JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. 7(2), p. 1171-1176

The Inhibitory Effects of *Lactobacillus casei* 1.2435-Fermented Milk against Angiotensin-I-Converting Enzyme

Wei Hou[1,2*], Xue-Gang Luo[1,2*], Zhao Han[1,2,3], Cai-Ju Zhou[1,2] and Tong-Cun Zhang[1, 2]

[1] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, College of Bioengineering, Tianjin University of Science and Technology, Tianjin, China.
[2] Tianjin Key Laboratory of Industrial Microbiology, Tianjin, China.
[3] Yirui Biological Engineering Co., Ltd. Tianjin 300457, China.

(Received: 13 February 2013; accepted: 10 April 2013)

Angiotensin-I-converting enzyme (ACE) is an important enzyme in the regulation of blood pressure. In recent years, several ACE inhibitory peptides had been isolated from the enzymatic digests of milk proteins, or fermented milk prepared with lactic acid bacteria. In this study, to analyze the inhibition mechanism of ACE by *Lactobacillus casei* 1.2435-fermented milk, the method of Cushman-Cheung was used to examine the change of ACE activity before and after being treated with the supernatant of fermented milk. In addition, the promoter of human ACE was also cloned and inserted into pGL3 luciferase reporter plasmid, and then the effect of the supernatant of fermented milk on the transcription of ACE was detected by luciferase reporter assay in COS-7 cells. The results showed that the ACE inhibitory rate of *L. casei* 1.2435-fermented milk was about 97.6%, and it would decrease to 73.6ÿ and 75.7ÿ after being treated with chloroform and boil, respectively. Besides, it seemed that the fermented milk and the lysis product of the bacteria also had an influence on the activity of ACE promoter.

Key words: *Lactobacillus casei*; Fermented milk; ACE; Luciferase assay.

Several risk factors are associated with stroke, including age, gender, elevated cholesterol, smoking, alcohol, excessive weight, race, family history, and hypertension[1]. Although some of these risk factors cannot be modified, one factor that can be controlled and has the greatest impact on the etiology of stroke is high blood pressure[2]. Hypertension is considered to be the central factor in stroke, with ~33 % of deaths due to stroke attributed to untreated high blood pressure[1]. Hypertension occurs in high frequency, and often accompanied with other diseases, such as hyperlipidemia, arteriosclerosis, and coronary heart disease. The treatment of hypertension is effective in reducing the risk of such diseases. The regulation of hypertension is associated with the rennin-angiotensin system, where angiotensin-I-converting enzyme (ACE) (dipeptidyl carboxypeptidase, EC3.4.15.1) is considered to be the core of the system[3,4]. In the rennin-angiotensin system, ACE cleaves the dipeptide portion of angiotensin I from the C-terminal and produces angiotensin II, a highly potent vasoconstrictor molecule, which induces release of aldosterone, and therefore causes the reabsorb of sodiumby kidney and elevates the blood volume, thus increasing blood pressure. In addition, ACE can also inactivates bradykinin, which is a vasodilatory molecule[5].

Synthetic ACE inhibitors (captopril, enalapril, benazepril, etc) are one group of drugs in the treatment of hypertension. However, these synthetic drugs have significant side effects, such

* To whom all correspondence should be addressed.
Tel.: +86-22-60600518;
E-mail: tony@tust.edu.cn

as dry cough and angioedema. The food-derived biologically active peptides could represent a healthier and natural alernative for the ACE inhibitory drugs. Because of this reason, the natural ACE inhibitory peptides have received more attention in nowadays[6].

Milk proteins are sources of many biological activities peptides, such as ACE inhibitory peptides. Since a casein-derived ACE inhibitory peptide named CEI 12 (FFVAPFPEVFGK) was first isolated and identified, several ACE inhibitory peptides have been isolated from the enzymatic digests of milk proteins, or fermentation of milk with lactic acid bacteria[7,8]. Examples of food products on the market containing ACE peptides are Ameal S (Calpis Co. Ltd., Tokyo, Japan), a sour milk tablet-form product based on the milk drink calpis, which contains two potent ACE inhibitors, VPP [β-casein f (84-86)] and IPP [β-casein f (74–76)], generated from casein using the proteolytic capabilities of *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae*[9,10]. A number of antihypertensive peptides that were tested in spontaneously hypertensive rats (SHR) have shown strong antihypertensive effects from a low orally administered dose, such as AVPYPQR (β-casein) and TTMPLW (α_{s1}-casein) or KVLPVPQ (β-casein) and YKVPQL (α_{s1}-casein)[11, 12]. Two other peptides (YP and KVLPLPQ) that were purified and characterized from fermented milk were also shown to have ACE inhibitory activity in SHR[12,13,14]. Milks fermented with Lactobacillus helveticus CPN4, R211, R289, and LP01; *Enterococcus faecalis* CE-CT 5827, 5727, 5728 and *Lactococcuslactis* subsp. *cremoris* LP25 have all been shown to contain ACE inhibitory peptides and to display antihypertensive activity in vivo[15,16,17].

