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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 which 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*. Gobbetti et al. (2000) isolated the ACE-inhibitory polypeptides LNVPGEIVE 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, a-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