A silk fibroin hydrolysate (SFH) exhibited a pronounced \textit{in vivo} blood pressure-lowering effect on spontaneously hypertensive rats (SHRs) accompanied with decreases in the plasma angiotensin II, endothelin (ET), TNF-\(\alpha\) and NO concentrations. We also observed a markedly decreased LPO level, a parameter of oxidative tissue damage, in the medium- and high-dose SFH-treated groups which was accompanied by an increased SOD level in erythrocytes. Our data suggest ACE inhibition together with an improved antioxidative status as the underlying antihypertensive mechanism for the silk fibroin hydrolysate. Since SFH could markedly lower the blood pressure and improve several physiological parameters involved in the occurrence of hypertension, it could be used as a possible supplement against cardiovascular diseases and as a functional food ingredient.

**Key words:** silk fibroin hydrolysate (SFH); antihypertensive; ACE inhibitor; antioxidant

Blood pressure is controlled by various regulatory factors in the body, including the angiotensin I-converting enzyme (ACE).\textsuperscript{1} Since ACE activity is closely associated with the development of hypertension and arteriosclerosis, \textit{in vitro} inhibition of angiotensin II formation is the most common strategy for screening therapeutic agents.\textsuperscript{2} There have been many natural ACE inhibitory peptides isolated from the hydrolysates of such proteins as cheese whey,\textsuperscript{3} casein,\textsuperscript{4} corn gluten,\textsuperscript{5} seaweed,\textsuperscript{6} pea protein,\textsuperscript{7} bovine skin gelatin,\textsuperscript{8} and porcine and chicken muscle.\textsuperscript{9,10} However, the blood pressure-lowering activities and the working mechanisms of most ACE inhibitory peptides derived from food proteins have not previously been investigated \textit{in vivo}.\textsuperscript{11}

Hypertension is always associated with deterioration of the vascular endothelial function.\textsuperscript{11} Substances that improve the vascular endothelial function can be expected to decrease the incidence of cardiovascular diseases. Vascular tone is regulated through the controlled release of endothelium-derived relaxing factors and endothelium-derived contracting factors.\textsuperscript{12} In our previous study, silk fibroin was hydrolyzed by Alcalase with a degree of hydrolysis (DH) of 17\%, and the resulting silk fibroin hydrolysate (SFH) showed significant \textit{in vitro} ACE inhibitory activity.\textsuperscript{13} Chronic administration to spontaneously hypertensive rats (SHRs) in the 600 mg/kg-d and the 1200 mg/kg-d groups exhibited significant hypotensive effects after 4 weeks of feeding.\textsuperscript{13} This present work investigates the possible underlying mechanisms for the blood pressure-lowering activity of SFH in SHRs, with emphasis on its \textit{in vivo} effects on the plasma parameters that may be involved in the occurrence of hypertension.

The alcalase hydrolysate from pure cocoon fiber was prepared as previously described.\textsuperscript{13} Male 8-week-old SHRs with a tail systolic blood pressure (SBP) of over 180 mm of Hg were individually housed at 22 \(^\circ\)C and 60\% humidity with a 12-h light-dark cycle. SHRs were assigned to three groups (8 rats/group) and fed with a standard diet plus SFH at three doses (100, 600, and 1200 mg/kg-d) by intubation for four continuous weeks. Saline served as a negative control, and nifedipine (1 mg/kg-d, Sigma, USA) served as a positive control. The body weight and systolic blood pressure (SBP) of each rat were measured once a week by the tail-cuff method and, after the last determination of blood pressure, venous blood samples from the rat tail were collected and centrifuged at 1500g for 10 min at 4 \(^\circ\)C. The plasma and erythrocytes were stored separately at −80 \(^\circ\)C until needed for analysis. Erythrocytes were resuspended in 1 volume of distilled water for lysis. The clear hemoglobin (Hb) layer was used for analyzing the antioxidative enzymes. There were no significant differences in the food intake and body weight between the control and test groups over the 4-week administration period (data not shown).

