Purification of novel angiotensin converting enzyme inhibitory peptides from beef myofibrillar proteins and analysis of their effect in spontaneously hypertensive rat model

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ABSTRACT

This study was conducted to purify the angiotensin converting enzyme (ACE) inhibitory peptides from beef myofibrillar proteins by using inexpensive enzymes alkaline-AK and papain. Different molecular weight peptides (< 3 and < 10 kDa) were obtained using ultrafiltration. The < 3 kDa peptides obtained by alkaline-AK (AK3K) digestion showed the highest ACE inhibitory activity (74.29%) as compared to other alkaline-AK peptides, and a strong antihypertensive effect of AK3K was observed in the spontaneously hypertensive rat (SHR) model. The AK3K treatment groups (400 and 800 mg/kg body weight) exhibited a decrease in systolic blood pressure (SBP) by 28 and 35 mmHg, respectively in the SHR model. The study demonstrated that the ACE inhibitory peptide obtained from beef myofibrillar proteins had the sequence Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val, and could be possibly used for lowering the SBP.

1. Introduction

Angiotensin converting enzyme (ACE) plays a key role in regulating blood pressure by causing blood vessels to constrict by converting angiotensin I to angiotensin II, which binds to AT1 and AT2 receptors in the smooth muscle cells of the blood vessels [1,2]. The ACE inhibitors have been represented as captopril, perindopril, aceon, trandolapril, univasc, and benzapril in the market. However, these ACE inhibitors have been found to have several side effects such as cough, headaches, fatigue, increase in blood potassium levels, fetal disorders, and taste disorder [3,4]. In contrast, bioactive materials derived from food and food proteins are stable and are associated with less side effects [5–7].

Bioactive peptides derived from hydrolyzed food proteins have been reported to exhibit diverse beneficial effects on human health due to their antioxidant, antihypertensive, antimicrobial, analgesic, immunomodulatory, antithrombotic properties, and these peptides also aid in the protection against memory impairment [8–12]. Among their beneficial properties, various reports showed that bioactive peptides with ACE inhibitory activity can provide cues for the development of commercial ACE inhibitors [13,14].

Spontaneously hypertensive rats (SHRs) are an experimental animal model for hypertension, and are widely used for determination of antihypertensive properties of drugs, because they show similar physiological characteristics as hypertension in humans [15]. The administration of peptides derived from diverse food sources has been shown to have antihypertensive activity in *in vitro* and *in vivo* studies [16–18]. For example, the administration of 0.1 mg/mL Met-Lys-Pro, a peptide with ACE inhibitory activity derived from bovine casein, which was hydrolyzed using subtilisin, bacillolysin and trypsin, significantly decreased systolic blood pressure (SBP) in the SHR model [19]. The pepsin-hydrolyzed peptide from Adlay glutelin was identified as Gly-Ala-Ala-Gly-Gly-Ala-Phe, and showed potent antihypertensive and ACE inhibitory activity in the SHR model at 15 mg/kg body weight [20]. Chen et al. (2015) and Huang et al. (2016) suggested that the peptides obtained using proteolytic enzymes alcalase and neutrase, from sardine and grass carp have strong ACE inhibitory and antihypertensive activities with a maximal drop in SBP by 23 and 43 mmHg, respectively in the SHR model [21,22]. However, no studies in the SHR model have been reported with peptide(s) obtained from beef hydrolysates using cost effective industrial enzymes. In our previous report, we obtained myofibrillar protein hydrolysates with different molecular weights from beef by using cost effective industrial enzymes and determined the ACE inhibitory of hydrolysates under *in vitro* conditions [8]. Therefore, the purpose of this study was to investigate the antihypertensive effect of novel peptides obtained from beef using inexpensive proteases in the SHR model, and to identify the sequence of these peptides.

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2. Materials and methods

2.1. Materials

The ACE and hippuryl-L-histydyl-L-leucine (HHL) were purchased by Sigma-Aldrich (St. Louis, MO., U.S.A.). The ultrafiltration membranes with a 3 kDa and 10 kDa molecular-weight-cut-off were purchased from Merck Millipore Corp. (Bedford, MA., U.S.A.). The thiobarbituric acid reactive substance assay (TBARS) kit was purchased by DoGen Bio Co. (Seoul, Korea). All other reagents were of the highest grade commercially available.

