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# Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340

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The aim of this study was to evaluate the antioxidant, antihypertensive and immunomodulatory characteristics of skim milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340. Supernatants obtained from the ferments after centrifugation were subjected to ultrafiltration and yielded four peptidic fractions of 10–5 kDa, 5–3 kDa, 3–1 kDa, and <1·0 kDa. Peptides in 5–3 kDa range exhibited a good antioxidant activity. The peptides (<1·0 k) was applied to Superdex-30 G column fractionation and produced six fractions (F1–6). Fraction F2 presented the highest angiotensin I-converting enzyme inhibition activity with IC50 of 67·71 ± 7·62 mg/ml. Moreover, fraction F6, which displayed a good immunomodulatory activity, had a positive effect on murine spleen lymphocyte proliferation with Stimulation Index of 0·729 ± 0·123. The present data showed the potential of the milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340 as a functional food, however, further research is needed to evaluate the biofunctional activity of this fermentation product *in vivo* using model animal.

**Keywords:** ACE inhibitory, antioxidant, immunomodulatory, fermented skim milk, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340.

Milk protein are precursors of many different biologically peptides such as antihypertensive, immunomodulatory and antioxidant peptides (Meisel, 2004). These peptides, which are inactive within the sequence of the milk protein, can be released and activated through different methods. One of them exploits the proteolytic potential of microorganisms. Previous studies have suggested that skim milk fermented by *Lactobacillus helveticus* showed beneficial effects in spontaneously hypertensive rats (SHR). The blood pressure lowering effect, allowed by the tripeptides Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP), was based on angiotensin-converting enzyme (ACE) inhibition (Nakamura et al. 1995a, 1996). In a human feeding trial, sour milk containing VPP and IPP was effective in lowering the blood pressure of hypertensive subjects, attributing to its resistance to degradation by gastrointestinal enzymes *in vivo* (Hirata et al. 2002). And several other ACE inhibitory peptides have been isolated from the ferments of milk with lactic acid bacteria (LAB). Ashar & Chand (2004) identified an ACE-inhibitory peptide β-casein f(72-81), Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile (SLVYPFPGPI), from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus*. It was markedly stable to digestive enzymes, acidic and alkaline pH, as well as during storage at 5 and 10 °C for 4 d. Quiros et al. (2007) isolated 4 *Enterococcus faecalis* strains from raw milk, which have stood out as producers of fermented milk with potent antihypertensive activity. They identified that the peptide β-casein f(133-138), Lys-His-Lys-Pro-Lys-Pro (LHLPLP), was one of the major peptides responsible for the activity of these fermented milk product, which showed distinct antihypertensive activity when administered orally to SHR rats.

Some immunomodulatory peptides, which could stimulate the proliferation of human lymphocytes, the phagocytic activities of macrophages, antibody synthesis and cytokine expression, were also isolated from the ferments of milk with LABs. Laffineur et al. (1996) reported that the sour milk fermented with *Lb. helveticus* exhibited immunomodulating effects on lymphocyte proliferation *in vitro* and the ability to stimulate the phagocytic activity of pulmonary macrophages, allowed by high proteolytic activity of the strain to

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cause the release of oligopeptides from digestion of milk proteins. LeBlanc et al. (2002) reported that the IgA-producing cell count increased significantly in mice administered with three peptidic fractions issued from milk fermented with *Lb. helveticus* R389, but the fibrosarcoma size decreased. It implied that the three peptidic fractions possessed good immunostimulatory and antitumor properties.

Many studies provide strong evidence that free radicals play an important role in aging and a variety of diseases (Beckman & Ames, 1998). The mechanism of antioxidant capacity might be involved in scavenging free radicals or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Halliwell & Gutteridge, 1990). So, antioxidative peptides might be applied as food ingredients to promote human health. Moreover, some antioxidant peptides were isolated from milk protein by fermentation with LABs. Kudoh et al. (2001) isolated an antioxidantive peptide κ-casein f(96-106), Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met (ARHPHPHLSFM), from milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* IFO13953. Virtanen et al. (2006) demonstrated that fermentation of milk with *Lb. jensenii* (ATCC 25258) or *Lb. acidophilus* (ATCC 4356) generated antioxidant activity in the whey fraction, allowed by peptides derived from the proteolysis of whey.

For above reasons, the potential proteolytic effect of microorganisms and the biological activities of peptides commend fermented milk proteins as good candidates for functional food production, targeted at heart and digestive system health as well as improving immune defense. There already are a few products supplemented with peptides with specific bioactivities on international markets. However, industrial scale isolation and purification of such peptides was too difficult to satisfy the requirement of markets in that the components of fermented products were very complicated.

