Identification of an Antihypertensive Peptide from Casein Hydrolysate Produced by a Proteinase from Lactobacillus helveticus CP790

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ABSTRACT
Casein hydrolysate, produced by an extracellular proteinase from Lactobacillus helveticus CP790, was fractionated by two-step reverse-phase HPLC. Only one fraction showed antihypertensive activity as measured by systolic blood pressure in spontaneously hypertensive rats after oral administration. Ten peptides in the fraction were further purified and identified by analysis of amino acid sequences. Each identified peptide was chemically synthesized, and the antihypertensive activity of each peptide was evaluated in spontaneously hypertensive rats. The synthetic peptide with a sequence of Lys-Val-Leu-Pro-Val-Pro-Gln, found in ß-casein, indicated strong antihypertensive activity from 2 to 10 h after oral administration of 2 mg of peptide/kg of BW, and the effect was maximal at 6 h after oral administration (-31.5 ± 5.6 mm Hg). Moreover, the antihypertensive effect of the peptide was dependent on the dosage of peptide from 0.5 to 2 mg of peptide/kg of BW. Interestingly, the antihypertensive peptide showed lower inhibitory activity of angiotensin I-converting enzyme, but the activity was increased after pancreatin digestion. (Key words: antihypertensive peptide, extracellular proteinase, angiotensin I-converting enzyme, Lactobacillus helveticus)

Abbreviation key: ACE = angiotensin I-converting enzyme, Hip-His-Leu = hippuryl-L-histidyl-L-leu, IC₅₀ = the concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity, SBP = systolic blood pressure, SHR = spontaneously hypertensive rats.

INTRODUCTION
Many inhibitors of angiotensin I-converting enzyme (ACE), which catalyze both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin, have been isolated from enzymatic digest of various food materials, including casein (6, 7, 8, 10), maize (9), and bonito (11, 19). Milk fermented with Lactobacillus helveticus CP790 and casein hydrolysate produced by the extracellular proteinase showed a potent antihypertensive effect in spontaneously hypertensive rats (SHR) by oral administration (17, 18). Recently, two ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, were purified from sour milk that had been fermented with L. helveticus and Saccharomyces cerevisiae (13, 14) with antihypertensive activity. Several peptides derived from a hydrolysate of αs₁-casein and ß-casein produced by the extracellular proteinase from L. helveticus CP790 exhibited ACE inhibitory activities (16, 18). However, the two tripeptides mentioned previously were not detected in the hydrolysate. In a preliminary study, we have synthesized some of these peptides in the casein hydrolysate and examined their antihypertensive activities in SHR after oral administration. However, these peptides did not show significant antihypertensive activities.

Therefore, we attempted to purify an antihypertensive peptide from casein hydrolysate produced by an extracellular proteinase from L. helveticus CP790, based on the antihypertensive activity in SHR.

MATERIALS AND METHODS

Materials and Microorganism
Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), ACE, pepsin, pancreatin, and carboxypeptidase A were purchased from Sigma Chemical Co. (St. Louis, MO). Whole casein was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation of the Peptides from Casein Hydrolysate
To purify the extracellular proteinase, 4 L of 9% (wt/vol) pasteurized reconstituted NDM were fermented with L. helveticus CP790 (16), and the pH
was maintained at neutrality by adding 5N NaOH. The cells were harvested at the midlog phase of growth. The proteins were released from the cell, and the extracellular proteinase was purified as previously described (16). To obtain the peptides, 1 g of casein was dissolved in 100 ml of 20 mM phosphate buffer, pH 6.7, and mixed with 10 μg of purified proteinase. After incubation at 42°C for 15 h, the hydrolysate was heated at 80°C for 5 min to stop the enzymatic reaction. Finally, 700 mg of peptides with molecular mass <10,000 Da were obtained by filtration (ultrafilter OMO10025; Fuji Filter Ltd., Tokyo, Japan) and used for further purification of antihypertensive peptides.