2.2. Preparation of peptides

Hanwoo (*Bos taurus coreanae*) beef shank (500 g) was purchased from the local market at Anseong-si, Gyeonggi, Korea. Ground beef (500 g) was mixed with 5 L distilled water (DW), and was washed 10 times to eliminate blood and fat. The extraction protein from the samples was processed with 2 L of 20 mM phosphate buffer (pH 7.4). The meat and buffer mixture was homogenized using a homogenizer for 60 s, and the used water was eliminated to obtain myofibrillar protein after centrifugation at $3000 \times g$ for 15 min. This study used two inexpensive enzymes, alkaline-AK and papain to hydrolyze the myofibrillar proteins. The protease alkaline-AK (180–200 KU/g solid) was obtained by fermenting soybean meal with *Bacillus methylotrophicus*, and papain was obtained from papaya plant (*Carica papaya*, EC 3.4.22.2). The myofibrillar protein was hydrolyzed by using 0.2% alkaline-AK (w/w) at pH 11 and 60 °C for 8 h; and 0.2% papain (w/w) at pH 6 and 60 °C for 4 h. The hydrolysates were heated at 90 °C for 15 min to inactivate alkaline-AK and papain. The ultrafiltration devices with 3 kDa and 10 kDa upper molecular weight cut-off membranes (Amicon® Ultra, Millipore, Billerica, MA, USA) were used to obtain peptides with molecular weight < 3 kDa and < 10 kDa from the beef hydrolysates. Afterwards, the < 3 kDa and < 10 kDa peptides obtained using alkaline-AK and papain digestion were lyophilized at −80 °C for 3 days and stored at −20 °C for further experiments. In this study, the used sample names are abbreviated as follows, AK3K, the < 3 kDa peptides obtained using alkaline-AK; AK10 K, the < 10 kDa peptides obtained using alkaline-AK; PA3K, the < 3 kDa peptides obtained using papain; PA10 K, the < 10 kDa peptides obtained using papain.

2.2.1. Fractionation and purification of peptides with ACE inhibitory activity

The peptides with the ACE inhibitory activity were subjected to a 2-step purification procedure using fast protein liquid chromatography (FPLC) on a gel permeation chromatography (GPC) column (HiPrep 26/60 Sephacryl S-100 HR, GE Health care Life Sciences, Chicago, IL, USA) at the flow rate of 2.0 mL/min. In the first step, the column was equilibrated with 50 mM Tris-HCl buffer (pH 6.8) and next, the peptides were eluted with 150 mM NaCl in the same buffer. The eluted peptide fractions were detected at 280 nm spectrophotometrically, and each fraction was analyzed for ACE inhibitory activity. The fractions with the highest inhibitory activities were concentrated using a rotary evaporator followed by lyophilization for 3 days in the next step.

In the second step, the fraction with ACE inhibitory was collected using RP-HPLC (HP Agilent 1100, Hewlett Packard Co., CA, USA) and HPLC system coupled with a fraction collector (Waters fraction collector II, Waters Co., MA, USA) on an Agilent Zorbax SB-C18 column (250 × 4.6 mm, 5 μm internal diameter). The fractionation was carried out using solvent A (water) and solvent B (0.1% TFA in acetonitrile) with an increasing linear gradient of B from 0 to 10% for 10 min, and a decreasing gradient from 10 to 0% for 25 min at a flow rate of 0.8 mL/min. The volume of the sample injected for analysis was 20 μL, and the detection wavelength was set at 200 nm. The highest ACE inhibitory of fractions was also determined. The most active fraction was subjected to a second round of RP-HPLC purification, using same conditions as

mentioned above. The purified peptide was further sent for the peptide sequencing.

2.3. Antihypertensive effect in the SHR model

The SHR model is considered to be the representative animal model for studying hypertension [23]. SHR model was generated by selective inbreeding of the Wistar-Kyoto rats (WKY) with a genetic basis for high blood pressure. In addition, SHR/Izm (Izumo) model was obtained from the stroke resistant SHR model with B substrain [24,25]. A total of 60 (20 mice/per experiment × 3 times) Male SHR/izm (10 weeks old) rats were purchased from Central Lab. Animal Inc. (Seoul, Korea). All the animals were housed in a 12 h day and night cycle and were fed a regular chow feed and tap water was provided *ad libitum*. All animal experiments were conducted following the Animal Care Ethics guidelines with protocols approved by the Animal Care Committee of the KPC Research Co., Ltd. of Korea (P182019). The *in vivo* experiments were performed in triplicates. After 1 week of adaptation, the SHRs were randomly divided in 4 groups, with 5 rats in each treatment group as follows, control (distilled water), captopril (20 mg/kg body weight), < 3 kDa peptide obtained by Alkaline-AK at 400 mg/kg body weight in distilled water (AK3K400), and < 3 kDa peptide obtained by Alkaline-AK at 800 mg/kg body weight in distilled water (AK3K800). All animals were administered drugs orally in a 1 mL solution using a disposable plastic syringe.