The aim of this study is to reveal the antioxidant, antihypertensive, and immnomodulatory activities of the peptidic fractions derived from the skim milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* LB340 *in vitro*. That will be helpful to evaluate the potential of milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340 as healthy food.

## Material and Methods

### Material and Chemicals

Skim milk powder (⩾ 32·4% protein, less than ⩽ 0·8% fat) was obtained from Fonterra Co-operative Group (Auckland, New Zealand). *Lb. delbrueckii* ssp. bulgaricus LB340 was from Danisco Company (Norrköping, Sweden). Vitamin C, 2,6-Di-tert-butyl-4-methylphenol (BHT) and EDTA were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). α-Tocopherol (vitamin E, $V_E$) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical was purchased from A Johnson Matthey Company (Malvern, USA). RMPI1640 incomplete culture medium was purchased from GIBCO Co. (Carlsbad, USA). 3-(4, 5)-dimethylthiahiazo (-2-y1)-3, 5-diphenytetrazoliumromide (MTT) was obtained from AMRESCO Inc. (Solon, USA). Bacidin, Bovine serum albumin (BSA), Hippuryl-l-histidyl-l-leucine (HHL) and Hippuric acid (HA) were obtained from Sigma Chemical Co. (Louis, USA).

### *Skim milk fermentation by* Lb. delbrueckii *ssp.* bulgaricus *LB340*

The milk mixture containing 12% (w/v) of skim milk powder was hydrated (40–45 °C, 30 min) and pasteurized (95 °C, 5 min). *LB. delbrueckii* ssp. *bulgaricus* LB340 was inoculated in skim milk at a ratio of 0·5:1000 (w/v). Fermentation was performed at 42 °C for 16 h under aerobic conditions without continuous stirring. The fermented milk was then stored at 4 °C for further analysis.

### Sample preparation

After fermentation, the insoluble part was removed by centrifugation at 6000 ***g*** for 15 min. The supernatant was ultrafiltered through a series of ultrafiltration membranes with molecular weight cutoffs of 10, 5, 3, and 1 kDa using a Minitan ultrafiltration system purchased from Millipore Co. (USA). The ultrafiltration runs were executed at room temperature under 1 MPa pressure. The membranes were recovered by rinsing for 10 min and cleaning with 0·1 M-NaOH according to the manufacturer's instructions after each ultrafiltration. The fraction with the molecular weight less than 1·0 kDa was lyophilized and stored at −80 °C for further analysis.

### Separation of peptidic fractions by Size Exclusion Chromatography (SEC)

The samples with the molecular weight less than 1·0 kDa were separated on a Superdex 30 prep grade (2·6 × 65 cm) column by using ÄKTA purifier 10 (GE Laboratories, Inc., USA). Phosphate buffer solution (PBS) (10 mM, pH 7·4) was used to equilibrate the column and to elute the peptides at a flow rate of 1·0 ml $min^{-1}$ and under the pressure of 0·2–0·4 MPa. A constant amount of sample (1·0 ml) with peptides concentration of 4 mg/ml was applied to the column, and each fraction was collected. The absorbance of the effluent was measured at 215 nm. Bacidin (1·4 kDa) was used as molecular standard. Each fraction from several runs was pooled and freeze dried, and protein content was measured using the modified Folin-phenol method using peptone as quantitative standard (Xing et al. 2009).

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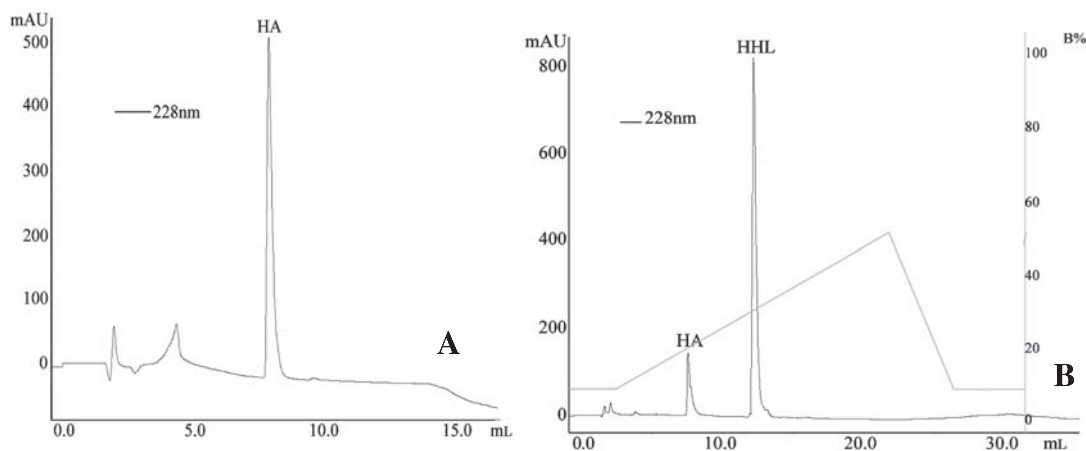


