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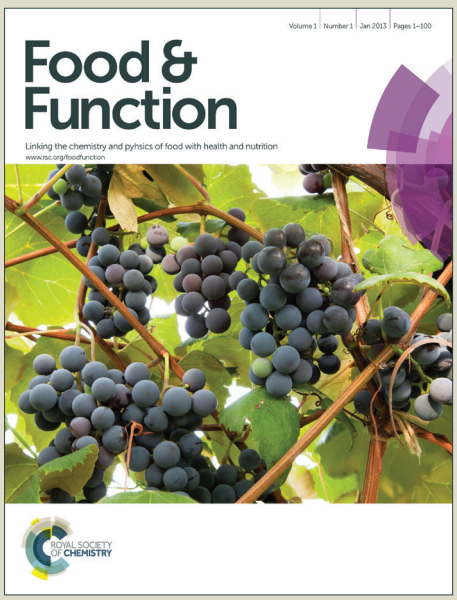

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Enrichment of ACE inhibitory peptides in navy bean (*Phaseolus vulgaris*) using lactic acid bacteria

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

The present study was conducted to explore a novel strategy to enhance ACE inhibitory activities of navy bean by preparation of navy bean milk (NBM) and then subjected to fermentation of respectively four lactic acid bacteria strains, namely, *Lactobacillus bulgaricus*, *Lactobacillus helveticus* MB2-1, *Lactobacillus plantarum* B1-6, and *Lactobacillus plantarum* 70810. With the exception of *L. helveticus* MB2-1, the other three selected strains had well growth performances in NBM with the viable counts increased to log 8.30-8.39 cfu/ml during 6 h of fermentation and, thus were selected for the following investigations. Protein contents of NBM significantly reduced for those treated with *L. bulgaricus* and *L. plantarum* B1-6, and the electrophoresis patterns showed the preferable proteins for LAB strains to hydrolyze were α and β type phaseolins, whereas γ-type phaseolin was resistant to hydrolysis. RP-HPLC analysis demonstrated all fermented NBM had higher intensities of peaks with retention time between 2.5-3.5 min indicative of formation of small peptides. All fermented NBM showed higher ACE inhibitory activity compared to the unfermented ones, in which 2h, 3h, and 5h were found to be the optimum fermentation periods for respectively *L. plantarum* 70810, *L. plantarum* B1-6 and *L. bulgaricus*, with IC_{50} values of 109±5.1, 108±1.1, 101±2.2 μg protein/ml. The subsequent *in vitro* gastrointestinal simulation afforded all fermented extracts reduced IC_{50} values and the extracts fermented by *L. plantarum* B1-6 exerted the lowest IC_{50} value of 21±2.1 μg protein/ml. The research has broadened our knowledge bases on the effect of lactic acid bacteria fermentation on the degradation of navy bean proteins and the capacity of releasing ACE inhibitory peptides. The approach was promising to obtain probiotic product with potential to be served as functional ingredients targeting hypertension.

Introduction

The increasing incidences of diet-related diseases nowadays have promoted many investigations to explore foodstuffs with nutraceutical functional properties. Hypertension, one of the world's most common high-risk diseases, affects about 22% of the world's population and is regarded as the main risk factor for cardiovascular diseases (CVD) such as stroke, myocardial infarction, heart failure and etc.[1] Elevation of blood pressure in human body has been considered closely related to the rennin angiotensin system (RAS)，where the angiotensin I-converting enzyme (ACE, EC 3.4.15.1) take a critical role responsible for converting the decapeptide angiotensin I to the octapeptide angiotensin II, which is a potent vasoconstrictor.[2] It also degrades bradykinin, a vasodilator, into inactive peptides.[3,4] This dual role enables the enzyme to elevate blood pressure.

There are available synthetic ACE inhibitors, the uses of which have achieved great success. For instance, captopril was the first ACE inhibitor successfully used in clinical practice.[5]However, captopril and the other known synthetics, have significant adverse effects such as cough, exanthema, taste alterations, skin rashes, gastric troubles and edema of lips.[6] Because of the adverse side effects of the synthetic compounds, there is intense interest in the exploration of ACE inhibitors from natural food sources to serve as substitutes or replacements which can curtail the reliance on synthetic compounds.[7] Fermentation, an ancient food manufacturing technique, has been introduced recently as an effective way to enhance nutraceutical properties of foodstuffs. Lactic acid bacteria (LAB) are probiotic strains and have been known to possess health claims when their populations are higher than 10^6 cfu/ml.[8] It has been well established that many LAB had developed proteolytic systems that capable of hydrolyzing exogenous proteins to liberate peptides and amino acids.[9,10] During this process, bioactive peptides might be released.[9] This phenomenon has been extensively studied in dairy products, such as milk fermented with *Lactobacillus helveticus*,[11,12] *Lactococcus lactis*,[13] *Lactococcus casei*, *Lactococcus acidophilus*, and *Lactococcus jensenii*.[14] However, few studies were carried out to explore the effect of LAB stains to release ACE inhibitory peptides from plant origin proteins. Tsai, *et al.* [15] have observed an increase of antihypertensive activities for soymilk after fermented with prozyme 6 and five LAB strains. Another study has shown fermentation of *Lactobacillus plantarum* CECT 748T has attributed lentil water-soluble extract potent ACE inhibitory activity.[16] It has also been reported pea proteins showed no ACE

inhibitory activities after treated with *Lactobacillus plantarum* 299v, however, the fermentation process facilitated the release of ACE inhibitory peptides during the following *in vitro* gastrointestinal digestion process.[1]

Navy bean, also named white bean or haricot bean, is a dry bean (*Phaseolus vulgaris*) variety popular in the United States and United Kingdom and ranked as the top dry bean variety produced in Canada.[17] Navy bean proteins demonstrated relatively stronger ACE inhibitory properties during enzymatic hydrolysis compared to other *Phaseolus vulgaris* beans.[18,19] Therefore, the current study was undertaken to investigate the effect of LAB fermentation on the degradation of navy bean proteins and the capacity of releasing of ACE inhibitory peptides. Four LAB strains, namely, *Lactobacillus bulgaricus*, *Lactobacillus helveticus* MB2-1, *Lactobacillus plantarum* B1-6, and *Lactobacillus plantarum* 70810 were selected for evaluations. The specific objective of the current study is to establish an effective fermentation process for generation of the ACE inhibitory peptides from navy bean milk (NBM).

Materials and methods

Materials

Whole navy bean seeds originated from Canada were purchased and were stored at 4 °C until use. Pepsin (P 6887), trypsin (T 0303), α-chymotrypsin (C 4129), ACE regent (A 6778), Hippuryl-His-Leu (HHL) (H 1635) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Molecular mass standard was from Bio-Rad Laboratories (Hercules, CA, USA). All other regents used were of analytical grade.

