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Antihypertensive Effects of Silk Fibroin Hydrolysate by Alcalase and Purification of an ACE Inhibitory Dipeptide

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Silk fibroin, which is normally discarded as an industrial byproduct in clothing plants, was hydrolyzed with alcalase. Angiotensin I converting enzyme (ACE) inhibitory activities of the silk fibroin hydrolysates (SFH) were investigated, and the hydrolysate with hydrolysis degree of 17% exhibited the highest ACE inhibitory activity. At the tested 600 mg/kg·d and 1200 mg/kg·d doses, SFH significantly lowered blood pressure of spontaneously hypertensive rats (SHR) after chronic oral administration. SFH was further purified using consecutive chromatographic methods on Sephadex G-15 column and reverse phase high-performance liquid chromatography (RP-HPLC) on an octadecylsilane column. After its purity was confirmed by analytical RP-HPLC and capillary electrophoresis, one ACE inhibitory dipeptide was isolated, and its molecular mass and amino acid sequence were determined as 238.2 Da and Gly-Tyr, respectively, by LC-ESI/MS. The results of this study suggest that silk fibroin byproducts have the possibility to become an effective source for ACE inhibitory peptides.

KEYWORDS: Silk fibroin; alcalase protein hydrolysates; ACE inhibitory peptide; antihypertensive effect; spontaneously hypertensive rat

INTRODUCTION

In recent years, there is a growing interest in obtaining biologically active compounds from natural sources. China is the major world cocoon and silk producer and supplier. During silk production process, some silk scraps (waste silk) will be inevitably produced and China has a vast annual resource of waste silk, which will not only pollute the environment, increase the cost of silk, but also is a waste of resources. Therefore, utilization of waste silk to produce high value-added products is important for the silk industry. Silk fibroin is an edible protein polymer, which has highly repetitive regions and functional amino acids (*1*). In silk fibroin, five amino acids including glycine (48%), alanine (32%), serine (11%), tyrosine (4.5%), and valine (2%) account for more than 97% of the total number of residues (*2*). Silk fibroin has been preferred in many biotechnological applications such as drug delivery and tissue engineering (*3*−*5*). Meanwhile, silk fibroin hydrolysate has been reported to possess multiple biological functions including blood pressure-depressing activity (*6*), antigenotoxic properties (*7*), and fibroblast growth-promoting activity (*8*).

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) acts as an exopeptidase that cleaves dipeptides from the C terminus of various oligopeptides (*9*). By converting the inactive decapeptide angiotensin I into the octapeptide angiotensin II and by inactivating the vasodilator bradykinin, ACE raises blood pressure (*10*). Currently, synthetic ACE inhibitors such as captopril, enalapril, alacepril, and lisinopril are often used as medicine against hypertension, but they can cause serious side effects such as cough and angioedema (*11*). Therefore, researchers are keen on searching for natural ACE inhibitors present in both animal and vegetable proteins as alternatives to synthetic ones for safe and economical use. So far, there have been many natural ACE inhibitory peptides isolated from the hydrolysis of various proteins such as cheese whey (*12*), casein (*13*), corn gluten (*14*), seaweed (*15*), pea protein (*16*), bovine skin gelatin (*17*), and porcine and chicken muscle (*18*, *19*). A recent review comprehensively summarized the findings of popular blood pressure-lowering nutraceuticals and functional foods with emphasis on their chemistry, production, application, efficacy, and mechanisms (*20*).

In a previous study, two types of peptides (GVGY and GVGAGY) prepared from the fibroin hydrolysate were shown to possess ACE-inhibitory activity (*6*). In the current study, using the discarded silk fabric as starting material, we modified a previously reported hydrolysis procedure for soluble fibroin powder (*21*) and employed a different purification methodology to purify the ACE inhibitory fraction. Further than the previous study (*21*), we demonstrated the in vivo antihypertensive property of the silk fibroin hydrolysates (SFH) through oral administration to spontaneously hypertensive rats (SHR). The amino acid sequence of this dipeptide was determined through consecutive chromatographic methods and determined its amino acid sequence by LC-ESI/MS.

MATERIALS AND METHODS

Materials and Chemicals. Natural cocoon fiber was donated by HaiAn Silk Co., Ltd (Jiangsu, China). Purified rabbit lung ACE and hippuryl-L-His-L-Leu (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was of HPLC grade, and all other chemicals and reagents used were of analytical grade. Alcalase 2.4 L serine-protease

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from *Bacillus licheniformis* was purchased from Novozymes (Bagsvaerd, Denmark).