**Fig. 1.** Reverse phase-HPLC chromatogram of ACE inhibitory activity.

*Purification of ACE inhibitors peptides from skim milk hydrolysate*

Separation of peptides was done using RP-HPLC. Forty milligram peptide fraction collected from SEC was loaded on a semi-preparative $C_{18}$ column (80 Å, 5 µm, 4·6 mm × 250 mm, Agilent). Elution was performed at a flow rate of 1 ml $min^{-1}$ with a binary gradient (Solvent A was a mixture of 20% acetonitrile (in water, v/v) containing 0·1% trifluoroacetic acid (TFA) (v/v), and solvent B was 70% acetonitrile (in water, v/v).). The gradient used was a linear gradient of solvent B in A going from 0 to 100% over 60 min at a flow rate of 1 ml $min^{-1}$. The elution profile was monitored at 215 nm and 280 nm and each sub-fraction from several runs was collected, freeze-drying and stored at − 80 °C for further analysis. The data-processing software was UNICORN version 5.11 (GE Laboratories, Inc, USA).

*DPPH radical scavenging activity*

Radical scavenging activity was determined using a 1.1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical scavenging assay with some modifications (Larrauri et al. 1998). One millilitre samples (3·5 mg/ml) from each SEC peptidic fractions was mixed with 4 ml of 75 µM-methanolic DPPH free radical. Mixtures were vortexed for 30 sec to homogenize, and left to react sufficiently for 60 min in the dark. Finally, absorbance was measured at 516 nm using 75 µM-methanolic DPPH free radical solutions as blank. The scavenging activity of the DPPH radical was expressed using the following equation: Scavenging activity (%) = 100 × (A0 − A1)/A0, where A0 is the absorbance of the methanolic DPPH free radical blank, and A1 is the absorbance of the final reaction mixture with peptides. Results were compared with the activity of BHT and vitamin E as positive control and deionized water as negative control.

*Preparation of ACE and determination of ACE inhibitory activity*

ACE extracts were prepared and the activity was determined according to the method of Nakamura et al. (1995b) and Wu & Ding (2002) with several modifications. Briefly, the fresh rabbit lung without connective tissues was cut into pieces and washed with precooled NaCl (0·7%) to get rid of blood. The treated lung was homogenized in precooled 100 mM -sodium borate buffer (BBS) (pH 8·3) at the ratio of 1:5 (w/v). Then, the mixtures were filtrated with 3-ply gauze followed by be ultracentrifuged for 40 min (40 000 ***g***, at 4 °C). The obtained clear light red-coloured supernatant containing the ACE activity was retained and stored at 5 °C (Cushman & Cheung, 1971). The ACE activity in these crude extracts was about 100 mU/ml.

Evaluation of ACE inhibitory activity was assayed using the RP- HPLC method described by Wu & Ding (2002). It is based on the hydrolysis of hippuryl-L-histidyl-L-leucine (HHL) by ACE to hippuric acid (HA) and histidyl-leucine (HL) as products (Fig. 1). The HA release from HHL is directly related to the ACE activity. Briefly, 30 µl ACE crude extracts were mixed with 75 µl of 100 mg/ml each peptide fraction. The mixture was incubated at 37 °C for 10 min. Then, 200 µl 5 mM-HHL solution substrate were added and the reaction mixture was incubated at 37 °C for 40 min. The reaction was stopped by the addition of 250 µl 1 M-HCl and 10 µl of solution were analyzed by reversed-phase HPLC and detected at the wavelength of $\lambda = 228$ nm. The column was eluted with 20% acetonitrile (in water, v/v) containing 0·1% (v/v) TFA on AKATA purifier 10 with flow rate of 1·0 ml $min^{-1}$. Controls with equal volume of PBS as sample substitute were included for each measurement, and the blank experiment was executed with premixing 250 µl 1 M-HCl and 30 µl ACE crude extracts to inhibit the activity of ACE, firstly (steps listed in Table 1).

