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ACE-inhibitory peptides from bovine caseins released with peptidases from *Maclura pomifera* latex

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ABSTRACT

In work reported here, a proteolytic extract prepared from *Maclura pomifera* latex was employed to hydrolyze bovine caseins. Densitograms of Tricine–sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) indicated that the caseins were considerably degraded after a 10-min reaction. The degree of hydrolysis determined by the 2,4,6-trinitrobenzenesulfonic-acid method was 17.1 ± 0.7% after 180 min of digestion. The concentration of small peptides increased with hydrolysis time, and analysis by reverse-phase high-performance liquid chromatography (RP HPLC) and mass spectrometry, revealed a virtually unchanged peptide profile. These results suggested that those proteases were highly specific, as only certain peptide bonds were cleaved. The hydrolysate of 180 min displayed the highest inhibition of angiotensin-converting enzyme (ACE) showing an IC_{50} of 1.72 ± 0.25 mg/mL, and the analysis of the peptide fractionation in this hydrolysate by RP HPLC exhibited two peaks responsible for that activity. Fragmentation analysis through the use of iterated matrix-assisted–laser-desorption-ionization–time-of-flight mass spectrometry (MALDI-TOF/TOF MS/MS) with the aid of bioinformatics tools enabled us to deduce two peptide sequences—one, YQEPVLGPVRGPFPIIV, having been previously reported as an ACE-inhibitor; the other, RFFVAPFPE, as yet undescribed. The presence of bioactive peptides in these casein hydrolysates argues for their potential use in the development of functional foods.

1. Introduction

Milk is a complex fluid containing literally hundreds of molecular species and providing energy (supplied by lipids and lactose), essential and nonessential amino acids (supplied by proteins), essential fatty acids, vitamins, inorganic elements, and water (O'Mahony & Fox, 2014). Cow milk possesses a protein concentration of approximately 32–38 g/L. This high-quality protein consists of about 20% whey proteins, whose major components are α-lactalbumin and β-lactoglobulin, and 80% caseins, divided into four major fractions, αS1-, αS2-, β-, and κ-casein, which are arranged in micelles (Gellrich, Meyer, & Wiedemann, 2014).

Among the different dairy products available on the market nowadays, protein hydrolysates constitute one of the most significant since they are obtained with the purpose of meeting special dietary needs (Clemente, 2000; Soares et al., 2006), increasing digestibility (Koopman et al., 2009), reducing allergenicity (Bu, Luo, Chen, Liu, & Zhu, 2013), or containing small peptides with biologic actions (Korhonen, 2009). To achieve those ends, proteolytic enzymes coming from various animal, plant, or microbial sources are used to digest food proteins (Aleixandre, Miguel, & Muguerza, 2008; Silva, Pihlanto, & Malcata, 2006).

A hydrolysate containing bioactive peptides can be considered as a functional food since it provides health benefits beyond basic nutrition and, being similar in appearance to conventional food, is therefore suitable for consumption as part of a normal diet (Siro, Kapolna, Kapolna, & Lugasi, 2008). Bioactive peptides are specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Pérez Espitia et al., 2012). The size of these peptides can vary from two to twenty amino-acid residues, and their specific activity is based on their sequence and-amino acid composition. Milk proteins are considered one of the richest sources of these compounds (Korhonen & Pihlanto, 2006). Bioactive peptides from bovine caseins comprise—among other species—the caseinophosphopeptides, involved in the transport and absorption of certain minerals; isracidin, an immunomodulator; the casomorphins and casoxins, respective opioid-receptor agonists and antagonists; and the casokinins, antihypertensive agents (Phelan, Aherne, FitzGerald, & O'Brien, 2009). The last of these inhibits the ACE that plays a central role in blood-pressure regulation through the renin-angiotensin-aldosterone system. Some of the

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bovine casokinins correspond to peptides f23–24, f23–27, and f194–199 from α_{S1}-casein, as well as to f177–183, and f193–202 from β-casein, among others (Silva & Malcata, 2005). Casokinins such as Ile-Pro-Pro and Val-Pro-Pro from β-casein are present in commercial dairy products with antihypertensive properties such as "Calpis" (Sour milk) from Calpis Co., Japan, and Evolus (a calcium-enriched fermented milk drink) produced by Valio Oy, Finland (Korhonen, 2009).

The Osage orange *Maclura pomifera* (Raf.) Schneid (Moraceae) is an ornamental species cultivated in Argentina, whose fruit latex contains unusual quantities of serine peptidases that have been used to hydrolyze soy and whey proteins (Bertucci, Liggieri, Colombo, Vairo Cavalli, & Bruno, 2015; Ortiz & Añón, 2001) and to clot bovine milk (Corrons, Bertucci, Liggieri, López, & Bruno, 2012). The aim of the present work was to prepare and characterize bovine casein hydrolysates through the use of peptidases from *M. pomifera* and to screen for and analyze the released bioactive peptides with ACE-inhibitory activity.

2. Materials and methods

2.1. Chemicals

Bovine-serum albumin (BSA), captopril, casein, rabbit-lung ACE, 2,4,6-trinitrobenzenesulfonic acid (TNBS), potassium persulfate, Tris(hydroxymethyl)aminomethane (Tris), and zinc chloride were purchased from Sigma Chemical Company (St. Louis, MO, USA); Coomassie Brilliant Blue G-250, acrylamide, bisacrylamide, molecular-weight standards and Tricine from Bio-Rad (Hercules, CA, USA); α-cyano-4-hydroxycinnamic acid (HCCA) from Bruker Daltonics (Billerica, MA); Abz-PheArgLys(DNP)Pro-OH from Bachem (Bubendorf, Switzerland), trifluoroacetic acid (TFA) from J.T. Backer (Philipsburg, NJ, USA), and L-leucine and trichloroacetic acid from Carlo Erba Reagenti (Rodano, MI, Italy). All other chemicals were obtained from local commercial sources and were of the highest purity available.

