Antihypertensive Effect of the Peptides Derived from Casein by an Extracellular Proteinase from *Lactobacillus helveticus* CP790

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**ABSTRACT**

Peptides derived from \(\alpha_1\)- and \(\beta\)-caseins by the *Lactobacillus helveticus* CP790 proteinase were investigated for their inhibitory activities against angiotensin I-converting enzyme. The antihypertensive effect of casein hydrolysates in strain SHR spontaneously hypertensive rats was also investigated. Both \(\alpha_1\)- and \(\beta\)-casein hydrolysates inhibited this enzyme. Some of these peptides showed enzyme inhibitory activity, and one of them from \(\beta\)-casein inhibited the enzyme greatly; the concentration of an angiotensin I-converting enzyme inhibitor needed to inhibit 50% of the enzyme activity was 4 \(\mu\)M. The hydrolysate of casein demonstrated antihypertensive activity in spontaneously hypertensive rats at an orally administered dosage of 15 mg/kg of body weight. Milk fermented with *L. helveticus* CP790, containing about 3% peptides, also showed antihypertensive activity in SHR rats with 5 ml/kg of body weight (15 mg of peptide/kg); however, the milk fermented with *L. helveticus* CP791, a variant defective for proteinase activity, did not show this activity. Results suggested that the peptides liberated from casein by the proteinase in the culture medium showed antihypertensive effect in SHR rats.

(Key words: antihypertensive effect, extracellular proteinase, *Lactobacillus helveticus*)

**Abbreviation key:** ACE = angiotensin I-converting enzyme, Hip-His-Leu = hippuryl-L-histidyl-L-leucine, IC\(_{50}\) = the concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity, SBP = systolic blood pressure.

**INTRODUCTION**

Angiotensin I-converting enzyme (ACE) catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin. Many inhibitors of these reactions have been recovered from an enzymatic hydrolysate of bovine casein (6, 8, 10, 11) or synthesized according to their sequence in casein (9). Lactic acid bacteria have a proteolytic system for cell growth (17, 18). Extracellular proteinases of lactic acid bacteria hydrolyze milk protein restrictively and liberate peptides in culture medium (14, 20). *Lactobacillus helveticus* has especially strong proteolytic activity. Recently, an extracellular proteinase was purified from *L. helveticus* CP790 (19). This enzyme preferentially hydrolyzed \(\alpha_1\)- and \(\beta\)-casein, but not \(\kappa\)-casein. The 25 main peptides liberated from \(\alpha_1\)- and \(\beta\)-casein by this purified enzyme were isolated and identified (19).

We tested the ACE inhibitory activity of each peptide isolated from the enzymatic hydrolysate of bovine casein. The antihypertensive activity of these peptides in spontaneously hypertensive rats (strain SHR) was investigated using trypsin hydrolysate as a control. The antihypertensive effect of the peptide liberated from milk protein by proteinase in the fermented milk was also investigated using the milk fermented with the variant of *L. helveticus* that was defective for proteinase activity as a control.

**MATERIALS AND METHODS**

**Materials**

Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), ACE, trypsin, and casein were from Sigma Chemical Company (St. Louis, MO).
Bacteria

Lactobacillus helveticus CP790 was prepared from a starter used in Japan; L. helveticus CP790 cells were spread on citrate milk agar (.4% sodium citrate, 4% skim milk, and 1.5% agar) plates, and a small colony was isolated from $1 \times 10^5$ parent cells and was designated L. helveticus CP791. The CP791 variant grew as well as the parent strain in milk medium containing .4% bacto-tryptone (Difco, Detroit, MI) and was designated as a variant defective in proteinase activity.

Rats and Measurement of Blood Pressure

Spontaneously hypertensive male rats (strain SHR) and male Wistar-Kyoto rats (strain WKY) were purchased from Charles River (Yokohama, Japan) and fed laboratory chow (CE-2; Clea, Tokyo, Japan). Blood pressures of 20- to 23-wk-old SHR rats (body weight, 330 to 360 g), and 18- to 20-wk-old WKY rats (body weight, 350 to 380 g) were measured as follows. Rats given each peptide or fermented milk by gastric intubation were kept at 45°C for 5 min; systolic blood pressure (SBP) was measured by the tail cuff with a programmed electro-sphygmomanometer (NARCO Bio-Systems, Austin, TX).

Measurement of ACE Inhibitory Activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (2) with some modification. Each 20 µl of inhibitor solution were preincubated with the Hip-His-Leu borate buffer (3.8 mM Hip-His-Leu, .1 M borate, and .3 M NaCl; pH 8.3) at 37°C for 5 min. Two milliunits of ACE were added and incubated at 37°C for 30 min. The liberated hippuric acid was extracted with ethyl acetate. One unit of enzyme produced 1 µM of hippuric acid/min from Hip-His-Leu at pH 8.3 and 37°C. The concentration of an ACE inhibitor was defined as that needed to inhibit 50% of the ACE activity (IC$_{50}$) under these conditions.