The evaluation of ACE inhibition was based on the measurement of the HA peak areas and the degree of ACE

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**Table 1.** Procedures of ACE inhibition experiments

| | Control (μl) | Sample (μl) | Blank (μl) |
|---|---|---|---|
| ACE | 30 | 30 | 30 |
| Sample | – | 100 | – |
| BBS | 100 | – | 100 |
| HCl | – | – | 250 |
| Incubation at 37 °C for 10 min | | | |
| HHL | 200 | 200 | 200 |
| Incubation at 37 °C for 20 min | | | |
| HCl | 250 | 250 | – |

inhibition (in %) was calculated according to the equation:

$$\text{ACE Inhibition } (\%) = 100 \times (A_{control} - A_{sample}) / (A_{control} - A_{blank}).$$

Where $A_{control}$ is the HA peak area of control (buffer added instead of test sample), $A_{blank}$ is the HA peak area of the reaction blank (HCl was added before HHL), and $A_{sample}$ is the HA peak area in the presence of sample. The $IC_{50}$ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assayed conditions.

### *Stimulation effect of murine spleen lymphocyte proliferation*

The peptide samples separated by Superdex 30 prep grade (2·6 × 65 cm) column were filtrated with 0·22 μm membrane (Millipore). The bioactivity of the peptides to stimulate murine spleen lymphocyte proliferation was tested by MTT assay *in vitro* (Mercier et al. 2004). Spleens excised from 6 to 8-week-old BALB/c mice were filtered through a 70 μm cell-strainer, and the splenic lymphocytes were isolated from splenocytes using RPMI-1640 medium. Then the cells were suspended at a final density of $2 \cdot 5 \times 10^6$ cells per ml for usage. One hundred microliter of $2 \cdot 5 \times 10^6$ cells per ml spleen cells were seeded into a 96-well plate. One hundred microliter of 12 ug/ml peptide samples were added to the cell suspension and the plate was incubated at 37 °C with 5% $CO_2$. After incubation for 24 h, 20 μl 5 mg MTT/ml was added to each well and the plate was further incubated for 4 h to allow MTT metabolism to formazan by the succinate-tetrazolium reductase only active in viable cells. Then, 100 μl dimethyl sulfoxide (DMSO) was added to stop the succinate-tetrazolium reductase activity, kill the cell and solubilize formazan crystals. The plate was swirled gently and then placed in the dark for 15 min at room temperature. The absorbance was measured at 570 nm wavelength using a micro-titer plate reader (Bio-TEK). BSA and PBS were used as control and blank sample, respectively. The Stimulate Index (SI) of proliferation was calculated by the formula as follows:

$$SI = (OD_{570\ trial} - OD_{570\ control}) / (OD_{570\ control} - OD_{570\ blank}).$$

### *Statistical analysis*

Three replicate trials for each experiment were performed. Analysis of variance was performed and means separated using software SPSS version 14.0. Analysis of variance (AOV) was done to determine the significance of the main effects. Significant differences ($P < 0 \cdot 05$) between means were identified using least significant difference (LSD) procedures.

## Results and Discussion

### *Milk fermentation and concentration of peptides*

The fermentation products smelled of yoghurt and tasted sour with pH 4·61. After centrifugation, the supernatants were separated by ultrafiltration membrane with molecular weight cutoffs of 10, 5, 3, and 1 kDa in succession. The result showed that the concentration of the peptide fraction was 6·22 mg/ml (10–5 kDa fraction), 4·06 mg/ml (5–3 kDa fraction), 3·89 mg/ml (3–1 kDa fraction), and 3·66 mg/ml (less than 1·0 kDa fraction).

