Studies on Anti-Hypertensive Peptides in Milk Fermented with *Lactobacillus helveticus*

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Milk fermented by *Lactobacillus helveticus*, a lactic acid bacterium with potent proteolytic activity, had an antihypertensive effect in animal model studies. During the culture process, the biogenic peptides valyl-prolyl-proline and isoleucyl-prolyl-proline were derived from milk protein. These two peptides have been shown to have an inhibitory effect on angiotensin-converting enzyme, which converts angiotensin I to angiotensin II, a strong vasoconstrictor. Pre-clinical and *in vitro* studies suggest that a portion of the orally ingested dose of these peptides can be absorbed in the intact form from the gastrointestinal tract, can inhibit the tissue renin-angiotensin system, and can produce significant reductions in blood pressure. In clinical studies, fermented milk containing biologically active peptides significantly decreased systolic and diastolic blood pressure in hypertensive subjects. In contrast, no significant effect on blood pressure was observed in normotensives. Based on these observations, it is possible that fermented milk containing valyl-prolyl-proline and isoleucyl-prolyl-proline which could provide a non-pharmacological approach to the management of hypertension. This report reviews a series of studies on antihypertensive peptides in milk fermented with *Lactobacillus helveticus* and also describes the results of clinical trials including the latest information.

Key words: anti-hypertensive effect; *Lactobacillus helveticus*; fermented milk; peptide; angiotensin-converting enzyme

INTRODUCTION

Milk fermented with lactobacilli is recognized as a pleasant sour-tasting drink, which has superior storage properties, making it good for human health. In recent years, through both epidemiological and experimental studies, fermented milk or the lactic acid bacteria themselves have been shown to have useful functions (51). In studies relating to the physiological effects, fermented milk prepared with lactobacilli has been reported to have many beneficial actions, including life-extending effects (2), inhibition of the evolution of cancer (43), stimulation of interferon production (5), reduction of blood cholesterol levels (25), and antibiotic activity (48). However, little is known about many of the key components involved, their mechanisms of action, or their use in humans.

Hypertension is considered to be a risk factor for the incidence of stroke and coronary heart disease (7, 24). The number of elderly people in Japan is rapidly increasing. In addition, the number of hypertensives is increasing progressively with age, with over half the hypertensive population over 65 years of age (13). Similarly, elevated blood pressure is a common prob-

lem in many countries. Almost 25% of the US adult population (approximately 50 million people) has hypertension, defined as systolic (SBP) and diastolic blood pressures (DBP) of 140/90 mmHg or higher and/or current use of antihypertensive medicines (36). More than half of all individuals aged 60 years or older in the United States have hypertension (4). Therefore it is important to prevent hypertension at an early stage, in addition to the treatment of hypertension. Monitoring of blood pressure and lifestyle modification are recommended for people with elevated blood pressure (10, 11, 17). Lifestyle modification includes dietary sodium reduction, weight control, moderation of alcohol consumption and cessation of smoking. Recently, the Dietary Approaches to Stop Hypertension (DASH) trial indicated that a diet rich in fruit, vegetables, and low-fat dairy foods with reduced saturated and total fat, could substantially lower blood pressure (23, 44). Thus, the utilization of foods having merits relating to blood pressure lowering has attracted attention as a new means of achieving the above objectives.

EFFECT OF FERMENTED MILK ON BLOOD PRESSURE

In 1908, it was claimed by Metchnikoff (31) that ingestion of yogurt prolongs the life span, on observing that lifespan was longer in those who regularly consumed yogurt. There has subsequently been great in-

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Fig. 1. Changes in survival ratio of mice administered sour milk or whole milk (2).

terest in the physiological effects of fermented milk prepared with lactobacilli, and many studies have been conducted. Arai et al. (2) studied the effect of Calpis sour milk on life span in ICR mice. Calpis sour milk (sour milk) is prepared by fermenting skim milk with a starter culture containing *Lactobacillus helveticus* as the predominant microorganism. Two hundreds and seventy mice were divided into three groups of 90 mice each, and were fed the diet containing pasteurized sour milk, whole milk, or the control basal diet. As shown in Fig. 1, the mean life span of mice was significantly longer in the sour milk group (91.8 weeks, $p < 0.05$) than that in the control (84.9 weeks) or whole milk groups (84.4 weeks). The main causes of death were renal atrophy, tumors, and pneumonia. These findings suggested that sour milk inhibited at least one of these pathological processes, and thus contributed to the extended life span in the mice. Furthermore, Wada et al. (55) reported that the progression of renal lesions induced by a high meat (HM) diet in APA hamsters was suppressed by including sour milk in the HM diet. From these observations, it can be concluded that sour milk has some effects on the circulatory system, possibly including blood pressure regulation.

