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Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk

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Abstract

Milk was fermented for up to 5 h at 43 °C with two lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*). A protease, flavourzyme, was added at the beginning of fermentation. The whey fraction was separated from the fermented milk and freeze-dried. During the 5 h of fermentation, the soluble protein content increased from 4.9 to 57.4 mg/g and peptide content increased from 2.1 to 32.8 mg/g, while inhibition of angiotensin I-converting enzyme (ACE) increased by a decrease of IC_{50} from 0.708 to 0.266 mg/ml, respectively. The whey was fractionated into four fractions by size exclusion chromatography on a Sephadex G-15 column. The fourth fraction of the whey showed the highest inhibitory efficiency ratio (IER) being 1329%/mg/ml. The amino acid sequence of the inhibitory peptide was Tyr-Pro-Tyr-Tyr, of which the IC_{50} was 90.9 μM. The whey showed mixed-type inhibition kinetics, while Captopril, the positive control showed competitive inhibition on ACE. Their K_i values were 0.188 mg/ml and 0.0067 μg/ml, respectively. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) was reduced to 15.9 and 15.6 mm Hg, respectively, in spontaneously hypertensive rat (SHR), after 8 weeks of oral administration of diluted whey (peptide concentration 4.9 mg/ml).

Keywords: Fermented milk; Angiotensin I-converting enzyme (ACE); Spontaneously hypertensive rat (SHR); Antihypertensive effect

1. Introduction

High blood pressure has been considered a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infraction) and end-stage renal disease. Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure and electrolyte homeostasis. ACE hydrolyzed an inactive form of decapeptide, Angiotensin I, to an octapeptide, Angiotensin II, is a potent vasoconstrictor, and inactivated catalytic function of bradykinin, which is a depressor. Angiotensin II has been implicated in the regulation of cellular lipoxygenases (LOX), which plays a role in atherogenesis by catalyzing oxidative modification of low-density lipoproteins (LDL) (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998). Angiotensin II and LOX-catalyzed oxidation of LDL are involved in hypertension and atherosclerosis. ACE inhibitors act as vasodilators, but the most obvious potential benefit is their effect on the renin–angiotensin–aldosterone system by reducing the levels of Angiotensin II. Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infraction or heart failure (Daemon, Lombardi, Bosman, & Schwartz, 1991; Geisterfer, Peach, & Owens, 1988).

Several ACE inhibitory peptides have been isolated from the enzymatic hydrolysis of milk proteins (Hernandez-Ledesma, Amigo, Ramos, & Recio, 2004; Maeno, Yamamoto, & Takano, 1996; Tauzin, Miclo, & Gaillard, 2002), and fermentation of milk with lactic acid bacteria (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Nakamura et al., 1995; Yamamoto, Maeno, & Takano,

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1999), or by chemical synthesis according to milk protein sequences (Kohmura, Nio, & Ariyoshi, 1990; Mullally, Meisel, & Fitzgerald, 1996). Moreover, these ACE inhibitory peptides (e.g. Val-Arg-Tyr-Leu) have been isolated from the enzymatic hydrolysis of milk proteins (Hernandez-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005), and Ile-Pro-Pro and Val-Pro-Pro from milk fermented with lactic acid bacteria (Gobbetti et al., 2000; Muguerza et al., 2006; Nakamura, Yamamoto, Sakai, & Takano, 1995). Ingestion of sour milk fermented by *Lactobacillus helveticus* that contained ACE inhibitory tripeptides (Ile-Pro-Pro and Val-Pro-Pro) seemed to lower blood pressure modestly in mild hypertensive subjects (Tuomilehto et al., 2004).

There are a number of products on the market manufactured by international food/food ingredients companies, aimed at exploiting the functional food potential of milk protein derived hypotensive peptides (IPP, VPP, FFVAP-FEVFGK and whey peptides) (Fitzgerald, Murray, & Walsh, 2004). These products are either in the form of fermented milk drinks or as milk protein hydrolysates. Therefore, the purpose of this study was to develop a combination method using lactic acid fermentation and proteolysis of milk to produce a milk product which is more abundant in bioactive peptides than the traditional fermentation or proteolysis products of milk. The bioactive peptides were examined on ACE inhibitory activity and stability by simulating gastrointestinal digestion. Furthermore, the inhibition type of the bioactive substances on ACE and the antihypertensive effect on spontaneously hypertensive rats were also evaluated.

