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Enterococcus faecalis strains from food, environmental, and clinical origin produce ACE-inhibitory peptides and other bioactive peptides during growth in bovine skim milk

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ABSTRACT

Enterococcus faecalis isolates from food and environmental origin were evaluated for their angiotensin-converting enzyme (ACE)-inhibitory activity (ACE-IA) after growth in bovine skim milk (BSM). Most (90% active) but not all (10% inactive) *E. faecalis* strains produced BSM-derived hydrolysates with high ACE-IA. Known ACE-inhibitory peptides (ACE-IP) and an antioxidant peptide were identified in the *E. faecalis* hydrolysates by reversed-phase high-performance liquid chromatography–tandem mass spectrometry (RP-HPLC–MS/MS). Antimicrobial activity against *Pediococcus damnosus* CECT4797 and *Listeria ivanovii* CECT913 was also observed in the *E. faecalis* hydrolysates. The incidence of virulence factors in the *E. faecalis* strains with ACE-IA and producers of ACE-IP was variable but less virulence factors were observed in the food and environmental strains than in the clinical reference strains. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) based analysis demonstrated that food and environmental *E. faecalis* strains were genetically different from those of clinical origin. When evaluated, most *E. faecalis* strains of clinical origin also originated BSM-derived hydrolysates with high ACE-IA due to the production of ACE-IP. Accordingly, the results of this work suggest that most *E. faecalis* strains of food, environmental and clinical origin produce BSM-derived bioactive peptides with human health connotations and potential biotechnological applications.

1. Introduction

Apart from their basic nutritional role many dietary proteins contain encrypted within their primary structure bioactive peptides with beneficial effects upon human health, once released by digestive enzymes during their gastrointestinal transit, by fermentation or ripening during food processing or by hydrolysis with specific proteolytic enzymes and food grade proteolytic preparations sourced from animals, plants, and microbes. Some of the released bioactive peptides exhibit antimicrobial, antioxidant, antithrombotic, antihypertensive, immunomodulatory, opioid, and antiproliferative activities, among others (López Expósito and Recio, 2008; Mills et al., 2011).

Among the compounds derived from dietary proteins those with angiotensin-converting enzyme (ACE)-inhibitory activity (ACE-IA), known as ACE-inhibitory peptides (ACE-IP), have been studied extensively due to their potential use in the treatment of elevated blood pressure and associated cardiovascular events (Phelan and Kerins, 2011). Therefore, ACE-IP derived from food substrates are of interest in the development of functional foods with ACE-IA (Chen et al., 2009; Hernández-Ledesma et al., 2011; Martínez-Maqueda et al., 2012; Sánchez et al., 2011).

Lactic acid bacteria (LAB) are able to produce ACE-IP during growth in milk by the action of extracellular proteases, cell wall proteinases and intracellular peptidases (Korhonen and Pihlanto, 2006; Otte et al., 2011; Yamamoto et al., 1994). For production of fermented milk with ACE-IA thermophilic and mesophilic LAB of dairy origin from the genera *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus* have been evaluated (Gobetti et al., 2000; Kilpi et al., 2007; Minervini et al., 2003; Muguerza et al., 2006; Nakamura et al., 1995). Other LAB such as *Lactobacillus animalis* DPC6134, isolated from the porcine small intestine, also produce ACE-IP after growth in

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Table 1
ACE-inhibitory activity (ACE-IA)[a] of supernatants of *E. faecalis* strains grown in bovine skim milk (BSM).

Strain	Origin	BSM culture modifications	IC_{50} (μg protein/ml)	Source or reference[b]
E. faecalis BCS27	Dry salted cod	Hydrolysate	28.4	DNBTA; Gómez-Sala (2006)
E. faecalis BCS53	Dry salted cod	Hydrolysate	26.3	DNBTA; Gómez-Sala (2006)
E. faecalis CECT184	Cow milk	Hydrolysate	16.1	CECT
E. faecalis CECT4039	Cow cheese	Flocculate	NA	CECT
E. faecalis CGV67	Norway lobster	Hydrolysate	24.9	DNBTA; Gómez-Sala (2006)
E. faecalis DBH18	Mallard ducks	Hydrolysate	23.8	Sánchez et al. (2007)
E. faecalis LG101	Buffalo milk	Hydrolysate	16.1	DNBTA, Citti (2005)
E. faecalis LG187	Buffalo milk	Hydrolysate	25.2	DNBTA; Citti (2005)
E. faecalis P77	Octopus	Curd	NA	DNBTA; Gómez-Sala (2006)
E. faecalis QA12	Aged buffalo cheese	Hydrolysate	22.8	DNBTA; Citti (2005)
E. faecalis QA21	Aged buffalo cheese	Hydrolysate	19.5	DNBTA; Citti (2005)
E. faecalis QA53	Aged buffalo cheese	Hydrolysate	24.8	DNBTA; Citti (2005)
E. faecalis QA127	Aged buffalo cheese	Hydrolysate	20.8	DNBTA; Citti (2005)
E. faecalis QF386	Buffalo mozzarella	Hydrolysate	21.4	DNBTA; Citti (2005)
E. faecalis QM38	Buffalo cheese	Hydrolysate	17.8	DNBTA; Citti (2005)
E. faecalis SDM37	Sardine	Hydrolysate	25.1	DNBTA; Gómez-Sala (2006)
E. faecalis SDP10	Sardine	Hydrolysate	26.3	DNBTA; Gómez-Sala (2006)
E. faecalis SMF10	Fresh salmon	Hydrolysate	23.5	DNBTA; Gómez-Sala (2006)
E. faecalis SMF19	Fresh salmon	Hydrolysate	20.8	DNBTA; Gómez-Sala (2006)
E. faecalis SMF54	Fresh salmon	Hydrolysate	27.3	DNBTA; Gómez-Sala (2006)

