His-His-Leu, an Angiotensin I Converting Enzyme Inhibitory Peptide Derived from Korean Soybean Paste, Exerts Antihypertensive Activity in Vivo

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It has been reported that soybean peptide fractions isolated from Korean fermented soybean paste exert angiotensin I converting enzyme (ACE) inhibitory activity in vitro. In this study, further purification and identification of the most active fraction inhibiting ACE activity were performed, and its antihypertensive activity in vivo was confirmed. Subsequently, a novel ACE inhibitory peptide was isolated by preparative HPLC. The amino acid sequence of the isolated peptide was identified as His-His-Leu (HHL) by Edman degradation. The IC_{50} value of the HHL for ACE activity was 2.2 μg/mL in vitro. Moreover, the synthetic tripeptide HHL (spHHL) resulted in a significant decrease of ACE activity in the aorta and led to lowered systolic blood pressure (SBP) in spontaneously hypertensive (SH) rats compared to control. Triple injections of spHHL, 5 mg/kg of body weight/injection resulted in a significant decrease of SBP by 61 mmHg ($p < 0.01$) after the third injection. These results demonstrated that the ACE inhibitory peptide HHL derived from Korean fermented soybean paste exerted antihypertensive activity in vivo.

Keywords: *Angiotensin I converting enzyme inhibitory peptide; fermented soybean paste; preparative HPLC; hypertension; spontaneously hypertensive rats*

INTRODUCTION

Hypertension is one of the major independent risk factors for arteriosclerosis, stroke, myocardial infarction, and end-stage renal disease. Angiotensin I converting enzyme (EC 3.4.15.1; ACE) is the key enzyme in renin−angiotensin systems. ACE increases blood pressure by both converting the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. Because ACE activity is closely associated with the development of hypertension and arteriosclerosis (*1*), in vitro inhibition of angiotensin II formation has been used for screening therapeutic agents such as ACE inhibitors against hypertension and arteriosclerosis. The discovery of peptides as ACE inhibitors in food proteins in 1979 (*2*) drew considerable attention to ACE inhibitory components in various foods from the view of prevention of hypertension (*3*). Recently, a number of ACE inhibitory peptides from food proteins, such as casein (*4*), tuna muscle (*5*), zein (*6*), sardines (*7*), corn gluten (*8*), and bovine blood plasma (*9*), have been reported. In addition, the occurrence of ACE inhibitors in some fermented foods such as soy sauce (*10*) and fermented milk (*11*) has also been reported. However, the blood pressure lowering activity of most ACE inhibitory peptides derived from food proteins has not been confirmed in vivo. Interestingly, the ACE inhibitory peptides derived from food proteins such as sour milk were shown to be resistant to digestive conditions and to be absorbed from the intestine into blood circulation. Moreover, the peptides were found to result in lowered blood pressure in vivo (*12*). ACE inhibitory peptides derived from daily food proteins would be useful in the development of a novel functional food additive for preventing hypertension as well as for therapeutic purposes.

Soybean protein has been shown to have beneficial effects on preventing hyperlipidemic or hypercholesterolemic lesions, which cause arteriosclerosis and hypertension (*13*). Our previous paper (*14*) demonstrated that soybean hydrolysate exerted an inhibitory activity of ACE in vascular tissue in vivo and lowered systolic blood pressure (SBP) in spontaneously hypertensive (SH) rats. Fermented soybean paste is one of the most widely and frequently taken foods in the Korean diet. We have already demonstrated that the fermented soybean paste fraction contains ACE inhibitory components and the fraction F53 exerts a strong ACE inhibitory activity in vitro (*15*). In this study, we isolated and identified the strong ACE inhibitory peptide of the F53 fraction obtained from fermented soybean paste. Moreover, we confirmed the ACE inhibitory activity and blood pressure lowering activity of the purified peptide in vivo.

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MATERIALS AND METHODS

Materials. A commercial fermented soybean paste was obtained from Se Woo Co., Ltd. (Ansan, South Korea). All solvents were of HPLC grade (EM Industries, Inc., Hawthorne, NY). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), ACE (from rabbit lung), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic tripeptide HHL (spHHL) was provided by the Protein Research Center at Pohang University of Science and Technology in South Korea. The purity was proved to be >95% by HPLC analysis.