However, the ACE inhibitory effects of by *L. casei*-fermented milk are still not very clear. In our previous study, the *L. casei* 1.2435 was identified by 16S rDNA sequence analysis, and in this study, we attempted to analyze the effects of *L. casei* 1.2435-fermented milk on the activity of ACE and the transcription of ACE promoter.

MATERIALS AND METHODS

Materials

The milk was produced by Mengniu dairy company; Hippuryl-L-histidyl-L-leucine (Hip-His-Leu, HHL, substrate against ACE) was purchased from Navy General Hospital; ACE was extracted from the lung of rat with the method of LIU Hong and CHEN Lan-ying[18]; Basic chemical reagents were purchased from Tianjin Jiangtian chemical Co.; Microorganism (*L. casei* 1.2435 and *E.coli* DH5α) and cells (COS-7 and MCF-7) were supplied by the Key Laboratory of Industrial Microbiology of the Ministry of Education.

Milk fermentation and sample preparation

Fermented milk was prepared as follow description. Briefly, preculture of *L. casei* 1.2435 was prepared in 5 mL MRS medium at 37°C for 20 h. Three percent (vol/vol) of the corresponding preculture was added to 5mL milk and cultured at 37°C for 20 h. After the two-step activation, the milk was inoculated with ten percent of the preculture, and was cultured at the same conditions.

The fermented milk was centrifuged at 12,000 ×g for 20 min at 4°C. The supernatant was concentrated by vacuum freeze-dry and re-dissolved with phosphate buffer saline (PBS). After filtering through a Millex 0.22 μm filter, the supernatant was used for further studies.

Besides, the lysis product of the bacteria cells were also prepared and used in the luciferase reporter assay. After centrifuging the fermented milk, the sedimentation was re-suspended and washed in 20 mL elution buffer (50mM Tris-HCl (pH=7.1), 30 mM $CaCl_2$). The suspension was collected by centrifugation at 5000 ×g for 20 min at 4°C. The cell pellets were washed three times and lysed with 7 mL of lysis buffer (50 mM Tris-HCl (pH=8.5), 100 mM NaCl, 1 mg/mL lysozyme) at 37°C for 3 h. The cell pellets were centrifuged at 7000 ×g for 20 min. The supernatant was the lysis product, and was filtered through a Millex 0.22 μm filter for further study.

Measurement of ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung with some modification[19,20]. Each 0.10 mL of supernatant were preincubated with 0.15mL ACE at 37°C for 5 min, then 0.10 mL HHL borate buffer (3.8 mM HHL, 0.1 M borate, and 0.3 M NaCl; pH 8.3) were added, and the mixture was incubated at 37°C for 60 min. The reaction was stopped with 0.25 mL of 1 N HCl; the hippuric acid liberated by ACE was extracted with 1.50 mL of ethyl acetate,

and after the ethyl acetate was removed by vacuum evaporation, the hippuric acid was dissolved in 3 mL of NaCl solution (1 mol/L) and determined spectrophotometrically at 228 nm.

Percent of the ACE inhibition was calculated as in (1).

ACE inhibitory rate (%) = (A -S) / (A-C) × 100 ...(1)

The S is the optical density of sample which is in the presence of both ACE and the supernatant, A is the density of ACE without the supernatant, and C is the control that is without ACE and the supernatant. The inhibition values reported were the means of three determinations.

Cloning of ACE promoter and construction of the luciferase reporter plasmid

In order to detect the effect of the supernatant of the fermented milk on the transcription of ACE, the promoter (-1,073 bp to 182 bp) of human ACE was cloned from the human genome, which was isolated from MCF-7 cells, using the polymerase chain reaction (PCR) method with the forward primer 5'-CCGCTCGAGCCA CGGTTCCTCCATTG-3' (the restriction site of *Xho*I was underlined) and the reverse primer 5'-CCCAAGCTTTGTAGCTCTGCGCGAAG-3' (the restriction site of *Hind*III was underlined). Because the high GC-content of the fragment, A 2 ×GC Buffer I (TAKARA Biotechnology (Dalian) Co.) was used in the PCR reaction. The digested PCR product by *Xho*I and *Hind*III was purified by Gel Extraction Kit (CWBIO Co.), and then inserted into the promoterless luciferase expression vector pGL3 basic by T4 DNA Ligase (Promega).