Preparation of Alcalase Hydrolysate. Pure cocoon fiber was boiled twice in 5 g/L of Na_2CO_3 (1:50, w/v) at 98−100 °C for 30 min to get rid of sericin. Refined silk fibroin was then added to 40% boiling $CaCl_2$ solution for 2−5 min and then cooled down with ice water and adjusted to 50 g/L concentration at pH 8.5. The silk solution was then hydrolyzed with alcalase (24 AU/kg solid) at 60 °C for 0−400 min (pH 8.5). Silk fibroin hydrolysate (SFH) was subsequently boiled for 10 min to inactivate the enzyme. The resultant SFH was centrifugated at 4000*g* for 15 min and desalted by sequential polysulfone ultrafiltration membrane (5 kDa cutoff, Dow, USA) and aromatic polyamide nanofiltration system (150 Da cutoff, Dow, USA). All recovered solution were lyophilized in a freeze spray drier to obtain SFH powder.

Determination of the Degree of Hydrolysis. The degree of hydrolysis (DH) was defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}) and calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis as DH (%) = $h/h_{tot} \times 100 = (B \times N_b)/M_P \times (1/\alpha) \times (1/h_{tot}) \times 100$, where B is the amount of NaOH consumed (mL) to keep the pH constant during the reaction. N_b is the normality of the base, M_P is the mass (g) of protein ($N \times 6.25$), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as $\alpha = 10^{pH-pK}(1 + 10^{pH-pK})$, where pH and p$K$ are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in a fibroin protein concentrate was calculated to be 12.4 mequiv/g.

Determination of ACE Inhibitory Activity of SFH. SFH powder obtained at different degree of hydrolysis was dissolved in distilled, deionized water, and after centrifugation at 7200*g* for 15 min, the supernatant was used for ACE inhibitory activity measurement. Sample solution of 10 and 40 μL substrate (2.17 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.3 M NaCl at pH 8.3) was preincubated at 37 °C for 6 min, and the mixture was incubated with 20 μL of ACE solution (100 munits/mL) for 30 min at the same temperature. The reaction was terminated by the addition of 80 μL of 1.0 M HCl. The resulting hippuric acid (HA) was microfiltered (0.45 μm) and quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Symmetry C_{18} column (3.9 mm ×15 cm) connected to a Waters 490 programmable multiwavelength detector. The sample was then eluted using 12% acetonitrile in 0.5% trifluoroacetic acid (TFA) (v/v) at a flow rate of 1.0 mL/min, and the eluate was followed at 228 nm. The average value from three determinations was used to calculate the ACE inhibition rate as equal to $[(E_c - E_s)/(E_c - E_b)] \times 100$, where E_s is absorbance with SFH added to the reaction mixture, E_c is absorbance with buffer added, and E_b is absorbance without ACE. The IC_{50} value was defined as the concentration of hydrolysate (mg/mL) required to reduce the hippuric acid peak by 50%.

Peptide Fractionation. To estimate the molecular weight (MW) range in SFH, it was subjected to high-performance size exclusion chromatography using a Waters 600 HPLC (Waters Corp., USA) equipped with a TSKgel G2000 SWXL column (7.8 mm ×300 mm) (Tosoh Corp. Japan). Aliquots of 10 μL samples in 45% aqueous acetonitrile with 0.1% TFA were injected onto the column, and elution was performed isocratically in the same TFA−acetonitrile buffer at 30 °C with a flow rate of 0.5 mL/min over 30 min. The UV detector was selected at 220 nm, and the molecular mass was estimated based on the elution time against those of molecular weight markers (Sigma-Aldrich, USA) including cytochrome C (12500 Da), bacitracin (1450 Da), glycine−glycine−tyrosine−arginine (451 Da), and triglycine (189 Da), which yielded a linear log MW vs elution time regression line ($R = 0.9934$). The relative content of each peptide fraction was expressed as the percentage area of its chromatogram peak.