L. plantarum 70810 was isolated from Chinese paocai fermented with cabbages,[20] *L. plantarum* B1-6 was isolated from Xingjiang Kirgiz boza, *L. helveticus* MB2-1 was isolated from traditional Sayram ropy fermented milk from Sayramtown in Xinjiang vygur autonomous region of China,[21] and *L. bulgaricus* was obtained from the China center of industrial culture collection (CICC), Beijing, China.

Inoculum preparation

The strains of *L. plantarum* B1-6 and *L. bulgaricus* were prepared for two successive transfers in de Man-Rogosa and Sharp broth (MRS, pH 6.2) at 37 °C for 24 h. The activated culture were again inoculated into MRS broth at 37 °C for 16 h. *L. plantarum* 70810 was prepared using the same condition as mentioned above except the incubation temperature was adjusted to 31 °C.

For inoculum preparation of *L. helveticus* MB2-1, 12% of milk whey reconstitution was used as medium and the incubation temperature was adjusted to 42 °C. All cells were harvested by centrifugation at 8500 rpm, 4 °C for 10 min, and were washed twice with sterilized physiological saline for further inoculum.

Navy bean milk preparation and fermentaion

Navy bean seeds were selected, rinsed and soaked in distilled water at room temperature for about 12 h. The soaked beans were then drained and dispersed in 8-fold of distilled water to make slurry using a homogenizer (BE601AB, Midea, China). The slurry was filtered with a 200-mesh screen cloth to remove the okara, and then maintained at 4 ºC for about 12 h to remove any precipitated starch. The supernatant, navy bean milk (NBM), was obtained and sterilized at boiling temperature for 5 min before inoculated respectively the four strains. Fermentations were carried out at the optimum growth temperatures, namely, 37 °C, 42 °C, 31 °C, and 37 °C for *L. bulgaricus*, *L. helveticus* MB2-1, *L. plantarum* 70810, and *L. plantarum* B1-6, respectively. Duration of the fermentation was 6 h for each strain based on the microbial growth curve and samples were collected at each 1 h interval for further analyses.

Microbiological analysis

The viable cell counts were determined by using the plate counts method with MRS agar (pH 6.2±0.2, Oxoid-CM0361, Unipath, Basingstoke, UK) according to the protocol reported in Li, *et al.*[22] Cell counts were expressed as log cfu/ml. A pH Meter (Schott Lab 850, Mainz, Germany) was used to evaluate the pH values.

Protein analysis

The protein contents were determined by the Bradford assay[23] using bovine serum albumin as a standard.

Electrophoresis

Aliquots taken at different fermentation durations were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out under reducing conditions (presence of 5% β mercaptoethanol [β ME]) by using a 12% polyacrylamide gel. SDS-PAGE was conducted on a Bio-Rad Miniprotein 3 unit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with voltage 60 V for stacking gel, and followed by 120 V for separating gel. Molecular mass standard was from Bio-Rad Laboratories (Hercules, CA, USA). Gels were scanned with Image Scanner III (GE Healthcare Biosciences, Uppsala, Sweden) and then analyzed using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analysis was performed with a ZORBAX Eclipse Plus C18 reversed-phase analytical column (4.60 x 250 mm, 5 µm particle size, Agilent). 20 µl samples were filtrated with 0.45 µm membrane and applied to the column. The samples were then eluted with mobile phases comprised two buffers: solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile [ACN]). The time program started at 5% B and then increased to 35% B in 20 min in a linear gradient and then maintained at 35% B for 5 min. The flow rate was 0.7 ml/min and the elution was monitored at 280 nm.

Angiotensin I-converting enzyme inhibitory properties

ACE inhibitory activity assay was performed using the protocol described previously by Barbana and Boye[24] by monitoring the formation of hippuric acid (HA) from Hippuryl-His-Leu (HHL) as substrate using reverse phase high-performance liquid chromatography (RP-HPLC). Briefly, 50 µl of 2.17 mM HHL, 10 µl of ACE (1.55 mU) and 10 µl of samples were prepared using borate buffer (100 mM, containing 300 mM sodium chloride, pH 8.3) and vortex to initiate the reaction. The reaction was conducted at 37 °C for 30 min with agitated incubation. After terminating the reaction by addition of 85 µl of 1 M HCl, 20 µl of the solution was injected into a 4.60 × 250 mm ZORBAX Eclipse

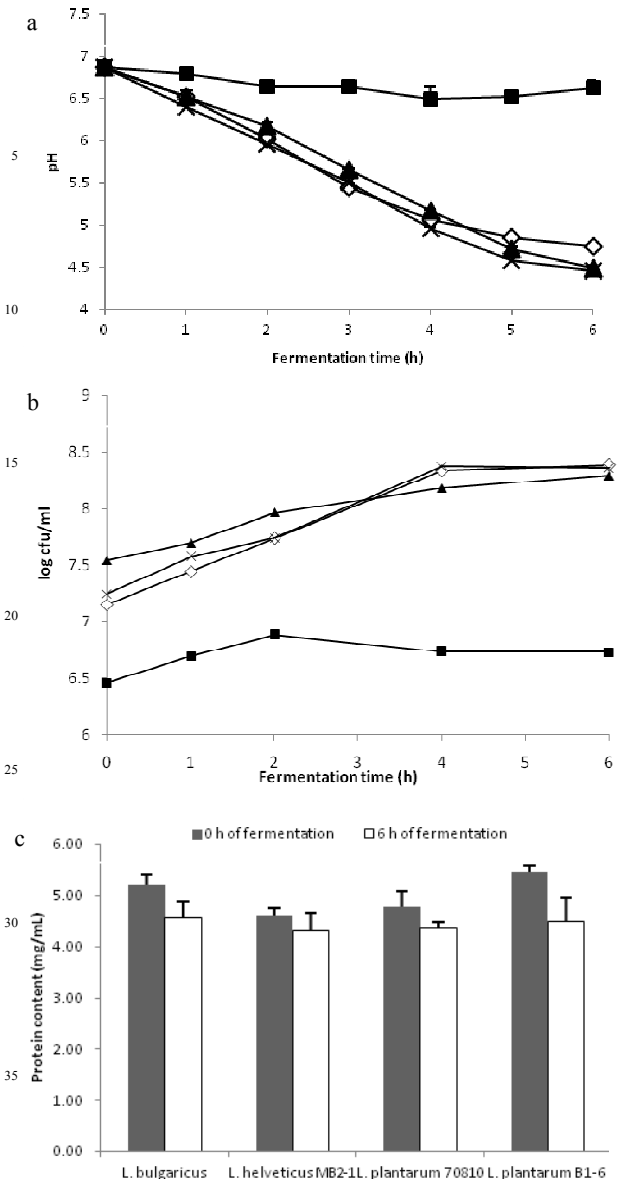

Fig. 1 (a) Decline of pH, (b) viable counts, (c) protein contents changes during growth of selected stains in NBM. *L. bulgaricus*(◇), *L. helveticus* MB2-1(■), *L. plantarum* 70810 (▲), *L. plantarum* B1-6(×).

