Characterization of angiotensin-converting enzyme inhibitory activity of fermented milk produced by *Lactobacillus helveticus*

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ABSTRACT

Hypertension affects up to 30% of the adult population in most countries. It is a known risk factor for cardiovascular diseases, including coronary heart disease, peripheral artery disease, and stroke. Owing to the increased health awareness of consumers, the application of angiotensin-converting enzyme (ACE)-inhibitory peptides produced by *Lactobacillus helveticus* to prevent or control high blood pressure has drawn wide attention. A total of 59 *L. helveticus* strains were isolated from traditional fermented dairy products and the ACE-inhibitory activity of the fermented milks produced with the isolated microorganisms was assayed. The ACE-inhibitory activity of 38 *L. helveticus* strains was more than 50%, and 3 strains (IMAU80872, IMAU80852, and IMAU80851) expressing the highest ACE-inhibitory activity were selected for further studies. Particularly, the gastrointestinal protease tolerance and thermostability of the ACE-inhibitory activity in the fermented milks were assessed. Based on these 2 criteria, IMAU80872 was found to be superior over the other 2 strains. Furthermore, IMAU80872 exhibited a high in vitro ACE-inhibitory activity at the following fermentation conditions: fermentation temperature at 40°C, inoculation concentration of 1×10^6 cfu/mL, and fermentation for 18 h. Finally, by using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry analysis, we observed changes of the metabolome along the milk fermentation process of IMAU80872. Furthermore, 6 peptides were identified, which might have ACE-inhibitory activity. In conclusion, we identified a novel ACE-inhibitory *L. helveticus* strain suitable for the production of fermented milk or other functional dairy products.

Key words: *Lactobacillus helveticus*, angiotensin-converting enzyme, fermented milk, peptide

INTRODUCTION

It has been reported that, as of 2000, >25% of the population worldwide (approximately 1 billion) had been affected by hypertension, and this figure is predicted to increase to 1.56 billion by 2025 (Kearney et al., 2005). The rennin-angiotensin-aldosterone system is a key factor in the maintenance of arterial blood pressure. In this system, angiotensin-converting enzyme (ACE; EC 3.4.15.1) cleaves the dipeptide portion of angiotensin I from the C-terminal and produces a potent vasopressor angiotensin II, which induces the release of aldosterone that causes the retention of sodium ions by the kidney and elevates the blood volume, thus increasing the blood pressure (Skeggs et al., 1956). Moreover, ACE also catalyzes the inactivation of bradykinin, which has an important vasodilation activity, leading to an elevated blood pressure. Therefore, ACE plays an important role in the regulation of arterial blood pressure, and inhibiting this enzyme can generate an antihypertensive effect. In fact, ACE-inhibitors are an excellent physiological target for clinical hypertensive treatment due to their involvement in the renin-angiotensin system and kinin-nitric oxide system.

Currently available ACE-inhibitors are synthetic pharmacological drugs and their use in healthy or low-risk populations is not advisable because of their adverse effects, such as dry cough, skin rashes, and angioneurotic edema. Thus, developing safe and natural ACE-inhibitors is necessary for future treatment and prevention of hypertension (Jao et al., 2012). These peptides have already been isolated from a variety of fermented dairy products, including cheese, yogurt, and fermented bovine milk (Donkor, 2007; Hartmann and Meisel, 2007; Qian et al., 2011). The fermented milk proteins, including both caseins and whey proteins, are rich sources of ACE-inhibitory peptides, as these peptides are embedded within their primary structures. They can be released by enzymatic hydrolysis during

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gastrointestinal digestion, food processing (FitzGerald et al., 2004), and microbial fermentation (Hajirostamloo, 2010).

Lactobacillus helveticus is a probiotic bacterium that is known to produce abundant intracellular proteolytic enzymes, including cell-envelope proteinases, endopeptidases, aminopeptidases, and the X-prolyl dipeptidyl aminopeptidase (PepX; Haandrikman et al., 1991; Exterkate, 1995). Some of these enzymes are capable of releasing ACE-inhibitory peptides into fermented milk drinks. Nakamura et al. (1995) reported the hypotensive effect of the tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), which were produced from milk fermentation with a combination of *L. helveticus* and *Saccharomyces cerevisiae*. Gobetti et al. (2000) isolated the ACE-inhibitory polypeptides LNPGEIVE and NVPGEIVE from yogurt-type products fermented with *Lactobacillus delbreukii* spp. *bulgaricus* and *Lactococcus lactis* spp. *cremoris*.