2.3.1. Measurement of blood pressure

The blood pressure and heart rate of SHRs were measured in a 40 °C chamber for 30 min by the tail-cuff method using blood pressure analysis systems (BP-2000 series II systems, Visitech systmes, NS, USA). After single oral administration as previously mentioned, the systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) were measured at the intervals of 0, 2, 6, 12 and 24 h. The rats were sacrificed after 24 h of oral administration, and blood and tissues (heart, kidney, spleen and liver) were collected, and stored at −80 °C for further analysis.

2.3.2. Organ index

After the oral administration for 24 h, the organs (liver, heart, kidney, spleen, and brain) of the SHRs were removed and washed with PBS, and were weighed using an analytical balance. The organ index was calculated by dividing the organ weight with the body weight.

2.3.3. Determination of oxidative status

The organs (liver, heart, kidney, spleen and brain) and serum of the SHRs were used for lipid peroxidation analysis. The lipid peroxidation was measured by TBARS kit according to manufacturer's instructions.

2.4. Determination of ACE inhibitory activity

The ACE inhibitory activity of fractions was measured by the Cushman and Cheung (1971) method [26] with slight modifications. Fifty microliters of sample was added to 50 μL of substrate (4 mM hippuryl-L-histidyl-L-leucine in 50 mM sodium borate buffer, pH 8.3) and was preincubated for 15 min at 37 °C, followed by incubation with 50 μL of ACE solution (0.025 U/mL) at 37 °C for 30 min. The control and blank solutions consisted of DW instead of peptides. The reaction was terminated by the addition of 0.25 mL of 1 M HCl. The hippuric acid in the samples where the reaction was terminated was extracted with 0.5 mL of ethyl acetate. After centrifugation at $5000 \times g$ for 5 min, 250 μL of the supernatant was removed and dried at 70 °C for 60 min. The extracted hippuric acid was dissolved in 2 mL of DW and the absorbance was detected at 228 nm using a spectrophotometer (Jasco, Tokyo, Japan). The ACE inhibitory activity was calculated as follows: ACE inhibitory activity (%) = $(C − S) / (C − B) \times 100$, where C is the control without sample, S is the sample, and B is the blank without

sample and the ACE solution.

2.5. Identification of ACE inhibitory peptide

In order to identify the peptide mass fingerprint of the AK3KF1-1 obtained by beef myofibrillar protein, nano LC–MS/MS analysis was performed with an Easy n-LC (Thermo Fisher Scientific, San Jose, CA, USA) and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray source. Samples were separated on a C18 nanobore column (150 mm × 0.1 mm, 3 μm pore size; Agilent Technologies, Santa Clara, CA, USA). The mobile phase A for LC separation was 0.1% formic acid, 3% acetonitrile in deionized water, and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 0%–32% B in 23 min, 32%–60% B in 3 min, 95% B in 3 min, and 100% A in 6 min. The flow rate was maintained at 1500 nL/min. Mass spectra were acquired using data-dependent acquisition with a full mass scan (350–1200 m/z), followed by 10 MS/MS scans. For full MS1 scans, the orbitrap resolution was 15,000 and the automatic gain control (AGC) was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

2.6. Statistical analysis

All the experiments were performed in triplicates and statistical analyses were performed using the one-way analysis of variance (ANOVA) using SPSS 20.0 (IBM, Armonk, NY, USA). Tukey's multiple comparisons test was used to determine significant differences between mean values, and evaluations were based on a significance level of $p < 0.05$.

3. Results and discussions

3.1. ACE inhibitory activity of novel peptides in an in vitro

The myofibrillar protein hydrolysates were extracted from beef by using commercial inexpensive enzymes, alkaline-AK and papain. These enzymes are more than 490 times cheaper than the commercially available purified trypsin and pepsin. Among the AK3K, AK10 K, PA3K and PA10 K fractions, the AK3K fraction showed the highest ACE inhibitory activity, followed by inhibitory activities in the following order, PA3K > PA10 K > AK10 K. The ACE inhibitory activities of the AK3K, AK10 K, PA3K, and PA10 K were 74.29 ± 1.12%, 60.62 ± 7.89%, 72.93 ± 1.26%, and 69.06 ± 0.55%, respectively (Table 1). The fractions with high ACE inhibitory activity from AK3K and PA3K were isolated by using FPLC on a GPC column (HiPrep 26/60 Sephacryl S-100 HR). Components of three isolated fractions of AK3K and PA3K are presented in Fig. 1. Each fraction was pooled and freeze-dried and its ACE inhibitory activity was determined. The ACE inhibitory activity of fractions obtained from AK3K at 20 mg/mL were 65.34 ± 7.26% for fraction 1 (F1), 36.95 ± 9.74% for fraction 2 (F2), and 52.46 ± 13.95% for fraction 3 (F3), respectively. The ACE