**Measurement of Blood Pressure**

Male SHR were purchased from Charles River Japan (Yokohama, Japan) and fed laboratory chow (CE-2, Clea Japan, Tokyo, Japan). Systolic blood pressure (SBP) of SHR, 18 to 25 wk of age (330 to 360 g of BW), were measured as follows. Rats that had been given each peptide by gastric intubation were kept at 45°C for 5 min, and the SBP were measured by the tail cuff with a programmed electro-sphygmomanometer (NARC0 Bio-Systems, Austin, TX). Phosphate-buffered saline (0.15 M NaCl and 0.01 M phosphate buffer; pH 7.4) containing 0.05% casein was used as a control in SHR, and the antihypertensive effects of the synthetic peptides dissolved in this buffer were measured.

**Measurement of ACE Inhibitory Activity**

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (3) with some modification. Each 20 μl of peptide solution were preincubated with the Hip-His-Leu borate buffer (3.8 mM Hip-His-Leu, 0.1 M borate, and 0.3 M NaCl; pH 8.3) at 37°C for 5 min. Two milliliters of ACE were added, and the mixture was incubated at 37°C for 30 min. One unit of ACE produced 1 μM of hippuric acid/min from Hip-His-Leu at pH 8.3 and 37°C. The liberated hippuric acid was extracted with ethyl acetate. The activity of an ACE inhibitory peptide was defined as that needed to inhibit 50% of the ACE activity (IC50) under these conditions.

**Purification of the Antihypertensive Peptide by Reverse-Phase HPLC**

The casein hydrolysate produced by the proteinase was subjected to reverse-phase HPLC, (models L4000, L6000, and L62000 instruments; Hitachi, Ltd., Japan), using a μ-Bondasphere C-18 column (3.9 mm × 150 mm; Nihon Millipore Ltd., Tokyo, Japan). The peptides were eluted by a linear gradient from solvent A (0.1% trifluoroacetic acid and H2O) to 40% of solvent B (0.1% trifluoroacetic acid and acetonitrile) over 60 min at a flow rate of 1.0 ml/min and were detected at 215 nm. To identify the amino acid sequence of a peptide, each peptide was purified by reverse-phase HPLC on the same column with a linear gradient from 10% of solvent A to 30% of solvent B over 30 min at the flow rate of 1.0 ml/min; detection was at 215 nm.

To determine the content of an antihypertensive peptide in the casein hydrolysate, the peptide was purified by reverse-phase HPLC with same procedure. The amount of the casein hydrolysate and all peptide mixtures obtained at each of the purification steps in this experiment were measured the dry weight.

**Digestion of the Antihypertensive Peptide with Gastrointestinal Enzyme**

Pancreatin digestion of the synthetic peptide was carried out at 37°C in 200 μl of 0.1 M Tris-HCl, pH 7.5, with 100 μg of peptide and 5 μg of enzyme. Pepsin digestion of the synthetic peptide was carried out at 37°C in 200 μl of 0.1 M Tris-HCl, pH 2.0, with 100 μg of peptide and 1 μg of enzyme. After 4 h of incubation, each digest was boiled for 10 min to stop the reaction. For carboxypeptidase A digestion, 15 μg of synthetic peptide were dissolved in 100 μl of reaction buffer (0.35 M KCl and 40 mM Tris-HCl; pH 7.5), and 10 units of carboxypeptidase A were added. After 10 h of incubation at 37°C, the reaction mixture was boiled for 10 min to stop the reaction and then analyzed by reverse-phase HPLC.

**Peptide Identification and Synthesis**

An amino acid sequence of purified peptide was identified by protein sequencer PPSQ-10 (Shimadzu, Kyoto, Japan). Peptides were chemically synthesized by peptide synthesizer PSSM-8 (Shimadzu).