One key factor that may affect the in vivo efficiency of ACE-inhibitory peptides is their bioavailability. To effectively administer the ACE-inhibitory peptides orally to hypertensive patients, it is important to ensure that these peptides pass through the digestive tract and are absorbed through the intestinal epithelium (Jao et al., 2012). Digestion of proteins and peptides starts in the stomach by the action of pepsin at acidic pH, and then the polypeptides are further truncated by the pancreatic proteases, trypsin, α -chymotrypsin, elastase, and carboxypeptidases A and B at a more alkaline pH (Jao et al., 2012). The gastric and intestinal enzymes confer preferential proteolytic activity to the substrates. For example, pepsin can damage leucine residue and C-terminal aromatic AA, whereas trypsin preferentially attacks positively charged C-terminal AA (such as Arg and Lys) and chymotrypsin targets at the aromatic or hydrophobic AA (such as Tyr, Phe, and Trp; Neurath, 1957; Auffret and Ryle, 1979). The combined proteolytic activity results in the release of potential bioactive peptides, which may exert a direct function at the gastrointestinal tract. Moreover, some common food-processing treatments also affect the efficiency of ACE-inhibitory peptide. For example, heat treatment is conventionally used to kill microorganisms and inactivate undesirable enzyme activity to extend the shelf life of food. However, heat treatment will also damage the nutrients and functional bioactive substances in the food products to some extent. Hannu et al. (1998) reported that the functional properties of peptides in the food matrix are highly influenced by their molecular structure, interactions with other components and the conditions of food processing. In particular, heat treatment of ACE-inhibitory peptides in fermented milk may destroy their hypotensive function.

Owing to the unique genetic makeup of each bacterial starter strain, the fermentation process and the final composition of the metabolites in the fermented products may vary. Metabolomics is an approach that has emerged over the last decade and it provides interesting potential to be applied in food science and microbiology (Fiehn, 2002). The metabolomic approach can either be targeted to specific compounds or metabolic pathways or untargeted for generating global chemical fingerprints of the samples. In either case, this approach aids in depicting and quantifying metabolites that are present within the food matrix. Specifically, it may also help to identify the hypertensive components of dairy products fermented by *L. helveticus*.

Thus, the first objective of the current study was to screen the fermented milk products of 59 previously isolated food-originated *L. helveticus* for potential strains with high ACE-inhibitory activity. Second, as the ACE-inhibitory peptides have to retain their activity after the food fermentation and ripening processes, as well as surviving the gastrointestinal digestive enzymes, the resistances of the ACE-inhibitory activity of the selected strains against heat treatment and gastrointestinal transit were evaluated. Third, the fermentation conditions (fermentation temperature, initial inoculum density, and fermentation time) required for producing fermented milk with a high ACE-inhibitory activity were assessed. Finally, the change in metabolomic profile during milk fermentation of the strain IMAU80872 was monitored by ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC-ESI-QTOF/MS).

MATERIALS AND METHODS

Original Sources of *L. helveticus* Strains

In our previous work (Zhang et al., 2012), 59 strains of *L. helveticus* were isolated from different traditional fermented dairy products (20 from Kurut, a traditional fermented yak milk; 23 from Qula, a traditional fermented yak milk crude cheese; 7 from fresh yak milk; 9 from yogurt whey) collected in Gansu Province of China. These strains were identified as *L. helveticus* by a combination of traditional physiological and biochemical identification methods together with 16S rRNA gene sequence analysis as published in a previous report (Bao et al., 2012).

Bacterial Stocks and Preparation of Fermented Milks

The 59 strains of *L. helveticus* were preserved as freeze-dried powder at the Lactic Acid Bacteria Culture

Collection (Inner Mongolia Agricultural University, China). The *L. helveticus* strains were first activated in reconstituted skim milk at 37°C overnight, followed by a second passage in MRS broth at 37°C overnight. They were then used as starter cultures in the fermentation experiments. The reconstituted milk was prepared by suspending 11% (wt/wt) skim milk powder (NZMP Ltd., Wellington, New Zealand) in sterilized distilled water at 95°C for 10 min.

Fermented milks were made in culture flasks by inoculating with seed cultures of *L. helveticus* strains at a concentration of 5×10^6 cfu/mL. Inoculated milk cultures were incubated at 37°C for 24 h. The titratable acidity (TA, °T) was determined by titration with NaOH using phenolphthalein as the indicator (National Standards of the People's Republic of China, 2010) and pH value of fermented milk samples was determined according to Method No. 947.05 of the Association of Official Analytical Chemists (AOAC, 1990). The bacterial growth was determined by spectrophotometric measurement of optical density at 600 nm (UV-1700, Shimadzu, Kyoto, Japan). The finished fermented milks were centrifuged at $6,000 \times g$ for 10 min at 4°C for supernatant collection, and the collected supernatants were stored at -20°C until further use in the determination of ACE-inhibitory activity, free amino nitrogen (FAN; Church et al., 1983), and VPP and IPP contents.

