular weight peptides were freeze-dried. The fraction <1 kDa was loaded onto a Biogel P-2 column (Bio-Rad, Richmond, CA) (2.5 × 75 cm) and eluted with 20 mM ammonium formate buffer (pH 6.0) and fractionated into five subfractions (fractions I, II, III, IV, and V). Fraction IV (F4) was fractionated by reverse phase column chromatography using a Varian C18 (Microsorb-MV 100-5 C18) (150 × 4.6 mm, 5 μm) HPLC system (Varian Prostar, Varian Inc., Palo Alto, CA) with fluorescence detection (Ex 220 nm, Em 320 nm). The gradient elution used water (solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient program was as follows: time zero, 10% solvent B, 10 min hold, then ramp to 35% in 50 min. The flow rate was 0.8 mL/min, and the total run time was 60 min.

Determination of Molecular Weight and Amino Acid Sequences. The sequences of the purified compounds were determined by using a protein sequencing system (Procise 491-Procise cLC 492, Applied Biosystems, Foster City, CA). Molecular weight was measured by matrix-assisted laser desorption ionization, time of flight/time of flight mass spectrometry (MALDI TOF/TOF MS; Bruker Daltonics, Bremen, Germany). The fractions were diluted in 5% acetonitrile/0.05% trifluoroacetic acid (TFA) (v/v) solution and analyzed by nanoelectrospray MS on a TOF mass spectrometer (Waters Micromass, Manchester, U.K.) in positive ion mode using a capillary voltage of 1000 V. The spray capillaries were produced in-house using a micropipet puller type P-87 (Sutter Instruments, Novato, CA) and were coated with a semitransparent film of gold in a sputter unit type SCD 005 (BAL-TEC, Balzers, Liechtenstein). The samples were fragmented by performing a collision offset plot from 1 to 50 V offset. The MS/MS spectra displayed represented the combination of several spectra. Within a set of peptide motifs the MS/MS spectra were combined over an identical range of medium offset values (15−25 V).

Statistical Analysis. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS), version 12.0 (SPSS Inc., Chicago, IL). The differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple-range tests. All data are reported as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Preventing carbohydrate absorption in the intestine is a reasonable way to decrease postprandial hyperglycemia in patients with diabetes because only monosaccharides can be absorbed in the intestinal lumen and transported into blood circulation. α-Glucosidase is an enteric digestive enzyme that degrades carbohydrates into monosaccharides and is considered to be an important factor for glucose homeostasis in diabetic subjects.[9,14] Thus, the α-glucosidase inhibitory activity of the purified peptide was determined.

Table 1. α-Glucosidase Inhibitory Activity of Silk Peptide E5K6 Fractions[a]

Fraction	Yield (%)	α-glucosidase activity, IC_{50} (mg/mL)
Silk peptide E5K6 (SP-0)	100.0	484.5 ± 14.5
Ultrafiltration		
>10 kDa	56.7	783.5 ± 23.3 d
10- 3 kDa	27.0	485.6 ± 12.0 c
3−1 kDa	8.8	455.4 ± 13.5 b
<1 kDa (SP-1)	7.5	213.6 ± 9.0 a
Biogel P-2		
F1 (28−33)	3.3	835.2 ± 6.1 d
F2 (34−39)	2.3	1999.2 ± 7.1 e
F3 (40−44)	1.0	101.5 ± 9.1 b
F4 (45−49) (SP-1-F4)	0.6	37.1 ± 1.1 a
F5 (50−56)	0.3	295.3 ± 12.7 c
Prep-HPLC		
SP-1-F4-1	0.2	2.7 ± 0.1
SP-1-F4-2	0.2	1.5 ± 0.1

[a] We used the positive control as acarbose, and its α-glucosidase inhibitory activity (IC_{50}) is 79.8 ± 4.4 μg/mL.

Silk from cocoon is composed of two kinds of proteins: insoluble fibrous protein (named fibroin) and soluble glue-like protein (named sericin), "cocoon" meaning whole. Silk peptides exert diverse bioactivities, such as hypocholesterolemic, antioxidant, immunoregulatory, antitumor, antiviral, and antibacterial effects.[3−5] Many earlier investigations demonstrated that silk peptide influences blood glucose and insulin.[6,7] Silk protein is insoluble in cold water; however, it is easily hydrolyzed using enzyme, whereby long protein molecules break down to smaller fractions, which are easily dispersed or solubilized in hot water. Our sample was made by boiling and protease N treatment.

