Purification and Characterization of an Antihypertensive Peptide from a Yogurt-Like Product Fermented by Lactobacillus helveticus CPN4

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ABSTRACT

Whey peptides in a yogurt-like product fermented by Lactobacillus helveticus CPN4 were fractionated by a Sep-pak C-18 cartridge followed by two-step reverse-phase HPLC. The antihypertensive activity was measured by systolic blood pressure in spontaneously hypertensive rats after oral administration of each fraction. Five major peptides in the final fraction were further purified by reverse-phase HPLC and were measured for these antihypertensive activities in spontaneously hypertensive rats. The only peptide in the final fraction that showed strong antihypertensive activity had a sequence of Tyr-Pro, which is found in αs1-casein (CN), β-CN, and κ-CN. The synthetic peptide Tyr-Pro yielded significant antihypertensive activity from 2 to 8 h after oral administration of 1 mg of peptide/kg of body weight, and the effect was maximal at 6 h after oral administration. The antihypertensive effect of the peptide was dependent on the peptide dosage from 0.1 to 10 mg of peptide/kg of body weight. The concentration of Tyr-Pro peptide increased during fermentation and reached about 8.1 mg/ml of whey in the pH 4.3 yogurt-like product. The antihypertensive peptide had a low inhibitory activity against angiotensin I-converting enzyme. The inhibition of 50% of the angiotensin I-converting enzyme (IC50) was 720 μM.

(Key words: yogurt-like product, Lactobacillus helveticus, antihypertensive peptide, angiotensin I-converting enzyme)

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

INTRODUCTION

Inhibitors of angiotensin I-converting enzyme (ACE), which catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin, have been isolated from enzymatic digests of various foods (3, 4, 5, 6). For related information, see review by Yamamoto (14).

Lactic acid bacteria have a proteolytic system that releases peptides from casein during fermentation (10, 11, 12). Lactobacillus helveticus generally has higher proteolytic activity than other lactic acid bacteria. The antihypertensive effect specific to milk fermented with L. helveticus has been reported (16) for spontaneously hypertensive rats (SHR). Recently, two antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, were purified from sour milk that was fermented with L. helveticus and Saccharomyces cerevisiae (8, 9). These two peptides demonstrate an antihypertensive effect by inhibiting ACE in target organs (7). Likewise, a yogurt-like product fermented with L. helveticus showed strong antihypertensive activity in SHR (18). However, the yogurt-like product did not contain the two antihypertensive tripeptides mentioned above and showed lower ACE inhibitory activity than did sour milk.

Therefore, because of the antihypertensive activity in SHR, we attempted to purify an antihypertensive peptide from the yogurt-like product fermented by L. helveticus and to understand the difference between antihypertensive components in the yogurt-like product and sour milk.

MATERIALS AND METHODS

Materials and Microorganism

Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), ACE, and casein were purchased from Sigma Chemical Co. (St. Louis, MO). An acid sensitive variant, L. helveticus CPN4 (18) derived from L. helveticus CP790 (15), was used from our culture collection.
Preparation of Fermented Milk Whey

*Lactobacillus helveticus* CPN4 (18) was cultured at 37°C in 100 ml of 9% (wt/vol) pasteurized reconstituted skim milk medium. The fermentation was stopped when the pH reached 4.3. The whey was collected by centrifugation at 20,000 × g for 10 min.

Measurement of Blood Pressure

Male SHR purchased from Charles River Japan (Yokohama, Japan) were divided into groups with 5 rats each and were fed laboratory chow (CE-2; Clea Japan, Tokyo, Japan). Systolic blood pressure (SBP) of SHR, 18- to 21-wk old (body weight: 300 to 330 g), was measured as follows. Test and control rats were held at 45°C for 5 min, and SBP were measured by a tail cuff with a programmed electrosphygmomanometer (NARCO Bio-Systems, Austin, TX). Peptide and fermented milk were orally administered by gastric intubation with PBS (0.15 M NaCl and 0.01 M phosphate buffer, pH 7.2) containing 0.05% (wt/vol) casein as a control. The antihypertensive activity of the synthetic peptide dissolved in PBS was determined in the same manner. Student’s *t* test was used for statistical analysis.

Measurement of ACE Inhibitory Activity

The ACE inhibitory activity was measured according to the method of Cuchman and Cheung (1) with modification (17). Twenty microliters 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 milliunits of ACE were added, and the mixture was incubated at 37°C for 30 min. The liberated hippuric acid was extracted with ethyl acetate. The activity of an ACE inhibitory peptide was defined as the amount needed to inhibit 50% of the ACE activity (IC50) under these conditions.