Plus C18 reversed-phase analytical column (4.60 x 250 mm, 5 µm particle size, Aglient). Samples were eluted using 50% (v/v) methanol in water with 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.7 ml/min for 15 min. The elution was monitored at 228 nm. Absorbance of the HA peak was measured. Blank samples were prepared without addition of enzyme, and control samples were prepared without the addition of bean protein hydrolysates.

ACE inhibitory activity was calculated using the following equation:

ACE inhibitory activity (%) = [(Ac-As)/(Ac-Ab)] × 100

Where Ac was the absorbance of the control in which 10 µl of borate buffer was added instead of the sample, Ab was the absorbance of the blank which was absent of the ACE, and As was the absorbance of the test sample. The IC_{50} values (half maximal inhibitory concentration) were determined by graphed ACE inhibition percentages versus semi-logarithmic values of sample concentrations following an earlier study.[25]

In vitro gastrointestinal digest simulation test

Simulation of human gastrointestinal digestion (GIS) was performed *in vitro* by sequential digestion using pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) according to a previous protocol.[26] Aqueous solutions of the fermented bean extracts were firstly underwent peptic hydrolysis with E/S 1/25 (w/w, based on protein content) at 37 °C for 2 h at pH 2.0. pH was then adjusted to 6.5 with 1 M NaOH to initiate the simulated intestinal digestion followed by adding trypsin and α-chymotrypsin with E/S 1/250 (w/w). Digestions were carried out at 37 °C for 2.5 h and were stopped by heating samples in boiling water for 10 min. Samples were stored at -20 °C and then analyzed for ACE inhibitory properties.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to determine the significant differences between means ($P<0.05$) using IBM SPSS Statistics.

Results and discussion

The growth and acidification performance of the selected strains

Changes of pH during navy bean milk fermentation using the four selected strains are shown in Fig. 1a. Declines were observed for pH values of fermented navy bean milk from 6.87, to 4.75, 4.49, and 4.46 during 6 hours of fermentation for *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6, respectively. The pH drop was more pronounced during the first 4 hours of *L. bulgaricus* fermentation, and the first 5 hours of *L. plantarum* 70810 and *L. plantarum* B1-6 fermentations, which have led to a significantly higher pH ($P<0.01$) for *L. bulgaricus* than that of the other two strains. The pH decline was slowed down thereafter for the three assessed strains until the end of fermentation which mainly due to the accumulation of the acids. *L. helveticus* MB2-1, compared to the previous mentioned three strains, demonstrated a dissimilar pattern that only a slight decline of pH value was observed (from 6.87 to 6.63).

For lactic acid bacteria, the rate of pH decline was indicative of growth performance of the strains. Previous study has also demonstrated that the pH decline was strain dependent.[27] This was mainly attributed to discrepancies in metabolic activity and the growth requirement of different strains.[28] *L. helveticus* MB2-1 was originally separated from Sayram ropy fermented milk which had a major dissimilarity in nutrient compounds composition than that of the navy bean milk (NBM). Lactose depleted, rich in oligosaccharides (especially raffinose and stachyose), different protein composition, and limited sulfuric amino acids might contribute to the growth retard of *L. helveticus* MB2-1 in NBM.

In accordance with the previous findings of the pH declines, the microorganism populations of the assessed four strains were log 8.39, 6.73, 8.30, and 8.36 cfu/ml at the end of fermentation for respectively *L. bulgaricus*, *L. helveticus* MB2-1, *L. plantarum* 70810, and *L. plantarum* B1-6 (Fig. 1b). LAB were considered

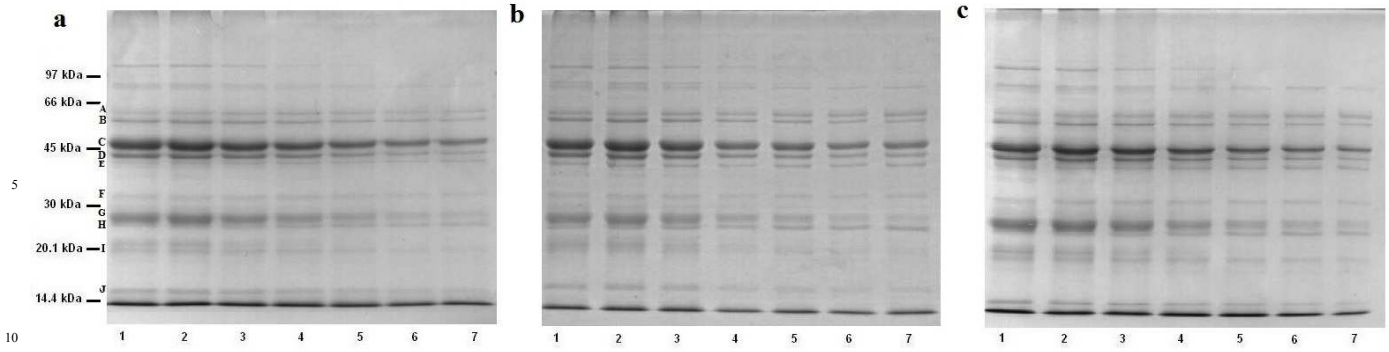

Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of NBM fermented for different periods. Lanes 1-7: 0h, 1h, 2h, 3h, 4h, 5h, 6h of fermentation by the selected strains. (a) *L. bulgaricus*, (b) *L. plantarum* 70810, (c) *L. plantarum* B1-6. A-J represented the major bands of NBM and was subjected to density analysis.

Table 1 Degradation of navy bean protein during the selected LAB fermentation

SDS-PAGE bands	MM (kDa)	Protein degradation rate (% hydrolyzed)		
		L. bulgaricus	*L. plantarum* 70810	*L. plantarum* B1-6
A	65.39	8%	-9%	5%
B	57.71	30%	38%	42%
C	45.75	64%	53%	61%
D	42.80	34%	45%	52%
E	40.74	-22%	-3%	6%
F	33.93	8%	4%	7%
G	29.34	59%	51%	60%
H	26.17	-8%	-16%	10%
I	22.86	33%	49%	44%
J	16.75	0%	12%	35%

A-J represented major bands as shown in the fig. 2 electrophoresis patterns of navy bean extracts.

to provide therapeutic effects at minimum concentrations of 10^6 cfu/ml,[8] therefore, all assessed strains have met the requirement in which the fermented NBM of *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 might exert better health claim than that of *L. helveticus* MB2-1 from this perspective.

