Available online at www.sciencedirect.com
ScienceDirect
International Dairy Journal 17 (2007) 641–647
INTERNATIONAL DAIRY JOURNAL
www.elsevier.com/locate/idairyj

Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation

Guan-Wen Chen, Jenn-Shou Tsai*, Bonnie Sun Pan

Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan, ROC

Received 27 May 2005; accepted 1 July 2006

Abstract

Fresh low-fat milk was fermented with five mixed lactic acid bacteria for up to 30 h at 42 °C. A protease, prozyme 6, was added 5 h after the beginning of fermentation. The whey was separated from the fermented milk and freeze-dried. As the fermentation time extended to 30 h, soluble protein content increased from 30.9 to 195.9 mg g^{-1}, free amino acid content increased from 2.8 to 192.8 mg g^{-1}, peptide content increased from 6.4 to 402.8 mg g^{-1} and γ-aminobutyric acid (GABA) increased from 0 to 80.6 mg 100 g^{-1}, while inhibition of angiotensin I-converting enzyme (ACE) increased as indicated by a decrease of IC_{50} from 1.18 to 0.24 mg mL^{-1}, respectively. The amino acid sequences of two ACE inhibitory peptides were Gly–Thr–Trp and Gly–Val–Trp, of which the IC_{50} values were 464.4 and 240.0 μM, respectively. The systolic blood pressure and diastolic blood pressure of spontaneously hypertensive rat (SHR) were reduced 22 and 21.5 mm Hg, respectively, after 8 weeks of oral administration of diluted whey (peptide concentration 5 mg mL^{-1}) from the 30 h fermentation.

Keywords: Fresh low-fat milk; Fermented milk; Angiotensin I-converting enzyme (ACE); γ-aminobutyric acid (GABA); Spontaneously hypertensive rat (SHR); Antihypertensive effect

1. Introduction

Milk proteins are sources of peptides with biological activities, such as angiotensin I-converting enzyme (ACE) inhibitory peptides, immunostimulating peptides, antimicrobial peptides, opioid peptides (Dionysius & Milne, 1997; Kayser & Meisel, 1996; Meisel, 1997; Pihlanto-Leppälä, 2001; Sato, Noguchi, & Naito, 1983). ACE (dipeptidyl carboxypeptidase, EC3.4.15.1) plays an important role in regulating blood pressure. In the rennin angiotensin system, ACE cleaves the dipeptide portion of angiotensin I from the C-terminal and produces a potent vasopressor angiotensin II, which induces the release of aldosterone, and causes the retention of sodium ions by kidney and elevates the blood volume, thus increasing blood pressure (Skeggs, Kahn, & Shumway, 1956). In addition, ACE also inactivates the vasodilator, bradykinin (Erdös, 1975). Consequently, ACE inhibitors may exert an antihypertensive effect.

Several ACE inhibitory peptides have been isolated from the enzymatic hydrolysis of milk proteins (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; 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., 1995a; Yamamoto, Maeno, & Takano, 1999), or chemical synthesis of peptides according to milk protein sequences (Kohmura, Nio, & Ariyoshi, 1990; Maruyama et al., 1987; Mullally, Meisel, & FitzGerald, 1996). A number of ACE inhibitory peptides have been shown to be effective in lowering blood pressure of spontaneously hypertensive rats after oral administration (Abubakar et al., 1998; Nakamura, Yamamoto, Skai, & Takano, 1995b; Sekiya, Kobayashi, Kita, Imamura, & Toyama, 1992; Yamamoto, Akino, & Takano, 1994b).

*Corresponding author. Tel.: +886 2 2462 2192 x 5126; fax: +886 2 2462 0105.
E-mail address: tsaijs@mail.ntou.edu.tw (J.-S. Tsai).

0958-6946/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2006.07.004

However, enzymatic hydrolysis of milk protein can release several peptides that usually have undesirable bitter tastes (Habibi-Najafi & Lee, 1996; Matoba, Hayashi, & Hata, 1970). Hydrolysis of the milk protein continues through the combined action of chymosin and starter of *Lactobacillus casei* which can promote development of the typical cheese flavor and reduce the bitterness (Lemieux, Puchades, & Simard, 1989). Debittering of enzymatic hrolysates of soy protein and milk casein was done by using an aminopeptidase from *Grifola frondoas* and *Lactococcus lactis* ssp. *cremoris* AM2 (Bouchier, O'Cuinn, Harrington, & FitzGerald, 2001; Nishiwaki, Yoshimizu, Furuta, & Hayashi, 2002).

