Continuous oral feeding of enzymatic hydrolysate of porcine skin collagen showed an antihypertensive effect in spontaneously hypertensive rats (SHRs). We isolated an angiotensin I-converting enzyme (ACE) inhibitory peptide, Gly-Phe-Hyp-Gly-Pro (IC_{50} = 91 \mu M), from the hydrolysate, but the ACE inhibitory activities of the other peptides isolated were weak. Although the ACE inhibitory activity of Gly-Pro (IC_{50} = 360 \mu M) was not potent, Gly-Pro exists in collagen as a large number of repeated sequences. We then examined the antihypertensive effect of Gly-Pro. Orally administered Gly-Pro at 500 mg/kg significantly decreased the blood pressure of SHRs, and at 50 mg/kg it also showed a tendency to lower the blood pressure. Oral administration of Gly-Phe-Hyp-Gly-Pro (10 or 30 mg/kg) also decreased the blood pressure of SHRs.

Key words: collagen hydrolysate; Gly-Pro; spontaneously hypertensive rat

ACE performs an important physiological role in controlling blood pressure; the enzyme catalyzes both the production of the vasoconstrictor angiotensin II and inactivation of the vasodilator bradykinin. Many antihypertensive ACE inhibitory peptides have been found in the enzymatic hydrolysates of food proteins.\(^1,2\) Collagen-derived ACE inhibitory peptides such as Gly-Pro-Hyp-Gly-Thr-Asp-Gly-Ala-Hyp, Gly-Pro-Hyp-Gly-Ala-Hyp, Gly-Pro-Leu, and Gly-Pro-Val have also been reported.\(^3,4\) An in vivo antihypertensive effect of collagen hydrolysate on animals through dietary ingestion has also been reported.\(^5\) Since then, the antihypertensive effects of collagen hydrolysates prepared from various sources and the isolation of several other ACE inhibitory peptides from those hydrolysate fractions have been reported.\(^6-9\)

An antihypertensive effect of LCP (Nitta Gelatin, Osaka, Japan), a fermented collagen hydrolysate derived from porcine skin, in SHRs by continuous oral feeding has been reported by Anzai et al.\(^10\) Since we also confirmed the antihypertensive effect of the hydrolysate of porcine collagen (SCP3100; Nitta Gelatin), which was hydrolyzed with a protease from Aspergillus oryzae and is commercially available on a large scale for foodstuffs, here we report the in vivo effect of SCP3100 and collagen-related peptides in SHRs.

The antihypertensive effect of continuous oral feeding of SCP3100 was examined in SHRs. Male SHRs (6 weeks old, purchased from Charles River Japan, Yokohama, Japan) were randomly divided into two groups of eight animals. During the experimental period, the animals were housed in an air-conditioned room at 22 ± 2 °C under artificial light controlled in 12-h light/darkness cycles. Both groups (n = 8) were given a standard laboratory diet (CE-2; Clea Japan, Tokyo) and distilled water ad libitum. Systolic blood pressure was measured by the tail-cuff method using a blood pressure monitor for rats (Model MK-2000; Muromachi Kikai, Tokyo) once a week after 2 weeks of age, and the body weights of the rats were recorded at the same time. After measurement at 16 weeks of age, only the test group (n = 8) was given a sample solution that contained 3.75% w/v of SCP3100 ad libitum instead of distilled water for the last 12 weeks. The sample solution was enclosed in a plastic bag which was attached with a backflow prevention nozzle through a sterile filter under aseptic conditions. All drinking fluids were completely replaced every 2–3 d. The experimental procedure was conducted in accordance with the Guidelines for Animal Experiments of AIST. During the 12-week feeding period, drinking-fluid consumption calculated from the weights of the fluid bags ranged from 30 to 70 ml/rat/d, and the average was about 50 ml/rat/d, and this was not different between the control and test groups. Data were analyzed by split plot design ANOVA performed using Staflexver. 5 (Artech, Osaka, Japan).

As shown in Fig. 1a, the systolic blood pressure of the SCP3100 feeding group was significantly (p < 0.05) lower than that of the control group. Systolic blood pressure at the end of the 12-week feeding was 216.0 ± 3.2 mmHg for the control group and 206.6 ± 2.9 mmHg for the test group. Thus continuous oral feeding of enzymatic hydrolysate from porcine skin collagen SCP3100 showed an antihypertensive effect in the SHRs. The average body weights of the two groups were not significantly different, though that of the test group was slightly larger (Fig. 1b).

