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Full Length Research Paper

Isolation and characterization of angiotensin I-converting enzyme inhibitor peptides derived from porcine hemoglobin

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In China, porcine blood has a long history as a food with medicinal effects and for treating strokes, an obvious clinical consequence of hypertension. However, the active ingredients are still unknown and need to be clarified. This study described the isolation, characterization and animal experiments of angiotensin converting enzyme (ACE)-inhibitor peptides derived from porcine blood. The highly active low-molecular-weight hydrolysates were obtained from pepsin digestion of discolored porcine blood. After isolation of the hydrolysate, an active fraction containing three peptides was obtained. These peptides were analyzed by MALDI-TOF-MS. They were WVPSV (P1), YTVF (P2), VVYPW (P3), with median inhibitory concentrations of 0.368, 0.226, 0.254 mg/ml, respectively. They were first found in porcine blood. Additionally, a digestion test was performed to verify the antihypertensive effect of the peptides *in vitro*. After digestion with gastrointestinal proteases, the ACE-inhibitor activity of these peptides was enhanced. When these peptides were administered orally to spontaneously hypertensive rats at a dose of 10 mg/kg, a temporary antihypertensive activity was observed at 3 and 15 h after administration. These findings suggested that the peptides in porcine blood might have potential as antihypertensive agents.

Key words: Angiotensin I-converting enzyme, inhibitor activity, peptide, porcine hemoglobin.

INTRODUCTION

Porcine blood has a long history as a food with medicinal effects or as a medicine in China. The Ming dynasty compendium of *Materia Medica* recorded that porcine blood has medicinal effects such as treating strokes (Li, 2004) which has been demonstrated as an obvious clinical consequence of hypertension. This effect of porcine blood can be found in ancient medical books such as the *M. Bielu* which contains the earliest records of the medicinal effects of porcine blood about two thousand years ago (Tao and Porcine, 1999). However, although more than 400 million pigs are currently slaughtered annually to provide meat in China alone (Nakamura et al., 1995), the porcine blood is usually discarded or little is used in industries while its medicinal functions are neglected. This is mainly caused by the uncertainty of the active ingredient in porcine blood. Therefore, determining its active ingredient is of particular significance. Angiotensin I–converting enzyme (ACE) plays an important physiologic role in regulating blood pressure (Suetsuna, 1998). It catalyzes the conversion of the inactive angiotensin I to the potent vasodilator, bradykinin. Hundreds of ACE-inhibitor drugs have been (Ondetti et al., 1977) reported such as captopril and cilazapril. Recent studies suggest that many peptides from enzymatic digests of various food materials

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including casein (Maeno, 1996; Silva et al., 2005), sardine muscle (Matsui et al., 1993), porcine skeletal muscle (Katayama et al., 2008), peanuts (Okamoto et al., 1995), rapeseed (Marczak et al., 2003), milk (Matsui et al., 1992) and fish proteins sources (Ichimura et al., 2003; Quist et al., 2009) have ACE-inhibitor function. Porcine blood is a rich source of proteins. Although some peptides have been isolated and characterized from porcine blood, including LGFPTTKTYFPHF (Yu et al., 2006), VVYPWT (Yu et al., 2006), PGLVVA (Mito et al., 1996), GLLVLG (Mito et al., 1996) and so on; it is essential to find novel peptides with ACE-inhibitor activity from porcine blood.

In this study, the isolation, purification and identification of novel ACE-inhibitor peptides from porcine blood were described. Furthermore, the three peptides were orally administrated in spontaneously hypertensive rats (SHR) to determine whether they were antihypertensive *in vivo.*

MATERIALS AND METHODS

Porcine blood was purchased from the market in Chengdu City, China. Hippuric acid-histidine-leucine (HHL) and ACE were obtained from the Sigma Chemical Co. Captopril was obtained from the Chengdu Institute for Drug Control. Acetonitrile was obtained from Thermo Fisher Scientific Inc. The SHRs were purchased from Slac Laboratory Animals Inc.