Protein contents

Protein contents of NBM collected at 0 h and 6 h of fermentations are shown in Fig. 1c. Decreased protein contents were observed for all investigated strains at the end of fermentation and statistically significant effects ($P<0.05$) were obtained for NBM fermented with *L. bulgaricus* and *L. plantarum* B1-6. This was, however, differed with the previous reports that increase of soluble protein contents were observed during the fermentation process. Slight increase of crude protein contents of 3.46%, 1.46%, 1.25%, and 1.38% were obtained for fermented lentil, chick pea, rice and wheat after 4 days.[29] Another study also presented an increase of the protein contents from 47.4 mg/g to 71.4 mg/g during 30 h of LAB fermentation of soymilk.[15] Increase of the proteins contents were mainly attributed to the accumulation of the microorganisms.[30] However, proteolysis might occur through the activation of cell-envelope proteinase (CEP) of LAB that had led to the degradation of the protein content.[9] It has been reported 1-2% of the proteins underwent hydrolysis during milk fermentation.[31] Aguirre, *et al.*[32] reported an average of 30.4% of soy protein degradation assayed for fermented with 12 LAB stains at 37 ^oC for 6 h. It should be noticed that the protein content alternation during fermentation of LAB strains were strain and time dependent.[15, 32] Results obtained in current study indicated pronounced proteolysis activities were presented by *L. bulgaricus* and *L. plantarum* B1-6, compared to the two other strains.

Based on the results of the growth performances as well as the changes of NBM protein contents, microbial counts of *L. helveticus* MB2-1 have changed little during 6 h of fermentation in NBM and a negligible protein content alternation was observed, which have indicated the poor growth capacity of *L. helveticus* MB2-1 in NBM. Therefore, *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 were selected to conduct the further studies of electrophoresis, RP-HPLC, and ACE inhibitory properties.

Electrophoresis

The protein profile changes during fermentation using the three selective strains are shown in Fig. 2, and the analyzed degradation percentages of major protein bands are presented in Table 1. In general, considerable alternations of protein profiles were obtained during the growth of *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6, which indicative of pronounced proteolytic activities. This was in accordance with our previous results.

Navy bean contained 54–79% of globulins which composed of a major fraction of phaseolin (7S) and a minor fraction of legumin (11S).[28] In the current observed electrophoresis patterns, bands A and B, with respectively molecular mass (MM) 65.39 kDa and 57.71 kDa, were likely breakdown products of 11S legumin as reported by previous studies.[34,35] However, bands A and B showed distinct profiles during proteolytic hydrolysis in which an intensive degradation of band B was demonstrated (i.e., 30-42% for *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 during fermentation) compared to that of band A. This was indicative of the existence of structural differences between the two proteins. Bands C, D and E represented the three subunits of phaseolin of navy beans, namely α, β, and γ-type. Navy bean had a characteristic "S" (Sanilac) type phaseolin that the three subunits were corresponded to the molecular masses of 45.75, 42.80, and 40.74 kDa.[36,37] Proteolytic systems of selected LAB have caused potent degradation on α and β type phaseolins.

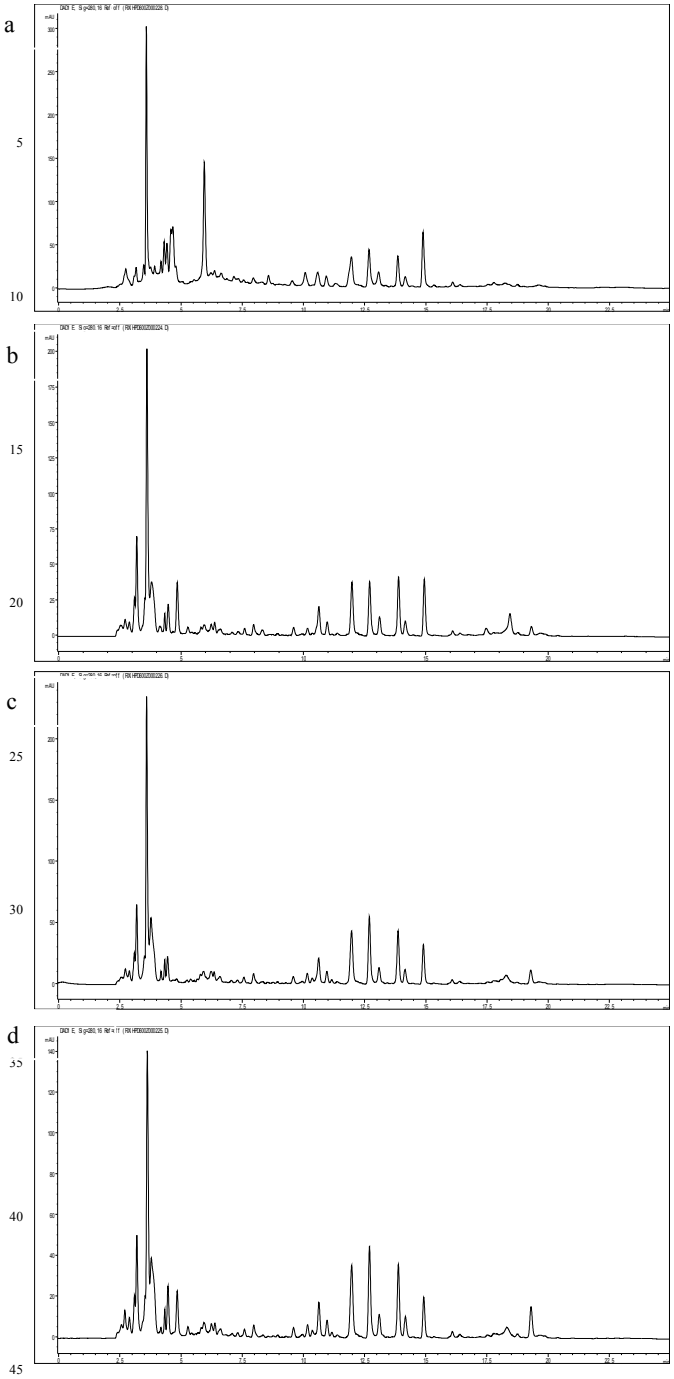

Fig. 3 Reverse phase high performance liquid chromatography (RP-HPLC) profiles of NBM at (a) 0h of fermentation, and 6h fermentation of (b) *L. bulgaricus*, (c) *L. plantarum* 70810, and (d) *L. plantarum* B1-6.