Since both LCP and SCP3100 inhibited the activity of ACE, with IC_{50} values of 0.7 mg/ml and 0.8 mg/ml

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respectively, we tried to isolate the ACE inhibitory peptides. ACE inhibitory activity was assayed by the method of Cushman and Cheung \(^1\) with minor modifications, using ACE from a rabbit lung (Sigma-Aldrich, St. Louis, MO) and a synthetic substrate hippuryl-L-histidyl-L-leucine (Peptide Institute, Osaka, Japan), as described in a previous report. \(^1\) Each collagen hydrolysate was applied to a Sephadex LH-20 (GE Healthcare, Buckinghamshire, UK) column (26 × 900 mm) eluted with 30% methanol. The active fractions eluted from the column were applied to a SP-Toyopearl 650M (Tosoh) column (18 × 650 mm) equilibrated with distilled water, and were also eluted with a linear gradient of NaCl (0 to 1 M). The active fractions eluted from the column were further applied to a DEAE-Toyopearl 650M (Tosoh, Tokyo) column (16 × 650 mm) equilibrated with distilled water, and were also eluted with a linear gradient of NaCl (0 to 1 M). The active fractions eluted from the column were further applied to a SP-Toyopearl 650M (Tosoh) column (16 × 650 mm) equilibrated with distilled water, and were also eluted with a linear gradient of NaCl (0 to 1 M). Each active fraction was finally applied to a \(\mu\) Bondasphere C18 column (3.9 × 150 mm; Waters, Milford, MA) for high-performance liquid chromatography (HPLC), and was eluted with a linear gradient of acetonitrile (0 to 63%) in 0.1% trifluoroacetic acid. The amino acid sequence of each purified sample was analyzed with a protein sequencer (Type 491, Applied Biosystems, Foster City, CA). ACE inhibitory peptides such as Gly-Phe, Gly-Pro-Arg, Val-Arg-Gly, Ala-Arg, Gly-Pro-Ala-Gly-Pro, and Gly-Ala-Hyp were isolated from LCP. Among these peptides, Gly-Phe-Hyp-Gly-Pro showed the strongest ACE inhibitory activity (IC\(_{50}\) = 91 \(\mu\)M). The IC\(_{50}\) values of the other peptides were more than 200 \(\mu\)M.

Thus, the ACE inhibitory activity of peptides contained in the hydrolysate was not strong. Although the ACE inhibitory activity of Gly-Pro was not strong (IC\(_{50}\) = 360 \(\mu\)M), Gly-Pro exists in collagen as a large number of repeated sequences such as -(Gly-Pro-X)n-. We then examined the antihypertensive effect of Gly-Pro. SHRs (14–16 weeks old) having systolic blood pressure of 190 mmHg or higher were divided into three groups of six rats each. All the groups were given a standard laboratory diet and tap water \textit{ad libitum}. A single oral administration of 50 and 500 mg/kg body weight (n = 6 for both groups) of Gly-Pro (Sigma-Aldrich) dissolved in distilled water at a concentration of 10 mg/ml and 100 mg/ml respectively was given. The control rats (n = 6) were given a corresponding volume of distilled water. As shown in Fig. 2, orally administered 500 mg/kg of Gly-Pro significantly decreased the blood pressure of the SHRs after 1 to 24 h, while 50 mg/kg of Gly-Pro also showed a tendency to lower blood pressure. In the distilled-water group, no significant change in blood pressure occurred over 48 h.

Another experiment carried out on 18–20 week-old SHRs (n = 6 for 500 mg/kg and n = 4 for 50 mg/kg of Gly-Pro) by the same procedure exhibited very similar profiles of systolic blood pressure over the investigation period (data not shown). On the other hand, there was no significant effect on blood pressure until 48 h after administration of the corresponding amounts of the glycine and proline mixture instead of 500 mg/kg of Gly-Pro (n = 6, data not shown).