NA: No ACE-IA detected.
[a] ACE-inhibitory activity (ACE-IA) as IC_{50} (μg protein/ml). Data are mean from two independent determinations in triplicate.
[b] Source abbreviations: CECT, Colección Española de Cultivos Tipo (Valencia, Spain); DNBTA, Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain).

bovine sodium caseinate (Hayes et al., 2007). Fermentation conditions and the proteolytic activity of the specific LAB strains seem to modulate the ACE-IA and production of ACE-IP (Nielsen et al., 2009; Otte et al., 2011). From studies in progress by our research group on the evaluation of LAB as potential cell factories for production of bioactive peptides, the aim of this work was to evaluate *Enterococcus faecalis* strains from different sources as producers of bovine skim milk (BSM)-derived hydrolysates with high ACE-IA.

2. Materials and methods

2.1. Microbial strains and growth conditions

Most of the *Enterococcus faecalis* strains evaluated in this work are shown in Table 1. *E. faecalis* P4 and *E. faecalis* P36 are medical isolates of human origin (Eaton and Gasson, 2001) and *E. faecalis* 3Er1 and *E. faecalis* H10 are human-clinical isolates of the multilocus sequence typing (MLST)-derived clonal complex 2 (CC2) and CC9, respectively (Ruiz-Garbajosa et al., 2006) obtained from the Servicio de Microbiología, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Hospital Universitario Ramón y Cajal, Madrid (Spain). Reconstituted 10% (w/v) bovine skim milk-powder (BSM) (Oxoid, Cambridge, UK), heated at 121 °C for 5 min, was seeded with a loop of the frozen stock culture (−80 °C) and the cultures were grown at 37 °C for 24–48 h. A 3% (v/v) of the grown culture was then added to freshly made reconstituted 10% (w/v) BSM and the cultures were further grown at 37 °C for 24 h. The cultures were then centrifuged at 8000 ×*g* for 10 min and their supernatants were filtered through 0.20 μm pore-size filters (Whatman Int. Ltd., Maidstone, UK) and stored at −20 °C before use.

2.2. Determination of the ACE-inhibitory activity (ACE-IA)

The ACE-IA of the supernatants of the *E. faecalis* strains grown in BSM was determined by a fluorometric assay (Sentandreu and Toldrá, 2006), with modifications (Quirós et al., 2009). The angiotensin converting enzyme (ACE, peptidyl-dipeptidase A, EC 3.4.15.1) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the ACE working solution was added to blank (B), control (C) or samples (S). The reaction was started by adding the fluorogenic substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-Phe (NO_2)-Pro-OH) (0.45 mM, Bachem Feinchemikalien, Bubendorf, Switzerland), and the reaction mixture was incubated at 37 °C. The fluorescence of the samples was measured in a Multiscan Microplate Fluorimeter (FLUOstar optima, BMG Labtech, Offenburg, Germany) with the FLUOstar (version 1.32 R2, BMG Labtech) control system for processing of the data. The ACE-IA was expressed as the protein concentration required to inhibit 50% of the ACE activity (IC_{50}). The percentage of ACE-inhibitory activity was calculated as: $100 \times (C - S) / (C - B)$. This parameter was plotted versus protein concentration and non-linear adjustment was performed to estimate the IC_{50} as previously described (Quirós et al., 2007). The protein concentration of the water-soluble extracts was determined by the Kjeldahl method or the BCA protein assay (Pierce, Thermo Fisher Scientific Inc., Rockford, IL USA).

2.3. Analysis of the BSM-derived hydrolysates by reversed-phase high-performance liquid chromatography–tandem mass spectrometry (RP-HPLC–MS/MS)

The RP-HPLC–MS/MS analysis of the supernatants obtained after growth of the *E. faecalis* strains in BSM was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) with column HiPore® (RP318 C_{18} column 250 × 4.6 mm, 5 μm of particle size; Bio-Rad, Richmond, CA, USA). The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source, as previously described (Hernández-Ledesma et al., 2004). Solvent A was a mixture of water–trifluoroacetic acid (1000:0.37, v/v) and solvent B contained acetonitrile–trifluoroacetic acid (1000:0.27, v/v). Peptides were eluted with a linear gradient of solvent B in A from 0% to 45% in 60 min at a flow rate of 0.8 ml/min. Different spectra were recorded over the mass/charge (m/z) range 100–1500. About 5 spectra were averaged in the MS and in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10,000 (i.e., 5% of the total signal) and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp from 0.35 to 1.4 V. The m/z spectral data was transformed to mass values by using the Data Analysis™ (version 3.0; Bruker Daltonik) control program. The BioTools (version 2.1; Bruker Daltonik) was used to process the MS(n) spectra for peptide identification and sequencing.