To study the effect of milk fermented by *L. helveticus* on blood pressure, diets containing 0.25–2.5% sour milk powder were administered to spontaneously hypertensive rats (SHR) (38) (Fig. 2). When the diet containing fermented milk was started at 7 weeks of age, the systolic blood pressure (SBP) of the rats was in the normal range (140 ± 5 SD mmHg), rather than in the hypertensive range. In comparison to the control group, the rats in the sour milk-added feed groups showed a tendency to have lower blood pressures from the age of 13 weeks, i.e. by the 6th week after the start of intake; this effect was also dose-dependent. Furthermore, those in the

Fig. 2. The changes of blood pressure during feeding the diet containing sour milk in spontaneously hypertensive rats (38). Means $\pm$ standard errors. $\circ$, control; $\square$, diet containing 0.25% (w/w) sour milk powder; $\triangle$, diet containing 1.25% (w/w) sour milk powder; $\bullet$, diet containing 2.50% (w/w) sour milk powder. Significant difference from control; * $p < 0.05$, ** $p < 0.01$.

2.5% sour milk-added feed group showed significantly lower blood pressures after the age of 21 weeks. Although feeding the diet containing sour milk was terminated at the age of 23 weeks, the lowered blood pressure value was maintained when measurements was conducted 48 hr after termination of feeding. The sour milk was also administered to SHR by single oral doses and changes in blood pressure were examined (40). The mean SBP was 213.7 ± 3.4 (SD) mmHg before administration of sour milk; it decreased significantly after 2 hr and a reduction of approximately 20 mmHg was maintained for a further 8 hr.

These findings demonstrate that sour milk showed a preventive effect following long-term consumption and showed blood pressure lowering after a single administration.

ANTIHYPERTENSIVE PEPTIDES IN FERMENTED MILK

A number of functions of peptides derived from milk proteins have been identified, including opioid (3), immunomodulation (42) and stimulation of mineral absorption (46). Maruyama et al. (26–28) identified several peptides produced by the tryptic hydrolysate of α_s - and β -casein, which are inhibitors of angiotensin-converting enzyme (ACE).

ACE (EC3.4.15.1), which is a zinc-metalloproteinase, is important in the regulation of blood pressure (Fig. 3). In the renin angiotensin system, dipeptidyl carboxypeptidase ACE acts on angiotensin I to hydrolyze His-Leu from its C-terminal and produce an 8-amino acid polypeptide angiotensin II (50). Angiotensin II acts not only as a vasoconstrictor on vascular smooth muscle,

Fig. 3. The renin-angiotensin and kinin-kallikrein systems.

but also acts on the adrenal cortex to increase the synthesis of aldosterone, which causes sodium retention, and thus increases blood pressure. In addition, in the kinin kallikrein system, ACE inactivates bradykinin, which exhibits vasodilatory action (8). Some ACE inhibitors show antihypertensive effects *in vivo* (41) and are currently used as pharmaceuticals. Some researchers have reported the presence of ACE inhibitory peptides in enzymatically hydrolyzed food materials (26–28, 56, 60), but not in milk fermented with lactobacilli.

ACE inhibitory activity in milk was monitored during fermentation with a Calpis sour milk starter (39) (Fig. 4). Reconstituted 9% (w/w) skim milk was pasteurized, inoculated with a starter containing *L. helveticus* as the predominant microorganism, and fermented at 37°C for 24 hr. The ACE inhibitory activity markedly increased from the late log phase to the stationary phase. Following a four-step HPLC procedure, two peaks were isolated from the sour milk. After purification, 74.4% of the initial ACE inhibitory activity was recovered in the two peaks, suggesting that most of the ACE inhibitory activity of the sour milk could be attributed to the two peaks produced during fermentation. The two peaks were identified as valyl-prolyl-proline (Val-Pro-Pro) and isoleucyl-prolyl-proline (Ile-Pro-Pro) by analysis of the amino acid composition and sequence and from ^1H and ^{13}C nuclear magnetic resonance spectra (39). The IC_{50} values (defined as the concentration of peptide required to inhibit 50% of the ACE activity *in vitro*) for Val-Pro-Pro and Ile-Pro-Pro were 9 and 5 μM , respectively.