Pre-treatment of porcine blood

Crude hemoglobin solution from porcine blood was obtained by centrifugation at 4,700 × g for 30 min. The crude globin solution was decolorized by enzymatic hydrolysis and absorption method (Ren et al., 2010). It was hydrolyzed with 8% pepsin, 18% substrate concentration at 35°C for 0.5 h. Then, the solution was adjusted to pH 3 and carbon (100:3 w/v) was added to react at 55°C for 0.5 h. After the removal of the insoluble fraction by centrifugation at 4,000 × g for 20 min, the supernatant solution was digested further with pepsin with a ratio of protein substrate to enzyme (1:3 w/w) at 37°C for 6 h. The hydrolysate was boiled at 90°C and the sediment was removed using centrifugation. The hydrolysate was stored at -20°C. Moreover, the molecular weight range was tracked by Glycine-SDS-PAGE (Coligan, 2007).

Isolation and purification of ACE-inhibitor peptides

A peptide with molecular weight of less than 3 kDa hydrolysate was isolated and concentrated using an Amicon ultra 3 K centrifugal filter device (4000 × g, 40 min). About 2 ml of the ultrafiltrate was separated on a Sephadex G-25 (Amersham Biosciences, Shanghai, China) gel filtration column (1.6 × 60 cm) and eluted with acetic acid–sodium acetate buffer (0.05 mol/L, pH 5.0) at a flow rate of 1.5 ml/min. Elution curves were obtained by measuring absorbance at 280 nm using an online spectrophotometer. In addition, the molecular weight range was tracked by Tricine-SDS-PAGE (Coligan, 2007). The fraction that showed high ACE-inhibitor activity was collected and filtered with a 0.45 μm-filter. Moreover, the most active fraction was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shim-pack VP-ODSC_{18} column (4.6 × 150 mm) at a flow rate of 0.5 ml/min using a water/acetonitrile (9:1) mobile phase and the eluent was monitored at 220 nm.

Assay of ACE-inhibitor activity

The determination of ACE-inhibitor activity was performed using the spectrophotometric method by Matzui with slight modifications (Matsui et al., 1992). For each assay, 12.5 μl of inhibitor and 25 μl of HHL (2.5 mM in borate buffer containing 200 mM NaCl at pH 8.3) were incubated with 25 μl of 25 mU ACE at 37°C for 1 h. The reaction was stopped by adding 63 μl 1M HCl and then the solution was adjusted to pH 9.2 by adding 14 μl Kolthoff buffer. Then, 12.5 μl TNBS in 0.1 M Na_2HPO_4 solution was added. After incubation at 37°C for 25 min, 2.25 ml 4 mM Na_2SO_3 in 0.2 M NaH_2PO_4 solution was added and the absorbance at 416 nm was measured on a Thermo Varioskan spectral scanning multimode reader.

Mass spectrometry analysis

MS and MS/MS measurements of the active peptide were performed in positive ion mode using MALDI-TOF/MS and ESIMS/MS, respectively.

Peptide synthesis

These peptides were chemically synthesized by the Shanghai Biotech BioScience and Technology Co., Ltd., Shanghai, China. The purity of the synthesized peptides was verified by RP-HPLC coupled with MS.

Digestion test

The stability of the ACE-inhibitor peptides against gastrointestinal proteases was assessed *in vitro*. The peptides were digested with pepsin and trypsin as previously described in the article. The digests were used for ACE-inhibitor activity determination.

Antihypertensive effects in spontaneously hypertensive rats

Animals used in this study were maintained through the method of Muguruma (Nakamura et al., 1995) with slight modifications: four 8-week-old SHRs were used as the controls and six as the experimental group. They were kept in a room with a 12 h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.). Temperature and humidity were controlled at 24 ± 1°C and 50 ± 10%, respectively. Food and tap water (0.45 μm) were available *ad libitum*. After 4 weeks of maintenance, the SHRs were used for experiment and the initial systolic blood pressures (SBP) were within 150 to 170 mmHg. The peptide was dissolved in distilled water (1 mg/ml) and orally administered to the SHRs at a dose of 10 mg/kg body weight (10 ml/kg). The control group was administered the same volume of distilled water. The SBP of the rats was measured at 0, 3, 15 and 24 h after administration of the peptides solutions using the tail method with a programmable electronic sphygmomanometer (BP-6; TME Technology Co., Ltd., Chengdu, China) after the rats had been warmed in a chamber maintained at 36°C for 25 min. The significance of differences in SBP before and after administration was analyzed using a paired Student's *t* -test.

