LWT - Food Science and Technology 64 (2015) 157–163

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Application of peptidases from *Maclura pomifera* fruit for the production of active biopeptides from whey protein

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ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form
23 April 2015
Accepted 22 May 2015
Available online 2 June 2015

Keywords:
Plant peptidase
Maclura pomifera
Whey hydrolysate
Bioactive peptide

ABSTRACT

A crude extract containing serine peptidases, was prepared from latex of *Maclura pomifera* fruits. Peptidases were isolated by precipitation with one volume of ethanol with a yield of 5.4 ± 0.4 Ucas per milligram of protein. This extract was used for hydrolysis of bovine whey proteins at 45 °C and pH 6.5. Proteolytic activity was 99% inactivated after 5 min of heat treatment (100 °C). Major whey proteins degradation profile was analysed by tricine SDS-PAGE. After 180 min of hydrolysis alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) were almost completely degraded. Hydrolysis degree was 31.3 ± 1.7% at 180 min of reaction and the peptides produced that were smaller than 3 kDa were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). Angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant capacity were detected in the hydrolysates and IC_{50} values for 180 min of hydrolysis were 0.53 ± 0.02 and 4.44 ± 0.44 mg/ml, respectively. One peptide sequence deduced from peptide masses in the 180 min filtered hydrolysate, coincided with an ACE-inhibitory peptide reported by other author. The results support the conclusion that, by the presence of ACE-inhibitory and antioxidant peptides, it would be possible to use these whey protein hydrolysates for functional food manufacturing.
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1. Introduction

Whey has historically been considered a by-product of cheese manufacturing process and some important regulations have prevented disposal of untreated whey. Nowadays whey is increasingly being viewed as more than a source of proteins with a particularly nutritive composition of essential amino acids (Smithers, 2008; Walzem, Dillard, & German, 2002). Whey protein accounts for about twenty percent of bovine milk protein and mainly consisting of α-LA, β-LG, bovine serum albumin (BSA), and immunoglobulins (Aoi et al., 2011); whereas minor components are lactoferrin, lactoperoxidase and various growth factors (Damodaran, 2000).

Hydrolysates are widely used in food technology due to their nutritional or functional properties and are produced from several protein sources by appropriate proteases, microbial fermentation or heating treatment with acid (Sarmadi & Ismail, 2010). Each hydrolysate is a peptide mixture of different chain length and a pool of free amino acids (Manninen, 2009). In enzymatic hydrolysis, the composition of hydrolysates depends on both, type of protease and source of protein used, as well as hydrolysis conditions set in reaction (Benitez, Ibarz, & Pagan, 2008).

Randomly, hydrolysis of food proteins could produce desirable effects, such as reducing allergenicity, achieving specific dietary requirements or improving functional properties (Van der Ven et al., 2002). In this way, studies support that using appropriate proteases, biologically active peptides encrypted in certain proteins could be released (Li-Jun, Chuan-he, & Zheng, 2008). Whey protein hydrolysates have great health improvement potentials including antimicrobial, immunomodulatory, antioxidant, antihypertensive and anticancer activities (De Carvalho-Silva et al., 2012).

Hypertension is a key factor in the development of cardiovascular diseases. In view of its prevalence and importance, changes in life-style, dietary approaches and pharmacological treatments are broadly applied to treat hypertension (López-Fandiño, Otte, & Van Camp, 2006). Antihypertensive biopeptides inhibit Angiotensin-converting enzyme (ACE), which plays an important role in the

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http://dx.doi.org/10.1016/j.lwt.2015.05.041
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regulation of blood pressure (Sabatini et al., 2008; Wang, Dong, Chen, Cui, & Zhang, 2010). Besides, antioxidant peptides from food proteins constitute an alternative use of synthetic antioxidants and can be employed to curtail free radical formation in food products thereby retarding lipid oxidation. Free radicals are known to be involved in the oxidation biomolecules and are believed to play a significant role in the occurrence of chronic diseases (Peng, Xiong, & Kong, 2009). Whey protein hydrolysates obtained employing commercial alcalase presented seven peptides showing strong antioxidant activity (Zhang, Wu, Ling, & Lu, 2013).

There are studies supporting that plant peptidases from *Carica papaya* and *Ananas comosus*, could be employed to obtain bioactive peptides from various food protein sources (Gao, Chang, Li, & Cao, 2010; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011; Salampessya, Phillips, Seneweera, & Kailasapathy, 2010). *Maclura pomifera* (Raf.) Schneid. (Moraceae) or Osage orange, a thorny dioecious tree, is an ornamental species cultivated in Argentina that possesses unusual quantities of serine peptidases in the latex of its fruits (Altuner, İşlek, Çeter, & Alpas, 2012). *M. pomifera* latex proteases have been used to hydrolyse soy protein (López, Natalucci, & Caffini, 1989) and to clot milk (Corrons, Bertucci, Liggieri, López, & Bruno, 2012). In the latter case, whey obtained displayed antioxidant and inhibitory ACE activities, which could be attributed to small peptides generated by action of proteases during milk-clotting. Therefore, the objective of the present study was to prepare whey hydrolysates employing peptidases from *M. pomifera*, to adjust reaction conditions and to investigate the formation of bioactive peptides with both mentioned activities.