Purification of ACE Inhibitory Peptide. The freeze-dried SFH from 17.1% DH digestion (2.4 g) was suspended in 5 mL of ddH_2O and then loaded onto a Sephadex G-15 gel filtration column (3.0 cm ×200 cm) equilibrated with 0.2 M sodium acetate buffer at pH 4. The column was then eluted with the same solution, and fractions were collected at a flow rate of 32 mL/h. Fractions were detected at 220 nm and analyzed for ACE inhibitory activity. The resultant fraction exhibiting the highest ACE inhibitory activity was further purified using a reversed-phase high-performance liquid chromatography (RP-HPLC) on a Waters μBONDAPAK C18 (7.8 mm ×300 mm) column with a gradient of aqueous acetonitrile (0% × 10 min, 0−50% × 10 min, 50% × 10 min, 50−0% × 10 min, 0% × 10 min) at a flow rate of 2.0 mL/min. The elution peaks were detected at 210 and 280 nm wavelength on a dual λ absorbance detector. The ACE inhibitory activities of the eluted peaks were determined and fractions from 30 replicate chromatography runs were pooled, lyophilized, and stored at −25 °C. After the pooled active peaks were further concentrated using a centrifugal evaporator, they were rechromatographed on a Zorbax 300 SB C_{18} (4.6 mm ×250 mm, Agilent) column with gradients of acetonitrile (0% × 10 min, 0−30% × 20 min, 30−100% × 20 min, 100−0% × 5 min, 0% × 5 min) containing 0.05% TFA at a flow rate of 1.0 mL/min.

Capillary Electrophoresis. A Beckman P/ACE5000CE system (Fullerton, CA) was used throughout capillary electrophoresis. Separations were run at 20 kV and 25 °C in bare fused standard capillary, 75 μm i.d. × 57 cm (50) (Waters, USA). The injection was done hydrodynamically by overpressure (3.45 kPa, 2 s), and the concentrations of the peptides were 0.1−0.3 mg/mL. The separations were run with positive polarity at the inlet, detection was done at 214 nm. Coating agents were added to the phosphate buffer (30 mmol/L NaH_2PO_4, pH 2.5).

Total Amino Acid Analysis. The determination of total amino acid composition was obtained after acid hydrolysis of the peptide solutions. The samples, acidified with 6 N HCl, were sealed in tubes under nitrogen and incubated at 110 °C for 24 h. For each sample, the whole digest brought to volume (25 mL) in a volumetric flask with deionized water. After filtration through two layers of Whatman no. 2 paper, 1 mL of filtrate was transferred into a 25 mL beaker and evaporated to dryness in a vacuum drier. The dry sample was redissolved in 0.02 N HCl for 30 min and applied to an Agilent HP1100 amino acid analyzer (Agilent Co., Palo Alto, CA) for amino acid analysis.

Structural Analysis by LC-ESI/MS. The purified peptide was subjected to LC/ESI-MS analysis using a Waters Platform ZMD 4000 system consisting of a Waters Micromass ZMD mass spectrometer coupled with a electrospray ionization (ESI) source and a Waters 996 HPLC (Waters, Milford, MA) equipped with a photodiode detector. MassLynx software version 4.0 was used for data analysis. Chromatography was performed at 35 °C using an Symmetry C-18 300A column (150 mm × 2.1 mm; Waters), and elution was made in the linear gradient mode from 0.1% formic acid to acetonitrile containing 0.1% formic acid (30 min) at a flow rate of 0.3 mL/min. Measurements were carried out in both positive ion and negative ion modes using the capillary voltage at 3.88 kV, desolvation temperature at 300 °C, and source block temperature at 120 °C.

Animals and Measurement of Blood Pressure. Male SHR with tail systolic blood pressures (SBPs) over 180 mmHg, 8 weeks old, 180 ± 30 g of body weight were purchased from Shanghai Institute of Hypertension (Shanghai, China), and all animal procedures were approved by Animal Care and Use Committee of same organization. The SHR were housed individually in steel cages in a room kept at 22 °C, 60% humidity, with a 12 h light−dark cycle and fed a standard laboratory diet and tap water. SHR were divided into three groups (8 rats/group) and fed with a standard diet plus SFH at three doses (100, 600, and 1200 mg/kg·d). The peptides were orally administered at a dose of 1 mL by intubation for four continuous weeks. Saline served as negative control, and nifedipine (1 mg/kg·d) (Sigma, USA), a dihydropyridine calcium channel blocker known as ACE inhibitor, served as positive control. The body weight and systolic blood pressure (SBP) of the rats was measured once a week by the tail-cuff method with a programmed electrosphygmomanometer (model UR-1000; Ueda Co., Ltd., Japan) after warming up rats in a 40 °C chamber for 10 min. The significance differences among each group were analyzed using the one-way analysis of variance (ANOVA) with posthoc analysis by Tukey's test.

