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Dairy yeasts produce milk protein-derived antihypertensive hydrolysates

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ABSTRACT

The potential of 20 dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species was examined for the production of milk protein-derived antihypertensive hydrolysates. For this purpose yeast strains were grown in microbiological medium with casein or lactoferrin as sole nitrogen source, and the inhibitory effects of casein and lactoferrin hydrolysates (CSHs and LFHs) on angiotensin I-converting enzyme (ACE) activity were determined. Based on the ACE-inhibitory activity, four CSHs and five LFHs were selected, and permeate fractions with molecular masses lower than 3 kDa (pCSHs and pLFHs) were obtained. *In vitro* ACE-inhibitory potencies (IC_{50}) of permeates varied from 18.8 to 87.6 μg/ml (pCSHs) and from 50.2 to 500 μg/ml (pLFHs). *K. marxianus* Km2 strain grown on either casein or lactoferrin produced the most potent permeates. pCSHs and pLFHs were orally administered to spontaneously hypertensive rats (SHRs) and exerted *in vivo* antihypertensive effect. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of selected yeasts to produce orally effective antihypertensive milk protein-derived hydrolysates.

1. Introduction

Food derived bioactive peptides are attracting increasing interest because of their variety and multifunctionality. Undoubtedly, those with blood pressure-lowering effects are receiving increasing attention due to the worldwide growing prevalence of hypertension (Kearney et al., 2005). One of the main targets for the treatment of hypertension is the renin angiotensin system (RAS), and its inhibition at three possible levels, angiotensin-converting enzyme (ACE), upstream renin activity or downstream angiotensin receptors, is the pharmacological basis for commonly used antihypertensive drugs (Fragasso et al., 2012). ACE, which hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive metabolite, is also the main target for antihypertensive food-derived peptides developed as an alternative to drugs (reviewed in Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012).

Nowadays milk proteins are the main source of antihypertensive peptides. Among the approaches for releasing ACE-inhibitory peptides from intact milk proteins, fermentation with proteolytic lactic acid bacteria (LAB) to partially digest the caseins during the manufacture of dairy products is a successful strategy (reviewed in Hernández-Ledesma, Contreras, & Recio, 2011). Moreover, milk fermented with *Lactobacillus helveticus*, containing the casein-derived peptides VPP and IPP, has shown significant antihypertensive effects in rats and humans (Hata et al., 1996; Nakamura, Yamamoto, Sakai, & Takano, 1995; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Seppo, Kerojoki, Suomalainen, & Korpela, 2002). Also enterococci from dairy origin are able to hydrolyse casein into peptides with ACE-inhibitory activity and antihypertensive effect (Chaves-López et al., 2011; Muguerza et al., 2006); however the pathogenic potential of some *Enterococcus* strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in food production.

Yeast products have been used for many years as ingredients and additives in food processing, although their potential bioactivity has been less investigated (Abbas, 2006; Dawson, 2002). Yeasts isolated from dairy environments have proteolytic character (Jakobsen & Narvhus, 1996) and thus potential for releasing bioactive peptides. *Kluyveromyces marxianus* was pointed out as a promise candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin, alone (Belem, Gibbs, & Lee, 1999) or in combination with *Lactobacillus rhamnosus* (Hamme, Sannier, Piot, & Bordenave-Juchereau, 2009; Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009). Recently *K. marxianus* isolated from

Abbreviations: ACE, angiotensin I-converting enzyme; CSH, casein hydrolysate; GRAS, generally recognized as safe; LAB, lactic acid bacteria; LFH, lactoferrin hydrolysate; pCSH, casein hydrolysate permeate with molecular mass lower than 3 kDa; pLFH, lactoferrin hydrolysate permeate with molecular mass lower than 3 kDa; RAS, renin angiotensin system; RP-HPLC, reversed phase-high performance liquid chromatography; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TFA, trifluoroacetic acid.

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Colombian kumis, a traditional low alcoholic fermented cow milk, was able to produce fermented milk with ACE-inhibitory activity (Chaves-López et al., 2012). However the *in vivo* antihypertensive effect of casein- and whey-derived bioactive peptides generated by yeast strains has not been demonstrated yet.

In contrast to the aforementioned milk proteins, the potential of bovine lactoferrin, a well-characterized component of milk whey, as a source of antihypertensive peptides has been much less explored. We have reported the inhibitory effects of enzymatic lactoferrin hydrolysates on ACE activity and also their antihypertensive effect in spontaneously hypertensive rats (SHRs), suggesting their potential application as nutraceutical in the treatment of hypertension (Fernández-Musoles et al., 2013; Ruiz-Giménez et al., 2012). However, there is a lack of information about the feasibility of using proteolytic microorganisms to generate new lactoferrin-derived peptides with antihypertensive effects.