To investigate the effects of Val-Pro-Pro and Ile-Pro-Pro on blood pressure, the synthesized peptides were administered to SHR and the blood pressure response was measured (40). Figure 5 shows the changes of SBP

Fig. 4. Changes of inhibitory activity of angiotensin-converting enzyme (ACE) of milk during fermentation by Calpis sour milk starter containing *Lactobacillus helveticus* as predominant microorganisms (39).

Fig. 5. Effect of single oral administration of Val-Pro-Pro or Ile-Pro-Pro on spontaneously hypertensive rats (40).

Means $\pm$ standard errors. $\square$, control; $\bullet$, Ile-Pro-Pro; $\circ$, Val-Pro-Pro; $\blacksquare$, sour milk. Significant difference from control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

after a single oral administration of sour milk (5 ml per kg body weight), Val-Pro-Pro (0.6 mg per kg body weight) or Ile-Pro-Pro (0.3 mg per kg body weight) to SHR. The peptide content was equivalent to the administration of 5 ml of sour milk per kg body weight, in terms of the extent of ACE inhibitory activity. The two tripeptides significantly decreased SBP from 2 to 8 hr after administration. The extent of the decrease in SBP due to sour milk and tripeptides was similar, several hours after administration. To study the relationship between dose of tripeptides and blood pressure response, changes in SBP were measured 6 hr after a single oral administration of several doses of Val-Pro-Pro or Ile-Pro-Pro (40). Antihypertensive activity of these two peptides was dose-dependent up to 5 mg per kg body weight. These data suggest that both Val-Pro-Pro and Ile-Pro-Pro play an essential role in the antihypertensive activity of sour milk.

With respect to safety, SBP changes were assessed following administration of excessive amounts of sour milk or tripeptides to normotensive Wistar Kyoto (WKY) rats (40). A single oral administration of the sour milk or tripeptides did not change SBP of normotensive WKY rats, even though the amounts of sour milk (25 ml per kg body weight) and tripeptides (mixture containing 10 mg of each peptide per kg body weight) administered were far higher than the dose found to be effective in SHR. These results suggested that the antihypertensive effect of sour milk and tripeptides was specific to the hypertensive state.

PRODUCTION OF PEPTIDES

Anti-hypertensive effects of fermented milks prepared with various lactic acid bacteria species were examined (57). Fermented milk was prepared with sixteen strains from seven species of lactic acid bacteria commonly used in fermented dairy products. A single oral administration of 5 ml of fermented milk per kg body weight to SHR was carried out. Many of the fermented milks prepared with *L. helveticus* strains showed significant antihypertensive effects 4 hr after administration. However, milk fermented by other species of lactic acid bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* did not show significant antihypertensive activity. Most of the milks fermented by *L. helveticus* strains showed higher ACE inhibitory activity compared to that of other species. Similar results were also observed with respect to the peptide content of fermented milk or proteolytic activity in the cell wall of lactobacilli. We found Val-Pro-Pro and Ile-Pro-Pro in the milk fermented with *L. helveticus* strains showed antihypertensive effects in SHR (unpublished data). These findings suggested that the ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, produced by *L. helveticus* showed antihypertensive activity in SHR.

The amino acid sequence Val-Pro-Pro is found in the primary structure of bovine β -casein (84–86). Also there is a sequence of Ile-Pro-Pro in bovine β -casein (74–76) and κ -casein (108–110). A cell wall-associated proteinase has been purified from *L. helveticus* CP790 and characterized by Yamamoto et al. (58). This proteinase was characterized as being of the PIII type that could hydrolyze α_{S1} -casein and β -casein, but not κ -casein. Therefore, Val-Pro-Pro and Ile-Pro-Pro are considered as being derived from β -casein. Several pep-

tides with ACE-inhibitory properties have been isolated from β -casein, which has been hydrolysed by the purified cell wall proteinase from *L. helveticus* CP790 (56). Although peptides containing Val-Pro-Pro and Ile-Pro-Pro sequences were observed, Val-Pro-Pro and Ile-Pro-Pro peptides were not found in hydrolysate of β -casein. Further steps such as cleavage by several peptidases would be needed to produce the Val-Pro-Pro and Ile-Pro-Pro peptides.