Transfection and luciferase assay

The COS-7 cells were grown in DMEM/ low glucose medium (Thermo) supplemented with 10% newborn calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd), and cultured in 24 well cell culture cluster at 37°C in a humidified atmosphere at 5 % CO_2, and were transfected with the pGL3-ACE constructs using TurboFect (Fermentas). Briefly, Cells were cultured in 24-well plates and transfected with 2 μg DNA. The total amount of DNA was kept constant by the inclusion of appropriate amounts of empty vectors. The cells was treated with the supernatant in different concentrations at 8, 12, 16 h after transfection. Cells were lysed with Luciferase cells lysis reagent (CCLR) at 24 hours after transfection, and the luciferase activity was evaluated using the Luciferase Reporter Assay System (Promega) by BioTek Synergy™ H4 multi-mode microplate readers (Gene Company Ltd.).

RESULTS AND DISCUSSION

ACE inhibitory activity of the *L. casei* 1.2435-fermented milk

To investigate the effect of *L. casei* 1.2435-fermented milk on the activity of ACE, the method of Cushman and Cheung was carried out. Captopril, a classic ACE inhibitory drug, was also analyzed as positive control. As shown in Fig. 1, the ACE inhibitory rate of *L. casei* 1.2435-fermented milk reached about 97.6 %, which was close to the rate of captopril (98.9 %). These results indicated that the fermented milk has a significant inhibitory effect on the activity of ACE.

To further confirming the species of active ingredients, we detected the influence of boil or chloroform on the ACE inhibitory activity of the fermented milk. The results showed that ACE inhibitory activity significantly reduced after being treated with either boil or chloroform. The ACE inhibitory rate was 73.6 % after being boiled which had reduced 24.5 % than former (97.6 %), and 75.7 % after being extracted by chloroform that had reduced 22.4 % (Fig. 1). These results suggested that the ACE inhibitory ingredients of the fermented milk might contain protein or polypeptide, which could be destroyed by heat or chloroform. Furthermore, the followed separation and SDS-

Table 1. The optimized components of PCR

Components	Volume	Components	Volume
Template DNA	<0.5μg	10mM dNTPs	2μl
Forward Primer	1μl	EasyTaq DNA Polymerase	0.5μl
Reverse Primer	1μl	ddH_2O to final volume	50μl
2×GC Buffer I	25μl		

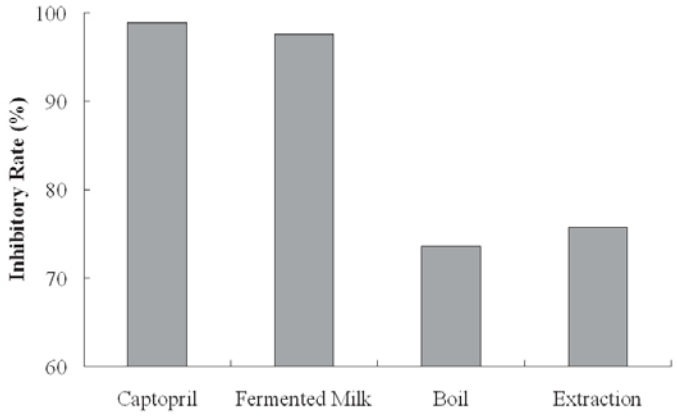

Fig. 1. The ACE inhibitory rate of *L. casei* 1.2435-fermented milk

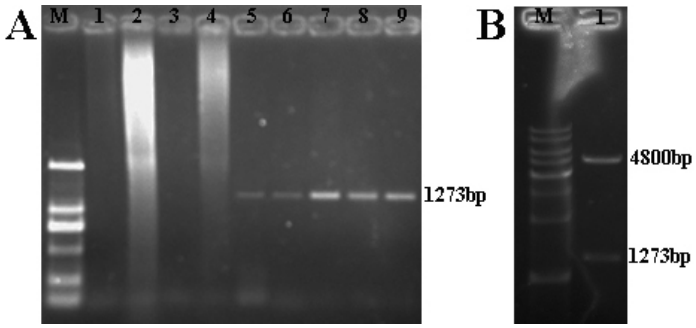

Fig. 2. Construction of the luciferase reporter plasmid driven by ACE promoter. (A) Optimization of cloning of ACE promoter by PCR. M: D2000 DNA Ladder; lane 1-9: PCR products with different PCR conditions. The product in lane 7 corresponds to the optimal conditions of PCR. (B) Identification of the constructed plasmid by restriction endonuclease digestion. M:1kb DNA Ladder; lane 1: the result of digestion verification