inhibitory activities of fractions obtained from PA3K at 20 mg/mL showed ACE inhibitory activities as F1 (38.14 ± 15.74%), F2 (67.05 ± 9.08%), and F3 (43.00 ± 16.53%), respectively (Table 1). Previous studies revealed that higher ACE inhibitory activities have been observed for low-molecular-weight peptides than for high-molecular-weight peptides [27,28]. Lee and Hur (2017a) also observed that the low-molecular-weight fraction of beef protein hydrolysate showed a higher ACE inhibitory activity than the high-molecular-weight fraction [8]. Moreover, other studies have also reported that low-molecular-weight peptides showed higher bioavailability than others due to an increased intestinal absorption [29,30]. It is because a shorter length/low-molecular-weight peptide can be easily absorbed from the intestine as well as easily binds to the active site of the ACE enzyme resulting in an almost complete inhibition of the ACE enzyme activity, thereby reducing to the conversion of angiotensin I to angiotensin II [31,32]. We also report that the low-molecular-weight (< 3 kDa) peptides can strongly inhibit angiotensin II formation by ACE compared to high-molecular-weight (< 10 kDa) peptides. Hence, we speculate that the low-molecular-weight (< 3 kDa) peptides show a higher ACE inhibitory activity due to rapid rates binding to the ACE active site.

We report in a previous study the beef myofibrillar hydrolysate obtained by alkaline-AK (exopeptidase obtained by fermenting soybean meal with *Bacillus methylotrophicus*) was mainly composed of aspartic acid, valine, leucine, isoleucine, and lysine [8,33], whereas papain is an endopeptidase that acts on amino acid residues of valine, arginine, and lysine to induce protein cleavage [34]. Several studies [35,36] reported that the peptides with ACE inhibitory activity generally show a high abundance of carboxylic or branched-chain amino acid residues such as aspartic acid, glutamic acid, leucine, and valine. Therefore, we reasoned that AK3K could have higher ACE inhibitory activity than the other fractions. In addition, the carboxylate from glutamic acid at the C-terminal chelates zinc, which is essential for the functioning of the ACE active site [37]. The abundance of branched chain and acidic amino acids in AK3K could be a potential reason for high ACE inhibitory activity than those of other fractions.

3.2. Antihypertensive effect of novel peptide in SHR model

The antihypertensive effects of AK3K were evaluated by measuring the changes in the SBP, DBP, and mean arterial pressure (MAP) in SHRs at 0, 2, 6, 12, and 24 h after the oral administration of 400 (AK3K400) and 800 (AK3K800) mg/kg of body weight (Fig. 2). After a 2 h administration of AK3K, SBP of AK3K400 and AK3K800 SHRs showed a decrease in SBP by 17 mmHg and 15 mmHg, respectively when compared with the control group. The SBP of AK3K400 and AK3K800 exhibited a maximal decrement by 28 mmHg and 35 mmHg after 12 h of AK3K administration. There were no differences in the antihypertensive effect in SHR rats between the AK3K and positive control group, which was administered with the antihypertensive drug captopril. Although, the DBP and MAP decreased by about 35 mmHg after 12 h of administration compared to 0 h, no significant differences were observed in the DBP and MAP among the treatment groups in this study.