MECHANISM OF ACTION OF PEPTIDES

Generation of angiotensin II in tissues such as vascular wall and kidney has been demonstrated (6). It has been suggested that inhibition of ACE in the vascular wall, kidney, brain and lung is important for the expression of antihypertensive activity of ACE inhibitory substances (52–54). To see if ACE inhibition occurs in the organs of SHR, sour milk containing Val-Pro-Pro and Ile-Pro-Pro peptides was administered and various organs were measured for ACE activity (29, 38). At 7 weeks of age, feeding the diet containing the sour milk powder (0.25 to 2.5%) and the standard laboratory diet was started (38). At 23 weeks of age, the average SBP of the 2.5% 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). Following excision of the aorta, lung, heart, liver, testes, kidney and brain, enzyme extracts were prepared to measure the ACE activity. ACE activity of the aorta (62.8 ± 12.6 mU/mg protein/min) was significantly lower ($p < 0.05$) in the sour milk group than that in the control group (127.7 ± 33.0 mU/mg protein/min). The ACE activities of plasma, heart, lung, liver, kidney, testes, and brain were not significantly different between the two groups.

Masuda et al. (29) reported that similar decreases take place in SBP and ACE activity in the abdominal aorta after a single oral administration of sour milk. At 23 weeks of age, 10 ml of sour milk or saline (per kg of body weight) were administered by gastric intubation and SBP was measured 6 hr after administration. In SHR, the SBP was significantly decreased by 24.6 ± 3.1 mmHg ($p < 0.01$) after administration of the sour milk. ACE activity was then measured in various organs in SHR and WKY rats after administration of sour milk or saline. ACE activity in abdominal aorta and lung extracts from SHR given saline was significantly higher ($p < 0.01$ and 0.05 , respectively) than activity from WKY rats given saline (Fig. 6). ACE activity in aortic extract from SHR given the sour milk was significantly lower ($p < 0.05$) than that from SHR given saline. These results suggest that inhibition of the tissue

Fig. 6. Angiotensin-converting enzyme (ACE) activity in organs of spontaneously hypertensive (SHR) or Wistar Kyoto (WKY) rats (29).

Means $\pm$ standard errors. Significant difference between two groups; * $p < 0.05$.

renin-angiotensin system is associated with the expression of the antihypertensive activity of sour milk.

It has been also reported by HPLC analysis that Val-Pro-Pro and Ile-Pro-Pro were present in the aorta of SHR after administration of sour milk (29). Small peptides, such as di- or tripeptides, are more easily absorbed in the intestine than are amino acids or larger oligopeptides (1, 30, 49). In addition, it is known that the Pro-Pro sequence is relatively resistant to degradation by enzymes (22, 35, 61, 62). Recently, Satake et al. (45) studied intestinal transport of Val-Pro-Pro by using monolayer cultures of human intestinal Caco-2 cells, which express many enterocyte-like functions. This study indicated that Val-Pro-Pro could be transported across the Caco-2 cell monolayer in an intact form mainly by paracellular diffusion. These results suggest that a portion of Val-Pro-Pro and Ile-Pro-Pro is absorbed from the intestine and delivered to the aorta, where these peptides inhibit ACE activity, and consequently exhibit antihypertensive activity.

CLINICAL STUDIES

Effect in Hypertensive Subjects

A series of clinical studies has been conducted to evaluate the effect of fermented milk containing Val-Pro-Pro and Ile-Pro-Pro on blood pressure in hypertensive patients (12) or subjects with elevated blood pressures (14, 16, 18, 20, 21, 34, 51).

A placebo-controlled study was conducted to evaluate the effect of sour milk on the blood pressure of 30 hospital outpatients who were hypertensive and taking antihypertensive medication (12). Subjects were randomly assigned to two groups. Sour milk (100 ml) or

unfermented placebo milk was administered daily for 8 weeks. Sour milk, but not the placebo milk, contained 1.5 mg Val-Pro-Pro and 1.1 mg Ile-Pro-Pro. In the test group, 4 and 8 weeks after ingestion, SBP decreased significantly by 9 and 14 mmHg, respectively. DBP showed the same trend.