However, no obvious hydrolysis of γ-type phaseolin was obtained, on the contrary, the density of which has increased after 6 h of fermentation by *L. bulgaricus* and *L. plantarum* B1-6. This interesting phenomenon might be due to the deglycosylation effects exerted by LAB thus had the glycosides removed from the larger phaseolins and to form γ-type phaseolin with reduced MM, since carbohydrate composition was accounted for an important reason to contribute to the MM diversity of different phaseolin subunits.[33] Aguiree *et al.*[32] has also reported similar results during soybean protein fermentation that α and α'-type of β-conglycinin were the preferred substrates for LAB proteolytic hydrolysis. Phaseolin was known to resistant to enzymatic hydrolysis thus have been considered as one of the major causes of the low digestibility of bean proteins.[38] Therefore, the current findings of the extensive hydrolysis of phaseolins by *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 might contribute to elevate of the nutritional value of bean protein through the potential to increase the digestibility. Band I with molecular mass of 22.86 kDa was probably corresponded to the primarily break down product of α-phasolin, which was reported in a previous study of navy bean proteins and evidenced by mass spectrometry.[36] Extensive hydrolysis of the protein was also carried out by the three LAB strains with the degradation percentages ranged from 33% to 49%. Regarding globulin hydrolysis, *L. plantarum* B1-6 exerted the highest proteolytic activity than the other two investigated LAB strains.

In addition, proteins with lower MM were also observed to be partly degraded during fermentation. *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 were able to partly hydrolyze bands G with MM of 29.34 kDa to the extents of 59%, 51% and 60%, respectively. This protein might correspond to one of the major antinutritional proteins phytohemagglutinin (PHA) according to the previous mass spectrum results of navy bean proteins.[36] Additionally, *L. plantarum* B1-6 was able to efficiently hydrolyze 35% of band J (MM: 16.75 kDa), which was considered to be α-amlyase inhibitor, whereas no such effect was observed by the incubation of *L. bulgaricus*. The capacity of LAB strains to degrade the antinutritional proteins of navy beans might result the navy proteins with reduced toxicity.

Reverse phase high performance liquid chromatogram(RP-HPLC)

RP-HPLC was considered as a fine tool to characterize the peptide profiles and was to separate the peptides based on their hydrophobicity/hydropholicity.[39] RP-HPLC profiles have afforded well separated 14 major peaks at retention times from 3 min to 20 min for both unfermented NBM and the fermented NBM using different LAB strains (Fig. 3). The fractions eluted at early retention times were considered to be peptides with low MM and high hydrophilicity.[40] For all LAB treated NBM, higher intensities of peaks at retention times from 2.5 to 3.5 min were observed indicative of the formation of several small hydrophilic peptides, whereas degradation of relatively larger and more hydrophobic peptides (peak 4 and 5) were obtained. *L. bulgaricus* and *L. plantarum* B1-6 exerted similar early eluted peptide patterns that a fraction at retation time of 4.84 min was presented, whereas this fraction was not shown for *L. plantarum* 70810. Fractions eluted later than 10 min were probably corresponded to larger proteins such as phaseolins and legumins according to the previous assignments of soy peptides.[32] Although peaks 6-14 were presented both before and after fermentation, the relative amount of each peak (except peak 12) was reduced after treated with LAB strains, which probably related to the degradation of globulins. Among the three strains, *L. plantarum* B1-6 demonstrated the highest proteolytic activities that an average degradation of 40.3% of the fractions 9-14 was obtained based on the reduction of peak area, followed by *L. bulgaricus* (35.3%)

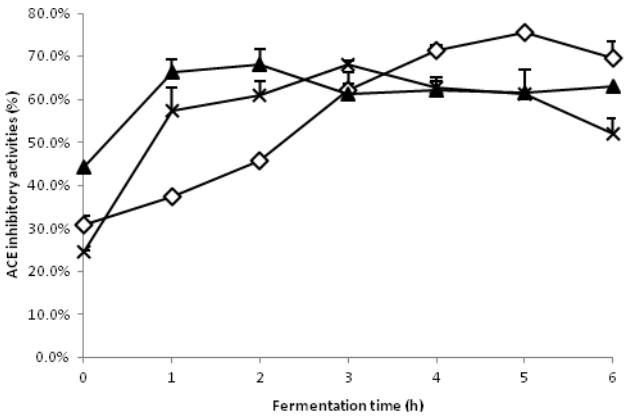

Fig.4 *In vitro* ACE inhibitory activities of NBM during growth of *L. bulgaricus*(◇), *L. plantarum* 70810 (▲), and *L. plantarum* B1-6(×).

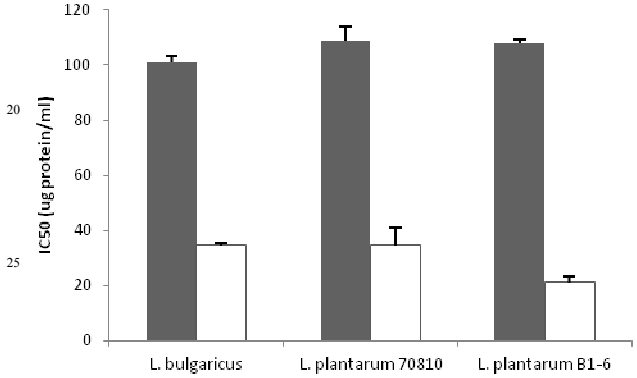

Fig. 5 IC_{50} values of NBM fermented using optimum conditions (■) and followed by in vitro gastrointestinal digestions (□). The optimum fermentation conditions were: *L. bulgaricus* (fermented at 37 °C for 5 h), *L. plantarum* 70810 (fermented at 31 °C for 2 h), and *L. plantarum* B1-6 (fermented at 37 °C for 3 h).

and *L. plantarum* 70810 (24.1%). This was in agreement with our previous findings. Additionally, the fermented NBM showed novel generated fractions with retention times of 18.2-18.4 min. Similar observations were reported during soy protein fermentation, in which the pattern was obtained for the *L. paracasei subsp. Paracasei* CRL 207 and the other eight LAB strains after 6 h of fermentation and was considered as the formation of hydrophobic peptides.[32]