Preparation of the Peptides and the Fermented Milks

Each 1 g of casein was hydrolyzed by .1 µg of trypsin or .5 µg of purified proteinase from L. helveticus CP790 (19) at 37°C for 3 h. Low heat skim milk medium (9% total solids) was fermented with L. helveticus CP790 at 37°C for 10 h, and L. helveticus CP791 was fermented in milk medium containing .4% bacto-tryptone and was used in oral administration.

Peptide Measurement

The peptide concentration of culture supernatant and the hydrolysate of casein by proteinase were measured by the o-phthaldehyde method (1).

RESULTS

ACE Inhibitory Activity of Peptides

The 25 main peptides isolated from casein hydrolysate (19) by proteinase from L. helveticus CP790 were measured for the ACE inhibitory activity as outlined in Materials and Methods and as summarized in Tables 1 and 2. As shown in Table 1, the hydrolysate of α$_{sl}$-casein inhibited this enzyme effectively (IC$_{50}$ = 11 µg/ml), but the 19 peptides and the mixture of these peptides isolated from α$_{sl}$-casein hydrolysate did not show potent inhibitory activity. Hence, another potent ACE inhibitory peptide in α$_{sl}$-casein hydrolysate may exist.

The hydrolysate of β-casein and the mixture of isolated peptides inhibited this enzyme effectively (IC$_{50}$ = 11 µg/ml) (Table 2). The peptide Asp-Glu-Leu-Gln-Asp-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser (β-15) isolated from β-casein inhibited ACE potently (IC$_{50}$ = 4 µM). The Leu-Leu-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (β-12; IC$_{50}$ = 21 µM), Pro-Pro-Gln-Ser-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Leu-Pro-Val-Pro-Glu (β-6; IC$_{50}$ = 25 µM), and Ser-Lys-Val-Leu-Pro-Val-Pro-Glu (β-5; IC$_{50}$ = 39 µM) from β-casein showed effective inhibitory activities. The trypsin hydrolysates of α$_{sl}$- and β-caseins did not show ACE inhibitory activity (IC$_{50}$ >1000 µg/ml).

The Antihypertensive Effect of Casein Hydrolysate

At 0, 2, 4, 6, 8 and 10 h after oral administration of the hydrolysates by the trypsin or the
### TABLE 1. The ACE\(^1\) inhibitory activity of peptides from \(\alpha_{s1}\)-casein.

<table>
<thead>
<tr>
<th>Peptides from (\alpha_{s1})-casein(^2)</th>
<th>(\text{IC}_{50}) (^3) ((\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp peptides</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Prot peptides</td>
<td>11</td>
</tr>
<tr>
<td>Peptides from prot peptides mixture, numbers 1 through 10</td>
<td>603 ((\mu)M)</td>
</tr>
</tbody>
</table>

1. Ala-Tyr-Pro-Ser
2. Arg-Pro-Lys-His-Pro-Ile
3. Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln
4. Ala-Tyr-Phe-Tyr-Pro-Glu
5. Phe-Val-Ala-Pro-Phe-Pro-Gln-Val
6. Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu
7. Gln-Leu-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro
8. Gln-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp
9. Gly-Ser-Glu-Asn-Ser-Glu-Lys
10. Gly-Ser-Glu-Asn

\(^1\)Angiotensin I-converting enzyme.
\(^2\)Trp peptides = Trypsin hydrolysate; prot peptides = hydrolysate from *Lactobacillus helveticus* CP790 proteinase.
\(^3\)The concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity.

### TABLE 2. The ACE\(^1\) inhibitory activity of peptides from \(\beta\)-casein.

<table>
<thead>
<tr>
<th>Peptides from (\beta)-casein(^2)</th>
<th>(\text{IC}_{50}) (^3) ((\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp peptides</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Prot peptides</td>
<td>24</td>
</tr>
<tr>
<td>Peptides from prot peptides mixture numbers 1 through 15</td>
<td>137 ((\mu)M)</td>
</tr>
</tbody>
</table>

1. Lys-Ala-Val-Pro-Tyr-Pro-Gln
2. Ala-Val-Pro-Tyr-Pro-Gln
3. Glu-Ser-Leu-Thr-Leu
4. Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu-Ser-Gln-Ser-Leu-Thr-Leu
5. Ser-Lys-Val-Leu-Pro-Val-Pro-Glu
6. Pro-Pro-Gln-Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Glu
7. Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe
8. His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Gln-Pro-Phe
9. Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro
10. Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
11. Leu-Pro-Gln-Asn-Ile-Pro-Leu-Thr-Gln-Thr-Pro-Val-Pro-Val-Pro-Leu-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys
12. Leu-Leu-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
13. Leu-Ser-Ser-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Ile-Glu-Glu-Arg-Glu-Ser-Glu-Glu-Ser-Glu-Glu-Glu
14. Leu-Ser-Ser-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Ile-Glu-Glu-Glu-Ser-Glu-Glu-Glu
15. Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Pro-Glu-Pro-Val-Leu-Gly-Pro-Val-Pro-Ser-Val-Pro-Ile-Ile-Val

