Note

Hypotensive Activity of Enzymatic α -Zein Hydrolysate

Shinsuke MIYOSHI, Toshiyuki KANEKO,
Yasuko YOSHIZAWA, Fumio FUKUI,
Hideoki TANAKA* and Susumu MARUYAMA*

Research and Development Center,
Showa Sangyo Co., Ltd., 2-20-2 Hinode,
Funabashi, Chiba 273, Japan

*Fermentation Research Institute, Tsukuba,
Ibaraki 305, Japan

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Many peptide inhibitors of angiotensin-converting enzyme [EC 3.4.15.1] (ACE) have been found as fragments of proline-rich proteins, i.e., gelatin,¹⁾ bovine²⁻⁵⁾ and human^{6,7)} casein, and γ -zein (a maize endosperm protein).⁸⁾ In the presence of a reducing agent, alcohol-soluble maize endosperm proteins can be separated into three fractions (α , β , and γ -zein) based on their solubility.⁹⁾ The γ -zein fragment Leu-Pro-Pro was reported as having strong ACE inhibitory activity, but it was not easily produced from γ -zein by enzymatic hydrolysis.⁸⁾ Therefore, we thought the peptides originated from another zein fraction that also contains many proline residues. In this study, α -zein was used as a substrate to be hydrolyzed with various proteases to examine their ACE inhibitory activity, and the resulting hydrolysate was tested for hypotensive activity on spontaneously hypertensive rats (SHR).

Zein (Sigma Chemical Co.) consisting primarily of α -zein was hydrolyzed with several different proteases in the following buffers: 50 mM Tris-HCl/5 mM CaCl_2 (pH 8.0) for thermolysin (Type X), 50 mM Tris-HCl (pH 8.0) for trypsin (type III), α -chymotrypsin (Type I-S), subtilisin (Carlsberg), and papain (recrystallized), and 0.075 N HCl for pepsin (crystallized). All the enzymes were purchased from Sigma Chemical Co. Each enzyme (50 mg) was added at 4°C to a 50-ml buffer suspension containing 0.5 g of α -zein. The mixtures were incubated with stirring at 37°C for 18 hr, and then adjusted to pH 7.0 with either 1 N HCl or 1 N NaOH. The reaction mixtures were heated to 100°C to inactivate the enzymes, and the precipitates were removed by centrifugation at 5,000 rpm for 10 min. The supernatants were ultrafiltered through a PM-10 membrane (Amicon Co.), and then the filtrates were lyophilized and weighed. Each buffer (50 ml) was also lyophilized and weighed, and the dry weight of the filtrate minus the dry weight of the hydrolysis buffer was defined as the net weight of the α -zein hydrolysate obtained.

ACE inhibitory activity was assayed by a modification

of the method of Cushman and Cheung¹⁰⁾ as described previously.³⁾ The sample and 5 mM hippuryl-L-histidyl-L-leucine were dissolved in a 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl, and incubated with 8 milliunits of ACE at 37°C for 30 min. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the above conditions was defined as IC_{50} , which was calculated using the net weight of α -zein hydrolysate. The hydrolysate resulting from hydrolysis with thermolysin showed the strongest activity (IC_{50} 21 $\mu\text{g}/\text{ml}$), and the other hydrolysates were weaker (IC_{50} 70–230 $\mu\text{g}/\text{ml}$).

For *in vivo* assays, the hydrolysis with thermolysin was done with no buffer on a much larger scale using the following procedures. α -Zein (100 g) and thermolysin (1 g) were suspended in deionized water (2,000 ml) at 4°C, and then the mixture was incubated with stirring at 65°C for 20 hr, while 5 N NaOH was used to keep the pH at 8.0. Thermolysin (1 g) was added again and the mixture incubated for another 20 hr. Forty ml of 5 N NaOH solution was used for hydrolysis pH control. The reaction mixture was autoclaved for 5 min at 105°C to inactivate the thermolysin, and then cooled to 4°C. The mixture was

Fig. 1. Changes in the Systolic Blood Pressure after an Intraperitoneal Injection of α -Zein Hydrolysate.

Values are the mean $\pm$ standard error ($n=6$). Significantly different from control (* $P < 0.05$, ** $P < 0.01$) by Student's *t*-test. $\circ$, control (saline); $\bullet$, α -zein hydrolysate (2.0 g/kg).

Fig. 2. Changes in the Systolic Blood Pressure after an Oral Injection of α -Zein Hydrolysate.

Values are the mean $\pm$ standard error ($n=8$). Significantly different from control (* $P < 0.05$) by Student's *t*-test. $\circ$, control (saline); $\bullet$, α -zein hydrolysate (5 g/kg).

centrifuged and ultrafiltered as described above. The filtrate was lyophilized to obtain a powder product (97.9 g), and the powder was examined for hypotensive activity on SHR by intraperitoneal and oral injection.

The SHR were warmed for 7 min in a 40°C thermostated box before measurement of blood pressure. Tail systolic blood pressure was measured using a plethysmographic tail apparatus (TK-350, Unicom Co.). Intraperitoneal experiments were conducted as follows: 9-week-old male SHR were fed a commercial nonpurified diet (CE-2, Japan CLEA) and water for 2 weeks *ad libitum*, and then either α -zein hydrolysate (2 g/kg) dissolved in saline or saline was injected intraperitoneally, and the blood pressure was measured at 0, 1, 3, 5, and 7 hr after the injection. As shown in Fig. 1, the blood pressure of the α -zein hydrolysate group was reduced at 3 and 5 hr, but in the saline group no change in the blood pressure occurred for 7 hr. Similar experiments were then done at different doses, *i.e.*, 0.4, 0.8, and 1.2 g/kg, and the blood pressure was measured 3 hr after the injection. The blood pressure was reduced in a dose-dependent manner (data not shown).

Oral experiments were done as follows: 9-week-old male SHR were fed twice a day for 2 weeks (from 9:00 to 10:00 a.m. and from 8:00 to 9:00 p.m.) and given water *ad libitum*. The α -zein hydrolysate (5 g/kg) was then orally injected (during 10:00 to 11:00 a.m.), and the blood pressure measured at 0, 3, 6, and 9 hr after the injection. Figure 2 shows that in 6 hr from the injection the blood pressure of the α -zein hydrolysate group became lower than that

of the saline group.

Further research will be focused on investigating the structure of peptides from this α -zein hydrolysate to define their amounts required for significant reduction of blood pressure.

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