Extraction and Purification of ACE Inhibitory Peptides. The fermented soybean paste sample was freeze-dried, ground to pass through a 20-mesh screen, and stored at 4 °C in a desiccator. The freeze-dried sample (300 g) was extracted, filtered (PM-10 membrane, Amicon, Beverly, MA), lyophilized, and then fractionated by two-step preparative HPLC as described previously (*15*). In the first step, the fractionation was carried out on a preparative HPLC model LC-20 (Japan Analytical Industry Co., Ltd., Tokyo, Japan) with a reversed phase column, JAIGEL-A-343-10 (250 × 20 mm i.d., Japan Analytical Industry Co., Ltd.). A step gradient system of water and acetonitrile was used as the first mobile phase: water/acetonitrile (98:2, v/v) at 5 mL/min for 5 min, water/acetonitrile (96:4, v/v) for 20 min, and water/acetonitrile (35:65, v/v) for 30 min. The elution was monitored at 214 nm with a UV detector. In the second step, an ion exchange column (JAIGEL-ES-502CP, 20 × 100 mm i.d., Japan Analytical Industry Co., Ltd.) was used, and the mobile phase was an isocratic system of 0.01 M sodium succinate buffer (pH 4.3)/acetonitrile (80:20, v/v) at 4 mL/min. All of the preparative HPLC was operated at room temperature.

The F53 fraction obtained in the previous experiment was lyophilized and stored at 4 °C in a desiccator. In this experiment, a further two-step purification of the F53 fraction was carried out on the same HPLC as mentioned above. The first step was performed with the reversed phase column, JAIGEL-ODS-A-343-10 (250 × 20 mm i.d., Japan Analytical Industry Co., Ltd.) and eluted isocratically with 0.05% trifluoroacetic acid/acetonitrile (95:5, v/v) at 5 mL/min. The elution was monitored at 214 nm with a UV detector. The fractions were collected for ACE inhibitory assay. The fraction showing the highest ACE inhibitory activity was concentrated and lyophilized (step 1 fraction). The step 1 fraction was applied on an ion exchange column, Shodex Asahipak ES-2502N-7C (100 × 7.6 mm i.d., Showa Denko, Tokyo, Japan) and eluted with 20 mM tris[hydroxymethyl]aminomethane−HCl buffer (pH 7.5) containing 125 mM sodium chloride at a flow rate of 1 mL/min. According to the above process, the fraction with the highest ACE inhibitory activity was collected and concentrated (step 2 fraction). The step 2 fraction was desalted on the same reversed phase column as mentioned above with water and then lyophilized.

Assay of ACE Inhibitory Activity. The ACE inhibitory activity was assayed according to the method of Cushman and Cheung (*16*) and repeated three times. Inhibition was expressed as the concentration of nitrogen that inhibits 50% of ACE activity (IC_{50}), and the total inhibitory activity (TIA) and the relative total inhibitory activity (RTIA) of each fraction was calculated as

$TIA_N =$

total nitrogen content of fraction N/IC_{50} of fraction N

$$RTIA = TIA_N/TIA_a$$

where "a" in TIA_a indicates the fraction with the highest ACE inhibitory activity. The nitrogen content was measured by an elemental analyzer, model Antek 7000B (Antek Instruments Inc., Houston, TX).

Molecular Weight Estimation. The desalted step 2 fraction was applied on a size exclusion column, Bio-Sil-SEC-125 (300 × 7.8 mm i.d., Cambridge, MA) and eluted with 0.5% SDS solution at a flow rate of 1 mL/min for estimating the molecular weight of the purified peptide. The molecular weight standard curve was obtained from the same column, and the standard molecular weight mixtures were as follows: angiotensin II (1046 Da), Met-enkephalin (573 Da), Leu-enkephalin (555 Da), Val-Tyr-Val (397 Da), and Gly-Tyr (238 Da).

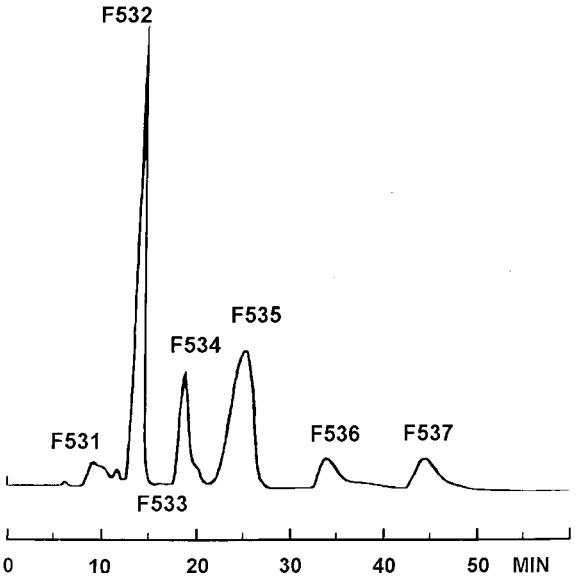

Figure 1. Preparative high-performance liquid chromatogram of fraction F53 on a reversed phase column (JAIGEL-ODS-A-343-10).