Purification of the Antihypertensive Peptide by Reverse-Phase HPLC

One hundred twenty milliliters of the whey prepared from the yogurt-like product fermented with the *L. helveticus* CPN4 was treated with 10 Sep-pak C-18 cartridges (Waters, Inc., Tokyo, Japan). Peptides were eluted with mixtures containing 10, 15, 20, 30, and 50% solvent B (0.1% trifluoroacetic acid in acetonitrile) in solvent A (0.1% trifluoroacetic acid in H2O). Eluent from the 10 cartridges was combined. Antihypertensive activity of each of the six fractions was measured in SHR, 6 h after oral administration, by use of 1 ml from 40 ml of concentrated peptides. The active fraction was concentrated with a centrifugal concentrator, diluted with PBS to 40 ml, and subjected to reversed-phase HPLC (models L4000, L6000, and L6200; Hitachi, Ltd., Japan) with 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 100% solvent A to 40% solvent B over 60 min at a flow rate of 1.0 ml/min, and absorbance was detected at 215 nm (first HPLC run). The fraction with the highest antihypertensive activity was concentrated by the procedure described above, was applied to the same reverse-phase column, and was rechromatographed by a linear gradient from 5 to 20% of solvent B over 60 min at a flow rate of 1.0 ml/min (second HPLC run).

Quantitative Analysis of the Antihypertensive Peptide

To evaluate the Tyr-Pro peptide content in the yogurt-like product, 100 μl of the whey were directly analyzed by reverse-phase HPLC using a μ-Bondasphere C-18 column. Peptides were eluted by a linear gradient of 5 to 20% solvent B with the same conditions as the purification procedure of the second HPLC run. The Tyr-Pro content in the yogurt-like product was determined with a standard curve prepared using a synthetic Tyr-Pro peptide as a control.

Peptide Identification and Synthesis

The amino acid sequence of the purified peptide was identified by a protein sequencer (PPSQ-10; Shimadzu, Kyoto, Japan). The amino acid composition of the purified peptide was determined by an amino acid analysis system (Japan Spectroscopic Co., Ltd.) after acid hydrolysis in 6.0N HCl for 20 h. The peptide was synthesized by a peptide synthesizer (PSSM-8; Shimadzu).

RESULTS

Purification and Identification of Antihypertensive Peptide

To purify an antihypertensive peptide, 120 ml of whey from a yogurt-like product was fractionated to six fractions via Sep-pak cartridges as shown in Table 1. Significant antihypertensive activities were observed in fractions 1, 2, and 5 (Table 1). The antihypertensive activity of fraction 2 (1 ml) was similar to the activity found in 3 ml prefractionated whey as assayed in SHR (corresponding to 1 ml of fraction-
TABLE 1. The antihypertensive activity of fractions from a Sep-pak cartridge.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluant1 (%)</th>
<th>Systolic blood pressure2 (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>X ( \pm ) SE</td>
</tr>
<tr>
<td>Prefractionated</td>
<td></td>
<td>–29.6*** 9.5</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>–9.6* 5.7</td>
</tr>
<tr>
<td>2</td>
<td>0–10</td>
<td>–22.3*** 3.2</td>
</tr>
<tr>
<td>3</td>
<td>10–15</td>
<td>–8.1 2.6</td>
</tr>
<tr>
<td>4</td>
<td>15–20</td>
<td>1.0 6.3</td>
</tr>
<tr>
<td>5</td>
<td>20–30</td>
<td>–8.8* 8.5</td>
</tr>
<tr>
<td>6</td>
<td>30–50</td>
<td>–7.6 5.3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.9 8.1</td>
</tr>
</tbody>
</table>

1Percentage of eluant B.
2Mean of five determinations.
*Different from the control (\( P < 0.05 \)).
***Different from the control (\( P < 0.001 \)).

Changes in SBP of SHR After Administration of the Peptide

At 0, 2, 4, 6, 8, and 24 h after oral administration of antihypertensive peptide Tyr-Pro, SBP of SHR was measured (Figure 3). No changes in SBP of the control rats occurred during the 24 h after administration. However, Tyr-Pro showed potent antihypertensive activity in SHR with a dosage of 1 mg of peptide/kg of BW at 4, 6 and 8 h after administration (–23.2 ± 6.7, –27.4 ± 7.1, and –19.4 ± 2.9, mm of Hg, respec-

Identification of the Peptide and the ACE Inhibitory Activity

To identify the antihypertensive peptide, the major peptide in the active fraction 2-2-2 was completely purified by reverse-phase HPLC (second HPLC run). The purified peptide in the fraction 2-2-2 was identified by analysis of the N-terminal sequence and amino acid composition. The peptide had a sequence of Tyr-Pro, and the amino acid composition was Tyr: Pro = 1:0.98. The peptide sequence was found in \( \alpha_{s1} \)-CN (f 146–147), \( \beta \)-CN (f 114–115), and \( \kappa \)-CN (f 58–59). The Tyr-Pro peptide showed low ACE inhibitory activity compared with other reported ACE inhibitory peptides, and the IC\(_{50}\) was 720 \( \mu \)M.

![Figure 1](image-url)
tively). The effect was maximal at 6 h after oral administration, and SBP of control and experimental rats were not significantly different at 24 h after administration (−4.8 ± 4.8 mm of Hg).