2.2. Preparation and characterization of plant proteolytic extracts

Ten mature fruits of *M. pomifera* were cut from a single tree (La Plata, Province of Buenos Aires, Argentina) and latex (5 mL) obtained by making incisions in the fruit and collecting the resulting drops of fluid in 80 mL of 0.1 M phosphate buffer (pH 6.6) containing 5 mM ethylenediaminetetraacetic acid and 5 mM cysteine, in order to avoid phenoloxidase activity and oxidation, respectively. The suspension was centrifuged at 16,000*g* and 4 °C for 20 min; and the resulting supernatant solution, referred to as pomiferin, was stored in aliquots of 1 mL at −20 °C. To remove phenolic compounds, partially purified extracts were prepared by precipitation with one volume of cold ethanol (Bertucci et al., 2015) and termed purified pomiferin extract (PPE). The proteolytic activity of PPE was determined on casein as a substrate and expressed as caseinolytic units per mL (Ucas/mL), as described by Priolo, López, Arribére, Natalucci, and Caffini (1991). The protein content was determined by the Bradford method (Bradford, 1976), with BSA as the standard. Specific activity was calculated as the ratio between caseinolytic activity and protein concentration (*i.e.*, Ucas/mg).

2.3. Casein-hydrolysates preparation

The bovine-casein substrate was prepared by dissolving 12.5 g casein in 1 L 0.1 M Tris-HCl buffer (pH 9.0), at 100 °C for 20 min with stirring. In order to remove protein aggregates, this hot suspension was then quickly filtered through cellulose filter paper. The hydrolysis of bovine-casein was performed by mixing 10 mL of PPE (1/5 dilution) with 90 mL of this casein suspension. Reactions were carried out at pH 9.0, 45 °C and stopped at different times (10, 30, 60, 90, and 180 min) by heat-shock inactivation of the peptidases at 100 °C for 10 min. Blanks were prepared with casein or PPE at the same dilution as in the reaction mixture through the addition of the appropriate amount of water only. Aliquots of each hydrolysate were filtered by centrifuging through 3-kDa Amicon Ultra-15 filter units (Millipore). The filtered and unfiltered hydrolysates were stored at −20 °C. In Addition, filtered hydrolysates were lyophilized and stored. The peptide concentration of the hydrolysates was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.4. Determination of degree of hydrolysis (DH)

The DH of the casein hydrolysates was determined spectrophotometrically by the TNBS method (Adler-Nissen, 1979), which assay is based on the reaction of the primary amino groups from the hydrolyzed caseins with this reagent. Hydrolysate samples were diluted with 0.213 M Na_2HPO_4 containing 1 g/100 mL sodium dodecyl sulfate (SDS), pH 8.2 to a maximum protein concentration of 1 mg/mL. L-Leucine was used as a standard (concentration range: 0–2.25 mM). Of the samples, 40 μL were mixed with 320 μL of the same phosphate buffer plus 320 μL of a solution of 0.5 mL/100 mL aqueous TNBS, followed by incubation for 1 h at 50 °C in the dark. The reaction was stopped by the addition of 640 μL 0.1 M HCl at room temperature in the dark and the absorbance measured at 340 nm 1 h later. The DH was expressed as the percent cleavage of peptide bonds with respect to the total number of peptide bonds per protein equivalent (Nielsen, Petersen, & Dambmann, 2001).

2.5. Tricine SDS-PAGE

Samples of the hydrolysates after different digestion times along with the appropriate blanks were analyzed by denaturing electrophoresis in Tricine SDS-PAGE (Corrons et al., 2012). The following molecular markers were employed: phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme; corresponding to 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa, respectively (Bio-Rad; low range). Electrophoresis was carried out in a Mini-Protean III cell (Bio-Rad Laboratories, Watford, UK) with a Tricine SDS-PAGE composed of a stacking gel, a separating gel, and a resolution gel; which combination proved especially suitable for resolving the mixture of peptides produced. The gels were placed in a fixative solution containing 50 mL of methanol, 10 mL of acetic acid, and 40 mL of water, and were then stained with Coomassie Brilliant Blue G-250 for 2 h at room temperature, followed by an overnight destaining. The electrophoretic profiles were analyzed by densitography through the use of the ImageJ software (Mac OS X, W. Rasband, National Institute of Mental Health, Bethesda, MD, USA).

2.6. Analysis and partial purification of peptides in hydrolysates by RP-HPLC

Unfiltered and filtered casein hydrolysates were applied to a Bondclone 10 C-18 column (00H-2117-C0, Phenomenex), operating at room temperature in an ÄKTA-Purifier chromatograph (GE, Uppsala, Sweden) at a flow rate of 2 mL/min. The column was equilibrated with 4 column volumes (CV) of 0.05 mL TFA in 100 mL water (Solution A) before each sample injection. Then, a basic linear gradient from 100% Solution A to 100% of 0.025 mL TFA in 100 mL acetonitrile (Solution B) was applied in 35 CV (61 min) to obtain a characteristic peptide profile. Alternatively, a step gradient from 100% Solution A to 30% Solution B in 29 CV (51 min) was used to separate groups of peptides at a flow rate of 2 mL/min. The elution was monitored by measurement of absorbance at either 280 or 215 nm.

2.7. Mass spectrometric analysis

The molecular weights of peptides in filtered hydrolysates were analyzed by MALDI-TOF MS. One microliter of each sample was mixed with 1 μL of crystallization-matrix solution (10 mg/mL HCCA in acetonitrile: water 1:2 with 0.1 g/100 mL TFA) and then deposited onto a