Determination of In Vitro ACE-Inhibitory Activity

The ACE-inhibitory activity was measured using the HPLC method described by Chen et al. (2010) with some modifications. The substrate, hippuryl-L-histidyl-L-leucine (HHL), and rabbit lung powder containing ACE were obtained from Sigma Chemical Co. (St. Louis, MO). Both HHL and ACE were separately dissolved in 100 mM Na-borate buffer (pH 8.3) containing 300 mM NaCl. The assay was performed by incubating a mixture of 50 µL of milk supernatant sample and 50 µL of HHL (10 mM) solution at 37°C for 2 min. Then, 50 µL of ACE (0.010 U/mL) solution was then added and the mixture was further incubated at 37°C for 30 min. The reaction was stopped by heating the mixture in 85°C water bath for 10 min to inactivate the enzyme. Afterward, 150 µL of deionized water was added before 20 µL of this solution was directly injected onto a Zorbax C₁₈ column (4.6 × 250 mm, particle size 5 µm, Agilent, Santa Clara, CA) to separate the product, hippuric acid, from HHL. The column was eluted with 75% acetonitrile in water (vol/vol) containing 0.1% trifluoroacetic acid at a flow rate at 1.5 mL/min using a pump; the eluent was monitored at 228 nm. The column temperature was controlled at 30°C.

The ACE-inhibition activity was calculated from the following equation:

$$\text{ACE-inhibition activity} = \frac{[(Cc - Cs)/(Cc - Cb)] \times 100\%,}$$

where Cc, Cb, and Cs were the concentrations of hippuric acid without the tested sample (control = Cc), without ACE (blank = Cb), and with both ACE and the tested sample (sample = Cs).

Characterization of Milk ACE-Inhibitors by Protease Digestion

To characterize the nature and the resistance of ACE-inhibitory materials in the fermented milks to gastrointestinal proteases, samples were digested alone either with pepsin (1:10,000, Sigma Chemical Co.) or trypsin (1:250, Sigma Chemical Co.), or sequentially with pepsin followed by trypsinase (Figure 1). Enzymatically digested samples were then assayed for ACE-inhibitory activity as described above. The procedures for enzyme digestion were modified from Chen et al. (2010).

Thermostability of ACE-Inhibitory Materials in *L. helveticus*-Fermented Milk

The ACE-inhibitory fermented milks produced by *L. helveticus* were treated with 3 different heating processes, namely 63°C for 30 min, 75°C for 25 s, and 85°C for 20 s. After the samples were immediately cooled down to 4°C in an ice bath, their pH was adjusted to 8.3. The ACE-inhibitory activity was measured.

Influence of Fermentation Temperature, Inoculum Concentration, and Fermentation Time on ACE-Inhibitory Activity of *L. helveticus*-Fermented Milks

Based on the ACE-inhibitory activity stability after protease digestion and heat treatment, selected strains were tested in milk fermentation experiments to determine how the fermentation temperature, inoculum concentration, and fermentation time affected the level of ACE-inhibitory activity during *L. helveticus*-fermented milk production. To assess the effect of fermentation temperature, the fermented milk was made in the flask culture by inoculating with 2% (vol/vol, 5×10^6 cfu/mL) of *L. helveticus* seed culture. The culture was incubated at 33, 37, and 40°C until the content reached pH 4.5. The ACE-inhibitory activity of the fermented milks was then measured.

To test the effect of inoculum concentration, the fermented milk was made in the flask culture inoculated

Figure 1. The procedures of fermented milk digestion by gastrointestinal proteases. ACE = angiotensin-converting enzyme.

with different concentrations (1×10^6 , 5×10^6 , 1×10^7 , or 5×10^7 cfu/mL) of *L. helveticus* seed culture. Inoculated cultures were fermented at 40°C until pH 4.5. The ACE-inhibitory activity, FAN, TA (°T), VPP, and IPP contents of the fermented milks were then measured.

To optimize the fermentation time, the milk was fermented with seed culture concentration of 1×10^6 cfu/mL at 40°C. These conditions were chosen based on the results from the optimization of inoculum density and fermentation temperature. The pH value, TA, FAN content, optical density at 600 nm, ACE-inhibitory activity, VPP, and IPP contents were determined at 0, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 42, and 48 h upon fermentation.