RESULTS AND DISCUSSION

Preparation of Silk Fibroin Hydrolysates (SFH) by Alcalase. In the present study, silk fibroin was first prepared by alcalase hydrolysis. Because many studies have suggested the importance of the degree of hydrolysis (DH) relating to ACE inhibitory activity (*22*), we first assessed the DH of the obtained protein hydrolysates and analyzed their corresponding ACE inhibitory activities. Unhydrolyzed silk fibroin protein has a minimum or

Table 1. Degrees of Hydrolysis (DH) and ACE Inhibitory Activities Related with Hydrolysis Period of Silk Fibroin Hydrolysates Obtained with Alcalase Treatments

hydrolysis time (min)	DH (%)	ACE inhibition (%)
0	0	0.3 ± 0.1
15	8.5 ± 0.1	13.1 ± 1.3
25	10.6 ± 0.1	22.5 ± 0.7
45	13.5 ± 0.3	33.4 ± 0.3
75	16.0 ± 0.2	56.8 ± 1.0
105	17.1 ± 0.1	72.5 ± 0.8
130	18.1 ± 0.2	68.3 ± 1.2
250	20.5 ± 0.1	62.6 ± 0.5
400	22.1 ± 0.2	57.4 ± 0.6

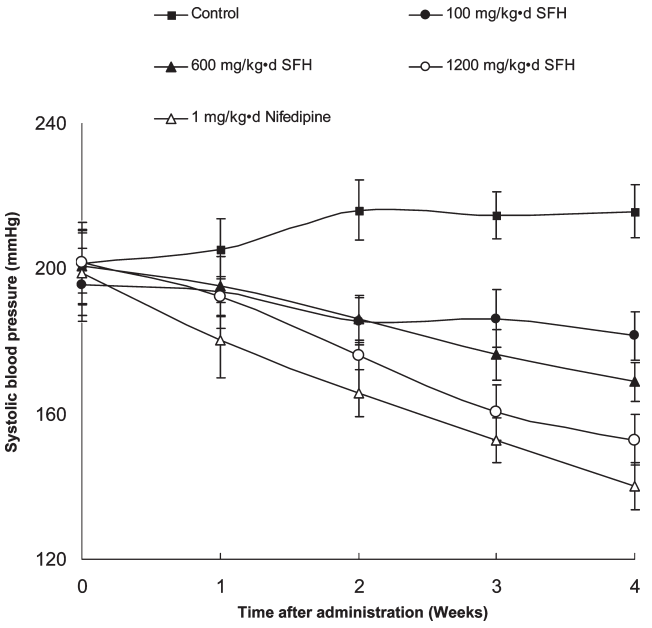

Figure 1. Decrease in SBP caused in SHR by the administration of saline, SFH (100 mg/kg·d), SFH (600 mg/kg·d), SFH (1200 mg/kg·d), and nifedipine (1 mg/kg·d). The data represent the mean values ± SEM for eight animals.

negligible amount of ACE inhibitory activity. High ACE inhibitory activity was observed only for SFH with high DH (**Table 1**). This is consistent with previous works reporting that low molecular weight peptides exhibited higher activity than high molecular weight peptides (*22*−*25*). Highest ACE inhibition activity (72.5% inhibitory rate with an IC_{50} of 0.34 mg/mL) was obtained with a DH of 17%, and further digestion resulted in a decrease in the inhibition level (**Table 1**). The difference between ACE inhibitory activities might be attributed to the different molecular weights and different amino acid composition of ACE inhibitory peptides present in the SFHs. Therefore, we used SFH with DH of 17% in the following studies.