2. Materials and methods

2.1. Materials and chemicals

Whole milk powder and skimmed milk powder were obtained from Wei Chuan Foods Corporation (Taiwan). Lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) in powder form were purchased from Danisco Niebull Gmbh. (Alemanha, Germany). Flavourzyme (from *Aspergillus oryzae*, activity labeled 1160 LAPU/g) was purchased from Novo Nordisk A/S Co. (Denmark). Pepsin, Pancreatin, Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), Angiotensin I-converting enzyme of rabbit lung, Captopril and chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Lactic acid fermentation facilitated by protease

The mixture of 4.5% (w/v) skimmed milk powder, 5.5% (w/v) whole milk powder and 7% (w/v) sucrose was pasteurized (95 °C, 30 min) then fermented with 0.1% (w/v) of lactic acid bacteria powder at 43 °C. At the beginning of fermentation, flavourzyme, filtered through a 0.2 μM membrane, was added to the milk at 0.14% (w/v) (the ratio of enzyme to milk protein was 0.45:100, w/w) and the fermentation continued for a total of 5 h. The fermented milk was then heated at 98 °C for 10 min to inactivate the protease and lactic acid bacteria. The insoluble material was removed by centrifugation at $6000\times g$ for 30 min. The supernatant was filtered (No. 2 filter paper; Toyo Roshi Kaisha, Ltd. Tokyo, Japan). The filtrate was defined as whey fraction (pH 4.47) and lyophilized to a powder form ready for analyses or fed to spontaneously hypertensive rats (SHR) at 150 ml/day with constant concentration.

2.3. Chemical analyses

The soluble protein content of the whey powder was determined by the Folin–Lowry method (Lowry, Resebrough, Farr, & Randall, 1951; Cooper, 1977). One millilitre of the sample was mixed with 1 ml of an alkaline-copper reagent and 3 ml of the Folin–Ciocalteu's phenol reagent (Merck, Germany) at tenfold dilution with deionized water. After the solution was allowed to stand for 30 min, the absorbance at 540 nm was measured with a spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The soluble protein content was quantified using bovine serum albumin as standard.

2.4. Measurement of peptide content

The peptide content of whey was measured by the method of Church, Swaisgood, Porter, and Catignani (1983) with some modifications. The whey solution (50 mg/ml) was filtered by 0.2 μM membrane and the filtrate passed through ultrafiltration membrane with molecular weight (M.W.) cut-offs of 5000 Da (Millipore, Bedford, MA, USA). This permeate was defined as small peptides (M.W. $<$ 5000 Da). Fifty millilitres of reagent was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (w/w) sodium dodecyl sulfate, 40 mg of *o*-phthaldialdehyde solution (dissolved in 1 ml of methanol) and 100 μl of β-mercaptoethanol and then adjusted to 50 ml with deionized water. Fifty microlitres of this permeate was mixed with 2 ml of reagent. The reaction mixture was incubated for 2 min at ambient temperature, and the absorbance at 340 nm was measured with spectrophotometer (Model UV-160A, Shimadzu, Kyoto, Japan). The peptide content was quantified using casein tryptone (Difco Laboratories, Sparks, MD, USA) as standard.

2.5. In vitro assay for ACE inhibitory activity

The inhibitory activity against ACE was determined using reverse-phase high performance liquid chromatography (RP-HPLC) modified for the spectrophotometric assay (Cushman & Cheung, 1971; Wu & Ding, 2002). Fifteen millimolar of Hippuryl-L-histidyl-L-leucine (HHL) was dissolved in 100 mM Na-borate buffer (pH 8.3) containing 300 mM NaCl. Rabbit lung ACE was dissolved in the same buffer at a concentration of 8 mU/ml. A mixture contain-