Table 1 shows the stepwise yield and IC_{50} values of α-glucosidase inhibitory activity for silk peptide E5K6. First, silk peptide E5K6 was separated using three types of ultrafiltration membranes (10, 3, and 1 kDa cutoffs) according to molecular size, and the following four fractions were obtained after filtration: >10, 10−3, 3−1, and <1 kDa. The yields of these fractions were 56.7, 27.0, 8.8, and 7.5% (dry basis), respectively. The IC_{50} values for α-glucosidase inhibitory indicated that the <1 kDa (213.6 mg/mL) fraction had a higher activity than the other fractions. Second, a Biogel P-2 column was used to further separate the <1 kDa fraction (Figure 1A). Fractions F1 (tubes 28−33), F2 (tubes 34−39), F3 (tubes 40−44), F4 (tubes 45−49), and F5 (tubes 50−56) were gathered from Biogel P-2 column chromatography. The yield of F1 was highest at 3.3%, and the yields of the F2, F3, F4, and F5 were 2.3, 1.0, 0.6, and 0.3%, respectively. The α-glucosidase inhibitory activity at IC_{50} indicated that F4 (37.1 mg/mL) had the highest activity compared to the other fractions. In addition, the IC_{50} values of the fractions were in the order of F4 > F3 (101.5 mg/mL) > F5 (295.3 mg/mL) > F1 (835.2 mg/mL) > F2 (1999.2 mg/mL). Third, we split F4 by HPLC as shown in Figure 1B. The two major peaks having α-glucosidase inhibitory activity greater than that of F4 were named F4-1 and F4-2, respectively, and the IC_{50} values of α-glucosidase inhibitory activity for these fractions indicated that F4-2 (1.5 mg/mL) had 2 times greater activity than F4-1 (2.7 mg/mL). The molecular weights of F4-1 and F4-2 were

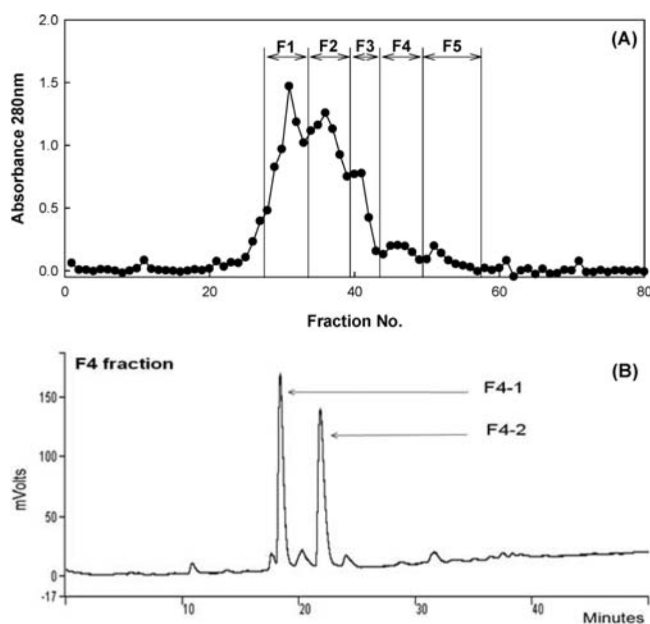
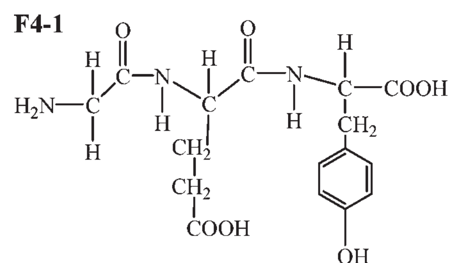
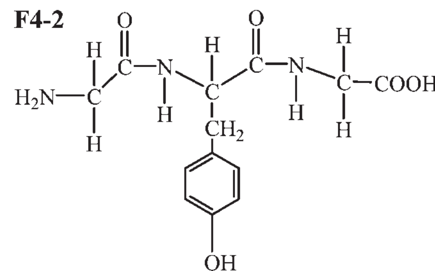


Figure 1. Purification of α-glucosidase inhibitory components by Biogel P-2 (A) and RS-HPLC (B). The fraction <1 kDa was loaded onto a Biogel P-2 column (2.5 × 75 cm), eluted with 20 mM ammonium formate buffer (pH 6.0), and fractionated into five subfractions. Fraction IV (F4) was fractionated by reverse phase column chromatography using a Varian C18 (150 × 4.6 mm, 5 μm) HPLC system with fluorescence detection (Ex 220 nm, Em 320 nm). The gradient elution used water (solvent A) and acetonitrile (solvent B) as the mobile phase. The flow rate was 0.8 mL/min, and the total run time was 60 min.



