Blood pressure increases were inhibited by feeding a diet containing sour milk fermented by a starter containing Lactobacillus helveticus and Saccharomyces cerevisiae to spontaneously hypertensive rats. In rats fed with the sour milk, the angiotensin I-converting enzyme activity of the aorta was significantly lower than that of rats fed with the control commercial diet.

Key words: sour milk; antihypertensive effect; peptide; angiotensin I-converting enzyme

Angiotensin I-converting enzyme (ACE) catalyzes the formation of the potent vasopressor angiotensin II from angiotensin I, and inactivates bradykinin, which has a vasodilatating action. Thus ACE is important in increasing blood pressure. Two kinds of ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, have been isolated and identified from Calpis sour milk, which was prepared by fermenting skim milk with a starter containing Lactobacillus helveticus and Saccharomyces cerevisiae. In the sour milk, most of the ACE inhibitory activity was represented by these two tripeptides. Furthermore, the sour milk and the tripeptides had antihypertensive effects by single oral administration in spontaneously hypertensive rats (SHR). These findings indicated that Val-Pro-Pro and Ile-Pro-Pro are essential in the antihypertensive activity of the sour milk. It is known that ACE exists in the vascular endothelium of various organs. In ACE inhibitory substances used as pharmaceuticals such as captopril, their antihypertensive activities are known to be related to the inhibition of ACE activity of organs, such as lung, kidney, aorta, and brain. Although many ACE inhibitory peptides were found from enzyme-digested food proteins, there have been few reports on the mechanism in vivo. In this study, we investigated the influence of long term feeding of the sour milk in SHR rats, and measured ACE activity in various organs to partly clarify the mechanism of the antihypertensive activity of the sour milk.

Calpis sour milk was prepared as previously reported. The sour milk was mixed with 9% (w/w) dextrin (Pinedex-R, Matsutani Chemical) and was lyophilized. The lyophilized sour milk powder was mixed with a standard laboratory diet (F-2, Funabashi Farms) to contain 0.25, 1.25, or 2.50% sour milk. Male SHR rats, 6 weeks of age (body weight: 125–155 g), were purchased from Charles River Japan and fed with the standard laboratory diet for one week. At 7 weeks of age, feeding the diet containing the sour milk (sour milk group) and the standard laboratory diet (control group) was started. At 23 weeks of age, the diet of 2.50% sour milk group was replaced by the control diet for 48 h before killing. The systolic blood pressure (SBP) and heart rate were measured by the tail-cuff method with a programmed electro-sphygmomanometer (PB98A, Softtron) every other week.

At 23 weeks of age, all animals in the 2.50% sour milk and control groups were killed by exsanguination from abdominal aorta under pentobarbital anesthesia. Aorta, heart, liver, testes, kidney, lung, and brain were excised and the enzyme extracts were prepared by the method described by Manjusri et al. with some modification. Each organ was chopped into small pieces and homogenized in 50 mm Tris-HCl (pH 7.9) containing 0.3 m NaCl (2 ml per g tissue in aorta, 4 ml per g tissue in heart, lung, liver, testes, and kidney) by an ultra disperser (LK-21, Yamato). The suspension was filtered through nylon mesh (No. 20, Abe Chemical), and 2 ml of filtered suspension was centrifuged at 44,000 × g for 90 min. The pellet obtained was resuspended in 2 ml of this buffer and centrifuged again at 44,000 × g for 90 min. The pellet was resuspended in 2 ml of 0.1 m sodium borate buffer (pH 8.3) containing 0.3 m NaCl and 0.5% Triton X-100. The suspension was centrifuged at 1000 × g for 10 min and the supernatant was used as the enzyme extract. ACE activity was assayed by the method of Cushman and Cheung. Enzyme extracts diluted with 0.5 m borate buffer (pH 8.3) containing 0.3 m NaCl (100 times for lung, 10 times for aorta and serum, and 4 times for other organs) was used in measurements of ACE activity. One unit (U) of enzyme activity was defined as the amount of enzyme that cleaves 1 mol of angiotensin I to angiotensin II per minute.