Amino Acid Analysis. The amino acid composition was analyzed with a Pico-Tag HPLC system (Waters Inc., Milford, MA) according to the method of Chang et al. (*17*). The amino acid sequence was analyzed by using a protein sequencer, a model Milligen 6600B (Milligen Co., Ltd., Bedford, MA).

Measurement of Blood Pressure. SH rats were obtained from the Institute of Asan Life Science Research Center (Seoul, South Korea). SH rats were housed in a temperature-controlled room (22 °C) under a 12 h light−dark cycle (8:00 p.m. to 8:00 a.m.) and had free access to tap water and standard laboratory rat chow (Samyang Purina Korea Inc., Seoul, South Korea). The care and treatment of experimental animals conformed to the NIH guidelines for the ethical treatment of laboratory animals. Male SH rats weighing 300 ± 10 g with an SBP of 170−190 mmHg were anesthetized with pentobarbital (250 mg/kg, intraperitonel). The carotid artery in SH rats was cannulated, and blood pressure was monitored continuously by using polygraph 1000 (Letica Scientific Instruments, Barcelona, Spain). The spHHL at 0.2−5 mg/kg of body weight (BW) in 0.5 mL of saline ($n = 4$ or 6) was injected into the femoral vein of the SH rats, and the change of SBP in the carotid artery was measured for 60 min. The spHHL was given in single or triple injections with an interval of 20 min. Control rats received an equal volume of the vehicle. The lowering efficacy of spHHL on SBP was compared to that of captopril (5 mg/kg of BW), which is widely used as an antihypertensive agent. ACE activity in the abdominal aorta and serum was also measured. Means in data were compared by one-way ANOVA and Student's *t* test.

RESULTS AND DISCUSSION

Purification of ACE Inhibitory Peptides. Fraction F53 was obtained from fermented soybean paste as described previously (*15*). The reversed phase column chromatography of the F53 fraction gave seven main peaks (from F531 to F537) as shown in Figure 1. For more effective purification of peptides, trifluoroacetic acid was used as an ion pairing reagent. Each fraction was collected and measured for ACE inhibitory activity. Among the fractions, F532−F536 showed high ACE inhibitory activity, ranging from IC_{50} = 2.3 to 3.5 μg/

Table 1. ACE Inhibitory Activity of Each Fraction Obtained from the First Reversed Phase Column

	fraction						
	F531	F532	F533	F534	F535	F536	F537
IC_{50}[a] (μg/mL)	14.0	2.8	2.6	3.5	2.3	2.4	16.7
RTIA[b]	0.1	1.1	0.2	1.3	1.0	0.9	0.2

[a] IC_{50}, defined as the concentration that inhibits 50% of the ACE activity. Relative standard deviation (RSD) < 2%. [b] RTIA, relative total inhibitory activity; RTIA = TIA_N/TIA_a; TIA_N = total nitrogen content of fraction N/IC_{50} of fraction N, where "a" in TIA_a indicates the fraction with the highest ACE inhibitory activity.

mL (Table 1). The ACE inhibitory activities of F535 and F536 were very similar, but F535 was selected for further work due to its simple composition of amino acid compared to F536. The total amino acid composition of F535 was Gly/His/Met/Leu/Phe = 17:63:2:11:7, whereas the total amino acid composition of F536 was Asp/Glu/Ser/Gly/His/Thr/Val/Ile/Leu/Phe/Lys = 8:8:4:41:4:10:5:5:8:4:3. It was supposed that F535 was easy to purify, as was the identification of the active peptide in it.