ACE inhibitory properties

ACE inhibitory activities of samples withdrawn at different fermentation periods are presented in Fig. 4. The intact NBM had no ACE inhibitory activities (data not shown), whereas at 0 h of fermentation, inoculations of different strains have led to increase of the ACE inhibitory activities to varied extents, i.e. 30.7%, 44.3% and 24.6% for *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6, respectively. Although cells were washed with sterilized saline before inoculation, some bioactive peptides might be brought into the NBM thus have caused the variations. Similar observations were reported during fermentation of lentils.[16] ACE inhibitory activities elevated rapidly during the first hour of fermentation, in which *L. plantarum* B1-6 exerted the highest improvement of 32.7%, indicative of intensive proteolysis. For samples incubated with *L. plantarum* 70810, the increase rate slowed down thereafter and the highest ACE inhibitory activities were obtained at the 2 h of fermentation with inhibitory rates of 68.2%. NBM fermented by *L. plantarum* B1-6 exhibited the most potent ACE inhibibitory properties at the 3 h of fermentation (68.14%), whereas that of *L. bulgaricus* had a steady increase curve for the first 5 h of incubation and peaked at 5 h of fermentation time (75.65%). It is known that libration of ACE inhibitory peptides during LAB fermentation was related to the action of the protease and several peptidases.[41] Therefore, ACE inhibitory peptides might be initially generated along with the growth of LAB but some of the peptides might later experienced degradation thus caused the overall decrease of the ACE inhibitory activity, since ACE inhibitory activity were known to be strongly affected by size and carboxyl terminal amino acid residues of the peptides. Similar to our results, decrease of ACE inhibitory activity was also demonstrated at the end of fermentation by milk fermented with *L. helveticus* strains Lh474.[28]

There were few reports regarding *L. plantarum* to generate ACE inhibitory peptides. *L. plantarum* 299v was introduced to treat pea seeds but no ACE inhibitory activities were obtained after fermentation.[1] However, in another study using *L. plantarum* CECT 748T in the fermentation of lentil water-soluble extract, potent ACE inhibitory activity was obtained.[16] Discrepancy between these results implied different LAB strains had influences on the capacity of releasing of ACE inhibitory peptides, meanwhile, legume protein profiles also had an effect on the ACE inhibitory activities. Torino, *et al.*[16] reported a pronounced increase of ACE inhibitory activity during the first 48 h of fermentation, whereas no further improvement occurred at 96 h of fermentation. In the current study, peptides with ACE inhibitory activity released much rapidly and the inhibition rates reached the highest within 6 hours fermentation. Similar to the previous findings, the bioactive peptides accumulation during fermentation was largely corresponded to the increase of microbial population.[16,28] It seemed that NBM was a proper environment for the growth of the selected microorganism, namely, *L. bulgaricus*, *L. plantarum* 70810 and *L. plantarum* B1-6 and this might facilitate the active action of proteolytic system of the strains on the navy bean proteins.

***In vitro* gastrointestinal simulation (GIS) and ACE inhibititory properties**

In vitro gastrointestinal simulation (GIS) was a useful way to predict the fate of the ACE inhibitory peptides when ingested.[42] The results of IC_{50} values of fermented NBM before and after *in vitro* GIS are shown in Fig. 5. NBM treated with different LAB strains possessed IC_{50} values ranged from 101 to 109 μg protein/ml. This was higher than the IC_{50} value of navy bean protein treated with subsequently enzymatic hydrolysis of alcalase and papain (68 μg/ml), whereas comparable with that of navy bean protein treated directly with GIS hydrolysis (IC_{50} value: 137 μg/ml).[18] NBM fermented by *L. bulgaricus* showed the lowest IC_{50} values (101±2.2 μg protein/ml), but no significant differences were observed between the three LAB strains ($P>0.05$). All fermented products demonstrated significantly lower IC_{50} values ($P<0.01$) when subjected to subsequent

digestion of pepsin, trypsin and chymotrypsin. Lowest IC_{50} values were obtained for *L. plantarum* B1-6 after *in vitro* GIS hydrolysis, i.e. 21±2.1 μg protein/ml, which was significantly lower ($P<0.05$) than that of *L. plantarum* 70810 and *L. bulgaricus*. The results demonstrated the NBM fermentation products librated new ACE inhibitory peptides after the treatment of the gastrointestinal enzymes, which suggested these products might have improved bioactivity when being ingested. This has implied a promising potential of fermented NBM to be served as functional foodstuffs.

Conclusions

The current study have investigated for the first time the impact of three LAB strains, i.e. *L. bulgaricus*, *L. plantarum* 70810, and *L. plantarum* B1-6 on the protein degradation and ACE inhibitory activities of navy bean milk (NBM). The results showed that the three strains had well growth performance during 6 h of fermentation of NBM, in which *L. plantarum* B1-6 exerted the most potent proteolysis to degrade navy bean proteins as shown by the results of total protein content, SDS-PAGE pattern and RP-HPLC chromatogram. Proteolytic hydrolysis by the three strains had preferably hydrolyzed α and β type phaseolins, whereas minor degradation was observed on γ-type phaseolin. The highest ACE inhibitory activities were obtained by NBM treated with *L. bulgaricus* for 2 h, demonstrated IC_{50} values of 101±2.2 μg protein/ml, whereas the lowest IC_{50} of 21±2.1 μg protein/ml was obtained by the *L. plantarum* B1-6 fermented NBM after *in vitro* GIS.

This study has reported a novel approach to produce a fermented NBM that are probiotic and with promising potential to be served as functional foodstuffs. However, although it is generally believed the release of ACE inhibitory peptides were closely related to the protease and peptidase of LAB proteolytic systems, it was not clear the specificity of CEP/peptidase on the liberation of ACE inhibitory peptides. Thus it will be very interesting to conduct future work to elucidate the internal mechanism of particular protease/peptidase released by LAB for libration of ACE inhibitory peptides.

Acknowledgements:

This work was co-financed by Key Projects in the National Science & Technology Pillar Program of China (No. 2013BAD18B01-4), High-Tech Research and Development Program of China (No.2011AA100903), National Natural Science Foundation of China (No. 31371807 and 31201422) and was also supported by the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Notes and references

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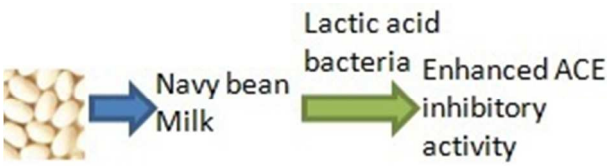