**RESULTS**

**Purification and Identification of Major Peptides in the Antihypertensive Fraction**

To identify an antihypertensive peptide, 10 mg of the casein hydrolysate were injected into reverse-phase HPLC, and the elution was divided into six fractions as shown in Figure 1A. This operation was
repeated 10 times, and each eluent was collected. Subsequently, antihypertensive activity of each of the six fractions in SHR was measured at 6 h after oral administration by use of 5% (wt/wt) of collected peptides from each SHR. Significant antihypertensive activity was observed only in fraction 3 (Figure 1B). Thereafter, 20 mg of the peptides in fraction 3 were further fractionated into 4 by HPLC, and the antihypertensive activity was measured by use of 5% (wt/wt) of collected peptides per SHR. Significant antihypertensive activity was observed only in subfraction 3D (Figure 2B).

Subsequently, seven major peaks in subfraction 3D (Figure 2A) were separated by reverse-phase HPLC. Each peak was further separated and collected by HPLC as described in the Materials and Methods (data not shown). Finally, 10 peptides in fraction 3D were purified and identified by analysis of the N-terminal amino acid sequences (Table 1). Sequences of four peptides were found in β-casein, sequences of four other peptides were in αs2-casein, and sequences of two peptides were in αs1-casein sequences (Table 1).

Figure 1. Preliminary separation and screening of peptides from casein hydrolysate; A) designation of fractions in eluant from reverse-phase HPLC using μ-Bondasphere C-18 column (Nihon Millipore Ltd., Tokyo, Japan) and B) antihypertensive activity of fractions as determined by changes in systolic blood pressure (SBP). Vertical bars represent standard errors (n = 5), and asterisks indicate that fraction 3 differed significantly from the control (**P < 0.01).

Figure 2. Fractionation and assay of fractions from fraction 3 of Figure 1; A) designation of fractions in eluant from reverse-phase HPLC using μ-Bondasphere C-18 column (Nihon Millipore Ltd., Tokyo, Japan) and B) antihypertensive activity of fractions as determined by changes in systolic blood pressure (SBP). Sequences of peptides associated with peaks are indicated by arrows. Vertical bars represent standard errors (n = 5), and asterisks indicate that fraction 3D differed significantly from the control (**P < 0.01).
PURIFICATION OF AN ANTIHYPERTENSIVE PEPTIDE

Figure 3. Antihypertensive effect of a single oral administration of peptide in spontaneously hypertensive rats (SHR). Each point is the mean of the changes of systolic blood pressure (SBP) of five SHR; the vertical bars represent standard errors. The control (○) was SBP containing 0.05% casein; treatment dose was 2.0 mg/kg of BW (●). Asterisks indicate the level of significant difference from control at each time interval: *P < 0.05, **P < 0.01, and ***P < 0.001.

Antihypertensive and ACE Inhibitory Activities of the Identified Peptides

Antihypertensive activity of each peptide was evaluated by measuring the change of SBP at 6 h after oral administration of 1 mg/kg of BW (Table 1). Potent antihypertensive effects were observed (−24.1 ± 7.8 mm Hg) for a peptide that had a sequence of Lys-Val-Leu-Pro-Val-Pro-Gln. No other peptide showed significant antihypertensive effects in SHR. The antihypertensive peptide content was measured by the o-phthalaldehyde method using the synthetic peptide as a standard as described in this paper. However, the total amount of peptides in the casein hydrolysate measured the dry weight directly.

The ACE inhibitory activity of each peptide was evaluated also as the IC_{50} value (Table 1). Unexpectedly, the antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln had lower ACE inhibitory activity (IC_{50} = 1000 µM). Conversely, a peptide Tyr-Lys-Val-Pro-Gln-Leu had a strong ACE inhibitory activity (IC_{50} = 22 µM), but this peptide showed no significant antihypertensive effect for SHR.