RTIA represents the relative value of total inhibition activity calculated by using the IC_{50} value and the amount of peptides contained in each fraction. As explained under Materials and Methods, because F535 showed the highest activity in Table 1, the TIA of each fraction was divided by the TIA of F535. On the basis of the highest ACE inhibitory activity (F535), the relative total inhibitory activities (RTIA) of F532, F534, F535, and F536 appeared to be nearly of the same quality, ranging from 0.9 to 1.3, showing that these fractions had high ACE inhibitory peptides. The F534 in Table 1 showed the highest RTIA, even though its activity is relatively low. RTIA may be an important consideration factor in the industry, because if we cannot get sufficient amounts of active peptides in any fraction, we may have some difficulties in applying it for industrial purposes even though it shows the highest activity.

Because of the highest ACE inhibitory activity, even though the difference was small, fraction F535 was further purified by an anion exchange column, Shodex Asahipak ES-2502N-7C, resulting in three distinct peaks (Figure 2). The inhibitory activity of each fraction (F535A, F535B, and F535C) revealed nearly the same quality, ranging from 2.1 to 3.0 μg/mL (Table 2), and was not much different from that of fraction F535. These results indicated that the purification of peptides was almost complete prior to the anion exchange chromatography. Among the fractions, F535A had distinctly bound amino acids. On the other hand, both F535B and F535C might contain nitrogenous non-amino acid compounds such as nicotinamine detected in soy sauce for ACE inhibitor (*18*), because their nitrogen contents derived from total amino acids by amino acid analysis were 10-fold lower than those obtained by total nitrogen analysis (data not shown). Therefore, F535A was selected for further work to identify the ACE inhibitory peptide.

In this experiment, the apparent degree of purification of F535A compared to F53 did not increase, whereas initial steps showed a high degree of purification (Table 3). It could be speculated that later purification steps removed only minor impurities such as free amino acids and other peptides from each fraction.

Identification of ACE Inhibitory Peptide. Even though there were some bound amino acids such as

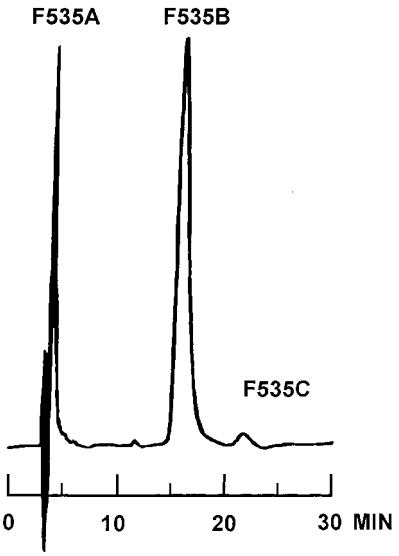

Figure 2. Preparative high-performance liquid chromatogram of fraction F535 on an ion exchange column (Shodex Asahipak ES-2502N-7C).

Table 2. ACE Inhibitory Activity of Each Fraction Obtained from the Ion Exchange Column

	fraction		
	F535A	F535B	F535C
IC_{50}[a] (μg/mL)	2.2	3.0	2.1
RTIA[b]	2.4	1.6	1.0

[a] IC_{50}, defined as the concentration that inhibits 50% of the ACE activity. RSD < 2%. [b] RTIA = TIA_N/TIA_a; TIA_N = total nitrogen content of fraction N/IC_{50} of fraction N, where "a" in TIA_a indicates the fraction with the highest ACE inhibitory activity.

Table 3. Purification of Inhibitors of ACE from Fermented Soybean Paste

purification step	IC_{50}[a] (μg/mL)	purification fold[b]
fermented soybean paste	276.3	1.0
ultrafiltrate	41.8	6.7
F5 fraction	6.8	41.2
F53 fraction	2.5	111.7
F535 fraction	2.3	121.7
F535A fraction	2.2	127.2

[a] IC_{50}, defined as the concentration that inhibits 50% of the ACE activity. RSD < 2%. [b] Relative value of reciprocal of ACE IC_{50}.

methionine and phenylalanine, fraction F535A showed its completely purified peptide mainly consisting of histidine and leucine due to the lack of free amino acids. The molar ratio of histidine to leucine was approximately 2:1. By sequence analysis, the amino acid sequence of F535A was found to be His-His-Leu, the molecular weight of which was calculated as ~405 Da. On the basis of the result of size exclusion chromatography using the standard curve between molecular weights of standards and their retention times, the molecular weight of the peptide was deduced as approximately between 400 and 500 Da (Figures 3 and 4), which nearly corresponded to the results above. The ACE IC_{50} value of His-His-Leu was 2.2 μg/mL. This was the first report on purification and identification of an ACE inhibitory peptide from fermented soybean paste. It was characteristic that the tripeptide found in this experiment had the same C-terminal sequence of His-Leu as that of the angiotensin I. Only two peptides with leucine at the C-terminal end, Leu-Val-Leu in porcine plasma (*19*) and Leu-Lys-Leu in sardines (*20*), have been reported so far. Because His-His-Leu had the same