The objective of the present study was to further investigate the potential of yeasts to generate milk protein-derived peptides with antihypertensive effect. For this purpose, different strains of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus* isolated from cheeses were screened for their ability to grow in media with casein or lactoferrin as sole nitrogen source and to produce protein hydrolysates containing ACE-inhibitory peptides. Selected casein and lactoferrin raw hydrolysates (CSHs and LFHs, respectively) were ultrafiltered to obtain permeates enriched in peptides of molecular weight lower than 3 kDa (pCSHs and pLFHs) which ACE-inhibitory potency was evaluated. Finally the antihypertensive effect of most potent permeates was assessed in SHRs.

2. Materials and methods

2.1. Materials

Bovine lactoferrin was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Casein (Promilk 85) was obtained from Ingredia (Arras Cedex, France). ACE from porcine kidney, captopril, and bicinchoninic acid protein assay kit were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). o-Aminobenzoylglycyl-p-nitrophenylalanylproline was provided by Bachem Feinchemikalien (Bubendorf, Switzerland).

2.2. Yeast strains and growth conditions

Twenty yeast strains belonging to *D. hansenii* (10), *K. lactis* (8) and *K. marxianus* (2) species isolated from artisanal ewes' and goats' milk cheeses were used in this study. Yeast strains were maintained on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar, pH 5.5).

2.3. Preparation of lactoferrin and casein hydrolysates

Stock solutions of lactoferrin and casein were sterilized by autoclaving at 121 °C, 15 min. Lactoferrin (1.5% lactoferrin, 2% glucose) and casein medium (2% casein, 2% glucose) were inoculated with 10^8 and 10^6 cells/ml, respectively, from pre-cultured strains on GPY (GPYA without agar), incubated at 28 °C and 100 rpm in an orbital shaker. For the initial screening, yeast strains were grown in 4 ml of casein or lactoferrin medium. Selected strains were cultured in five 200 ml-batches of casein and lactoferrin medium. At the end of the incubation period (7 days for casein medium and 14 days for lactoferrin medium), yeast cells were eliminated by centrifugation (13,000 rpm, 10 min), and the supernatants were considered as casein and lactoferrin hydrolysates (CSHs and LFHs). Proteolysis and ACE-inhibitory activity of hydrolysates were determined as specified further.

Selected CSHs and LFHs were subjected to ultrafiltration through a VivaFlow 50 crossflow cassette with a cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France). Resulting permeates, enriched in peptides of molecular weight lower than 3 kDa were named pCSHs and pLFHs. Protein concentration of permeates was estimated by the bicinchoninic acid method (BCA) using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.4. Determination of extent of proteolysis

CSHs and LFHs were analyzed by reversed phase-high performance liquid chromatography (RP-HPLC) using a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector and a 717 plus Autosampler. Hydrolysates (90 μl) were applied to a Symmetry® C_{18} 5 μm, 4.6 × 150 mm column (Waters). The column was developed at a flow rate of 1 ml/min at 40 °C. Elution was performed with a linear gradient of solvent B (acetonitrile with 1% TFA) in solvent A (water with 1% TFA) from 0 to 80% in 60 min. Detection of peptides and proteins was carried out at 214 nm. Extent of proteolysis, expressed as percentage, was calculated from the chromatographic peak areas of either casein or lactoferrin in hydrolysates at the end of the incubation time *versus* peak areas at time zero.

2.5. In vitro assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of CSHs and LFHs was measured using the fluorescent method described by Sentandreu and Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitrophenylalanylproline by the action of ACE.

Effects on ACE activity of hydrolysates (20 μl) were expressed as percentage of ACE activity inhibition calculated with respect to a control without hydrolysate. Duplicate assays were done and ACE-inhibitory activity was expressed as mean percentage ± SEM.

The IC_{50} value of pCSHs and pLFHs was defined as the protein concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 10.0 (SPSS Inc., Chicago, IL, USA).

2.6. In vivo assay of antihypertensive effect in SHRs

Ten male SHRs weighing 250–300 g were used (Charles River Laboratories, Barcelona, Spain). Rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles and consumed tap water and standard diets *ad libitum*. Experimental procedures were conducted in accordance with the Spanish legislation on 'protection of animals used for experimental and other scientific purposes' and to the Directives of the European Community on this subject. The study was approved by the 'Ethics Committee for Animal Welfare' of Hospital 'La Fe'.

Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted non-invasive blood pressure equipment (LE5001 unit with LE5160R cuff & transducer, Panlab Harvard Apparatus, Cornellá, Barcelona, Spain). Permeates (200 mg/kg) were orally administered by gastric intubation in 750 μl of physiological saline. Before the measurements, rats were kept at 37 °C during 15 min to make the pulsations of the tail artery detectable. The SBP was measured before peptide intake (zero time), 1, 2, 3, 4 and 24 h after intake. Physiological saline (750 μl) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the absolute difference (in mm Hg) with respect to the basal values of measurements obtained just before starting the treatments.