Quantification of VPP and IPP by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

Separations of VPP and IPP in the supernatants from the fermented milk samples were performed on an ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) connected to a quadrupole time of flight instrument (Q-ToF, Waters, Manchester, UK). The UPLC system was equipped with a binary gradient pumping system, a photo diode array detector set at

220 nm, and an automatic injector (Waters). The 2.1 $\times$ 100 mm BEH-C₁₈ column (Waters) was used in these experiments. The injection volume was 4 µL. Solvent A was a mixture of water-formic acid (100:0.1, vol/vol) and solvent B contained acetonitrile-formic acid (100:0.1, vol/vol). Peptides were eluted with a linear gradient of solvent B in A going from 5 to 30% over 5 min at a flow rate of 0.4 mL/min.

Data were acquired from m/z 100 to 700 using a desolvation temperature of 350°C, source temperature of 100°C, cone voltage of 30 V, and collision energy of 6 eV. The nitrogen desolvation and nebulizer gas flow rates were set to 600 and 50 L/h, respectively. The mass spectrometer was calibrated across the m/z range of 100 to 1,500 using a solution of sodium formate. Data were centroided during acquisition using an external reference comprising a 2-ng/mL solution of Leu-enkephalin infused at 10 µL/min, generating an [M+H]⁺ ion at m/z 556.2771.

The peptides IPP and VPP were prepared by a conventional Fmoc solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystems Inc., Uberlingen, Germany). The purity of the synthesized peptides was verified by analytical HPLC coupled to MS.

Metabolic Profiling by UPLC-ESI-QTOF/MS Analysis

The metabolites of *L. helveticus* IMAU80872 over 48 h of milk fermentation were analyzed by UPLC-ESI-QTOF/MS (Waters) according to the method of Zhang et al. (2013). In short, an aliquot of 5 μ L of sample solution was injected onto an Acquity UPLC BEH C18 column (100 mm $\times$ 2.1 mm, 1.7 μ m, Waters) at 40°C with the flow rate of 0.4 mL/min. The optimal mobile phase consisted of a linear gradient system of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile; 2% B was used from 0 to 0.5 min; from 0.5 to 24 min, B was increased from 2 to 100% linearly; from 24 to 27.5 min, 100% B was constant; then, B was decreased to 2% and kept for 2 min. The column temperature was controlled at 30°C.

Mass spectrometry was operated by electrospray ionization in the positive ionization mode. The eluent was subject to analysis by a high-definition mass spectrometer (Waters) with analysis conditions as follows: the source temperature was 100°C, desolvation gas temperature was 350°C, cone gas flow was 1 L/h, desolvation gas flow was 800 L/h, the capillary voltage was 3.2 kV, the sampling cone voltage was 32 V, microchannel plate voltage was 2,300 V, and extraction cone voltage was 3.0 V. The mass spectrometry data were collected in the range of 100 to 1,000 (m/z).

Processing of Metabolomics Data and Identification of Differential Components

Data from UPLC/ESI-QTOF/MS measurement were imported into the MarkerLynx Application Manager (V4.1, Waters) software for peak detection and alignment. The intensity of each ion was normalized with respect to the total ion count to generate a data matrix that consisted of the retention time, m/z value, and the normalized peak area (10,000). The multivariate data matrix was analyzed by EZinfo software 2.8 (Waters). All the variables were mean-centered and Pareto scaled before principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). A PCA allows visualization of variation of metabolic profiles of samples collected at different fermentation time points, whereas PLS-DA calculates models that differentiate between sample groups.

The identification of differentiated chemical components along the fermentation process was based on the the PLS-DA loading plot and the S-plot of variable importance projection (VIP) confidence interval (VIP > 1). Differentiated fermentation metabolites were chosen based on their contribution to the variation and correlation within the data set. The MassFragment Application Manager (Waters) was used to facilitate the

MS/MS fragment ion analysis process by chemically intelligent peak-matching algorithms. The identities of the specific metabolites were confirmed by comparison of their mass spectra and chromatographic retention times. The ion spectra of metabolites of interest were matched with the structure message of metabolites acquired from open biochemical databases, such as HMDB (<http://www.hmdb.ca/>), METLIN (<http://metlin.scripps.edu/>), MassBank (<http://www.massbank.jp/>), and KEGG (<http://www.genome.jp/kegg/>).

Statistical Analysis

All experiments were performed in triplicate. Statistically significant differences between sample groups were evaluated with ANOVA. *P*-values of less than 0.05 were considered statistically significant differences between sample groups. Analysis of variance and correlation analysis were performed with SAS software (SAS version 9.00, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

During the fermentation of milk, the major milk proteins are degraded into peptides due to the action of indigenous milk enzymes (mainly plasmin), added coagulants, and microbial enzymes originated from the starter and nonstarter lactic acid bacteria (LAB). Among the LAB, the species *L. helveticus* has the highest extracellular proteinase activity and, thus, the highest ability to release bioactive peptides, including the hypotensive, ones to the fermented milk (Yamamoto et al., 1994). The principal aim of our paper was to screen 59 candidate *L. helveticus* strains and identify potential hypotensive strain that is suitable to be applied in milk fermentation.