### *Antioxidant effect*

The process of antioxidation was always coupled with the hydrogen donation to free radicals or the radical scavenging (Siriwardhana et al. 2003). The relatively stable DPPH free radical has been widely applied as a substrate to evaluate the antioxidant activity (AOA) of compounds acting as free radical scavengers or hydrogen donors (Blois, 1958). The results shown in Fig. 2 revealed that the peptide fractions with the molecular weight less than 10 kDa exhibited a good AOA, and the AOA was correlated with the molecular weight ranged from 0–5 kDa. But, AOA of the peptide fraction ranged from 10–5 kDa declined a little to 54·12 ± 0·42%. The DPPH free radical scavenging effect of the peptide fraction ranged from 5–3 kDa was about 65·98 ± 2·21%, which was not significantly different from that of the peptide fraction ranged from 3–1 kDa (63·83 ± 0·68%) ($P > 0 \cdot 05$). Most importantly, the DPPH free radical scavenging effect of the peptide fraction less than 1·0 kDa reached 54·72 ± 0·28% although it was significantly different from that of other peptide fraction ($P < 0 \cdot 05$). Due to the mucus layer in the small intestine, which was equivalent to a thickness of 100–150 μm molecular filters with exclusion molecular weight of 0·6–0·8 kDa, and the peptidase in the intestinal epithelial cells, the AOA of the peptide fraction less than 1·0 kDa might represent that of fermentation better *in vivo* (Cao & Zhang, 2006). Peng et al. (2009) reported that the hydrolysis of whey protein isolation (WPI) obtained by alcalase digestion were separated into four fractions with molecular weights >40, 2·8–40, 0·1–2·8, and <0·1 kDa. The free radical scavenging capacity of these small peptide components was related to their molecular weight, of which 0·1–2·8 kDa fraction showed the strongest free radical scavenging

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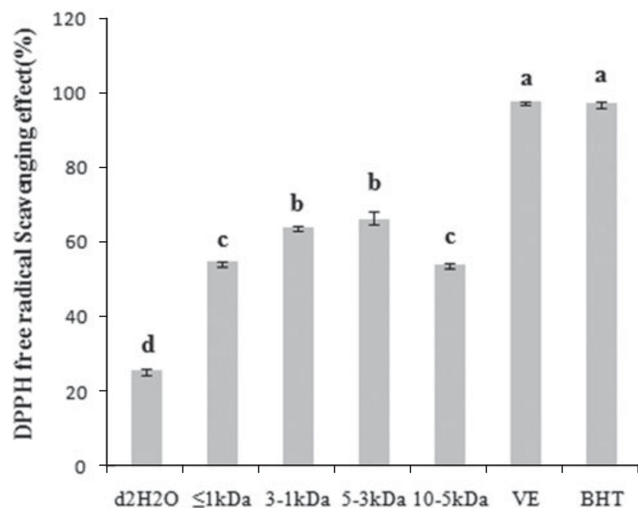


**Fig. 2.** Relative radical scavenging effect (RSE) of the peptide fractions separated by molecular membrane ultrafiltration.

capacity. This is more in line with our results in this experiment. Blanca et al. (2005) revealed that the small peptide components with the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium radical cation (ABTS*) scavenging capacity were also isolated from the commercial fermented milk consumed widely in Spain, and its scavenging ability was related to the molecular weight. Kudoh et al. (2001) also isolated a small peptide, 96–106 amino acid of κ-casein, with anti-oxidative activity from fermented milk using *Lb. delbrueckii* ssp. *bulgaricus* IFO13953. The role of free radicals and active oxygen species in various diseases, including aging, cancer, inflammation and the toxicity of numerous compounds, has been well documented (Ames et al. 1993). So, anti-oxidative peptides obtained in this study might be applied as food ingredients to promote human's health.

Moreover, Jain et al. (2009) reported that three LAB strains, *Lb. casei, Lb. acidophilus* and *Lactococcus lactis*, showed potent dose dependent free radical scavenging activity in terms of DPPH scavenging potential, allowed by the intact LABs and their metabolites produced in fermentation. We could not exclude that the peptidic fractions with molecular weight less than 1·0 kDa contained those low molecular weight metabolites, which might contribute to the anti-oxidant activity of this fraction.