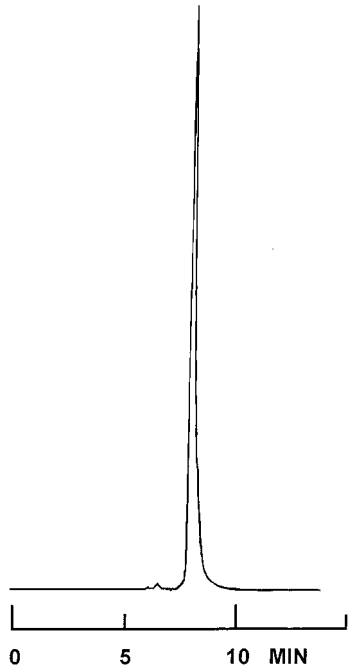

Figure 3. Size exclusion chromatogram of fraction F535A.

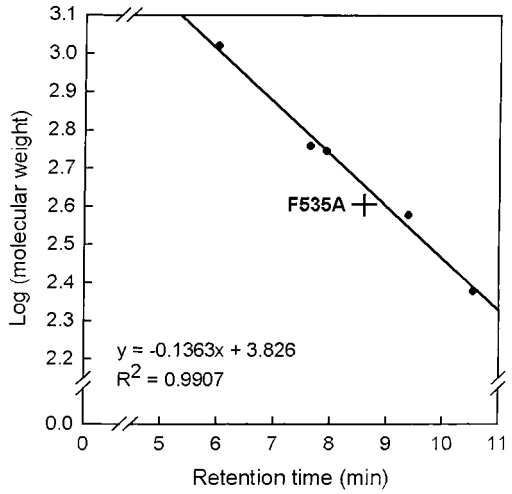

Figure 4. Relationship between molecular weight and retention time on the size exclusion chromatograph.

C-terminal end sequence as angiotensin I, His-Leu, it might be important for the function of ACE. Further studies are necessary to elucidate the formation process of His-His-Leu and the inhibitory mechanism against ACE.

Antihypertensive Activity of HHL. It is extremely laborious and time-consuming to obtain large amounts of purified HHL from fermented soybean paste, so the tripeptide HHL was synthesized chemically. The synthetic HHL (spHHL) was confirmed to exert the same inhibitory activity of ACE in vitro (IC_{50} = 2.2 μg/mL) as HHL derived from soybean paste. To clarify whether the spHHL exerts a lowering activity on SBP, spHHL was injected into the femoral vein of SH rats and the change of SBP in the carotid artery was monitored. Single or multiple injections of HHL at 0.2 mg/kg of BW did not alter the SBP of SH rats (data not shown). However, a single injection of spHHL at a dose level of 5 mg/kg of BW into the femoral vein of SH rats significantly ($p < 0.01$) reduced the SBP by 32 mmHg after the injection, and the lowered SBP was maintained

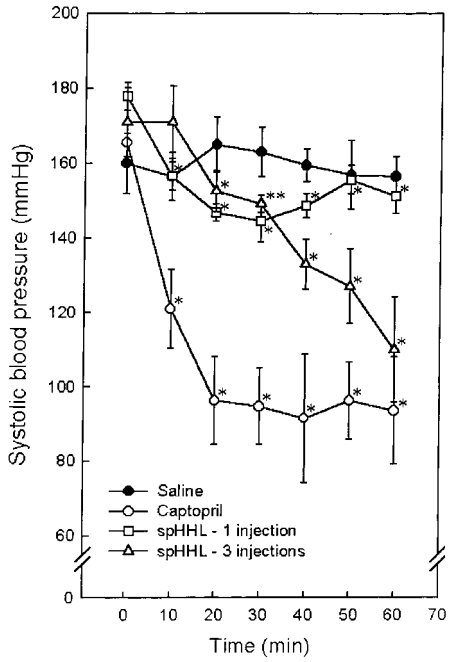

Figure 5. Effect of spHHL on the change of SBP in SH rats. SH rats were anesthetized, and spHHL was injected into the femoral vein. The change of SBP in carotid artery was measured for 60 min. The total dose of spHHL was given in a single injection (5 mg/kg of BW) or triple injections (2 mg/kg of BW × 3 times) with an interval of 20 min. Control received an equal volume of the vehicle. The lowering efficacy of spHHL on SBP was compared to that of captopril (5 mg/kg of BW). Body weights were 358 ± 29 g (saline), 329 ± 14 g (captopril), 310 ± 10 g (spHHL, single injection), and 322 ± 40 g (spHHL, triple injections). Data are expressed as the mean ± SEM (n = 6). *, $p < 0.01$; **, $p < 0.05$: significantly different from the initial SBP.