Selection of *L. helveticus* Strains Based on Fermentation Characteristics and ACE-Inhibitory Activity

The pH value, FAN, TA, and ACE-inhibitory activity of 59 *L. helveticus* fermented milks (after 24 h of fermentation) are presented in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-9382>). To assess the acidification capacity of these strains, 2 parameters, including pH and TA, were measured. The pH value and TA ranged from 3.35 to 5.22 and 49.00 to 240.42°T, respectively, suggesting that the acidification capacity varied widely among strains. The growth of the strains was reflected by the absorbance at 600 nm, ranging from 0.01 to 0.68, whereas the FAN content fell between 1.65 to 10.5 nmol/L. Thirty-eight out of the 59 tested strains showed a relatively high ACE-inhibitory activity

Table 1. Physical parameters of fermented milks produced by 3 *Lactobacillus helveticus* strains with highest angiotensin-converting enzyme (ACE)-inhibitory activity (mean $\pm$ SD)¹

Strain No.	pH value	TA ($^{\circ}$ T)	OD ₆₀₀	FAN (mmol/L)	ACEI (%)
IMAU80851	3.51 $\pm$ 0.01	178.46 $\pm$ 4.85	0.205 $\pm$ 0.35	8.88 $\pm$ 0.14	81.71 $\pm$ 0.00
IMAU80852	3.36 $\pm$ 0.00	240.42 $\pm$ 2.43	0.337 $\pm$ 0.06	8.90 $\pm$ 0.31	81.68 $\pm$ 0.01
IMAU80872	3.37 $\pm$ 0.01	225.93 $\pm$ 1.35	0.226 $\pm$ 0.03	7.22 $\pm$ 0.17	76.64 $\pm$ 0.04

¹TA = titratable acidity; OD₆₀₀ = absorbance at 600 nm; FAN = free amino nitrogen; ACEI = ACE-inhibitory.

of over 50%. Interestingly, the ACE-inhibitory activity was significantly ($P < 0.01$) and positively correlated to the acidity and FAN in the fermented milk (Figure 2). This may reflect that the ACE-inhibitory activity was produced during the process of fermentation and microbial metabolism, in particular the proteolytic action on the milk proteins.

As all these strains were originally isolated from traditionally fermented foods, our results so far have shown 2 important points. First, based on the assessed fermentation properties, namely acidification capacity, bacterial growth, and FAN, it can be concluded that there was a large intraspecies variation in these properties. Thus, a screen approach is necessary for selecting appropriate strains suitable for food industrial application. Second, all these strains originated from traditionally fermented products, suggesting that these products are good sources for isolating hypotensive *L. helveticus* strains. Furthermore, potential exists to use these strains in dairy industry to generate products of additional health benefit (Muguerza et al., 2006). Based on the ACE-inhibitory activity, 3 strains (IMAU80872, IMAU80852, and IMAU80851) showing the highest ACE-inhibitory activity (76.64 $\pm$ 0.04, 81.68 $\pm$ 0.01, and 81.71 $\pm$ 0.00%, respectively) were chosen for further characterization (Table 1).

Figure 2. Correlation between angiotensin-converting enzyme (ACE)-inhibitory activity and 4 physical parameters of *Lactobacillus helveticus* fermented milk. ** $P < 0.01$.

ACE-Inhibitory Activity of Milk After Digestion by Protease

Approximately 90% of the absorption of peptides in the gastrointestinal tract takes place in the region of the small intestine (Regazzo, 2010). However, it is known that the in vitro ACE-inhibitory activity and in vivo hypotensive effect may not be directly correlated. One of the reasons is the gastrointestinal resistance of these peptides; of equal importance is whether the peptides require an additional proteolytic activation step to fully release its bioactivity. The ACE-inhibitory peptides from different sources have been isolated, including yogurt, cheese, ovalbumin, koumiss, and meat (Pihlanto-Leppälä et al. 1998; Foltz et al., 2007; Chen et al., 2010; Darewicz et al., 2014). It was found that the activity of ACE-inhibitory peptides may be higher or lower after proteolytic digestion. Thus, to investigate the effect of gastrointestinal proteases on the ACE-inhibitory activity, fermented milk samples produced by IMAU80872, IMAU80852, and IMAU80851 were digested with pepsin, trypsinase, or pepsin followed by trypsinase, and the ACE-inhibitory activity of untreated and treated samples were compared (Figure 3). It was found that the ACE-inhibitory activity of these 3 *L. helveticus*-fermented milks significantly increased ($P < 0.05$) after digestion by proteases, regardless of a single or sequential protease treatment, suggesting that the ACE-inhibitory materials were resistant to gastrointestinal proteases. The sequential pepsin-trypsinase treatment of IMAU80872-, IMAU80852-, and IMAU80851-fermented milks increased the ACE-inhibitory activity from 76.84 $\pm$ 0.29, 76.84 $\pm$ 0.29, and 79.58 $\pm$ 0.31% to 89.68 $\pm$ 0.19, 87.27 $\pm$ 0.34, and 89.34 $\pm$ 0.27%, respectively ($P < 0.05$, comparing the untreated and treatment samples in each case). Our results also show that a proteolytic activation step was indeed required to release the ACE-inhibitory activity fully.