We observed that AK3K showed antihypertensive effects similar to the ACE inhibitory drug, captopril, especially on the SBP of rodents. Although the main mechanism for antihypertensive effect of AK3K is unclear, we hypothesized multiple possible causes of AK3K action. First possible mechanism of the antihypertensive effect could be the presence of hydrophobic amino acids in the peptide. This is in line with the observation that the peptides with hydrophobic amino acids may play a key role in the antihypertensive activity [38,39]. Previous studies have indicated that ACE inhibitors suppress the angiotensin I conversion and activation of bradykinin *in vitro* and *in vivo*, due to high affinity for active site of ACE, and are also composed of hydrophobic amino acids such C-terminal tripeptide residues [40]. Wu et al. (2006) found that ACE inhibitory activity and the inhibition of ACE I conversion rate by a peptide increased significantly with a greater percentage of the
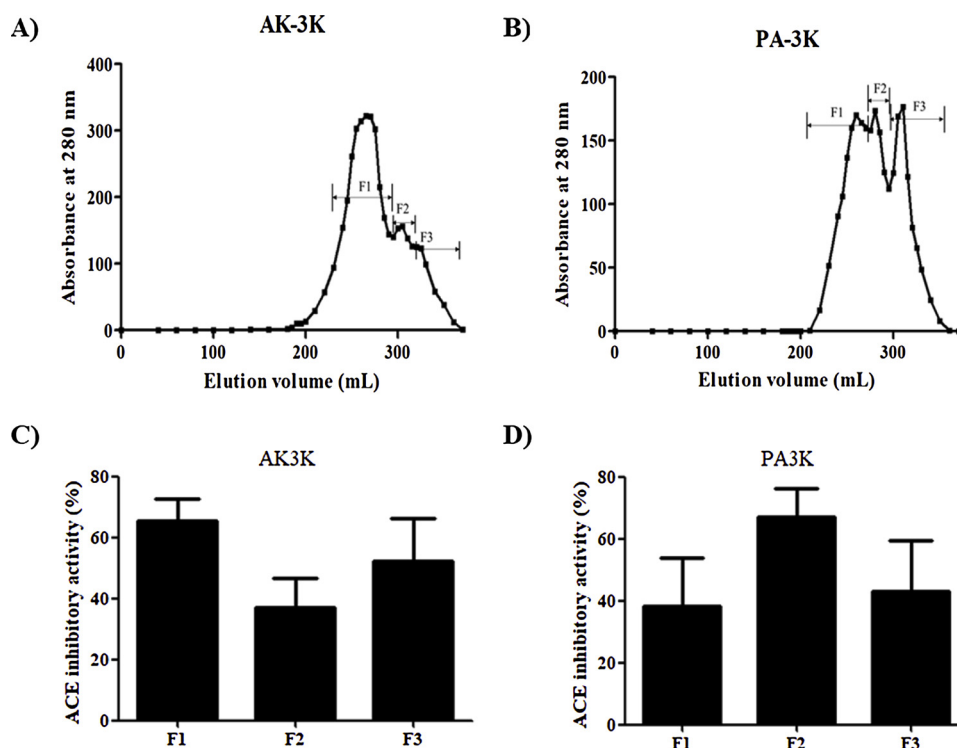
Table 1
ACE inhibitory activity of peptides obtained from alkaline-AK and papain digestion, and their subfractions.

Samples	ACE inhibitory activity (%)	Samples	ACE inhibitory activity (%)
AK3 K [1]	74.29 ± 1.12	PA3 K [1]	72.93 ± 1.26
AK10 K [1]	60.62 ± 7.89	PA10 K [1]	69.06 ± 0.55
AK3K-F1 [1]	65.34 ± 7.26	PA3K-F1 [1]	38.14 ± 15.74
AK3K-F2 [1]	36.95 ± 9.74	PA3K-F2 [1]	67.05 ± 9.08
AK3K-F3 [1]	52.46 ± 13.95	PA3K-F3 [1]	43.00 ± 16.53
AK3K-F1-1 [2]	56.87 ± 1.24	PA3K-F2-1 [2]	56.02 ± 7.86
AK3K-F1-2 [2]	46.84 ± 3.83	PA3K-F2-2 [2]	35.13 ± 9.51

[1] Peptide concentration: 20 mg/mL [2] Peptide concentration: 4 mg/mL.

Fig. 1. Elution profile (A and B), and ACE inhibitory activity of the peptides < 3 kDa (C and D) obtained by digestion of beef myofibrillar proteins by alkaline-AK and papain by fast protein liquid chromatography on GPC column (HiPrep 26/60 Sephacryl S-100 HR).

hydrophobic amino acids [41]. Our previous study also found that the AK3K contained high amounts of hydrophobic amino acids such as alanine, isoleucine, leucine and methionine [33]. Thereby, ACE inhibitory activity of the peptide can be explained by the abundance in the hydrophobic amino acids regulating action of the enzyme site (ACE) *via* strong binding competition.

Another possible mechanism for the antihypertensive effect of AK3K could be based on the peptide size. The strong ACE inhibitory peptides are characterized by small size and a capability to resist protease digestion, which also facilitates easier absorption from the intestine, and as a result these peptides display an effective antihypertensive effect after oral administration [42]. Similar observations have been reported wherein, short-chain peptides with low molecular weight show a better rate of absorption from the intestine and result in a better antihypertensive effect than long-chain peptides [8,32], because the small-sized peptides can easily bind with the substrates of the enzyme. Thus, antihypertensive effects exerted by AK3K, the ACE inhibitory peptide with hydrophobic amino acids and small size could be due to a high binding affinity with the substrate of ACE, or by blocking the active site of ACE.

3.3. Measurement of organ index and determination of oxidative status in serum and organs

As shown in Table 2, the body and organ weight of the heart, kidney, spleen, and liver showed no significant change across all the dietary groups, indicating that AK3K administration may not largely influence the organs.

The concentrations of malondialdehyde (MDA) were measured as an indication of lipid oxidation in the serum of the SHRs. As shown in Fig. 3, the lipid oxidation levels as measured by the concentration of MDA in the serum were significantly lower in the AK3K at 400 and 800 mg/mL and captopril treated groups than the control group ($p < 0.05$).