RESULTS

Isolation of ACE-inhibitor peptides

The decolorized porcine blood was digested with pepsin and the ACE-inhibitor activity of the crude hydrolysate

Table 1. ACE-inhibitor activity and yield of hydrolysate, fraction I and three peptides.

	Sequence	Recovery yield (%)	Purification fold	IC_{50} (mg/ml)
Hydrolysate				4.370
Fraction I				0.397
P1	WVPSV	0.11	166	0.368
P2	YTVF	0.13	125	0.226
P3	VVYPW	0.08	182	0.254

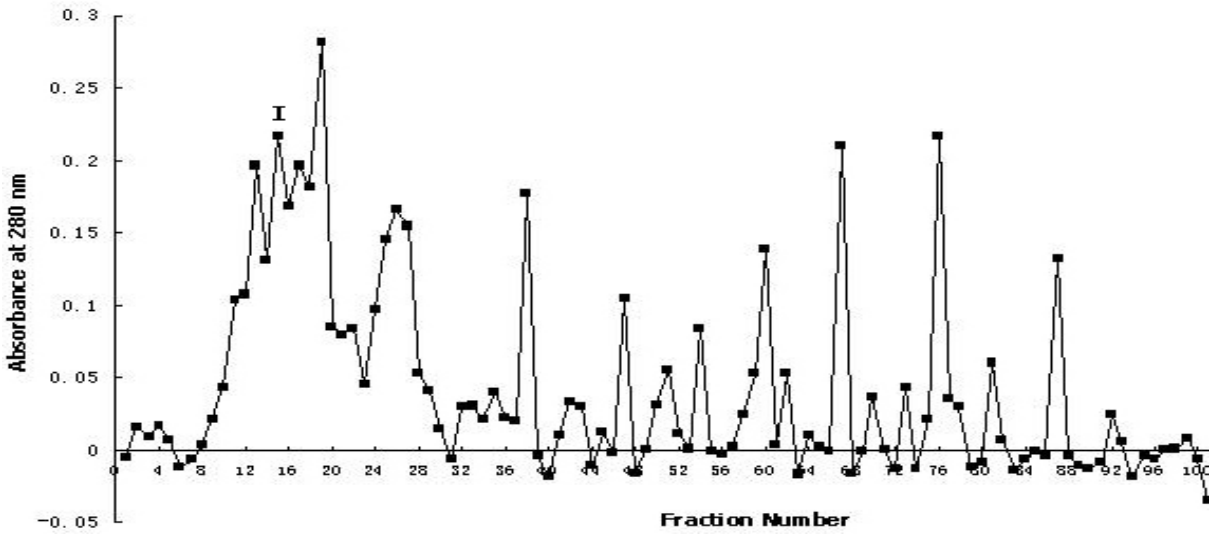

Figure 1. Gel filtration chromatography of peptic hydrolysate on a Sephadex G-25 column with acetic acid-sodium acetate buffer (0.05 mol/L, pH 5.0) at a flow rate of 1.5 ml/min. I represented fraction I.

was determined (Table 1). The median inhibitory concentrations (IC_{50}) of the peptides showed high activity (4.370 mg/ml). Then, the hydrolysate was fractionated on Sephadex G-25 gel filtration chromatography. As shown in Figure 1, there were major absorbance peaks at 280 nm and the fractions associated with the peaks were collected for the ACE-inhibitor activity assay. ACE-inhibitor activity was observed in all fractions, however, fraction I showed the highest activity with IC_{50} (0.397 mg/ml) as shown in Table 1. The lower the molecular weight of the peptides, the more detrimental to the direct absorption by the gel. Thus fragments that were eluted early (Fraction I) were low molecular weight peptides. Fraction I was further separated by RP-HPLC on a Shim-pack VP-$ODSC_{18}$ column. Three peptide (P1, P2 and P3) showing ACE-inhibitor activity were obtained and each peptide was further purified in the same RP-HPLC conditions with different gradients.