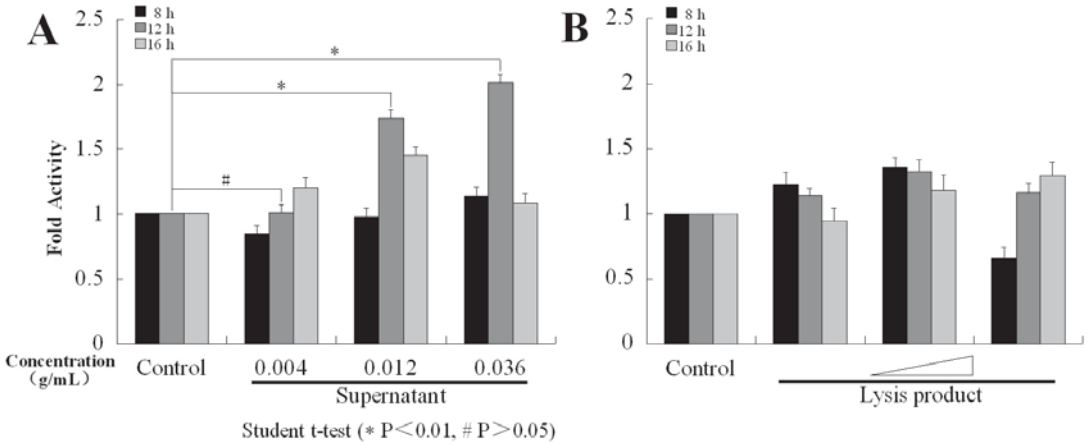

Fig. 3. Transcriptional activity of ACE promoter affected by the supernatant of fermented milk and lysis product of the bacteria. The activity of different treating time was shown in the fig with different colours. The control was treated with PBS at same time. The three wells of samples was added with a concentration gradient which was noted in the fig. A or shown as the shape of triangle in fig. B. Data are means SD (bars) values for six times of the determinations. * P<0.01, # P<0.05 VS Control

PAGE analysis found that the ACE inhibitory components contained some protein larger than 5 kDa (Data not shown), which had not been reported up to now. However, there might also have some non-protein ACE inhibitory substances existing in the fermented milk, which needed to be studied in the future.

Cloning of ACE promoter and construction of the luciferase reporter plasmid

The condition and reaction system of PCR was optimized at first (Fig. 2A). By the results, we have define the best condition as Tab. 1, and the opitimized PCR was performed as follows: denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1.5 min, and cycle three times; denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1.5 min, and cycle 27 times. The taget bandwas purified and inserted into the pGL3 basic plasmid. The plasmids extracted from the positive clone of *E. coli* DH5α were then verified by the digestion with *Xho*I and *Hin*dIII, and a 1273 bp band could be observed in the gel (Fig. 2B). At last, the sequenceing confirmed that the nucleotide sequence inserted in the plasmid was identical with the ACE promoter, suggesting that the luciferase reporter plasmid was constructed successfullly.

Effect of *L. casei* 1.2435-fermented milk and lysis product of the bacteria on the transcriptional activity of ACE promoter

To confirm whether *L. casei* 1.2435-fermented milk and the bacteria itself could also change the transcription of ACE gene despite its inhibitory function on the enzymic activity, the effect of the supernatant of fermented milk and the lysis product of *L. casei* 1.2435 on the activity of ACE promoter was detected by luciferase reporter assay in COS-7 cells. As shown in Fig. 3, both the supernatant of *L. casei* 1.2435-fermented milk and the lysis product of the bacteria could enhance the transcriptional activity of ACE promoter after treated for 8-16 h. Especially, the wells treating with 0.012 and 0.036 g/mL supernatant had a significant difference (*P*-0.01) to the control. Furthermore, it seemed that there existed a dose-effect relationship when the supernatant of fermented milk was administrated as 0.004, 0.012 and 0.036 g/mL for 12h (Fig. 3A). These results laid the foundation for the further research of the inhibition mechanism of ACE by *L. casei* 1.2435-fermented milk.