### *The effect of antihypertensive of peptides*

The peptide fractions with the molecular weight less than 1·0 kDa collected from ultrafiltration was applied to SEC. The results in Fig. 3(A) show that these peptide components were successfully separated into 6 obvious peaks according to their molecular weight, named as F1–F6, which were collected and lyophilized into powder. The samples (100 mg/ml) reconstituted from the freeze dried powder were applied to ACE inhibitory activity test. The results shown in Fig. 4(A) revealed that the peptidic fraction F2 showed highest ACE inhibitory activity (66·42 ± 1·82%) followed by F1 (50·62 ± 1·37%), F3 (20·00 ± 1·60%), and F4 (6·15 ± 0·36%) compared with the control sample with no ACE inhibitory activity. However, the peptidic fractions F5 and F6 showed the negative ACE inhibitory activity. The content of peptidic fractions from F5 and F6 peak was relatively low in this study, so the samples derived from their vacuum freeze-dried power contained more salt, especially the $Na^+$ salt, which may result in promoting ACE activity (Zhao et al. 2001). We further investigated and found that the $IC_{50}$ of ACE inhibition of F2 peptide fraction was about 67·71 ± 7·62 mg/ml (Fig. 4(B)), which was higher than that of the reported purified peptide which ranged from 100 to 500 μM. (Hayes et al. 2007). To further investigate the peptide components with ACE-inhibition activity, the sample from F2 fraction was applied to semi-preparative RP-HPLC and produced 4 obvious peaks, named as F2-I – F2-IV (Fig. 3(B)). The results of ACE inhibitory activity analysis showed that the magnitude of ACE inhibitory activity followed the order: F2-II > F2-I > F2-III > F2-IV, which represented 24·20 ± 0·20%, 22·14 ± 0·06%, 21·53 ± 0·17%, and 19·33 ± 0·80%, respectively (Fig. 4(C)). The results implied that the ACE inhibitory activities of F2 fractions were composed of those of the samples further isolated by RP-HPLC. From the relationship between their concentration and ACE activity inhibition efficiency, we could conclude that these small peptides collected from RP-HPLC did not show functional accumulation or synergism, which might be attributed to the interaction between these small peptides, or purity of the sample collected from SEC not better than that from RP-HPLC. Many antihypertensive milk-borne peptides have been isolated and identified (Hannu & Anne, 2006). Among them, VPP and IPP derived from β-casein and κ-casein have been well illustrated and commercialized (Hannu et al. 2006), such as "Calpis" sour milk (Calpis Co., Japan) and "Evolus" sour milk (Valio Ltd., Finland). A quantitative structure–activity study showed a positive correlation between the hydrophobicity of the ultimate C-terminal amino acid and the ACE-inhibitory activity of small peptides (Pripp, 2004). Therefore, the release of many peptides with aromatic or other hydrophobic amino acids, or Pro, at the C-terminal was expected to result in peptidic fractions with high ACE inhibition. In the RP-HPLC, the components of peptidic fractions F2 showed some degree of hydrophobic character (Fig. 3(B)), which might allow the components to be resistant to the digestive enzyme. The exclusion molecular weight of the mucus layer in the small intestine is about 0·6–0·8 kDa, and there are some peptidases in the intestinal epithelial cells (Cao & Zhang, 2006). Therefore, it could be concluded that the ACE inhibitory activity of the peptidic fractions less than 1·0 kDa shown in this study could represent the activity of the fermented milk *in vivo* in the main. It needs to be further confirmed.

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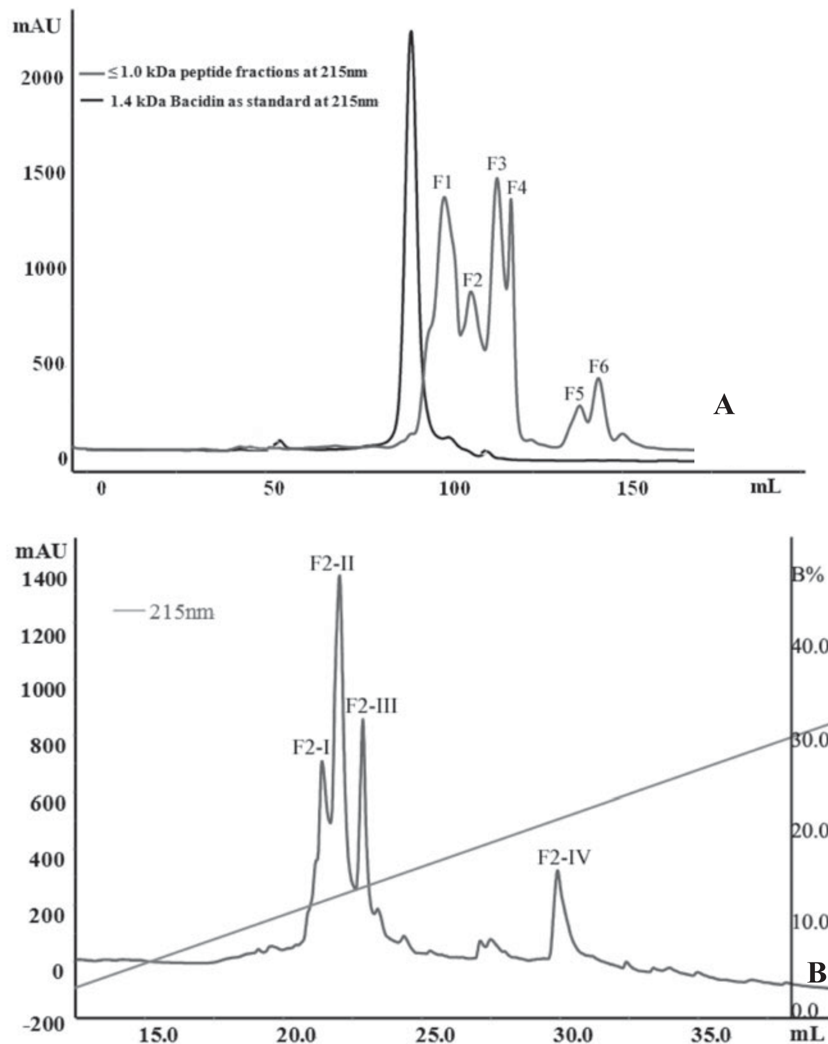