Characterization of ACE-inhibitor peptides

These three peptides were identified by MALDI-TOF-MS from fraction A. Figure 2 showed peptide 1 with the amino acid sequence WPYVV. Peptide 2 was identified as shown in Figure 3. Figure 4 shows the amino acid sequence of peptide 3. These were all novel ACE-inhibitor peptides and were the first purified from porcine blood with IC_{50} values 0.368, 0.226 and 0.254 mg/ml, respectively (Table 1).

Digestion test of ACE-inhibitor peptides

The digestion test of P1, P2 and P3 (1 mg/ml) with gastrointestinal protease was used to predict the antihypertensive effects *in vitro*. As shown in Table 2, the ACE-inhibitor activity of P1 was increased by the hydrolysis of pepsin, whereas its activity changed little by the hydrolysis of trypsin. The ACE-inhibitor activity of P2 was increased by the hydrolysis of trypsin, but it changed little by the hydrolysis of pepsin. The ACE-inhibitor activity of P3 was little changed by the hydrolysis of trypsin and pepsin, respectively. Moreover, the ACE-inhibitor activity of P1 and P2 increased by the hydrolysis of pepsin and trypsin together, but P3 activity changed little.

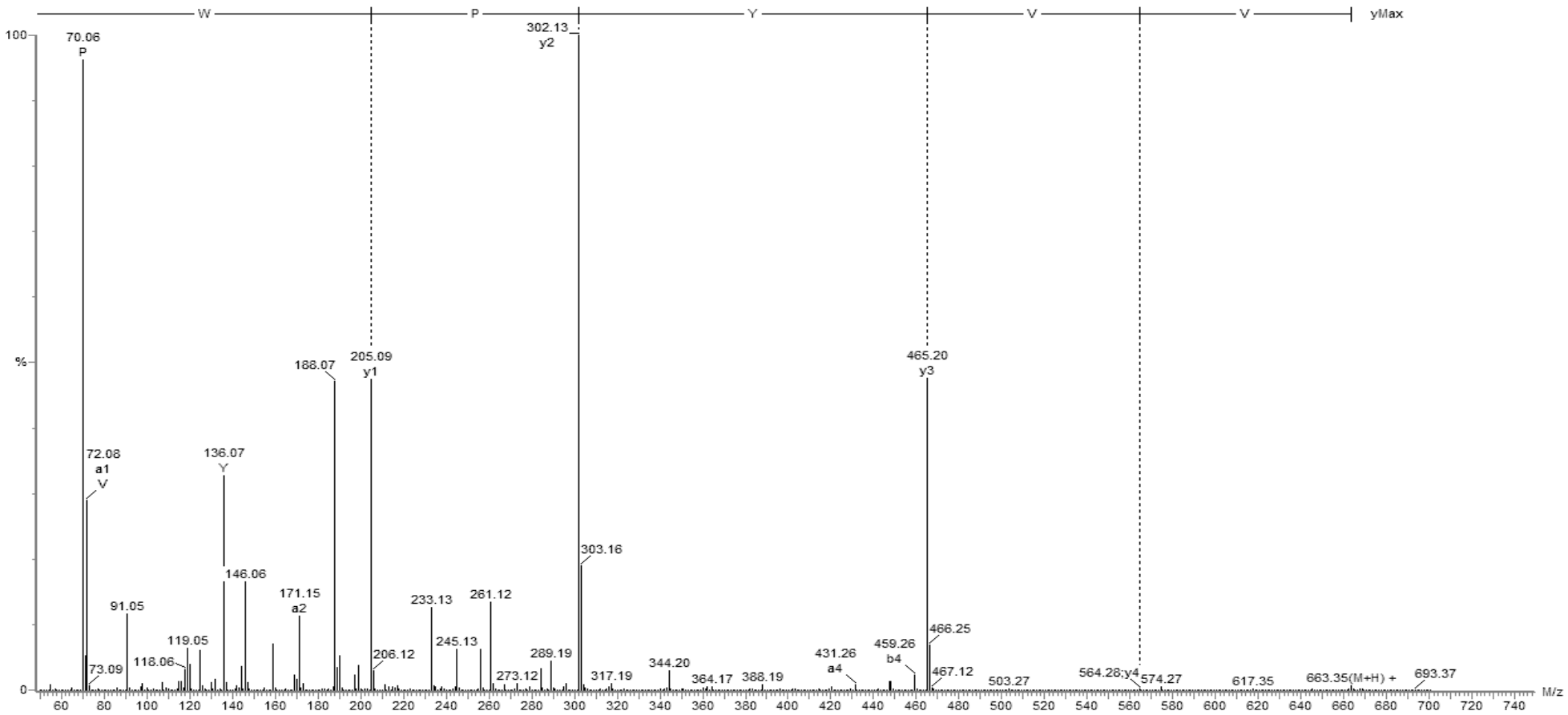

Figure 2. MALDI-TOF/MS of the peptide P1 and the sequence of the peptide were showed on the top of the figure.

Antihypertensive effects of the identified peptides on spontaneously hypertensive rats

As the three synthetic peptides showed strong ACE-inhibitor activity *in vivo*, they were orally administered to the SHRs. The SBPs of the rats at 3 h after administration of the peptides decreased significantly ($p < 0.05$) compared with those of the control sample. After administration of P1 and P2, the SBP of the SHR decreased by 22.5 and 18.5 mmHg, respectively, whereas those after administration of P3 were lower (decreased by 9.6 mmHg), as illustrated in Figure 5. This indicated that P1 and P2 had greater antihypertensive activity. 15 h later, the values of SBP decreased significantly ($p < 0.05$) after administration of P1 and P2 (decreased by 9.6 and 14.75 mmHg), but P3, change value was only 4.9 mmHg.

After administration for 24 h, the values of SBP of P1 and P3 groups had almost returned to the initial values except in the P2 group, which showed that P2 might take longer to be metabolized. Consequently, the blood pressure system treated with ACE was restored to almost