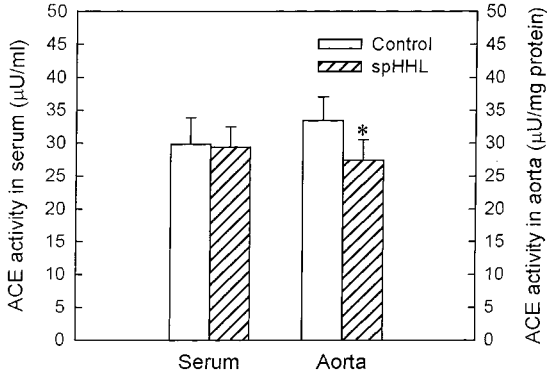

Figure 6. Effect of spHHL on the activity of ACE in serum and aorta of SH rats. SH rats were anesthetized, and the spHHL (5 mg/kg of BW) was given in triple injections and monitored SBP for 60 min. Serum and aorta were isolated for measuring ACE activity after the measurement of blood pressure in SH rats. ACE activity in the samples was expressed in units per milligram of protein. Data are expressed as the mean ± SEM (n = 6). *, significantly different from control, $p < 0.05$.

for the rest of the monitoring (Figure 5). Triple injections of HHL at 5 mg/kg of BW/injection also resulted in a significant decrease of the SBP by 61 mmHg ($p < 0.01$) after the third injection (Figure 5). The lowering efficacy of spHHL (5 mg/kg of BW × 3 injections) on SBP was the same as that of captopril. ACE activity in the abdominal aorta treated with spHHL was significantly ($p < 0.05$) lower than that of the control (Figure 6), but not in the serum. The lowering activity of spHHL on

SBP could be associated with its inhibitory activity on ACE in the vascular tissue.

It would be noteworthy that there was a small decrease in ACE activity accompanied by a strong reduction of blood pressure, although spHHL exerted significant inhibitory activity on ACE in vascular endothelial tissue compared to control (Figures 5 and 6). Several antihypertensive agents have been suggested to lower blood pressure through mechanisms that go beyond their primary (e.g., hypotensive or hypocholesterolemic) actions such as ACE inhibitors or HMG-CoA reductase inhibitors (*21*). In addition, vasoregulatory properties of biological peptides in the endothelium-dependent relaxation that regulate peripheral resistance have been suggested to be involved in the blood pressure lowering (*22*). These findings suggest that spHHL might play an important role in restoring endothelium-dependent relaxation in the small arteries that regulate peripheral resistance in the SH rats, besides the inhibitory activity on ACE.

Unlike spHHL injection, the efficacy of spHHL in oral administration might be altered by the accompanying digestive modification. It has been demonstrated that di- and tripeptides are absorbed by a transport system separate from those responsible for mediating absorption of free amino acids (*23*). Recent study has also demonstrated that small (di- and tripeptides) and large (10−51 amino acids) peptides generated in the diet can be absorbed intact through the intestines and produce biological effects progression (*24*). An ACE inhibitory peptide derived from sour milk was resistant to digestive condition and exerted a blood pressure lowering activity (*12*). Taking these together, we postulate that orally administered spHHL might be absorbed from the small intestine into blood circulation and could exert antihypertensive activity. Further study is necessary to determine the impact of digestive condition on the spHHL.

Because soybean protein is considered in the prevention of cardiovascular diseases including hypertension and atherosclerosis, the antihypertensive property of HHL, which is originally derived from soybean hydrolysates, might have an important implication in the development of a novel functional food as well as therapeutic application.

ABBREVIATIONS USED

His-His-Leu, histidyl-histidyl-leucine; ACE, angiotensin I converting enzyme; RTIA, relative total inhibitory activity; SBP, systolic blood pressure; spHHL, synthesized tripeptide HHL; SH, spontaneously hypertensive; RSD, relative standard deviation.

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