Gly-Glu-Tyr (GEY, M.W. 367)
α-glucosidase inhibitory activity
IC_{50} : 2.7 mg/mL



Gly-Tyr-Gly (GYG, M.W. 295)
α-glucosidase inhibitory activity
IC_{50} : 1.5 mg/mL

Figure 2. Amino acid sequences of fractions F4-1 and F4-2. The sequences of the purified compounds were determined by using a protein sequencing system. Molecular weight was measured by MALDI TOF/TOF MS.

measured by MALDI TOF-TOF MS and were 367 and 295 Da, respectively. The amino acid sequences of the fractions indicated that F4-1 was composed of Gly-Glu-Tyr (GEY) and F4-2 was composed of Gly-Tyr-Gly (GYG) (Figure 2).

Previous studies reported that silk peptide influenced blood glucose and insulin in vivo,[6,7] but the mechanism of antidiabetic activity has remained unclear as a principle compound has not yet been defined. Therefore, the purpose of the present investigation was to evaluate the effects of silk peptide E5K6 used for diabetes treatment on the inhibition of intestinal α-glucosidase. The substances having α-glucosidase inhibitory activity were selected and separated from the peptide, and fractions were further isolated and treated with enzymes to increase substance activity. The potential antidiabetic effects of the fractions were ultimately evaluated. Hu et al.[11] showed that silk fibroin hydrolysate using elastase has α-glucosidase inhibitory activity; however, these silk fibroin hydrolysates were siginificantly different in hydrolysis time and enzyme content.

α-Glucosidase inhibitors (such as acarbose, voglibose, and miglitol) are prescribed to manage blood sugar levels in type 2 diabetes mellitus, and these drugs lower blood sugar levels by slowing or decreasing carbohydrate breakdown in the intestine.[15] The potential antidiabetic activity of silk peptide E5K6, with its inhibitory behavior on α-glucosidase, was investigated in this study. Here, we purified and elucidated two peptides, GEY and GYG, as potential antidiabetic compounds (Figure 2).

Fibroin protein is composed of 45.9% Gly, 30.3% Ala, 12.1% Ser, 5.3% Tyr, 1.8% Val, and only 4.7% of 15 other amino acids. Most of the sequence is of low complexity and forms a Gly-X dipeptide motif covering 94% of the sequences: X is Ala in 64%, Ser in 22%, and Tyr in 10% of the repeats.[16,17]

Some researchers have reported that several amino acids acted as α-glucosidase inhibitors. Suzuki et al.[18] had initially tested the inhibitory effects of Gly or Ser and showed some α-glucosidase inhibition activity. Various in vitro assays have shown that many plant polyphenols possess carbohydrate-hydrolyzing enzyme inhibitory activities. Some compounds such as green tea polyphenols, sweet potato anthocyanins, the soy isoflavone genistein, and several flavonoids inhibit α-glucosidase and α-amylase activities.[19−22] These inhibitory activities of plant phytochemicals against carbohydrate-hydrolyzing enzymes contribute to lowering postprandial hyperglycemia. α-Glucosidase inhibitors such as acarbose competitively bind to the oligosaccharide binding site of α-glucosidase or α-amylase, which prevents the binding and enzymatic hydrolysis of the oligosaccharide substrate.[23] It is therefore possible, but not proven, that the α-glucosidase inhibitory activities of these compounds result from the phenol group of acarbose and polyphenols bind to these enzymes. The IC_{50} values for α-glucosidase inhibitory activity showed that GYG (1.5 mg/mL) had 2 times higher activity than GEY (2.7 mg/mL) (Table 1). These results agree with the higher α-glucosidase inhibitory activity of GYG compared to GEY. Voglibose, in contrast to acarbose, has fewer of these side effects and is hence preferred lately. It is also more economical compared to acarbose. Therefore, further studies are needed on the side effect using animals or humans for industrial application.

Silk peptide E5K6 as a peptide mixture of hydrolyzed silk, or peptides derived from silk including GYG and GEY, may be a new strategy in the development of antidiabetic peptide nutraceuticals. Although we are now just exploring the detailed molecular

mechanism underlying the effects of GYG, further studies may lead to new therapeutic approaches in the treatment and/or prevention of type 2 diabetes. Our results are the first report on purified compound with antidiabetic potential isolated from cocoon hydrolysate. This study used background that supports the synthetic process of peptide with potential antidiabetic activity.

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