Previous reports have suggested that the occurrence of coronary

heart disease, such as hypertension is linked to the oxidative stress and the development of lipid peroxides [43,44]. Vascular oxidative stress has been determined in spontaneous genetic animal models, such as the SHR model. In addition, increased oxidative stress levels have also been demonstrated in mesenteric vessels and aorta of SHRs [45,46]. The serum MDA levels which serve as an indicator of lipid oxidation and oxidative status show an increase in hypertensive patients. MDA levels also show a concomitant decrease upon administration of anti-hypertensive therapy [47,48]. In this study, reduction of lipid oxidation levels after AK3K treatment could be correlated with an improvement in the physiology of the SHRs. Our previous study [8] also found that AK3K administration enhanced iron chelation and nitrite scavenging activity under *in vitro* conditions. Therefore, the decrease in MDA levels, associated with an alleviation of oxidative stress by administration of AK3K could be another possible mechanism of action for the anti-hypertensive effect of AK3K in SHRs.

3.4. Identification of the ACE inhibitory peptide

Fraction F1 of AK3K and fraction F2 of PA3K, which showed the highest ACE inhibitory activity, were further separated into 2 subfractions, F1-1 and F1-2 by RP-HPLC. The ACE inhibitory activities of AK3K F1-1, AK3K F1-2, PA3K F2-1, and PA3K F2-2 at 4 mg/mL were 56.87 ± 1.24%, 46.84 ± 3.83%, 56.02 ± 7.86 and 35.13 ± 9.51%, respectively. Among these fractions, AK3KF1-1 showed the highest ACE inhibitory activity. Following the identification of the fraction with the highest ACE inhibitory activity, we sought to identify the specific peptide with the ACE inhibitory activity (Table 1). The amino acid composition of the ACE inhibitory peptide from above F1-1 fraction was determined by LTQ Orbitrap XL mass spectrometer, and the sequence of ACE inhibitory peptide was identified as Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val (Fig. 4). We report for the first time that Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val, obtained from the beef myofibrillar protein showed a high ACE inhibitory activity in SHRs. Previous studies have revealed that there is a relationship between ACE inhibition and the peptide structures
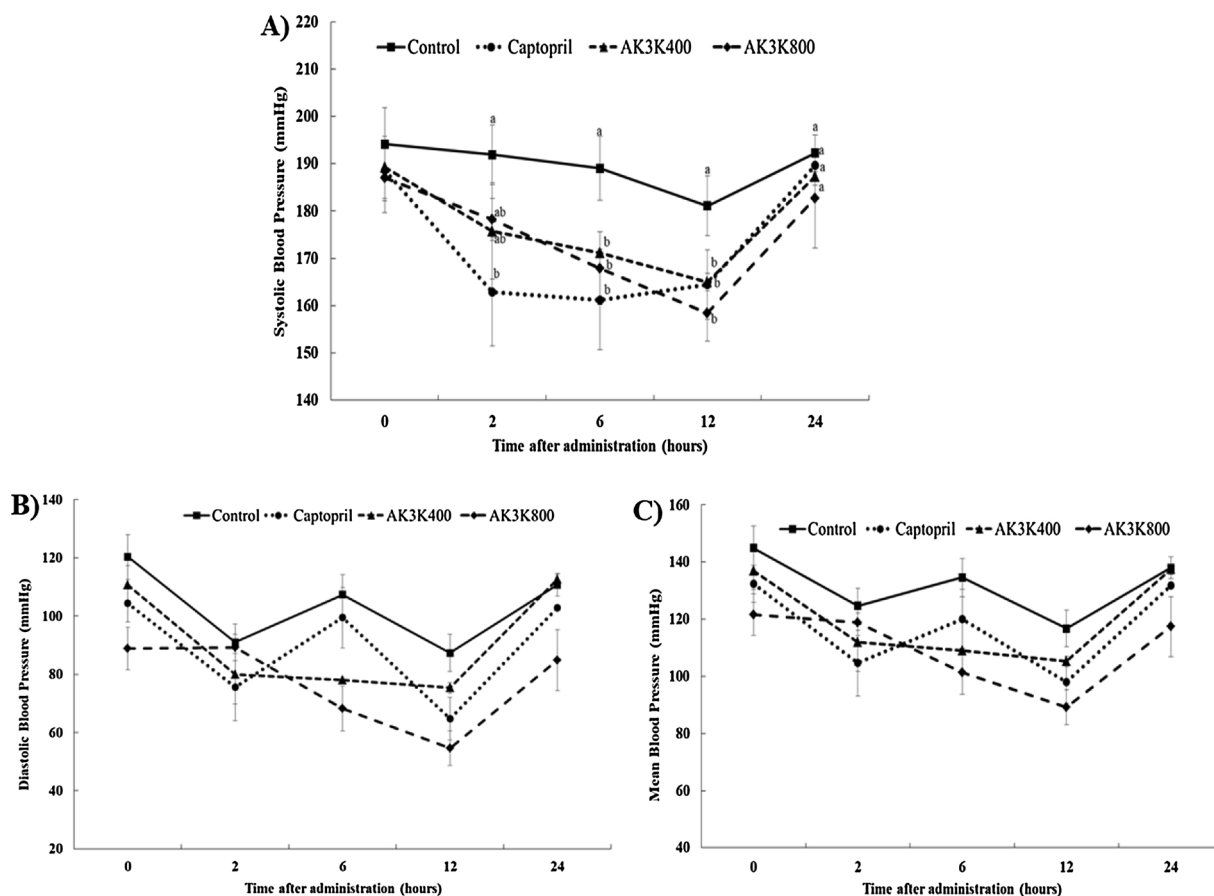
Fig. 2. Antihypertensive effects of AK3K peptides on the SHR model. Captopril (20 mg/kg body weight) and peptides < 3 kDa obtained by alkaline-AK digestion, AK3K400 and AK3K800 mg/kg body weight, respectively on (A) systolic blood pressure, (B) diastolic blood pressure, and (C) mean blood pressure in SHR model after 0, 2, 6, 12, and 24 h of a single oral administration. Data are presented as mean ± SD. [a-b]Different letters indicate statistically significant differences ($p < 0.05$) in the different groups.