**Fig.** The Body Weight (A) and Change of Blood Pressure (B) during Feeding the Diet Containing Sour Milk in Spontaneously Hypertensive Rats.

Feeding the diet containing sour milk was started at 7 weeks of age of rats. Each point indicated the mean of six animals and vertical bars represent the standard errors.

**Abbreviations:** SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; ACE, angiotensin I-converting enzyme.
of substrate per min. The specific activity was expressed as the number of units per mg of protein. The protein content was measured by the method of Lowry et al., using bovine serum albumin (Type V, Sigma) as a standard. Serum of each rat in both the groups was collected to measure total protein, A/G ratio, BUN, creatinine, uric acid, total cholesterol, high density lipoprotein cholesterol, β- lipoprotein, triglyceride, ZTT, total bilirubin, GOT, GPT, LDH, AL-P, γ-GTP, amylase, and Fe. Difference between control and sour milk groups was analyzed statistically by Student’s t-test.

There was no significant difference in the body weight of rats in each group (Fig. A). Diet intake during experimental period did not differ much in all groups (17.1 ± 0.2, 17.6 ± 0.3, 17.8 ± 0.2, and 17.3 ± 0.2 g rat per day for the control, 0.25%, 1.25%, and 2.50% sour milk groups, respectively). The SBP of rats gradually increased in the course of experiment (Fig. B). The SBP of rats in the sour milk group tended to be lower than in the control group after 13 weeks of age. In the 2.50% sour milk group, the SBP was significantly lower than that of the control group after 21 weeks of age. The antihypertensive activity of the sour milk tended to be dose-dependent (Fig. B). At 23 weeks of age, the average SBP of the 2.50% sour milk group (201.2 ± 5.8 mmHg) was significantly lower (p < 0.05) than that of the control group (220.3 ± 3.4 mmHg), even at 48 h after the substitution of the diet to the control diet. During the experimental period, the average of heart rate was not significantly different in all the groups.

At death, the weight of heart, liver, testes, kidney, and spleen for body weight were not significantly different between the control and 2.5% sour milk groups (data not shown). The ACE activity of various organs are shown in Table. The ACE activity of aorta was significantly lower in the sour milk group than that in the control group. The ACE activity of plasma, heart, lung, liver, kidney, testes, and brain were not significantly different. All the serum markers measured were not significantly different (data not shown).

Recently, an antihypertensive effect of the sour milk by a single oral administration was reported. The SBP decreased significantly at several hours and returned to the initial level 24 h after administration. In this study, we found that the development of hypertension in SHR rats was inhibited by long term feeding with sour milk (Fig. B). Lower SBP in rats fed with 2.5% sour milk was observed even 48 h after the replacement of the sour milk diet by the control diet. These results suggest that sour milk has not only a temporary antihypertensive effect by single oral administration but also a long-lasting effect on the hypertensive stage by long term feeding in SHR rats. Unlike blood pressure, sour milk did not affect the heart rate, body weight, organ weight, and eighteen kinds of markers in serum measured.

In ACE activity of various organs measured, the activity in aorta was significantly lower in the 2.5% sour milk group. Unger et al. reported that the prolonged antihypertensive action of ACE inhibitors might be related to persistent ACE inhibition in tissue such as vascular wall and kidney. Ikemoto et al. reported that the ACE activity of aorta in aged stroke-prone spontaneously hypertensive rats were significantly higher than with normotensive Wistar Kyoto rats. These suggest that the decrease of ACE activity in aorta may be important in expression of antihypertensive activity of the sour milk in SHR rats.

It is known that small peptides as di- or tri-peptides are easily absorbed in the intestine and Pro-Pro sequence are resistant to enzyme degradation. One possible explanation for the decrease of ACE activity is that the tripeptides are absorbed, reach the target organ, and decrease ACE activity. Decrease of ACE activity might take place by inhibitory activity of the tripeptides, or by other mechanisms, including the repression of the enzyme. However, further studies are necessary to demonstrate the absorption of these peptides, and clarify the mechanism in the decrease of ACE in the aorta.

References