Antihypertensive Activity of SFH in SHR. The inhibitors of angiotensin converting enzyme (ACE) represent one of the best known classes of antihypertensive peptides (*26*−*28*). Because SFH showed a potent in vitro ACE inhibitory activity, we investigated its antihypertensive effect at different doses upon chronic administration to SHR. The time course of the changes in SBP is shown in **Figure 1**. The SBP gradually increased and decreased throughout the course of the experiment in the control and low-dose (100 mg/kg·d) group, respectively, but no significant changes have been detected (**Figure 1**). The high-dose (1200 mg/kg·d) and nifedipine group exhibited significant differences from the control group after 2 weeks of feeding on the test diets ($p < 0.05$), and the medium-dose (600 mg/kg·d) group exhibited significant difference from the control group after 3 weeks ($p < 0.05$). These differences were maintained throughout the rest of the feeding period. After 4 weeks of feeding on the test diets, SBP values (mmHg) were 215.8 ± 7.2 in the control group compared with 152.9 ± 7.0 in the high-dose group and 140.1 ± 6.6 in the nifedipine group. Over the 4-week administration peroid, there were no significant differences in food intake and body weight between the control and the test groups (data not shown). In a previous report (*29*), fibroin alcalase hydrolysate released two major active peptides showing ACE-inhibitory activity on Sephadex G-25 column (identified as GVGY and GVGAGY) and their synthetic samples could decrease the SBP significantly in the SHR 2 h after oral administration.

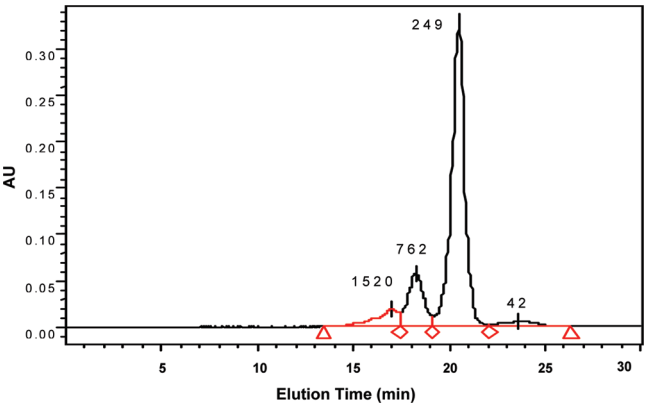

Figure 2. Elution profile of high-performance gel filtration chromatography of SFH on a TSKgel G2000 SWXL column to identify its molar mass distribution. Peptide fractions with determined MW are indicated by arrows.

Peptide Fractionation. High-performance gel filtration chromatography was run to separate peptide fractions in the SFH and identify their molecular weight (MW) distributions. The SFH yielded four fractions on the TSKgel G2500PW column, with peak molecular masses of 1520, 762, 249, and 42 Da (**Figure 2**). These fractions centered around these peaks represented 7.9, 15.5, 72.9 and 3.7% of the total mass, respectively. Peak 3 representing a significant portion of SFH consisted of dipeptides. No peptide/amino acids were detected in eluents collected before 15 and after 25 min.

Purification and Characterization of ACE Inhibitory Substances from SFH. SFH was then separated by gel filtration chromatography on a Sephadex G-15 column and the ACE inhibitory activities of individually collected fractions were measured by HPLC method (*30*). As reported in **Figure 3A**, six fractions were separated on Sephadex G-15 column for SFH and they were designated as SFH P1−P6. Each fraction was pooled and freeze-dried, and its ACE inhibitory activity was determined. All fractions (at 0.2 mg/mL) displayed ACE inhibitory activity as shown in **Figure 3B**. Of the six fractions collected, fraction P6 exhibited the highest level of ACE inhibitory activity as 49.1%, followed by 40% of fraction P3 (**Figure 3B**). Consistent with previous reports for chickpea and sunflower protein hydrolysates (*31*, *32*), SFH peptide fraction P6 that eluted last from the reverse phase column had the highest ACE inhibitory activity. It has been suggested that ACE inhibitory peptides are rich in hydrophobic amino acids (V, I, L, M, F, W, and C), which results in a higher retention in hydrophobic chromatography column (*33*).