Table 2
The body weight, organ weight, and organ index of each treatment group.

Treatment Group	Weight (g)					
	Body	Liver	Heart	Kidney	Spleen	Brain
Control	264.30 ± 10.05	10.31 ± 0.94	1.02 ± 0.01	2.00 ± 0.12	0.37 ± 0.05	1.83 ± 0.04
Captopril	260.08 ± 5.23	9.89 ± 0.54	1.03 ± 0.02	2.02 ± 0.06	0.38 ± 0.01	1.84 ± 0.01
AK3K 400	270.74 ± 5.68	10.12 ± 0.41	1.05 ± 0.01	2.12 ± 0.03	0.38 ± 0.02	1.86 ± 0.05
AK3K 800	266.11 ± 6.32	9.78 ± 0.29	1.08 ± 0.01	2.13 ± 0.13	0.37 ± 0.01	1.86 ± 0.02

Values represent mean ± SD (n = 3). The groups are control (distilled water), captopril (20 mg/kg body weight), AK3K400 (400 mg/kg body weight), and AK3K800 (800 mg/kg body weight).

[49,50]. The ACE inhibitory peptide is mainly composed of short chain peptides with 2–12 amino acids, mainly a mixture of different amino acids including acidic amino acids (Asp and Glu), basic amino acids (lysine and arginine), and hydrophobic amino acids (phenylalanine, valine, isoleucine, leucine, proline, and tryptophan) which have a strong influence on ACE-binding [8,51,52]. A possible reason for the antihypertensive activity of Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val is its amino acid composition. Hydrophobic amino acid residues with aliphatic side chains, such as Gly, Ala, Val, Leu, and Ile at C-terminus have been associated with a significant increase in the ACE inhibitory activity due to higher binding ability with ACE than the other amino acids [53]. Studies also report that peptides with sulfhydryl (SH) group cause an improvement in hypertension by the free radical scavenging activity of the thiol group relieving the oxidative stress [54,55]. Moreover, cysteine has a potent antihypertensive effect, because thiol group of cysteine shows an antioxidant activity resulting in reduction of

oxidative stress by modulating the levels of nitric oxide [56]. In addition, arginine is the precursor for nitric oxide, which has a potent ACE inhibitory activity and causes a reduction in blood pressure [57]. Furthermore, the above-mentioned amino acids with small or low molecular weight are more effective inhibitors of ACE due to strong binding with ACE, and higher absorption rates in the intestine. Therefore, we assume that peptides (Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val) obtained from beef myofibrillar protein by using alkaline AK in this study may show antihypertensive effect due to a combination of the above-mentioned mechanisms. Therefore, the < 3 kDa novel peptide obtained by alkaline-AK could be potentially used as safe ACE inhibitory material.