Changes of SBP of SHR After Administration of the Peptide

At 0, 2, 4, 6, 8, 10, and 24 h after oral administration of the antihypertensive peptide, Lys-Val-Leu-Pro-Val-Pro-Gln, the SBP of SHR was measured (Figure 3). No changes of SBP of the control rats occurred during the 24 h after administration. However, Lys-Val-Leu-Pro-Val-Pro-Gln had potent antihypertensive effects in SHR at a dosage of 2 mg/kg of BW at 2, 4, 6, 8, and 10 h after administration (−20.2 ± 2.9, −26.3 ± 1.6, −31.5 ± 5.6, −25 ± 8.3, and −22.7 ± 2.8 mm Hg, respectively). However, SBP between control and experimental rats were not significantly different at 24 h after administration (−2.9 ± 2.4 mm Hg).

Antihypertensive Activity with Different Dosages of the Peptide

The antihypertensive activity at different dosages of the peptide Lys-Val-Leu-Pro-Val-Pro-Gln was in-

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Origin</th>
<th>Systolic blood pressure ( \overline{X} ) (mm Hg)</th>
<th>IC_{50} (µM)</th>
</tr>
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<tbody>
<tr>
<td>1. Lys-Val-Leu-Pro-Val-Pro-Gln</td>
<td>( \beta ) : 169-175</td>
<td>−24.1* 7.8</td>
<td>1000</td>
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<tr>
<td>2. Leu-Gln-Ser-Trp</td>
<td>( \beta ) : 140-143</td>
<td>−1.6 3.4</td>
<td>500</td>
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<tr>
<td>3. Arg-Glu-Leu-Glu-Glu-Leu</td>
<td>( \beta ) : 1-6</td>
<td>1.3 3.0</td>
<td>&gt;1000</td>
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<tr>
<td>4. Thr-Lys-Val-Ile-Pro</td>
<td>( \sigma_{g2} ) : 188-202</td>
<td>−9.2 2.7</td>
<td>400</td>
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<tr>
<td>5. Ala-Met-Lys-Pro-Trp</td>
<td>( \sigma_{g2} ) : 189-192</td>
<td>−4.6 5.8</td>
<td>580</td>
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<tr>
<td>6. Tyr-Lys-Val-Pro-Gln-Leu</td>
<td>( \sigma_{s1} ) : 104-109</td>
<td>−12.5 5.2</td>
<td>22</td>
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<tr>
<td>7. Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys</td>
<td>( \sigma_{g2} ) : 190-197</td>
<td>−2.9 5.9</td>
<td>300</td>
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<td>8. Leu-Leu-Tyr-Gln-Gln-Pro-Val</td>
<td>( \beta ) : 191-197</td>
<td>1.2 4.4</td>
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<td>9. Ala-Tyr-Phe-Tyr-Pro</td>
<td>( \sigma_{s1} ) : 143-147</td>
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<td>600</td>
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1Origin and the position in each casein molecule are indicated.
2Mean of five determinations.
3The concentration of peptide needed to inhibit 50% of the ACE activity.
*Different from control \((P < 0.05)\).
Investigated at 6 h after oral administration (Figure 4). The antihypertensive effect was dose-dependent for dosages from 0.2 to 2 mg of peptide/kg of BW, but the effect was not significant with the dosage of 0.2 mg of peptide/kg of BW.