The purpose of this study was to develop a combination method using lactic acid fermentation and proteolysis on milk to produce a milk product that has no bitter taste but more abundant in bioactive peptides than the traditional fermentation or proteolysis products of milk. Furthermore, the antihypertensive effect of bioactive substances was also evaluated in spontaneously hypertensive rats (SHR) and the major ACE inhibitory peptides were purified and identified to confirm the bioactivities of these peptides derived from fermented milk whey.

2. Materials and methods

UHT fresh low-fat milk (Ying Chuan Co., Ltd. Taiwan) was purchased from local markets in Keelung, Taiwan. The mixed lactic acid bacteria (*Lb. casei*, *Lb. acidophilus*, *Streptococcus thermophilus*, *Lb. bulgaricus* and *Bifidobacterium*) in powder form were purchased from Lyo-San Inc. (Lachute, Quebec, Canada). Prozyme 6 (from *Aspergillus*; activity labelled 60,000 U g^{-1}) was purchased from Amano Pharmaceutical Co. (Nagoya, Japan). Hippuryl- L-histidyl-L-leucine (Hip–His–Leu), ACE of rabbit lung and other chemicals of analytical grade were purchased form Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Protease facilitated lactic acid fermentation

The UHT fresh low-fat milk was fermented with 0.1% (w/v) of lactic acid bacteria powder at 42 °C for 30 h. After 5 h of fermentation, Prozyme 6 filtered through a 0.2 μm membrane was added to the milk at a ratio of 1: 100 (w/w). The fermented milk was then heated at 98 °C for 10 min to inactivate the protease and lactic acid bacteria. Insoluble material was removed by centrifugation at 6000 × *g* for 30 min. The supernatant was filtered (No. 2 filter paper; Toyo Roshi Kaisha, Ltd. Tokyo, Japan). The filtrate was the whey fraction and lyophilized to a powder form ready for analyses or fed to SHR source-biological supplier at 50 mL day^{-1}.

2.2. Chemical analyses

The soluble protein content of the sample was determined by the Folin–Lowry method (Cooper, 1977; Lowry, Resebrough, Farr, & Randall, 1951).

The peptide content of whey was measured by method of Church, Swaisgood, Porter, and Catignani (1983) with some modification. Fifty milliliters of reagent were 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, the volume was then adjusted to 50 mL with deionized water. After filtration with a less than 5000 Da molecular mass cut-off filter (Millipore, Bedford, MA, USA) fifty microliters of the sample 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 a standard.

The free amino acid content of the sample was quantified using a modified method of Doi, Shibata, & Matoba (1981). The 0.5 mL of the sample was mixed with 1 mL of the Cd-ninhydrin solution (0.8 g of ninhydrin dissolved in a mixture of 80 mL of 99.5% ethanol and 10 mL of acetic acid, followed by the addition of 1 g of $CdCl_2$ dissolved in 1 mL of water) in a tube and rapidly transferred into a water bath at 84 °C for 5 min. After cooling to 25 °C, the absorbance at 507 nm was measured with a spectrophotometer (model UV-160A, Shimadzu, Kyoto, Japan). The free amino acid content was quantified using L-tyrosine as standard.

2.3. Assay for ACE inhibitory activity

The determination of ACE inhibitory activity was performed with reversed-phase high-performance liquid chromatography (RP-HPLC) modified from the spectrophotometric method described by Cushman and Cheung (1971) and Wu and Ding (2002). Five hundred milligram hippuryl-L-histidyl-L-leucine (HHL) was dissolved in 77.6 mL of 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 53.2 mU mL^{-1}. A mixture containing 75 μL of ACE solution and 75 μL of filtered sample (Conc. = 150 mg mL^{-1}) was incubated at 37 °C for 10 min, then 75 μL of HHL solution was added and incubated for 30 min. The reaction was stopped by addition of 250 μL of 1 M HCl. Ten μL of this solution was injected directly onto a Luna C_{18} column (4.6 × 250 mm, particle size, 5 μm; Phenomenex, Torrance, CA, USA) to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50% methanol in water (v/v) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.8 mL min^{-1} using a pump (model L-7100, Hitachi Co. Ltd., Tokyo, Japan) and the detector was monitored at 228 nm (UV/Visible detector 118, Gilson Medical Electronics, Villiers-le-Bel, France). The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity}\,(\%) = [(\text{Ec-Es})/(\text{Ec-Eb})]100, \quad (1)$$