According to Fujita et al. (2000), ACE-inhibitory peptides in vitro can be classified into 3 groups: (1) true inhibitor type, with 50% inhibition values of peptides that are not affected by preincubation with ACE or gastrointestinal proteases; (2) substrate type, with

Figure 3. Angiotensin-converting enzyme (ACE)-inhibitory activity of fermented milks after gastrointestinal protease digestion. Statistical differences between groups are represented by different letters (a–c; $P < 0.05$). Error bars indicate SD.

peptides that are converted to true inhibitors by ACE or gastrointestinal proteases, resulting in peptides with weaker activity; and (3) prodrug type, with peptides that are converted to true inhibitors by ACE or gastrointestinal proteases. Our results suggest that the ACE-inhibitors in the fermented milks produced by these 3 strains were of the prodrug type or a mixture of the prodrug and the true inhibitor types.

Similar to our study, Matsui et al. (1999) reported that the ACE-inhibitory activity of the small peptide (isoleucine-valine-tyrosine) isolated from wheat germ increased by 27% after enzyme hydrolysis by a mixture of pepsin, trypsin, and chymotrypsin. Qureshi et al. (2013) reported a significant increase in the ACE-inhibitory activity of Gamalost and Norvegia cheeses ($P < 0.05$) upon digestion by human gastrointestinal and duodenal juices (healthy volunteers), as the cheese was enzymatically digested to produce several active β -CN-derived peptides and AA.

Thermostability of ACE-Inhibitory Fermented Milk by *L. helveticus*

During modern dairy processing, yogurts are subjected to various kinds of heat treatments. Therefore, to ensure the heat stability of ACE-inhibitory activity, the fermented milks of IMAU80872, IMAU80852, and IMAU80851 were heat treated at different conditions simulating to industrial dairy pasteurization processes, and the effect of such processes on the ACE-inhibitory activity was monitored (Figure 4). Only the ACE-inhibitory activity of IMAU80872-fermented milk was not significantly affected by any of the tested heating

process ($P > 0.05$). The ACE-inhibitory activities of IMAU80852- and IMAU80851-fermented milks were significantly reduced after heat treatments at 75°C for 25 s and 80°C for 20 s, respectively ($P < 0.05$). These results suggest that the ACE-inhibitory activity of IMAU80872-fermented milk had the highest heat stability. Other heat-stable ACE-inhibitory materials have previously been reported. For example, no significant change was noted in the ACE-inhibitory activities of peptides separated from tuna and soy even after 100°C heat treatment ($P > 0.05$; Wu and Ding, 2002; Hwang, 2010). Owing to the high heat stability of the ACE-inhibitory activity of IMAU80872-fermented milk and its great potential for industrial use, we further established the optimum fermentation conditions (including the fermentation temperature, time, and initial inoculum density) of this strain in relation to its ACE-inhibitory activity.

Influence of Fermentation Temperature, Inoculum Density, and Fermentation Time on ACE-Inhibitory Activity of *L. helveticus* IMAU80872-Fermented Milk

Lactobacillus helveticus IMAU80872 was allowed to ferment at 33, 37, and 40°C. No significant difference was found in the resultant ACE-inhibitory activity in the fermented milks (77.30 ± 0.13 , 79.65 ± 0.20 , and $81.19 \pm 0.19\%$, respectively; $P > 0.05$), although an increasing trend of ACE-inhibitory activity was observed with an increase in the fermentation temperature (Figure 5). Similar results were reported by Otte

Figure 4. Effect of different heat treatments on angiotensin-converting enzyme (ACE)-inhibitory activity of fermented milk. Statistical differences between groups are represented by different letters (a–c; $P < 0.05$). Error bars indicate SD.