Antihypertensive Activity with Different Dosages of the Peptide

The antihypertensive activity of different dosages of the synthetic peptide Tyr-Pro was investigated 6 h after oral administration (Figure 4). The mean SBP was 227.0 ± 3.9 mm of Hg before administration. After single oral administrations of 0.1, 0.3, 1, 3, and 10 mg of Tyr-Pro/kg of BW, SBP was decreased by 10.2 ± 4.0 (P < 0.05), 20.8 ± 4.1 (P < 0.001), 27.4 ± 7.1 (P < 0.001), 30.5 ± 6.7 (P < 0.001), and 32.1 ± 7.4 mm of Hg (P < 0.001), respectively. The antihypertensive effect was dose-dependent from 0.1 to 10 mg of peptide/kg of BW.

Quantitative Analysis of the Peptide in the Fermented Milk

The peak height of peptide corresponding to Tyr-Pro elution was measured at 19.5 min. Concentration of Tyr-Pro increased, and pH decreased during milk fermentation by *L. helveticus*. The concentration of the Tyr-Pro reached 10 mg/ml at 10 h after inoculation when the pH was 4.1 (Figure 5). The Tyr-Pro peptide concentration was approximately 8.1 mg/ml of whey of the yogurt-like product (pH 4.3).

**DISCUSSION**

In this study, a potent antihypertensive peptide, Tyr-Pro, was purified and identified from a yogurt-like product fermented with *L. helveticus* CPN4. A Tyr-Pro peptide from sake and sake lees was antihypertensive (19). The antihypertensive effect of Tyr-Pro peptide from a fermented yogurt-like product was studied in detail in this study. A strong anti-
hypertensive effect (–29.6 ± 9.5 mm of Hg) was observed in SHR by oral administration of 3 ml of whey from a yogurt-like product (Table 1). Three milliliters of the yogurt-like product corresponded to a dosage of about 0.1 mg of Tyr-Pro peptide/kg of BW. However, lower antihypertensive activity (–10.5 ± mm of Hg) was observed when Tyr-Pro peptide was given to SHR at a dosage of 0.1 mg of peptide/kg of BW compared with dosage of 3 ml of whey (See Figure 4). The Tyr-Pro peptide was purified as the major antihypertensive component from the whey fraction of the yogurt-like product. However, other minor antihypertensive component(s) might exist in the product.

In a previous study, two kinds of antihypertensive peptides, Ile-Pro-Pro and Val-Pro-Pro, were purified from sour milk fermented by a starter culture containing L. helveticus and S. cerevisiae. Both peptides had strong ACE inhibitory activities and were produced in the sour milk during fermentation (8). However, the ACE inhibitory activity in the yogurt-like product (pH near 4.3) was at the basal level (8). This result suggests the yogurt-like product does not contain these two antihypertensive peptides. The amino acid sequences of Ile-Pro-Pro and Val-Pro-Pro occur at three positions in bovine caseins (β-CN, f 74–76; β-CN f 84–86; and κ-CN, f 108–110). The Tyr-Pro amino acid sequence was found at more C-terminal regions of bovine caseins (αs1-CN, f 146–147; αs1-CN, f 159–160; β-CN, f 114–115; and κ-CN, f 58–59). The proteinase of L. helveticus CP790 preferentially hydrolyzes the casein molecules at the C terminus (14). These results suggest that the Tyr-Pro peptide can easily be released from the caseins by proteolytic action at the beginning of growth in the fermented milk (yogurt-like product).

Many ACE inhibitory peptides have been isolated from hydrolysates of various food materials (3, 4, 5, 6, 14). However, the ACE inhibitory activity of the peptides did not correlate with the antihypertensive activity found in SHR. In the current study, the ACE inhibitory activity of the Tyr-Pro peptide (IC50 = 720 μM) was lower than the activities of the peptides Ile-Pro-Pro or Val-Pro-Pro (5 and 9 μM, respectively) (8). However, the Tyr-Pro peptide showed the same antihypertensive activity as the activities of the peptides Ile-Pro-Pro and Val-Pro-Pro (9). Recently, the role of chymase as a major angiotensin II-forming enzyme in the heart and its vessels was reported (13). Chymase hydrolyzes peptide bonds at the carboxyl termini of hydrophobic aromatic residues (such as Phe and Trp) and efficiently forms the potent vasoconstrictor angiotensin II by cleaving the Phe8-His9 bond in angiotensin I. More characterization of Tyr-Pro peptide, which has a low ACE inhibitory activity, is necessary to understand the mechanism of the antihypertensive effect.
Recently, the antihypertensive effect of sour milk was shown in hypertensive patients (2). The systolic blood pressure of patients decreased significantly, by 14.1 ± 3.1 mm of Hg, after daily oral administration of 95 ml of sour milk over 8 wk (2). The systolic blood pressure decreased about 25 mm of Hg with a dosage of 5 ml of sour milk. In this study, a similar antihypertensive effect was observed with a dosage of 3 ml of the yogurt-like product (−29.6 ± 9.5 mm of Hg). The yogurt-like product is possibly a functional food product for hypertensive patients according to these results.

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REFERENCES