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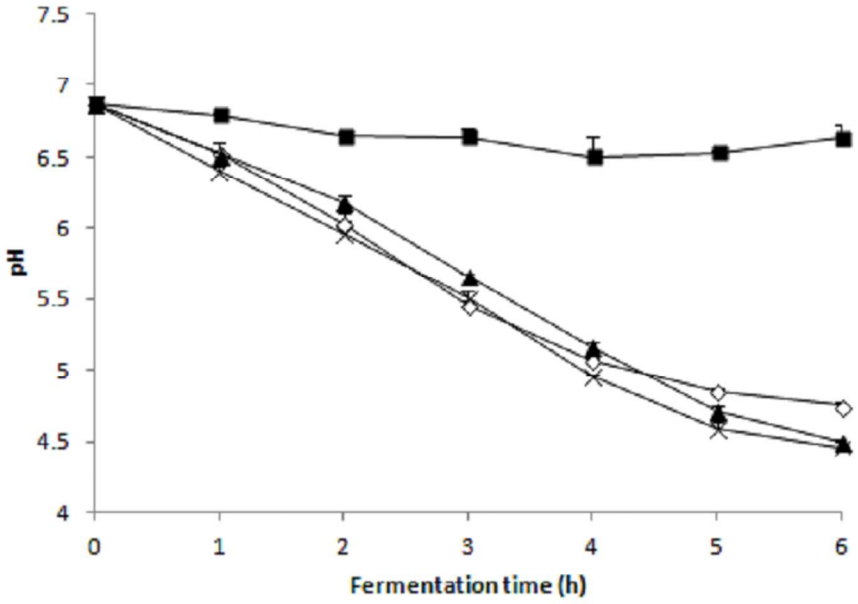

Fig. 1 (a) Decline of pH, (b) viable counts, (c) protein contents changes during growth of selected stains in NBM. L. bulgaricus(◇), L. helveticus MB2-1(■), L. plantarum 70810 (▲), L. plantarum B1-6(×).
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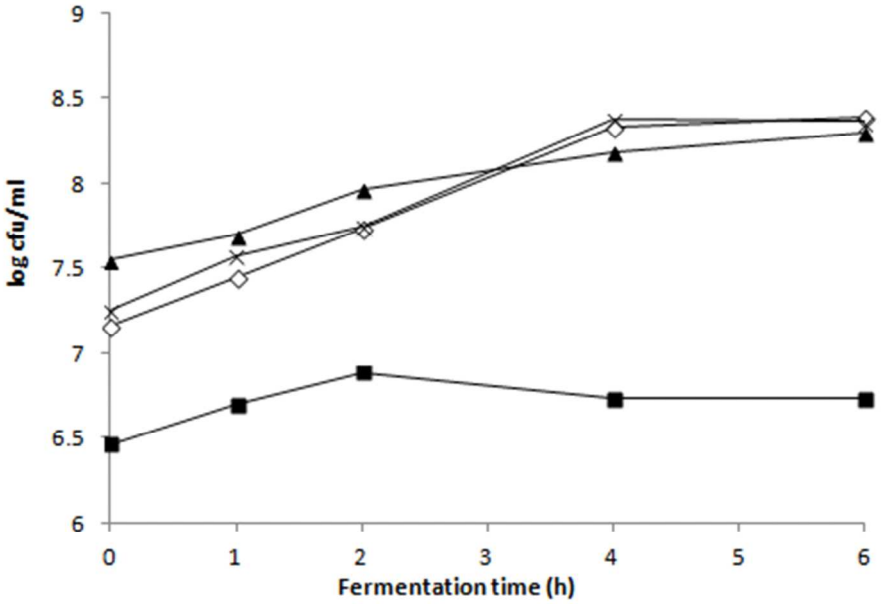

Fig. 1 (a) Decline of pH, (b) viable counts, (c) protein contents changes during growth of selected stains in NBM. L. bulgaricus(◇), L. helveticus MB2-1(■), L. plantarum 70810 (▲), L. plantarum B1-6(×).
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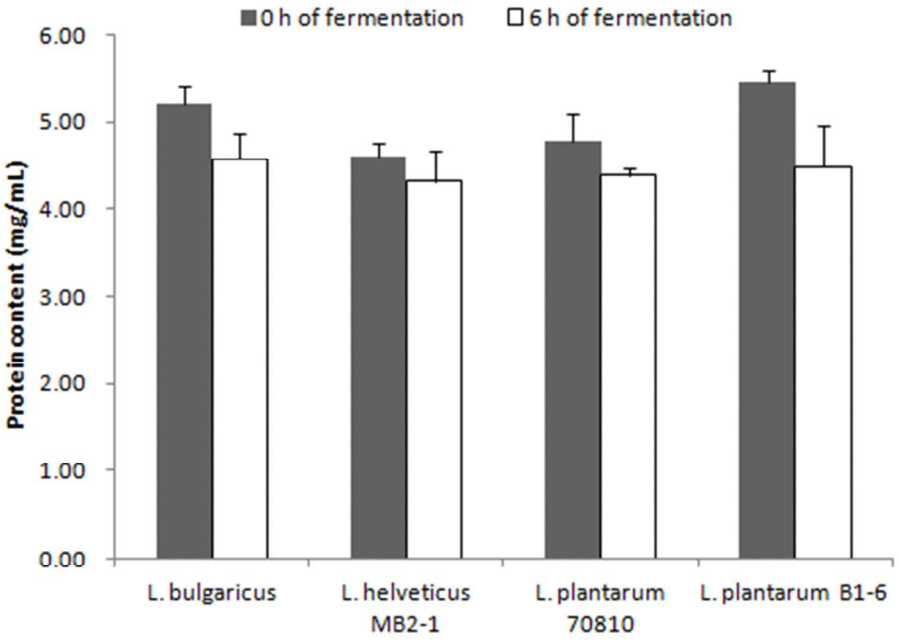

Fig. 1 (a) Decline of pH, (b) viable counts, (c) protein contents changes during growth of selected stains in NBM. L. bulgaricus(◇), L. helveticus MB2-1(■), L. plantarum 70810 (▲), L. plantarum B1-6(×).
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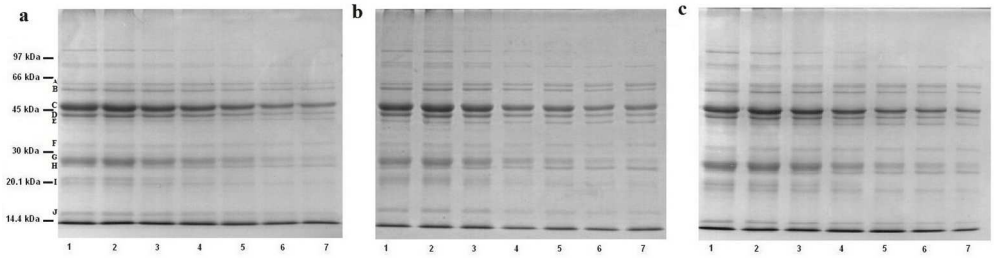

Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of NBM fermented for different periods. Lanes 1-7: 0h, 1h, 2h, 3h, 4h, 5h, 6h of fermentation by the selected strains. (a) L. bulgaricus, (b) L. plantarum 70810, (c) L. plantarum B1-6. A-J represented the major bands of NBM and was subjected to density analysis.
823x233mm (96 x 96 DPI)

