Blood Pressure-Depressing Activity of a Peptide Derived from Silkworm Fibroin in Spontaneously Hypertensive Rats

Kiharu Igarashi,1† Kosuke Yoshioka,1 Kenji Mizutani,2 Masazumi Miyakoshi,2 Toshiyuki Murakami,2 and Toshifumi Akizawa3

1Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka, Yamagata 997-8555, Japan
2Research Center, Maruzen Pharmaceuticals Co., Ltd., 1089-8 Sagata, Shin-ichi, Fukuyama, Hiroshima 729-3102, Japan
3Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-0101, Japan

Received January 14, 2005; Accepted September 27, 2005

Peptides showing inhibitory activity against the angiotensin I-converting enzyme (ACE) were investigated from the fibroin fraction of discarded silk fabric. Fibroin, which was hydrolyzed with alcalase after partial hydrolysis with hot aqueous 40% CaCl2, released two major active peptides showing ACE-inhibitory activity. The two peptides were identified as glycyl-valyl-glycyl-tyrosine (GVGY) and glycyl-valyl-glycyl-alanyl-glycyl-tyrosine (GVGAGY) by analyses with a protein sequencer and LC/MS/MS. GVGY, whose ACE-inhibitory activity has not previously been reported, showed a blood pressure-depressing effect on spontaneously hypertensive rat (SHR).

Key words: fibroin; peptide; blood pressure; spontaneously hypertensive rats (SHR); angiotensin I-converting enzyme

Silk fabric is valued for its beautiful appearance and suitable clothing properties such as its humidity-holding characteristics and protection of the skin from ultraviolet light. As the end of a silk thread used for weaving cannot be incorporated into silk fabric because it remains on the machine, it is almost always discarded without use. Hirabayashi et al. and Igarashi et al. have attempted to use the thread ends as raw food material after partial hydrolysis with calcium chloride or hydrochloric acid, and have shown that they could indeed be used as food and that it had a hypocholesteremic effect.1,2 These reports prompted us to investigate functional materials from discarded thread that would be useful for health. Since there are many reports dealing with the isolation and identification of hypotensive or angiotensin I-converting enzyme (ACE)-inhibitory peptides from food protein3 as well as silk protein,4 we directed our work to the preparation of new hypotensive peptides from silk protein. The relationship between ACE-inhibitory activity and oligopeptide characteristics has been reported by Kawakami et al.5 They suggested that hydrophobic amino acid as the N-terminal and such aromatic amino acid as tyrosine or phenylalanine as the C-terminal were strongly concerned with ACE-inhibitory activity.

We investigate in this present study new peptides showing a blood pressure-depressing effect on spontaneously hypertensive rats (SHR) that was obtained from fibroin prepared from discarded silk thread.

Silk yarn was boiled in an aqueous 1% sodium bicarbonate solution for 30 min to remove dirt and sericin. After discarding the sodium bicarbonate solution, the remaining part was treated in the same way twice more to prepare fibroin, this being washed with distilled water and dried at 40–50 °C. The dried fibroin (10 g) was added to 200 ml of a hot aqueous 40% CaCl2 solution (w/v) and heated at 85–88 °C for 8 h to dissolve and partially hydrolyze the fibroin. The resulting fibroin solution was cooled to about 40 °C, transferred to a cellulose tube with a molecular cut of about 10,000 (Sanko Junyaku Co., Ltd., Tokyo, Japan), and dialyzed with tap water for 3 days to remove CaCl2.