Figure 5. Effect of fermentation temperature and inoculum density on the angiotensin-converting enzyme (ACE)-inhibitory activity of *Lactobacillus helveticus* IMAU80872 fermented milks. Significant differences in the ACE-inhibitory activity between fermented milks produced with different temperature and inoculum densities are represented by different letters (A–C and a–c, respectively; $P < 0.05$). Error bars indicate SD.

et al. (2011) using the strains *L. helveticus* MI1198 and CHCC4080. The slight increase of ACE-inhibitory activity may be related to a higher efficiency of the proteolytic enzyme in releasing bioactive peptides. Pan and Guo (2010) studied the correlation between the fermentation temperature of *L. helveticus* LB10 and its cell wall proteinase, aminopeptidase, prolyl dipeptidyl aminopeptidase enzymes, as well as the ACE-inhibition rate, and found that the cell wall proteinase and ACE-inhibitory activity, but not other enzymes, were closely related.

The effect of different initial inoculum density of *L. helveticus* IMAU80872 on the fermented milk ACE-inhibitory activity was also tested. Interestingly, the fermented milk ACE-inhibition rate was related to the initial inoculum density, as the fermented milk produced with the inoculation of 1×10^6 cfu/g had significantly higher ACE-inhibitory activity than those made with higher starting *L. helveticus* concentrations (74.97 ± 0.33 vs. 69.95 ± 0.29 , 69.40 ± 0.23 , and $67.04 \pm 0.15\%$ corresponding to higher initial inoculum levels; $P < 0.05$; Figure 5). This phenomenon was probably related to the growth conditions of the starter bacteria, as a lower initial bacterial density might have offered a less competitive environment in terms of nutrients and metabolites for bioactive peptide synthesis. As the inoculum concentration increased, a rapid growth might have caused an excessive acid production, which in turn suppressed the proteolytic activity that was required for the generation of bioactive peptides.

Along the milk fermentation of *L. helveticus* IMAU80872, various parameters changed (Figure 6). In the logarithmic phase (6–10 h; Figure 6), bacterial cells grew exponentially. Most likely, during this time, bacterial cell wall proteinases were produced, accumulated, and in turn enhanced protein hydrolysis (essentially casein), leading to the release of peptides and free AA. The global increase in free AA in the medium fulfilled the AA requirements for the growth of the bacteria (Juillard et al., 1995). Thus, a rapid increase in FAN and fermented milk peptides was observed, and this was followed by an increase in ACE-inhibitory activity (Figure 6). Our results are in line with those reported in Pan and Guo (2010) that *L. helveticus* LB10-produced ACE-inhibitory peptides and bacterial cell wall proteinases during the logarithmic and stationary phases. With the extension of the stationary phase (10–30 h), more organic acids accumulated, which caused the continuous drop of the milk pH value. This then decreased the bacterial activity and slowed down the elevation of ACE-inhibitory activity and release of FAN (Figure 6). However, the FAN content turned to a downward trend after 18 h of fermentation, which may be due to the deprivation of nutrients available to the microbes. Otte et al. (2011) found that there was no absolute correlation between LAB fermented milk ACE-inhibition rate with the bacterial intracellular enzyme, PepX. In our study, the absorbance of fermented milk declined after 30 h of fermentation, suggesting that the *L. helveticus* IMAU80872 cells started to lyse. In that case, the bacterial intracellular enzymes would also be released. However, the ACE-inhibitory activity of the fermented milk remained stable, indicating that such activity was not directly relating to the bacterial intracellular enzymes, confirming the results of Otte et al. (2011).

The changes of VPP and IPP concentrations and the ACE-inhibitory activity during milk fermentation of *L. helveticus* IMAU80872 is shown in Figure 6. Interestingly, the ACE-inhibitory activity of the fermented milk rapidly increased after around 10 h of fermentation, although the VPP and IPP contents were almost undetectable. The fact that the ACE-inhibitory activity did not correlate well with the appearance of VPP and IPP and that the VPP and IPP peptides appeared relatively late may suggest that the ACE-inhibitory activity was largely not based on the VPP and IPP peptides. Moreover, even when more VPP and IPP were detectable after 12 h of fermentation, no change was observed in the fermented milk ACE-inhibitory activity, which further supports our speculation that the ACE-inhibitory activity was likely conferred by mechanisms other than VPP and IPP peptides. Similarly, Bütikofer et al. (2007) also found poor correlation between the sum of the 2 tripeptides, VPP and IPP, and

Figure 6. Changes of angiotensin-converting enzyme (ACE)-inhibitory activity, free amino nitrogen (FAN), Val-Pro-Pro (VPP), and Ile-Pro-Pro (IPP) concentrations, absorbance at 600 nm (OD_{600}), and pH during *Lactobacillus helveticus* IMAU80872 milk fermentation. Error bars indicate SD.

the 50% inhibition values of ACE-inhibitory activity in soft cheese samples. Finally, after 36 h of fermentation, the fermented milk VPP and IPP concentrations were slightly reduced, which might be due to the depletion of both nutrients and casein substrate in the fermented milk. Thus, the release of VPP and IPP naturally reduced and the bacteria might have started to use other nutrient sources in the environment including these readily digestible peptides.