To further purify ACE inhibitory peptides from SFH, fraction P6 was applied to a semipreparation C18 reverse phase column

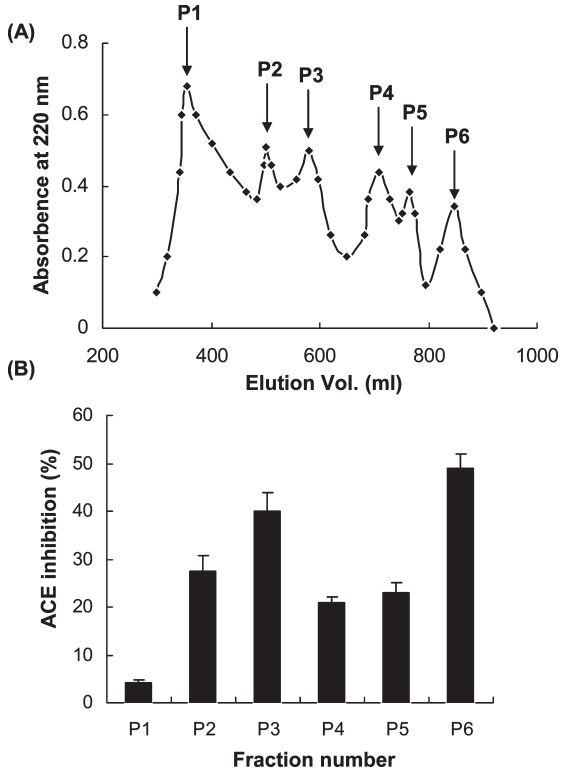

Figure 3. Elution profile of SFH on Sephadex G-15 column (**A**) and the ACE inhibitory activities of the separated fractions (P1−P6) (**B**). Bars represent the average of three assays.

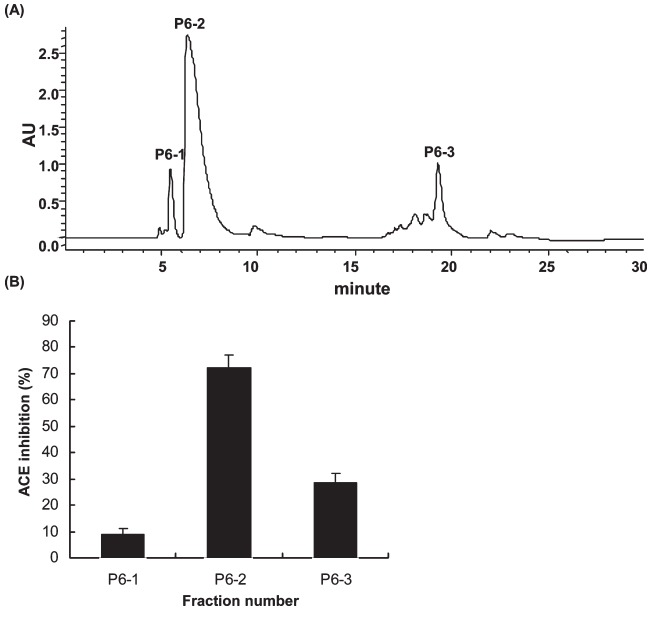

Figure 4. Purification of ACE inhibitory peptides in the SFH P6 by reversed-phase HPLC chromatography on a μBONDAPAK C18 (7.8 mm × 300 mm) column (**A**). ACE inhibitions of three major fractions after primary RPLC (**B**). Bars represent the average of three assays.

and the chromatographic profile at 210 nm revealed three major subfractions designated P6−1 to P6−3 present in fraction P6 (**Figure 4A**). These three fractions were collected individually, and their ACE inhibitory activities were determined (**Figure 4B**). Although ACE inhibitory activity was observed in all three subfractions, P6−2 exhibited the highest inhibitory activity as 72.1% followed by 28.5% for P6−3 and 8.8% for P6−1 (**Figure 4B**).

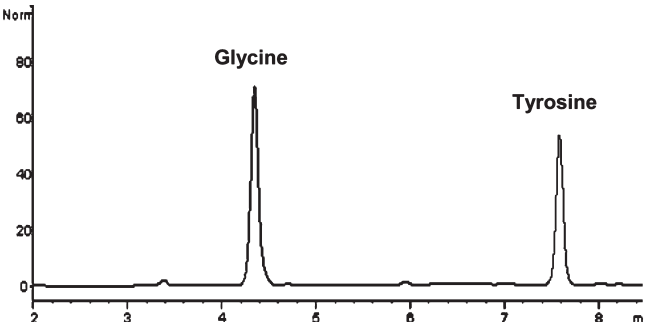

Figure 5. Amino acid composition analyses of SFH P6-2 on an Agilent HP1100 amino acid analyzer.