**Fig. 3.** (A) UV chromatogram of Size exclusion chromatography of ultrafiltration less than 1·0 k from fermented milk supernatants. Collected fractions were F1–F6. (B) UV chromatogram of semipreparative RP-HPLC of water-soluble extract from F2 peak fraction collected from (A). Collected fractions were F2-I–F2-II.

### *Stimulation effect of peptides on murine spleen lymphocyte proliferation*

After ultrafiltration, the samples with molecular weight less than 1 kDa were applied to SEC and produced 6 peaks F1–F6 (shown in Fig. 3(A)). The fractions were collected and concentration standardized to 12 µg/ml to test the effect on murine spleen lymphocyte proliferation. The results showed in Fig. 5 demonstrated that four fractions have a positive impact on murine spleen lymphocyte proliferation, namely F1, F3, F4 and F6, with Stimulation Indices (SI) of $0{\cdot}014 \pm 0{\cdot}003$, $0{\cdot}096 \pm 0{\cdot}048$, $0{\cdot}646 \pm 0{\cdot}209$, and $0{\cdot}729 \pm 0{\cdot}123$, respectively. However, F5 fraction presented a negative regulation on mouse spleen lymphocyte proliferation, with SI $-0{\cdot}499 \pm 0{\cdot}137$. Many studies showed that immunomodulation activity of milk-borne peptides usually manifested in two aspects: one was to stimulate the vitality of lymphocytes and promote proliferation, in which peptides were mainly derived from β- and κ-casein; the other is to inhibit lymphocyte activity and reduce proliferation, in which most peptides derived from α-casein acted as this role (Sütas et al. 1996). However, both these two types of immune regulation had certain biological significance. A positive regulator of the immune system could promote immune cells to secrete a number of immune factors, such as IgA, IL-6 and so on, to enhance human's immunity. When a negative regulator inhibits lymphocyte proliferation, it would up-regulate the expression of IL-10 and down-regulate the expression of IL-2, which would reduce human's hypersensitivity response (Politis & Chronopoulou, 2008). This might be another key reason that yogurt is suitable for allergic people. Tellez et al. (2010) confirmed the immunomodulating effect of compounds derived from milk fermented by *Lb. helveticus* (LH-2) *in vitro*.