Gastrointestinal Digestion of the Peptide

To test the resistance of the antihypertensive peptide to gastrointestinal enzymatic digestion, the antihypertensive peptide was incubated with pepsin, pancreatin, and carboxypeptidase A. The ACE inhibitory activity of the reaction mixture was increased by pancreatin or carboxypeptidase A but not by pepsin (data not shown). To determine the ACE inhibitory peptide in the reaction mixture, the peptide digested by carboxypeptidase A was analyzed by reverse-phase HPLC. A peptide, which had a sequence of Lys-Val-Leu-Pro-Val-Pro and lacked the C-terminal glutamine residue of the Lys-Val-Leu-Pro-Val-Pro-Gln, was identified in the digest (Figure 5). The short peptide was also detected in the digest by pancreatin (data not shown). On the basis of these findings, the hexapeptide, Lys-Val-Leu-Pro-Val-Pro, was chemically synthesized, and the ACE inhibitory activity and the antihypertensive activity were measured. The peptide had a strong ACE inhibitory activity (IC$_{50}$ = 5 μM) and a strong antihypertensive activity in SHR at a dosage of 1 mg of peptide/kg of BW by 6 h after oral administration (−32.2 ± 1.4 mm Hg; P < 0.001). The ACE inhibitory peptide, Tyr-Lys-Val-Pro-Gln-Leu, which was partially hydrolyzed to Tyr-Lys-Val-Pro by pancreatin, exhibited low ACE inhibitory activity (IC$_{50}$ > 1000 μM).

DISCUSSION

In this study, a potent antihypertensive peptide, Lys-Val-Leu-Pro-Val-Pro-Gln, was purified and identified from the casein hydrolysate produced by a protease from L. helveticus CP790. From measurement of the peptide content in the casein hydrolysate, the antihypertensive peptide seemed to be generated efficiently from casein by the protease, although the antihypertensive peptide was not found in a previous study (16). This result was probably due to the difficulty in purifying this peptide from other peptides with similar retention times in reverse-phase HPLC.

In a previous report using SHR, antihypertensive activity was strong at 6 h after oral administration of the casein hydrolysate produced by the L. helveticus CP790 protease but not by trypsin using a dosage of 15 mg of peptides/kg of BW (18). The concentration of antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln was at least 390 μg/15 mg of the casein hydrolysate formed by the L. helveticus CP790 protease. Considering these results and the dose-response experiment in this study, Lys-Val-Leu-Pro-Val-Pro-Gln is likely to exert a major antihypertensive effect in the casein hydrolysate produced by L. helveticus CP790.

Unexpectedly, the antihypertensive peptide did not have strong ACE inhibitory activity. However, a hexapeptide, Lys-Val-Leu-Pro-Val-Pro, which had a
stronger ACE inhibitory activity, was liberated from the heptapeptide, Lys-Val-Leu-Pro-Val-Pro-Gln, by pancreatic digestion. Antihypertensive activity of the ACE inhibitory peptide was similar as to that of the heptapeptide. The ACE inhibitory hexapeptide may be generated from heptapeptide by gastrointestinal digestion. Antihypertensive activity of the hexapeptide, Lys-Val-Leu-Pro-Val-Pro, was stronger ACE inhibitory activity, was liberated from the heptapeptide by gastrointestinal digestion and therefore exhibit antihypertensive activity in SHR. Absorption of small peptides from intestine is known to occur (4, 5, 12). Further studies on the conversion of the heptapeptide in the intestine to the ACE inhibitory hexapeptide and the absorption from intestine of the hexapeptides in SHR are necessary to understand the mechanism of the antihypertensive effect of the heptapeptide.

Several ACE inhibitory peptides that had a proline residue as the C-terminus were reported (1, 11, 13, 15). A proline residue was also observed in the ACE inhibitory peptide, Lys-Val-Leu-Pro-Val-Pro. However, two peptides, Thr-Lys-Val-Ile-Pro and Ala-Tyr-Phe-Tyr-Pro with proline residue at the C-terminus, did not show strong ACE inhibitory activities. These results suggested that both the proline residue in the C-terminus and the amino acid sequence might be important for ACE inhibition of the hexapeptide. Evaluation of ACE inhibitory activity of the deletion derivatives from the N- or C-terminus of the hexapeptide, would be also important to understanding the ACE inhibitory mechanism of the hexa-peptide.

The potential for the antihypertensive peptide Lys-Val-Leu-Pro-Val-Pro-Gln in functional foods to mitigate hypertension would seem to merit further investigation.

REFERENCES