2. Materials and methods

2.1. Chemicals

BSA, captopril, casein, rabbit lung ACE, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,20-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), butylated hydroxytoluene (BHT), potassium persulphate, Tris(hydroxymethyl)aminomethane (TRIS), and zinc chloride were purchased from Sigma Chemical Company (St. Louis, MO), Coomasie Brilliant Blue R-250 and G-250, low-range molecular weight standards and tricine from Bio-Rad (Hercules, CA), Abz-PheArgLys(DNP)Pro-OH from Bachem (Bubendorf, Switzerland), San Regim skim milk powder from San-Cor (Santa Fe, Argentina), chymosin (Chy-Max Extra, 2080 IMCU/g) from Hansen Argentina SAIC (Quilmes, Argentina), trifluoroacetic acid (TFA) from J.T. Backer (Philipsburg, NJ, USA), L-leucine and trichloroacetic acid from Carlo Erba Reagenti (Rodano, MI, Italy). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.2. Crude extract

M. pomifera fruits were collected in La Plata, Argentina in autumn. Latex was obtained by making incisions in fruits and received on phosphate buffer 0.1 mol L^{-1} (pH 6.6) containing ethylenediaminetetraacetic acid 5 mmol L^{-1} to avoid phenoloxidase activity (Ryan et al., 2011). The suspension was centrifuged at 16,000× *g* and 4 °C during 20 min. The filtered solution was named pomiferin, fractionated and stored at −20 °C.

2.3. Partial purification of pomiferin by fractionation with ethanol

To remove phenolic compounds, partially purified extracts were prepared by precipitation with ethanol. Fractions of 1 mL pomiferin were treated with increasing volumes (1–6 mL) of cold ethanol and left to precipitate for 30 min at −20 °C prior to centrifugation (16,000× *g*, 30 min). The final ethanol precipitates were redissolved with 1 mL of the extraction buffer and frozen.

2.4. Specific activity of plant extracts

Proteolytic activity was determined on casein substrate and activity was expressed as Ucas mL^{-1}, an arbitrary enzyme unit defined by Corrons et al. (2012). Protein content was determined by the Bradford's method (Bradford, 1976), using BSA as standard.

Specific activity was calculated as the ratio between caseinolytic activity and protein concentration. The ethanolic precipitate redissolved (EPR) that showed the major specific activity was named EPP (Ethanol Purified Pomiferin) and employed to hydrolyse whey proteins.

2.5. Thermal inactivation of EPP

To determine time needed for EPP proteases inactivation, samples were heated for 0, 2.5, 5, 7.5, 10 and 15 min at 100 °C, after what residual caseinolytic activity was measured as was described in 2.4.

2.6. Whey preparation

Skim bovine powder (12.5 g) was dissolved at 35 °C in 100 ml of 0.01 mol L^{-1} calcium chloride solution and treated with 2.25 IMCU mL^{-1} (International Milk Clotting Units) chymosin. Clots were left for 1 h at room temperature, cut, and centrifuged at 16,000× *g* (4 °C, 15 min). Drained whey was fractionated and stored at −20 °C.

2.7. Hydrolysis of whey proteins employing EPP

EPP was used to prepare bovine whey protein hydrolysates. Reactions were carried out mixing an enzyme: whey volume ratio of 1:9 (pH 6.5, 45 °C). Hydrolysates were collected at variable time intervals. Reactions were stopped by heat treatment (100 °C, 10 min). Blanks were prepared with EPP or whey in the same dilution of reaction mixture. Aliquots of each hydrolysate were filtered using 3 kDa Amicon Ultra–15 centrifugal filter units (Millipore) afterwards, filtered and unfiltered hydrolysates were stored at −20 °C. Filtered hydrolysate were lyophilised and stored. Hydrolysate peptide concentration was determined by micro-Kjeldahl method (Wiles, Gray, & Kissling, 1997).

2.8. Tricine SDS-PAGE

Hydrolysate samples were analysed on denaturing electrophoresis in tricine gels according to Bruno et al. (Bruno et al., 2010), in a Mini-Protean III dual slab cell (Bio-Rad Laboratories, Watford, UK). Electrophoretic profiles were analysed by densitography employing ImageJ software (W. Rasband, National Institute of Mental Health, Bethesda, MD, USA).

2.9. Degree of hydrolysis (DH) determination

DH of whey hydrolysates was determined spectrophotometrically at 340 nm using the TNBS method (Adler-Nissen, 1979). L-leucine was used as standard (concentration range: 0–2.25 mmol L^{-1}).

2.10. RP-Chromatographic profile of hydrolysates

Each filtered hydrolysates was analysed by reverse phase high performance liquid chromatography (RP-HPLC) using a Bondclone

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