\(^1\)Angiotensin I-converting enzyme.
\(^2\)Trp peptides = Trypsin hydrolysate; prot peptides = hydrolysate from *Lactobacillus helveticus* CP790 proteinase.
\(^3\)The concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity.
TABLE 3. Oral administration of fermented milks to strain SHR spontaneously hypertensive rats.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Administration</th>
<th>Systolic blood pressure&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (ml/kg)</td>
<td>Peptide (mg/kg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>18</td>
<td>&lt;.3</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> CP790</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td>Supernatant</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td>Precipitate</td>
<td>18</td>
<td>54</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> CP791</td>
<td>18</td>
<td>59</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean of five determinations.

*Significant difference from the control ($P < .05$).

**Significant difference from the control ($P < .01$).

The fermented milk was centrifuged at 20,000 × g for 10 min; the supernatant (whey fraction) and the precipitate (milk proteins and cell debris fraction) were also tested. The whey fraction, containing about 3% of peptides, also showed the antihypertensive effect in SHR rats with 54 mg of peptides/kg of body weight, but...
the suspension of the precipitate from equal volume of fermented milk and milk (control) did not show an antihypertensive effect in SHR rats. We concluded that the peptides that were liberated from casein by the extracellular proteinase in culture medium were responsible for the potent antihypertensive activity in SHR rats.

The milk fermented with _L. helveticus_ CP791, the variant with defective proteinase activity and derived from _L. helveticus_ CP790, did not show the antihypertensive activity on SBP of SHR rats.

The peptides liberated from casein by the proteinase from _L. helveticus_ CP790 (75 mg of peptides/kg of body weight) and the milk fermented with _L. helveticus_ CP790 (18 ml of milk/kg of body weight or 54 mg of peptides/kg of body weight) did not show antihypertensive activity in WKY rats during the 10 h following oral administration (Figure 2). In our opinion, the ACE was overproduced in SHR rats, but not in WKY rats, and these peptides reduced the hypertension of SHR rats.

**DISCUSSION**

The proteinase from _L. helveticus_ CP790 has been purified and characterized before (19), and the 25 main peptides liberated from αs1- and β-casein by this enzyme were identified (19). Some of these peptides showed potent ACE inhibitory activity, and these peptides, when administered orally, demonstrated antihypertensive activity on SBP of SHR rats. However, administration of 75 mg of trypsin hydrolysate of the casein/kg of body weight did not show strong antihypertensive activity in SHR rats. A number of ACE-inhibitory peptides have been reported (6, 8, 10, 11) from the enzymatic hydrolysate of bovine casein, but these peptides did not show any potent antihypertensive activity in SHR rats. Some of the peptides had a proline-rich sequence in the C-terminal portion. The ACE inhibitors reported first from snake venom also had a proline-rich sequence in their C-terminal part (4, 15), but the peptides released by the proteinase of _L. helveticus_ CP790 did not have any special sequence in the C-terminus. Another consensus sequence may inhibit the potency of ACE.

The fermented milk, containing about 3% of peptides, showed a potent antihypertensive effect on SBP in SHR rats at 18 ml of milk/kg of body weight (54 mg of peptides/kg). The whey fraction of the fermented milk also showed antihypertensive activity. The antihypertensive compounds were purified from an extract of _Lactobacillus casei_ cell lysate (5, 16). However, in our study, the precipitate (milk proteins and cell debris) and the milk fermented with _L. helveticus_ CP791, the variant defective for proteinase activity, were not effective on SBP of SHR rats. This result indicated the possibility that the peptides in the culture medium and liberated from casein by the proteinase showed antihypertensive effects in SHR rats. Lactobacilli usually demonstrate a stronger proteolytic activity than lactococci (18), but the resulting peptides are thoroughly hydrolyzed by some peptidases (3, 12). Recently, aminopeptidases were purified and characterized from _L. helveticus_ LHE-511 and _L. helveticus_ CNSHRZ 32 (7, 13). Thus, a future step is to compare the peptides in milk fermented with _L. helveticus_ CP790 to the peptides released by an extracellular proteinase.
and to identify the main peptide showing strong antihypertensive effect in SHR rats. We would like to apply these peptides as a healthy food for hypertensive patients.

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REFERENCES