Thirty non-medicated mild or moderate hypertensive volunteers were recruited for a double-blind placebo controlled study with a similar protocol (21). The decrease in SBP was significantly greater in the test group than that in placebo group at 4, 6, and 8 weeks after ingestion ($p < 0.05$, $p < 0.05$, and $p < 0.01$). In the test group, there was a significant decrease in SBP from a baseline value of 157.8 ± 13.0 mmHg to 147.5 ± 11.9 mmHg at 2 weeks after intake ($p < 0.001$), and reached 144.1 ± 8.7 mmHg after 8 weeks ($p < 0.001$).

Similar results have been reported with hypertensive subjects by several researchers using different foods in liquid or tablet form (14, 16, 18, 20, 34).

Effect in Subjects with High-Normal Blood Pressure

Recently, a placebo-controlled, double-blind study was conducted to investigate antihypertensive effects of liquid yogurt containing Val-Pro-Pro and Ile-Pro-Pro in 106 individuals with high-normal blood pressure (37). The test group ($n = 53$) ingested liquid yogurt containing 2.26 mg of Val-Pro-Pro and 1.48 mg of Ile-Pro-Pro for 12 weeks. The placebo group ($n = 53$) ingested the same amount of placebo drink. The differences between SBP of the test and placebo groups at 4 ($p < 0.05$), 6, 10 and 12 weeks (all $p < 0.01$) after ingestion were statistically significant (Fig. 7). In the test group, SBP was significantly decreased from $134.1 \pm$

Fig. 7. Changes of systolic and diastolic blood pressure of subjects with high-normal blood pressure by ingestion of product containing Val-Pro-Pro and Ile-Pro-Pro for 12 weeks (37). ○, control group; ●, test group. Means $\pm$ standard deviation. Significant difference from control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.6 mmHg at 0 week to 131.0 ± 6.5 mmHg at 2 weeks after ingestion, and reached 128.0 ± 5.8 mmHg 12 weeks later. DBP showed a similar trend. No adverse effect, including cough, was observed in this study.

The reduced SBP/DBP at 8 weeks after ingestion were 14/9 mmHg in the study on mild hypertensive subjects (20) and 5/4 mmHg in the study on high-normal blood pressure (37), respectively. Thus, there tended to be a positive correlation between the initial blood pressure value and the reduced blood pressure value.

The occurrence of coughing associated with ACE inhibitors (47) is thought to involve the accumulation of substances such as bradykinin and substance P (15). In each of the above studies, no adverse events, including cough, were observed. Since the levels of Val-Pro-Pro and Ile-Pro-Pro peptides in the sour milk ingested were relatively low and their inhibitory activity towards ACE was mild, this is likely to be the reason for the high degree of safety with respect to coughing.

Effect in Normotensive Subjects

Twenty six normotensive volunteers were subjected to the 8 week continuous ingestion study using sour milk (containing 1.5 mg of Val-Pro-Pro and 1.1 mg of Ile-Pro-Pro) (16). No changes were seen in SBP, DBP, or any other blood parameters, either in the sour milk

or the placebo group. To evaluate the effect of a large intake of Val-Pro-Pro and Ile-Pro-Pro (17.7 and 11.5 mg, respectively), Yasuda et al. (59) conducted a double-blind, placebo controlled study using tablet form products on 20 normotensive subjects. Subjects ingested test or placebo tablets for one week. Similarly, a study of 43 normotensive subjects, involving 4 weeks continuous intake of large amounts of tablets (containing 7.9 mg of Val-Pro-Pro and 4.5 mg of Ile-Pro-Pro, respectively) was conducted (19). In both studies, no significant changes were observed in SBP, DBP, or any other blood parameters in either group.

CONCLUSIONS

During fermentation, Val-Pro-Pro and Ile-Pro-Pro, which had ACE-inhibitory properties, were produced by an *L. helveticus* protease. From *in vitro* and animal model studies, a portion of orally ingested peptide could be absorbed in the intact form from the intestine and acted directly on the tissue renin-angiotensin system to inhibit ACE activity, and thereby reduce blood pressure. Terms such as probiotics, prebiotics, and biogenics are used to define mechanisms of microorganism function (9, 32, 33). Biogenics refers to food ingredients which directly exert a health benefit on the host without involving intestinal microflora. The present results indicate that the antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, work on the host as biogenics. In clinical studies, fermented milk products containing Val-Pro-Pro and Ile-Pro-Pro showed a significant reduction in blood pressure in subjects with elevated blood pressure. No adverse events were observed in a series of studies.

These results suggest that food containing Val-Pro-Pro and Ile-Pro-Pro is useful in the management of hypertension without causing adverse events.

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