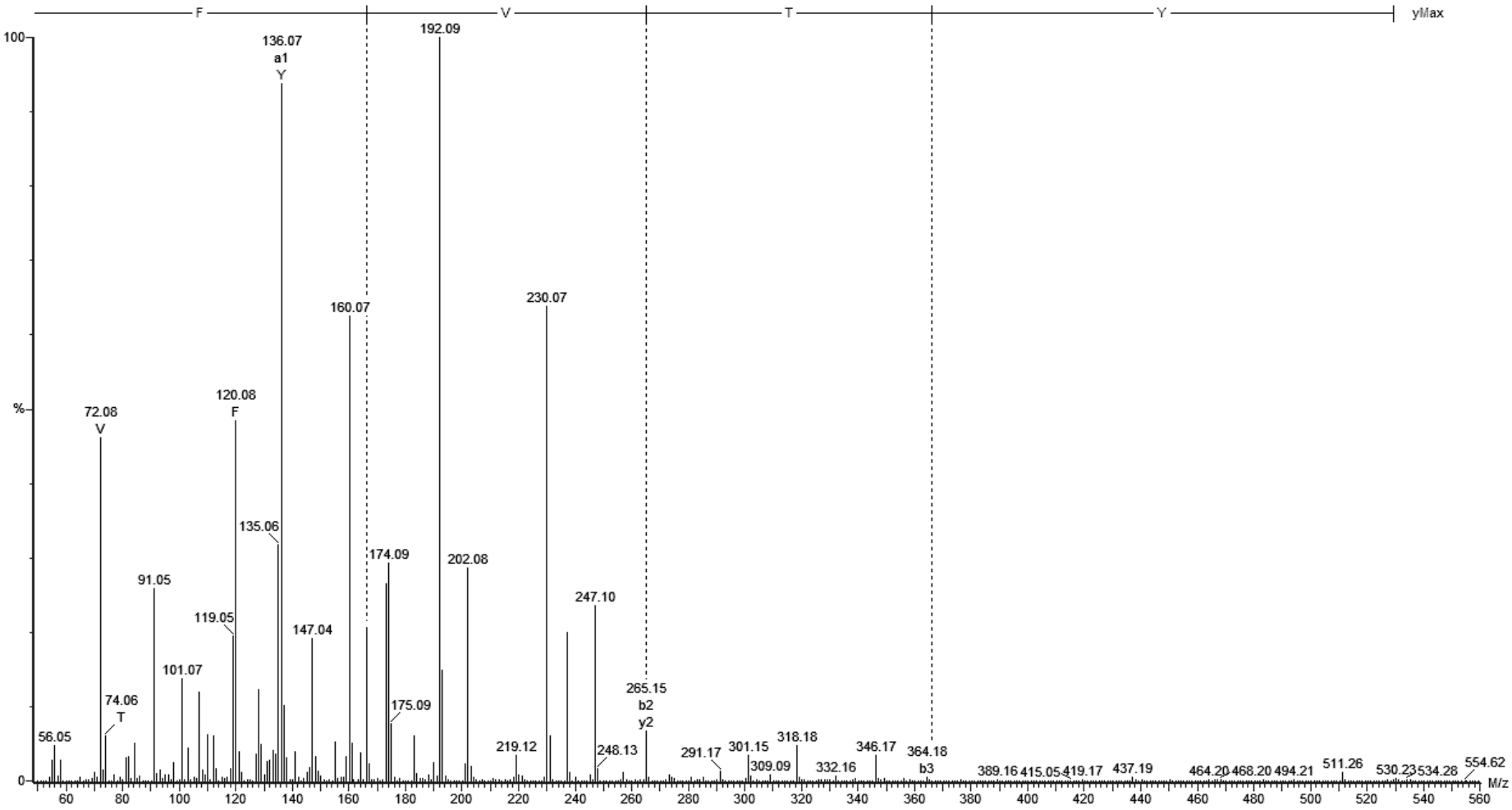

Figure 3. MALDI-TOF/MS of the peptide P2 and the sequence of the peptide was showed on the top of the figure.

the pre-administration state of the peptides within 36 h after administration.

DISCUSSION

To digest the crude discolored porcine blood, four proteases were used. Given that low-molecular-weight peptides are easy absorbed by the human body, the content of the low-molecular-weight peptides in the hydrolysate was maximized as much as possible. In addition, the proteases were used to simulate the *in vitro* digestion conditions. Tricine-SDS-PAGE was used to trace the molecular weight range of the hydrolysate, which indicated that pepsin was the optimal protease. The ACE-inhibitor activity was all enhanced after digestion of the three peptides with gastrointestinal protease. This suggested that