CONCLUSION

The present study demonstrates that the *L. casei* 1.2435-fermented milk has a potent inhibition to ACE which the inhibitory rate reached 97.6%. It seemed that the main active components might include, but were not limited to be proteins. In addition, both the supernatant of *L. casei* 1.2435-fermented milk and the lysis product of the bacteria could promote the transcriptional activity of ACE promoter after treated for 8-16 h. Taken together, *L. casei* 1.2435-fermented milk and the probiotics itself could be applied as a candidate for the development of novel functional foods and drugs that provide benefits to patients with hypertension.

5 Acknowledgments

This study was financially supported by the 863 (Hi-tech research and development program of China) program under contract No. 2008AA10Z336 and No.SS2012AA023408, and the foundation (No. 200911) of Tianjin Key Laboratory of Marine Resources and Chemistry (Tianjin University of Science & Technology), P. R. China.

REFERENCES

1. Mark, K.S., Davis, T.P.. Stroke, development, prevention and treatment with peptidase inhibitors. *Peptides.*, 2000; **21**(12): 1965-73.
2. Dunbabin D.. Cost-effective intervention in stroke. *Pharmacoeconomics*, 1992; **2**(12): 468-99.
3. Cooper R, McFarlane-Anderson N, Bennett FI, Wilks R, Puras A, et al. ACE, angiotensinogen and obesity: a potential pathway leading to hypertension. *Journal of Human Hypertension*, 1997; **11**(2):107-11.
4. Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N., Suzuki, H.. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agric. Biol. Chem*,1987; **51**:1581-86.
5. Fitzgerald, R.J., Murray, B.A.. Bioactive peptides and lactic fermentations. *Int. J. Dairy Technol*, 2006; **59**: 118-25.
6. Vermeirssen, V., Camp, J.V., Verstraete, W.. Bioavailability of angiotensin-I-converting enzyme inhibitory peptides. *Br. J. Nutr.*, 2004; **92**(4): 357-66.
7. Minervini, F., Algaron, F., Rizzello, C.G, Fox, P.F., Monnet, V., et al. Angiotensin-I-converting enzyme inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-

hydrolyzed caseins of milk from six species. *Appl. Environ. Microbiol.*, 2003; **9**(9): 5297-305.
8. Maruyama, S., Su-zuki, H.. A peptide inhibitor of angiotensin I-converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem*, 1982; **46**: 1393–4.
9. Nakamura, Y., Yamamoto, N., Sakai, K., Takano, T.. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors of angiotensin-I-converting enzyme. *J. Dairy Sci.*, 1995; **78**(7): 1253-7.
10. FitzGerald, R.J., Meisel, H.. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *British Journal of Nutrition*, 2000; **84**(Suppl. 1): 33-7.
11. Kohmura, M., Nio, N., Kubo, K., Minoshima, Y., Munekata, E., et al. Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agric. Biol. Chem.*, 1989; **53**(2): 2107-14.
12. Maeno, M., Yamamoto, N., Takano, T.. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.*, 1996; **79**(10): 1316-21.
13. Yamamoto, N., Maeno, M., Takano, T.. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J Dairy Sci.*, 1999; **82**(9): 1388-93.
14. Muguerza, B., Ramos, M., Sanchez, E., Manso, M.A., Miguel, M., et al. Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. *Int. Dairy J.*, 2006; **16**(1): 61-9.
15. Fitzgerald, R.J., Murray, B.A.. Bioactive peptides and lactic fermentations. *Int. J. Dairy Technol*, 2006; **59**: 118-25.
16. Meisel, H., Goepfert, A., Gu¨nther, S.. ACE-inhibitory activities in milk products. *Milchwissenschaft*, 1997; **52**: 307-11.
17. Oshima, G., Shimabukuro, H., Nagasawa, K.. Peptide inhibitors of angiotensin-I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochim. Biophys. Acta*, 1979; **556**:128-37.
18. Hong, L., Lan-ying, C.. Purification and characterization of angiotensin converting enzyme. *Chinese Journal of Biochemistry and Molecular Biology*, 2000; **16**(16): 788-92.
19. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin converting enzyme of rabbit lung. *Biochem Pharmacol*, 1971; **20**: 1637-48.
20. Vermeirssen, V., Camp, J.V., Verstraete, W.. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioact ive peptides. *J. Biochem. Biophys. Methods*, 2002; **51**: 75-87.