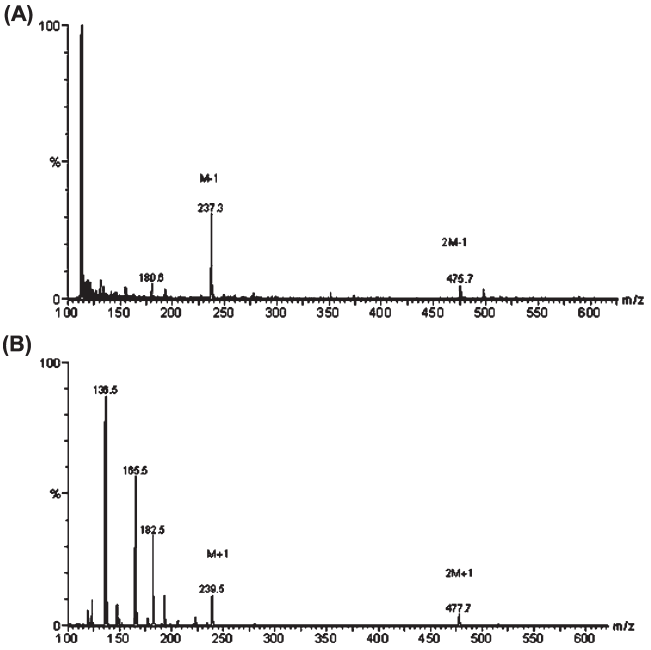

Figure 6. Mass spectrum of purified SFH P6-2 carried out with the Waters platform ZMD 4000 system. The electrospray ionization (ESI) was used in negative mode (**A**). The electrospray ionization (ESI) was used in positive mode (**B**).

The purity of P6−2 fractions was analyzed by analytical reversed-phase HPLC (Figure S1A, Supporting Information) and capillary electrophoresis (Figure S2B, Supporting Information), both of which showed a single peak, thus comfirming the purity of P6−2 fraction. We then performed amino acid analysis, and the P6−2 was shown to be composed of glycine and tyrosine in a molar ratio of 1.25: 1 (**Figure 5**). The purified SFH P6−2 peptide was then analyzed for its molecular weight and primary structure using LC-ESI/MS. As shown in **Figure 6**, $(M - H)^-$ 237.3 and $2(M - H)^-$ 475.7 appeared in the negative ion mode (**Figure 6A**) and $(M + H)^+$ 239.5 and $2(M + H)^+$ 477.7 appeared in the positive ion mode (**Figure 6B**). The amino acid sequence of the completely purified SFH P6−2 peptide was identified to be Gly-Tyr (238.2 Da) by computation analysis of the LC-ESI/MS system. Gly-Trp has been also identified as one of the ACE inhibitory substances purified from fermented soybean seasoning in which study the precursor ion of Gly-Tyr was detected at m/z 238.43, and the fragment ion was selected at m/z 164.95 by means of a standard reagent (*34*). Other ACE inhibitory dipeptides have been well summarized by SATO et al. (*15*) that various dipeptides have been proven to possess ACE inhibitory activities including Val-Tyr from sake (*35*), Ile-Tyr from

wheat germ (*36*), Ala-Trp from human serum albumin (*37*), Phe-Tyr from R-zein (*38*), Val-Trp from sake lees (*35*), Ile-Trp from fish sauce (*39*), and Leu-Trp from ovalbumin (*18*). In addition, Val-Tyr, Ile-Tyr, Ala-Trp, Val-Trp, and Ile-Trp have been confirmed to have ACE inhibitory activities by Cheung et al. (*40*). So far, the structure−activity correlations among the various ACE inhibitory peptides still remain ambiguous. On the basis of some common structure patterns, it has been suggested that the most favorable amino-terminal residues are branched amino acids such as Val and Ile and the most preferred carboxyterminus residues are among Trp, Tyr, Pro, or Phe (*37*).

In conclusion, we obtained a silk fibroin hydrolysate through alcalase digestion and demonstrated its in vitro ACE inhibitory activity and in vivo antihypertensive activity in SHR. After purification by consecutive chromatographic methods, a single dipeptide was purified and sequenced as Gly-Tyr by LC-ESI/MS. Following this procedure, we demonstrated that silk fibroin, an inexpensive byproduct of silk fabric production, could be used for producing a value-added food component with health promoting characteristics.

Supporting Information Available: RP-HPLC C18 rechromatography of the SFH P6-2 fraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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