The inner part of the dialyzate (about 1 l) was mixed with an alcalase solution (alcalase 2.4 L FG, Novo Nordisk, Denmark), after adjusting the pH value to 7.5–8.5 with 0.05 N NaOH, and incubated at 52 °C for 17 h. The filtrate obtained by filtering the cooled hydrolyzate through No. 2 filter paper (Advantec, Tokyo, Japan) was passed through a column of Amberlite XAD-2 resin (Organ Co., Ltd., Tokyo, Japan) column to adsorb the peptides. The adsorbed peptides in the column were eluted with 50% EtOH, after washing the column with H2O. The freeze-dried eluate was fractionated into four

† To whom correspondence should be addressed. Fax: +81-235-28-2812; E-mail igarashi@tds1.tr.yamagata-u.ac.jp

Abbreviations: ACE, angiotensin I-converting enzyme; SHR, spontaneously hypertensive rats; LC, liquid chromatography; MS, mass spectrometry
fractions (A–D) by Sephadex G-25 column chromatography, using H$_2$O as the eluent. Fraction B showed the strongest inhibitory activity against ACE when measured with 30 μl of each fraction, 250 μl of a hippuryl-l-histidyl-l-leucine solution (7.6 mM in a borate buffer (pH 8.3) containing 608 mM NaCl) as a substrate and 100 μl of an ACE solution with activity of 60 munits/ml (rabbit lung; Wako Pure Chemicals Ind., Osaka, Japan), according to the method of Saito et al.$^6$ Fraction B was further fractionated by reversed-phase HPLC with a Develosil C30-UG-5 column (25 × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan), using 5% MeCN and 20% MeCN as developing solvents (a linear gradient of 5% MeCN to 20% MeCN in 180 min) to obtain three major fractions (Fig. 1). Fractions 1 (peak 1 corresponding to compound a) and 2 (peak 2 corresponding to compound b) showed stronger ACE-inhibitory activity than fraction 3 (corresponding to peak 3) when each fraction (2.7 mg dry weight/ml in 30 μl of H$_2$O) was measured for its ACE-inhibitory activity, so these two fractions were further purified by gel filtration HPLC, using a Develosil 300 Diol-5 column (10 × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan) and H$_2$O as the eluent to obtain compounds a and b for determining their amino acid compositions, amino acid sequences and mass spectra.

Purified compounds a and b were hydrolyzed with 6 N HCl at 110 °C for 24 h, before being analyzed for amino acid content by an Atto MLC 703 instrument with detection by the ninhydrin method. The amino acid sequences of these compounds were determined by an LC/MS/MS analysis with an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan) used in the electrospray ionization mode. The molecular weights of these compounds were confirmed by an ESI mass spectral analysis with the same mass spectrometer.

Compounds a and b were respectively composed of glycine, valine and tyrosine in a molar ratio of 2:1:1, and of glycine, valine, alanine and tyrosine in a molar ratio of 3:1:1:1 from the results of the amino acid analysis. The amino acid sequences of compounds a and b were glycyl-valyl-glycyl-tyrosine (GVGY), and glycyl-valyl-glycyl-valyl-glycyl-tyrosine (GVGAGY), respectively, when determined by the protein sequencer. Compounds a and b respectively gave pseudomolecular ion peaks at $m/z$ 395(M + H)$^+$, 393(M – H)$^-$ and 417(M + Na)$^+$, and at $m/z$ 523(M + H)$^+$, 521(M – H)$^-$ and 545(M + Na)$^+$ in the ESI mass spectrum, indicating respective molecular weights of 394 and 522. The MS/MS analysis confirmed the amino acid sequences of GVGY in compound a and GVGAGY in compound b. Compounds a and b were identified from these data as GVGY and GVGAGY, respectively. Since it is known that the fibroin heavy (H) chain contains the sequences GVGAGY and GVGY, respectively, it may be reasonable to consider that these two compounds were released from the fibroin heavy chain by alcalase. Although it has already been reported that the hydrolysis of silk cocoons with protease, by which the cocoons were treated with HCl at 50 °C for 3 h and subsequently neutralized with NaOH before enzymic hydrolysis, released ACE-inhibitory GVGAGY, the isolation and identification of GVGY as an ACE-inhibitory compound has not previously been reported.$^8$