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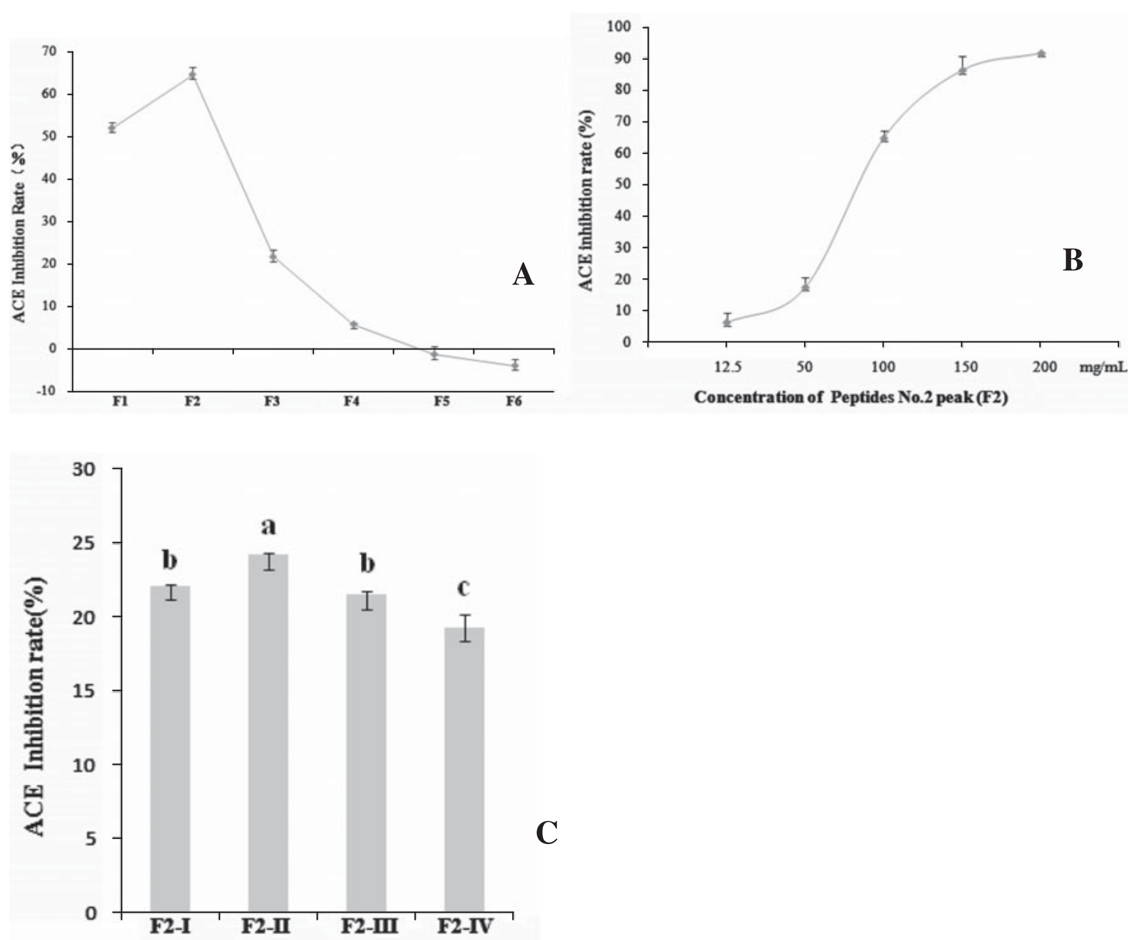


**Fig. 4.** (A) The ACE inhibition rate of each peptide fraction F1–F6 collected from SEC. (B) ACE IC50 calculation curve of F2 peptide fractions. (C) ACE inhibition rate of each RP-HPLC fraction at concentration of 2 mg/mL.

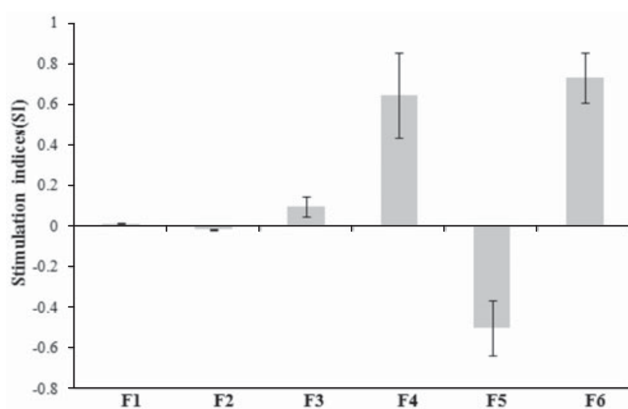


**Fig. 5.** Effect of the peptide components collected by SEC on proliferation of Murine spleen lymphocytes.

The cell-free supernatant obtained from the fermented milk and its fractions could stimulate macrophages to produce more cytokines, such as IL-6, TNF-α and IL1-b, and nitric oxide (NO) than the control.

The potential of peptidic fractions in fermented milk to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused increasing scientific and commercial interest. The size of bioactive peptides may vary from 2 to 20 amino acid residues. Undoubtedly, it was well known that the dipeptides and tripeptides could be absorbed intact in man by mechanisms independent of the specific amino acid entry mechanisms. And there was dispute to whether tetrapeptides, pentapeptides, or hexapeptides could be taken up intact (Jauregi, 2009). However, the concept that the bioactivity of peptide with less molecular weight *in vitro* would in some degree represent that *in vivo* was being accepted (Cao & Zhang, 2006). The peptidic fractions, exhibiting antioxidant, immunomodulatory and ACE inhibitory activity in this study, was low molecular weight less than 1·0 kDa, suggesting that milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* LB340 has potential as a functional food. Further research is needed to evaluate the biofunctional activity of this fermentation product *in vivo*.

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