The vascular endothelial function is one of the key factors for the progression of cardiovascular diseases. Many ACE inhibitors have been reported to improve the vascular endothelial function.\textsuperscript{14,15} Among the markers of the endothelial function, endothelin, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and Ang II are three pathogenic factors, since the inhibition of their synthesis/release could cause endothelium-dependent vasodilatation.\textsuperscript{16} The plasma endothelin (ET) concentration was measured in the current study by a commercially available radioimmunoassay kit (Clinical Assay, Beijing, China) and is expressed as pg/mL. The level of TNF-\(\alpha\) in each serum sample was determined by using a commercial ELISA kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol. Total plasma nitric oxide was quantified with a Nitric Oxide Colori-
metric Assay Kit (R&D Systems), which involves the conversion of nitrate to nitrite by the nitrate reductase enzyme. Total nitrite is then determined as a colored azo dye product of the Griess reaction. The concentration of NO was determined by a standard curve and is expressed as μmol/L.

The respective plasma Ang II concentration in the medium- (600 mg/kg-d) and high-dose groups (1200 mg/kg-d) was significantly decreased by 26% and 66% (Table 1). Endothelin (ET) is one of the strongest vasoconstrictive substances in the body and plays an important role in the change of peripheral vascular resistance. The medium- and high-dose groups in this study could induce a similar and significant decrease in ET concentration to the nifedipine group (Table 1). TNF-α is an adipocytokine that contributes to the progression of arteriosclerosis by inducing insulin resistance and accelerating the production of various adhesion factors and growth factors by acting on the vascular endothelial cells. The serum levels of TNF-α in the medium- and high-dose SFH-treated SHR groups in the present study were significantly decreased in comparison with the control group (Table 1). This result demonstrates that SFH needs to be administered in a relatively high dose to protect endothelial cells against the alteration induced by TNF-α. Nitric oxide (NO) is also an important bioregulatory molecule which has a number of physiological effects, including control of the blood pressure. The endothelial dysfunction in SHRs is also hallmark by reduced NO availability. The endothelial dysfunction of vascular endothelial function. Meanwhile, SFH modulates the activity of the endogenous antioxidant, SOD, of LPO in the medium- and high-dose groups (Table 2). Superoxide dismutase (SOD) is one of the scavengers of radical oxygen species and plays a role in vascular disease prevention. The SOD activity of erythrocytes was examined as previously described. (25) The enzyme activity is expressed as units/mL, and 1 unit of SOD activity is defined as the amount of enzyme required to oxidize 1 μmol of o-dianisidine in 1 min. Our data indicate that supplementation with SFH not only decreased the oxidative stress, but also significantly increased the concentration of SOD in the medium- and high-dose groups (Table 2); this suggests that the increased endogenous antioxidative activity might have been partly responsible for the decline in blood pressure in SFH-fed SHRs. A silk fibroin hydrolysate has been reported in the literature to possess multiple biological functions, including blood pressure-depressing activity, antioxidative properties, and fibroblast growth-promoting activity. Although the antioxidative activity of SFH has been previously reported, it may have originated from the various oligo-peptide and functional free amino acids present in SFH.

In conclusion, the findings from this study potentially elucidate the basic mechanisms underlying the antihypertensive effects of SFH on SHRs and provide new evidence for the potential of SFH as a functional food ingredient with therapeutic benefits for preventing and treating hypertension. SFH could prevent the development and progression of hypertension via modulation of the serum ET, Ang II, TNF-α and NO levels, which in turn could control blood pressure by improving the vascular endothelial function.
which could decrease the LPO content and contribute to the amelioration of hypertension. A single dipeptide purified by consecutive chromatographic methods and sequenced as Gly-Tyr has been demonstrated in our previous paper to possess the highest \textit{in vitro} ACE inhibitory activity among the SFH fractions. The \textit{in vivo} antihypertensive activity and physiological effects of SFH described here probably were mainly due to this fraction. However, silk fibroin has highly repetitive regions, and functional 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.\textsuperscript{29} It is highly possible after hydrolysis that there are other active oligo-peptide and functional free amino acids to exhibit multiple bioactivities, including antihypertension and antioxidation.

References