GVGY and GVGAGY samples used for measuring the ACE-inhibitory activities (IC$_{50}$ values) and antihypertensive activities were synthesized by solid-phase synthesis with the Fmoc method, using a Applied Biosystems 433A peptide synthesizer. The deprotected sample was purified by HPLC in a reverse-phase column (Capcell Pack C18, 15 × 250 mm; Shiseido Co., Tokyo, Japan). Linear-gradient elution with 0–100% of solvent B (50% MeCN in 0.1% trifluoroacetic acid) in solvent A (H$_2$O) over the course of 30 min was used. The purified GVGY and GVGAGY samples showed...
good agreement with compounds a and b according to the results of analytical HPLC with the same developing solvents as those used for the purification of these compounds, but the column was changed to one of analytical size (4.6 × 250 mm).

The amounts of GVGY, GVGAGY, glycyl-tyrosine (GY) and captopril a compound known to show a strong suppressive effect on the systolic blood pressure (SBP) needed to inhibit 50% of ACE activity (IC$_{50}$) were 35, 68, 15 and 0.7 μM, respectively. The ACE-inhibitory activity commonly observed in GVGY, GVGAGY and GY may indicate that the GY part in GVGY and GVGAGY was concerned with this ACE-inhibitory activity.

To measure the blood pressure, male spontaneous hypertensive rats (SHR), 9 weeks old and each weighing 220–230 g, were purchased from SLC (Shizuoka Laboratory Animal Center, Shizuoka, Japan). The animals were kept under the following conditions: 12 h light and 12 h dark (6:00–18:00 light), a temperature range of 22–24°C, and a relative humidity of 40–60%. The rats were fed on a basal diet composed of 20% casein, 65.5% α-cornstarch with sucrose (α-cornstarch:sucrose = 2:1), 5.0% cellulose powder, 5.0% corn oil, 3.5% mineral mixture (AIN-93G-MX), and 1.0% vitamin mixture (AIN-93-VX) for 2 days. The rats now weighing 230–240 g were subsequently deprived of the diet for 10 h before the oral administration of 5 mg (22 mg/kg of body weight) or 10 mg (44 mg/kg of body weight) of synthesized compound a, or 5 mg (22 mg/kg of body weight) of captopril as a positive control (Wako Pure Chemicals Ind., Osaka, Japan) dissolved in 0.3 ml of physiological saline. Tap water was freely available.

The systolic blood pressure (SBP) of the rats was measured by the tail cuff method with a BP-98 programmed electrophysymomanometer (Softron Co., Tokyo, Japan) after placing for 10 min in a heated box set at 38°C. Physiological saline was orally administered instead of the sample solution to the control group.

The animals were cared for at all times according to the institutional guidelines of Yamagata University.

The oral administration of compound a (GVGY) decreased the systolic blood pressure significantly and in a dose-dependant manner when compared to the control group administered with physiological saline (Fig. 2). The maximal decreases of SBP in the rats administered with 5 mg (22 mg/kg of body weight) and 10 mg (44 mg/kg of body weight) of GVGY were found 2 h after administration (−19 ± 2 and −33 ± 2 mm of Hg, respectively). On the other hand, SHR administered with 5 mg of captopril (22 mg/kg of body weight) showed the maximal decrease 4 h after administration (−17 ± 5 mm of Hg), indicating the possibility that lasting effect of GVGY was shorter than that of captopril.

Although it has been reported that the enzymic hydrolysis of silk fibroin by alcalase produced glycyl-tyrosine (GY), which inhibited the ACE activity during in vitro experiments, the isolation of compound a has not previously been reported. It may in future be necessary to determine antihypertensive activity of compound b (GVGAGY) which also showed ACE inhibitory activity.

Aromatic amino acid residues such as tryptophan, tyrosine and phenylalanine at the C-terminal of peptides which show a blood pressure-depressing effect have been reported to bind with the active center of ACE, which could also show its blood pressure depressing effect through interaction of the tyrosine residue with the active center of ACE.
References