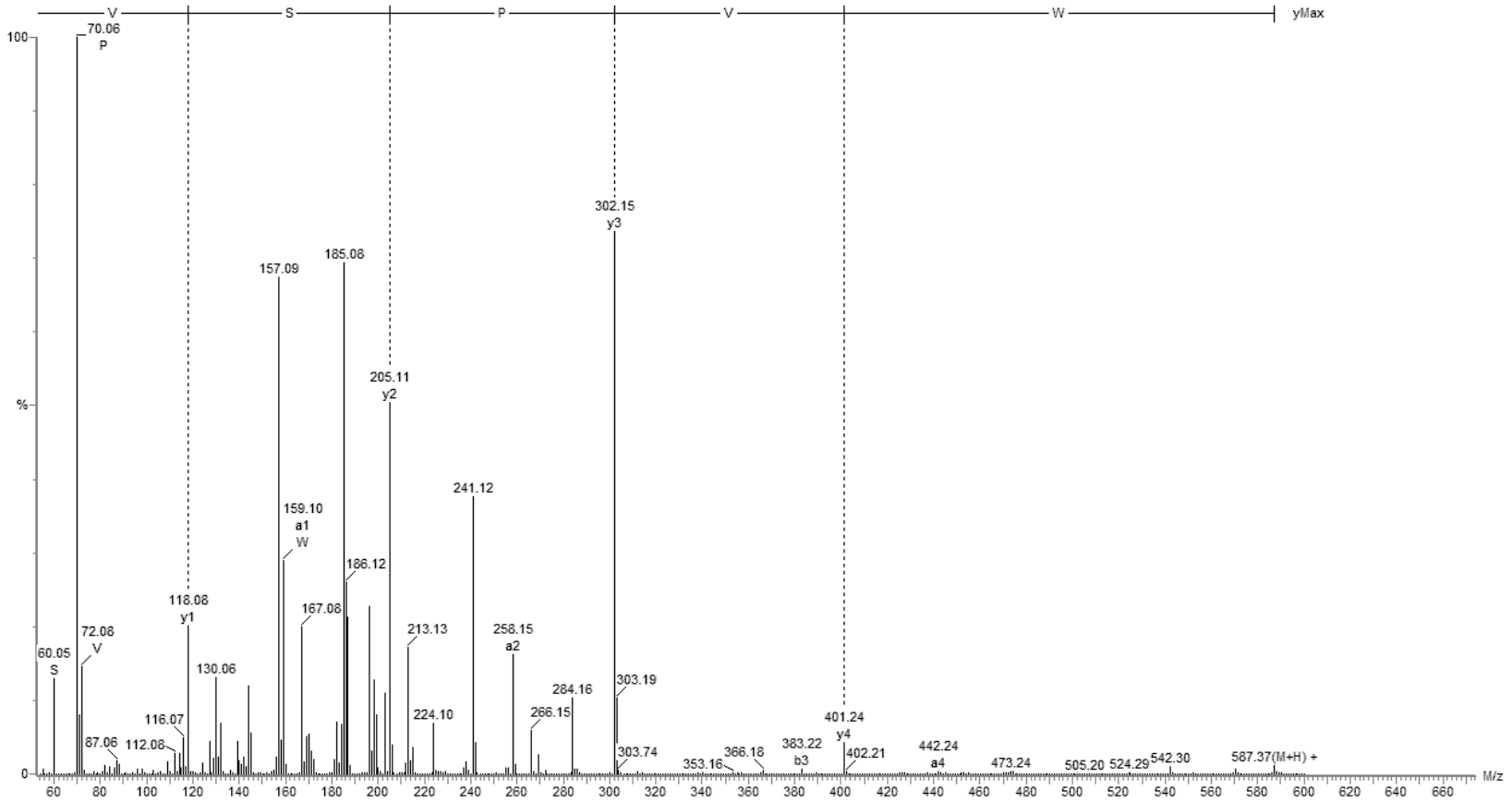

Figure 4. MALDI-TOF/MS of the peptide P3, and the sequence of the peptide was showed on the top of the figure.

Table 2. ACE-inhibitor activity of peptides following digestion with gastrointestinal protease.

Enzyme	Relative ACE-inhibitory activities (%)		
	P1	P2	P3
Control	100	100	100
Pepsin[1]	120	101	101
Trypsin[2]	102	112	102
Pepsin+ trypsin[3]	122	113	105

[1]Pepsin digestion (2 h); [2]trypsin digestion (2 h) and [3]trypsin digestion (2 h) after pepsin digestion (1 h).

