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Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk

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Abstract

A total of 231 microorganisms were isolated from raw cow milk samples and the angiotensin-converting enzyme-inhibitory (ACEI) activity of the resultant fermented milk produced with the isolated microorganisms was assayed. Forty-six of these microorganisms were selected on the basis of high ACEI activity. Four *Enterococcus faecalis* strains stood out as producers of fermented milk with potent ACEI activity (IC_{50} (the protein concentration that inhibits 50% of ACE activity): 34–59 $\mu g\,mL^{-1}$). Single doses (5 $mL\,kg^{-1}$) of the whey fraction obtained from these fermented milk samples were administered to spontaneously hypertensive rats (SHR) and to normotensive Wistar-Kyoto (WKY) rats in order to investigate their possible antihypertensive activity. Highly significant decreases in the systolic blood pressure (SBP) and in the diastolic blood pressure (DBP) were observed when the fermented milk was administered to SHR. Nevertheless, the fermented milk did not modify the SBP and the DBP of the WKY rats. Raw cow milk is an excellent source of wild lactic acid bacteria able to produce fermented milk with antihypertensive activity and antihypertensive activity of milk fermented by *Enterococcus faecalis* strains was associated with peptides different from Ile-Pro-Pro and Val-Pro-Pro.

Keywords: Fermented milk; *Enterococus faecalis*; Antihypertensive effect; ACE-inhibitory activity

1. Introduction

Hypertension is an important problem in our society given its high prevalence and its role in cardiovascular diseases. The rennin–angiotensin–aldosterone system is a key factor in the maintenance of arterial blood pressure. One of the main components of this system is angiotensin-converting enzyme (ACE) [EC 3.4.15.1] (Ondetti, Rubin, & Cushman, 1977) which catalyzes the conversion of angiotensin I, an inactive decapeptide, into angiotensin II, an octapeptide with a potent vasoconstrictor action (Skeggs, Kahn, & Shumway, 1956). Moreover, ACE catalyzes the inactivation of bradykinin, which has an important vasodilation activity. Therefore, ACE plays an important role in the regulation of arterial blood pressure and inhibition of this enzyme can generate an antihypertensive effect. In fact, ACE-inhibitory (ACEI) drugs are commonly used to control arterial blood pressure.

Milk fermentation has been shown to be a successful strategy to produce ACEI and/or antihypertensive peptides (see reviews of Korhonen & Pihlanto-Lepälä, 2003; Gobbetti, Minervini, & Grizello, 2004; Silva & Malcata, 2005). In particular, different strains of lactic acid bacteria, such as *Lactobacillus helveticus* and *Lb. delbrueckii* subsp. *bulgaricus*, have been shown to

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produce fermented milk with high ACEI activity. An example is the production of antihypertensive milk fermented by *Lb. helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995a, b). The antihypertensive effect of this fermented milk, which has been commercialized in Japan (Calpis, Calpis Co. Ltd., Tokyo, Japan), has been demonstrated in spontaneously hypertensive rats (SHR) (Nakamura et al., 1995b) and in a clinical study with hypertensive patients (Hata et al., 1996). Similarly, milk fermented with *Lb. helveticus LBK-16H* has demonstrated significant antihypertensive effects in humans (Seppo, Kerojoki, Suomalainen, & Korpela, 2002; Seppo, Jauhiainen, Poussa, & Korpela, 2003). In both cases, the peptides Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP) have been identified as responsible for most of the ACEI activities of these fermented milk samples. IPP and VPP have also been found in casein hydrolysates produced by an extracellular proteinase of *Lb. helveticus* CP740 (Yamamoto, Akino, & Takano, 1994). Moreover, other peptides with ACEI activity have been isolated and characterized from milk fermented with *Lb. delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *cremoris* (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Ashar & Chand, 2003).

Several studies have reported the importance of measuring the antihypertensive activity in animal models, as in vitro ACEI activity is not always accompanied with antihypertensive activity (FitzGerald & Meisel, 2000).

The aim of this study was to select wild strains of bacteria from raw cows' milk which were able to produce fermented milk with inhibitory ACE activity. Moreover, we also investigated the antihypertensive effect of fermented milk produced with four selected strains of *E. faecalis* on the arterial blood pressure of SHR and normotensive Wistar-Kyoto rats (WKY).

2. Materials and methods

2.1. Substrates and chemicals

Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), rabbit lung powder containing ACE (5U) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Skim milk powder used for fermentation was FDA-Grade A milk. Distilled water used was obtained from a Millipore Milli-Q® system.

2.2. Screening of microorganisms and selection criteria

Selection criteria for the preliminary screening of microorganisms included the odor characteristics of raw milk samples after incubation at 42 °C for 48 h. Initially, incubated raw milk samples that presented an acceptable yoghurt or cheese flavour and visible coagulation were chosen for microorganism isolation. Gram staining and the catalase test were carried out for all isolated bacteria.

After this initial selection, microorganisms were chosen for their ability to generate fermented milk with high ACEI activity. Colony type, cellular morphology by Gram staining and fermentative characteristics using the API method (BioMerieux SA, Marcy l'Etoile, France) were also determined. The ACE inhibition assay was performed on the supernatant of the fermented milk after centrifugation at $20000 \times g$ for 10 min. An ACEI activity higher than 70% of the 50% diluted whey fraction was used as a selection criterion. Values of IC_{50} (the protein concentration that inhibits 50% of ACE activity) were determined for promising strains in terms of odour characteristics. The last selection criterion was based on the highest inhibitory activity and the absence of IPP and VPP peptides.

Selected microorganisms were incorporated to the Grupo Leche Pascual S.A. collection and some were deposited in the Spanish Type Culture Collection (*Colección Española de Cultivos Tipo*, CECT).

2.3. Isolation of microorganisms

Selected bovine milk aliquots of different sources were inoculated into MRS agar (Oxoid Ltd, Basingstoke, UK), specific for *Lactobacillus* genera, or into M17 agar (Biokar Diagnostics, Beauvais, France), specific for *Streptococcus* genera. Plates were incubated for 48 h at 42 °C in anaerobic conditions (AnaeroGenTM, Oxoid Ltd, Basingstoke, UK) or at 30 °C in aerobic conditions, respectively. Isolation of the different colonies was performed by consecutive re-inoculation. Pure bacterial cultures of isolated strains were maintained as stock cultures in spare tubes stored at 4 °C and subcultured periodically.

2.4. Preparation of fermented milk

Pre-cultures of isolated strains were prepared on reconstituted 10% (w/w) skimmed milk powder and sterilized at 100 °C for 20 min. The milk was inoculated with a loop of the corresponding stock culture to yield an initial bacterial concentration of 10^5–10^7 colony forming units (CFU) mL^{-1}. Incubation was performed overnight at 30 or 42 °C depending on the strain. Fermented milk batches were prepared with sterile reconstituted 10% (w/w) skimmed milk powder. A corresponding pre-culture (3%, v/v) was added and fermentation was carried out for 48 h at the same temperature as the analogous pre-culture. The fermentation process was stopped by pasteurization of the fermented milk at 75 °C for 1 min. At the end of the fermentation, the pH of the fermented milk was directly