Identification of Differential Metabolites During Milk Fermentation

To identify which metabolites contributed to the ACE-inhibitory activity in the fermented milk sample, we monitored the change in metabolic profile of fermented milks of IMAU80872 at different time points of fermentation by UPLC/ESI-QTOF/MS analysis. A total of 11,365 types of metabolites (each signal was represented by a different m/z detected at the same retention time) were collected over the 48 h. A PCA was performed based on the whole data set, including triplicate samples taken at each time point (Figure 7). The first 2 principal components accounted for 76 and 8% of the variation, respectively. Samples from 0 to 12 h showed a clear pattern of shift from the left to the right side of the PCA score plot, suggesting gradual

changes of the chemical composition during the milk fermentation. Meanwhile, samples collected beyond 18 h of fermentation clustered closely, indicating that no significant chemical changes in the metabolic profile occurred after this time point, which might correspond to the stationary phase of the bacteria growth (Figure 6).

Due to the results of the PCA, further analysis at 2 and 12 h of fermentation was performed with PLS-DA (S-plot is shown in Figure 8). Each dot represents a different type of metabolite on the S-plot, with the x- and y-axis representing contribution coefficient and correlation, respectively. Metabolites far from the cross section of x- and y-axes were the more relevant ions for discrimination between groups. Based on cutoff of confidence level or VIP >1, 6 differential metabolites showing significant difference between these 2 fermentation time points could be identified. Interestingly, all 6 metabolites were peptide in nature (Table 2). The identities of these peptides were confirmed by cross checking their MS/MS data at public chemical data-banks, including HMDB, METLIN, MassBank, and KEGG databases. Some of these peptides, including ALPM, VAGTWY, and IPI, have previously been reported to show ACE-inhibitory activity (references in Table 2), whereas the ACE-inhibitory capacity of the other 3 identified peptides remains to be evaluated in future work.

Figure 7. Principal component analysis score plot showing the dynamics of metabolic profiles during fermentation. Triplicate samples were taken at each time point. Symbols of different shapes correspond to the fermentation time in hours.

CONCLUSIONS

Our study preliminarily assessed the ACE-inhibitory activity of 59 *L. helveticus*-fermented milks. Thirty-eight

of the tested fermented milks had an ACE-inhibitory activity of more than 50%. The strains IMAU80872, IMAU80852, and IMAU80851 possessed the highest ACE-inhibitory activity. Subsequent assessments found

Table 2. Differential peptides identified at 2 and 12 h of IMAU80872 milk fermentation

Peptide No.	Peptide sequence	Measured mass	<i>m/z</i>	Source of peptide	Angiotensin-converting enzyme-inhibitory activity reported previously
1	LKP	365.5042	33.8677	β-LG f(61–63)	—
2	ALPM	430.6	8.4736	β-LG f(158–161)	Mullally et al. (1997)
3	PGPIHD	634.8022	14.086	Unidentified	—
4	VAGTWY	695.8347	19.623	β-LG f(31–36)	Pihlanto-Leppälä et al. (1998)
5	DN	247.2045	22.5127	Unidentified	—
6	IPI	341.5	27.0878	κ-CN f(25–28)	Chiba and Yoshikawa (1991)

Figure 8. S-plot of partial least square-discriminant analysis of metabolomes after 2 and 12 h of fermentation. X- and y-axes correspond to the contribution coefficient and the correlation, respectively. Each dot represents 1 type of metabolite. The 6 differential metabolites showing significant difference (variable importance projection >1) between the 2 time points marked.

that IMAU80872 was superior to the other 2 strains, as the fermented milk produced by IMAU80872 was the most resistant to both protease and heat treatments. The fermentation for 18 h at 40°C with the initial inoculum density of 1×10^6 cfu/g were the optimal conditions for IMAU80872 to produce fermented milks with high ACE-inhibitory activities. Finally, we identified 6 differential peptides that were significantly enriched after milk fermentation of 12 h by IMAU80872. To conclude, our study identified a novel bioactive ACE-inhibitory *L. helveticus* strain that is suitable to be used in antihypertensive fermented yogurt or other functional dairy products.

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