We further examined the antihypertensive effect of the ACE inhibitory peptide Gly-Phe-Hyp-Gly-Pro. Single oral administration of 10 mg/kg (n = 5) and 30 mg/kg...
(n = 6) of Gly-Phe-Hyp-Gly-Pro (custom-made by the Peptide Institute.) dissolved in distilled water at concentrations of 2 mg/ml and 6 mg/ml respectively was performed on 17–20 week-old SHRs. As shown in Fig. 3, Gly-Phe-Hyp-Gly-Pro significantly decreased the blood pressure of the SHRs at both 30 and 10 mg/kg. As with Gly-Pro, 24 h after single oral administration of the peptide, blood pressure was significantly lower than that of the control group.

In the present study, we found a blood pressure-lowering effect of SCP3100 and its constituent peptides, Gly-Pro and Gly-Phe-Hyp-Gly-Pro. Oligopeptides which consist of Pro and Gly are generally resistant to proteolysis in vivo. Gly-Pro is known to be a good substrate for peptide transporters related to intestinal absorption, and it is fairly stable in the blood stream. Type I collagen contains Gly-Pro sequences at concentrations of 2 mg/ml and 6 mg/ml respectively was performed on 17–20 week-old SHRs. As shown in Fig. 3, Gly-Phe-Hyp-Gly-Pro significantly decreased the blood pressure of the SHRs at both 30 and 10 mg/kg. As with Gly-Pro, 24 h after single oral administration of the peptide, blood pressure was significantly lower than that of the control group.

In the present study, we found a blood pressure-lowering effect of SCP3100 and its constituent peptides, Gly-Pro and Gly-Phe-Hyp-Gly-Pro. Oligopeptides which consist of Pro and Gly are generally resistant to proteolysis in vivo. Gly-Pro is known to be a good substrate for peptide transporters related to intestinal absorption, and it is fairly stable in the blood stream. Type I collagen contains Gly-Pro sequences at approximately 20% by weight, though the free form of Gly-Pro was not a major component of SCP3100 (approximately 30 nmol/g of SCP3100). It is possible that the collagen hydrolysate showed antihypertensive activity through gastrointestinal digestion and absorption of small peptides such as Gly-Pro. In addition, it has been reported that orally administered 14C-labeled collagen hydrolysate was absorbed from the intestine in the high molecular weight form of the peptide in mice. Gly-Phe-Hyp-Gly-Pro and other peptides having weak ACE inhibitory activity, detected in SCP3100, might also be transported in the blood stream and exhibit antihypertensive activity. On the other hand, Iwai et al. reported that food-derived hydroxyproline-containing collagen peptides, such as Pro-Hyp, Ala-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, and Phe-Hyp have been identified in human blood after oral ingestion of certain gelatin hydrolysates, and that no hydroxyproline-containing peptides larger than Pro-Hyp-Gly were detected. These peptides might also be candidates for antihypertensive active peptides, because it is known that peptides containing a proline or hydroxyproline residue at the C-terminal position often have ACE inhibitory activity, and that Phe-Hyp can be released from Gly-Phe-Hyp-Gly-Pro. Presumably, other mechanisms to lower blood pressure are involved in the antihypertensive effect of the collagen hydrolysate, since blood pressure is controlled by a number of different biochemical substances besides ACE. Hence further research is necessary to determine the contribution ratio of Gly-Pro and Gly-Phe-Hyp-Gly-Pro in the antihypertensive effect of the collagen hydrolysate. Several researchers have reported that certain antihypertensive peptides exhibited long-term antihypertensive effects at 24 h after single oral administration in SHRs. Sato et al. found a long-lasting antihypertensive effect in a peptide fraction extracted from an edible mushroom, Mycopoliodonoides aitchisonii.  Suetsuna et al. identified long-term antihypertensive peptides such as Lys-Tyr, Phe-Tyr, and Ile-Tyr, from sea alga (wakame). In this study, both Gly-Pro and Gly-Phe-Hyp-Gly-Pro also showed a tendency to show long-term efficiency, and this is another point that makes further research attractive.

References

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Fig. 3. Changes in Systolic Blood Pressure of SHRs under Administration of Gly-Phe-Hyp-Gly-Pro.

Single oral administration was performed at doses of 0 (●, control, n = 6), 10 (▲, n = 5), and 30 (▲, n = 6) mg/kg of body weight, and systolic blood pressure was measured 0, 1, 2, 4, 6, 8, 24 and 48 h after administration. Data are expressed as mean ± SE. Different from control at *p < 0.05, **p < 0.01 by the Tukey test.