4. Conclusions

This study determined the antihypertensive effect of novel peptides obtained from beef protein hydrolysates by alkaline-AK and papain
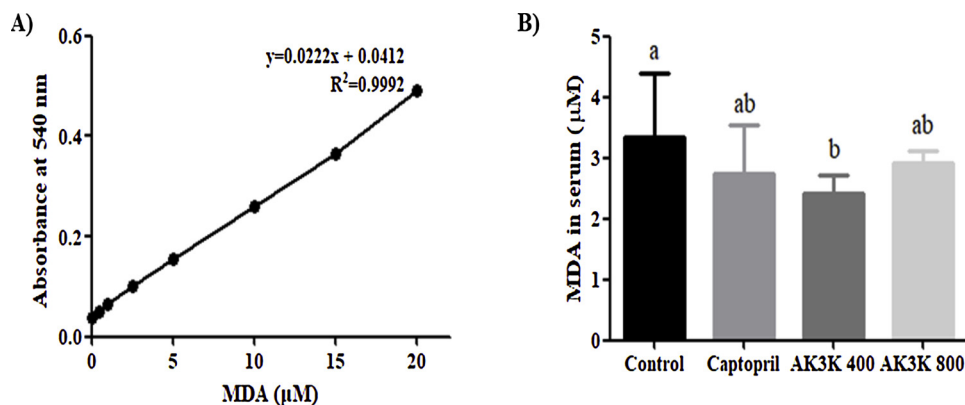
Fig. 3. TBARS assay results of (A) standard graph for the estimation of lipid peroxidation by malondialdehyde (MDA), and (B) MDA in serum. Values represent mean ± SD (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) in organ weight between different treatment groups.
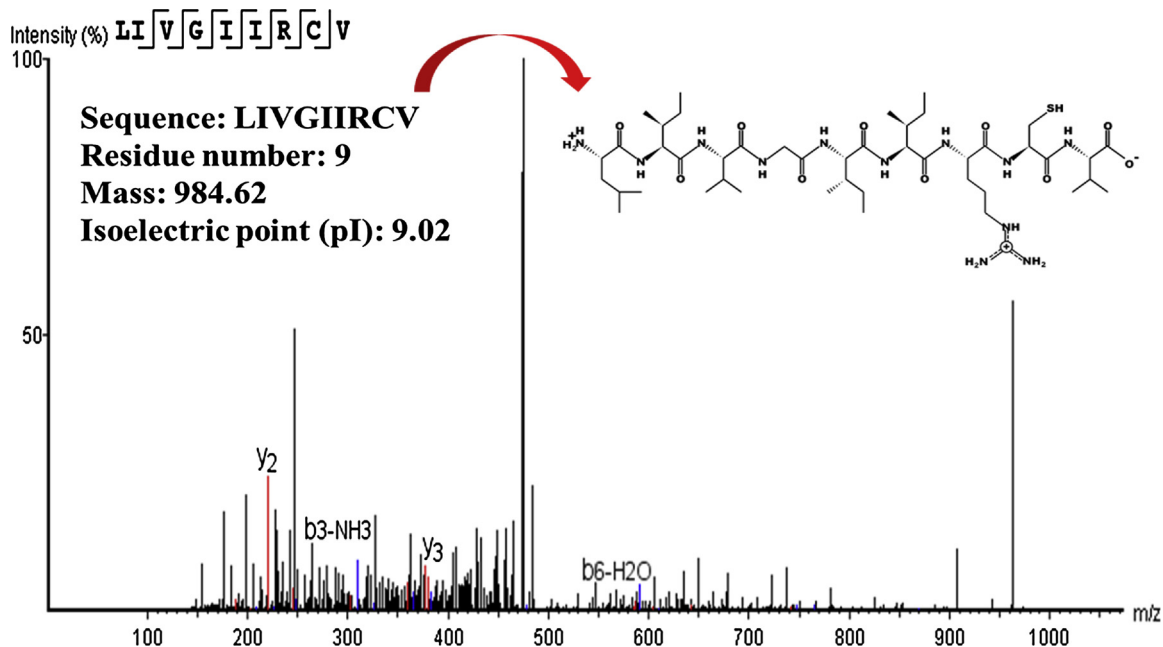

Fig. 4. Identification of molecular mass and amino acid sequence of ACE inhibitory peptide in peak obtained from AK3KF1-1. MS/MS was performed on a LTQ Orbitrap XL mass spectrometer.

digestion in the SHR model. The peptide sequences were identified by FPLC and LC–MS/MS. The < 3 kDa novel peptide from the alkaline-AK fraction had effective antihypertensive activity in SHRs, which could contribute to the reduction of SBP and MDA levels in the serum. Furthermore, the < 3 kDa novel peptide, Leu-Ile-Val-Gly-Ile-Ile-Arg-Cys-Val, obtained by alkaline-AK digestion of beef myofibrillar protein was identified. This peptide could act as a new potent natural material for treatment of symptoms of cardiovascular disease.

Ethical statement

All animal experiments were conducted following the Animal Care Ethics guidelines with protocols approved by the Animal Care Committee of the KPC Research Co., Ltd. of Korea (P182019).

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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