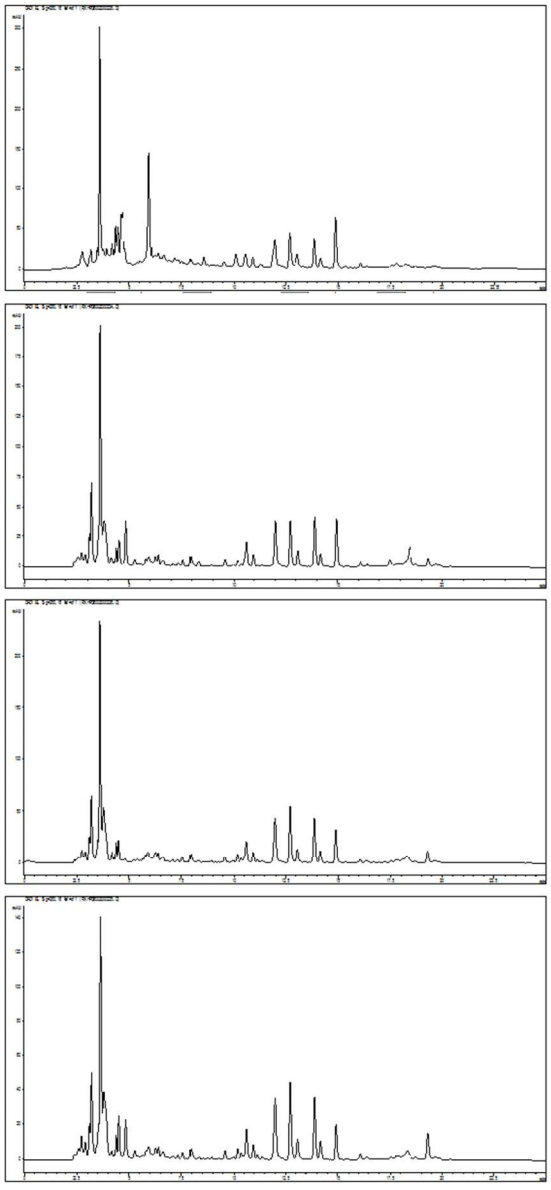

Fig. 3 Reverse phase high performance liquid chromatography (RP-HPLC) profiles of NBM at (a) 0h of fermentation, and 6h fermentation of (b) L. bulgaricus, (c) L. plantarum 70810, and (d) L. plantarum B1-6. 973x2044mm (96 x 96 DPI)

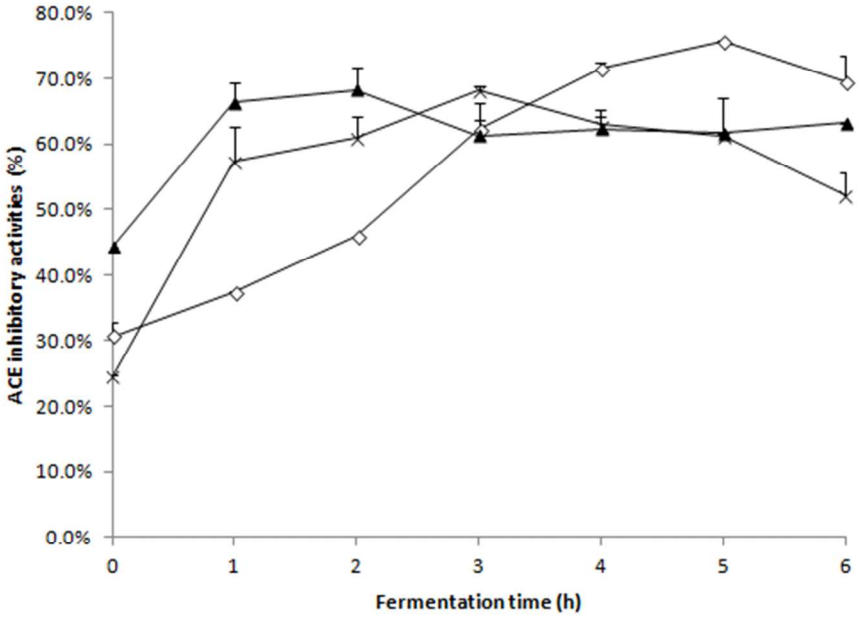

Fig.4 In vitro ACE inhibitory activities of NBM during growth of L. bulgaricus(◇), L. plantarum 70810 (▲), and L. plantarum B1-6(×).
973x640mm (96 x 96 DPI)

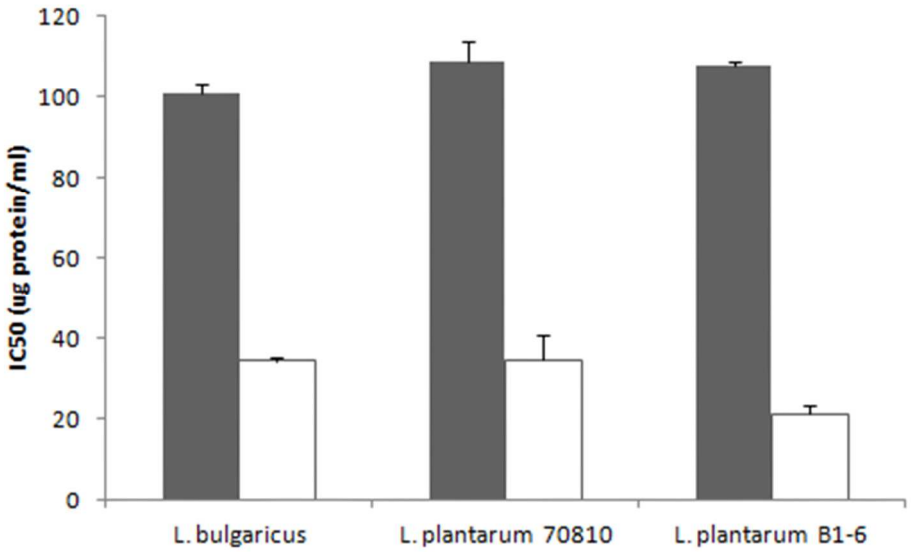

Fig. 5 IC50 values of NBM fermented using optimum conditions (■) and followed by in vitro gastrointestinal digestions (□). The optimum fermentation conditions were: L. bulgaricus (fermented at 37 oC for 5 h), L. plantarum 70810 (fermented at 31 oC for 2 h), and L. plantarum B1-6 (fermented at 37 oC for 3 h).
819x509mm (96 x 96 DPI)