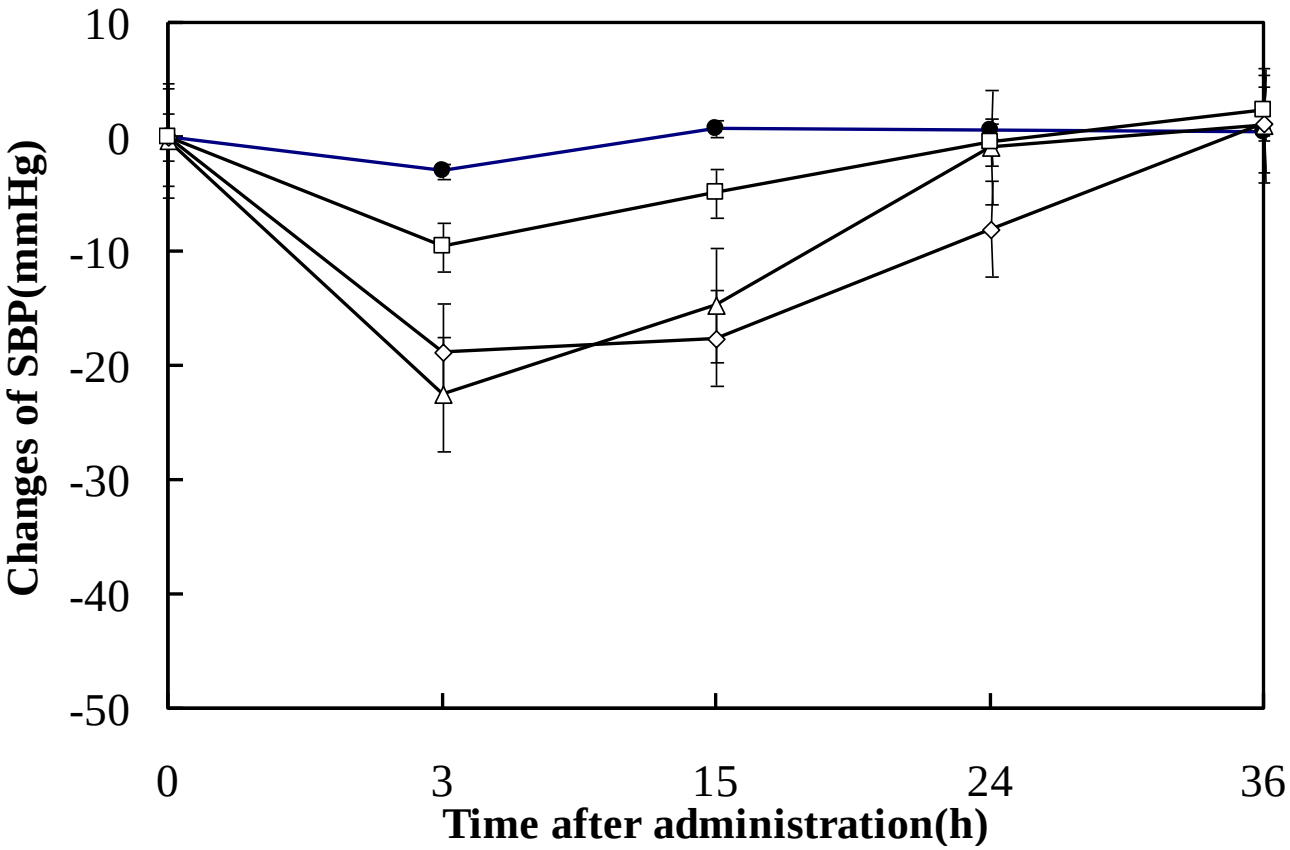

Figure 5. Effect of a single oral administration of peptides on SHR. Changes in systolic blood pressure (SBP) from zero time were expressed as means and the vertical bars represented the standard deviations. Closed circles represented the control treatments (distilled water), triangles represent the values for P1 (10 mg/kg), diamonds represented the values for P2 (10 mg/kg) and squares represented the values for P3 (10 mg/kg). *Significant differences from the control ($p < 0.05$).

in vitro, the three peptides could be digested into smaller molecular weight peptides which were much easier or directly absorbed by the body. This certainly agreed with a previous article that indicated that oligopeptides could be directly absorbed by the intestinal villi cells (Silveria et al., 2004). At 3 h after oral administration of the ACE-inhibitor peptides to the SHRs, their SBPs were decreased significantly.

Within 36 h, their SBP were restored to pretreatment levels. This proves the antihypertensive activity of the peptides *in vivo*. However, their mechanism of action *in vivo* still needs further studies for verification.

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