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

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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.

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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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

10 C-18 Phenomenex column (3.5 mL) at room temperature in ÄKTA-Purifier (GE, Uppsala, Sweden), flow rate of 1 ml min^{-1}. Column was equilibrated with solvent A (0.50 mL TFA + 99.5 mL water). Linear gradient from 100% solvent A to 100% solvent B (0.25 mL TFA + 99.75 mL acetonitrile) was applied in 35 column volumes.

2.11. Molecular weight distribution profile

Molecular weights of filtered hydrolysate peptides were investigated by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Samples were mixed with matrix (sinapinic acid dissolved in 1 g/100 mL TFA). Mass spectrum was determined using Bruker Ultraflex spectrometer equipped with a nitrogen laser (337 nm with pulses of 1–5 ns) and an ion source acceleration of 25 kV; bovine trypsinogen was used as internal calibrator. Molecular masses obtained were analysed with FindPept software tool (http://web.expasy.org/findpept/) and compared with the sequences of major whey proteins.

2.12. Determination of ACE-inhibitory activity

Lyophilised samples of filtered hydrolysates were dissolved in demineralised water at 1:5 of initial volume and were subjected to ACE inhibition analysis using the fluorogenic substrate Abz-PheArgLys(DNP)Pro-OH (Carmona, Schwager, Juliano, Juliano, & Sturrock, 2006). The reaction was performed in a spectrofluorometer (model RF-1501, Shimadzu Corporation, Kyoto, Japan), recording the increase of fluorescence (λex: 320 nm; λem: 420 nm). Controls were carried out employing captopril. Data were expressed as the inhibition percentage and IC_{50} values of hydrolysate with highest inhibitory ACE activity and of captopril, were determined by regression analysis using Sigmaplot software 10.0 (SPSS Inc, Chicago, IL, USA).

2.13. ABTS radical scavenging activity

ABTS radical cation was generated according to Luo et al. (2013). Aliquots (1 mL) of this radical were mixed with 10 μL of different unfiltered hydrolysates. Ten minutes later, the absorbance was measured at 734 nm. Trolox and BHT were employed as antioxidant standards. The following equation was used to calculate the ABTS scavenging activity:

$$\text{ABTS scavenging activity}(\%) = [(AB_{0min} - AS_{10min}) - (AB_{0min} - AB_{10min})] \times 100/AB_{0min}$$

where AB_{0min} and AB_{10min} are the absorbances without sample at 0 and 10 min, respectively, and AS_{10min} was the absorbance with sample at 10 min. Percent values were transformed in Trolox Equivalens (TE) by using of calibration curve (0.005–0.5 mg/mL Trolox).

IC_{50} values of the hydrolysate with highest antioxidant activity, Trolox and BHT were obtained by linear regression analysis using Sigmaplot software 10.0.

2.14. Statistical analysis

All data were reported as mean ± standard deviation of three replicates. The results of antioxidant and ACE inhibitory activities were compared by one-way ANOVA test (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA 92037 USA). Significant differences between the means of parameters were determined by Tukey's test ($P < 0.05$).

3. Results and discussion

3.1. Characteristics of enzyme extracts

In a previous work, a proteolytic extract (14.1 ± 0.8 Ucas/mL) from latex of *M. pomifera* fruits was used as clotting agent on bovine milk (Corrons et al., 2012). In the present work, crude extract from latex of *M. pomifera* fruits presented 8.04 ± 0.21 Ucas/ml of caseinolytic activity. This variability is a common fact in proteolytic activity of plant extracts, because this parameter would depend on the amount of latex recovered, fruit size and quantity of protease present in fruits, and varies according to many aspects of plant physiology and development along with environmental factors (Schaller, 2004).

Partially purified extracts were prepared by precipitation with different ethanol volumes. Specific activity of enzyme extracts (Table 1) decreased when the amount of solvent was increased, reaching a maximum value with one volume of ethanol. Thus, EPR prepared with one volume of ethanol was named EPP and retained higher specific activity (5.4 ± 0.4 Ucas/mg) than the CE (4.4 ± 0.2 Ucas/mg).

Thermal inactivation conditions constitute a required parameter in industrial processes to stop the reactions. Some studied proteases as bromelain retained about 20% of original activity after incubation at 105 C° for 30 min (Poh & Abdul Majid, 2011). In the present study, EPP conserved only 1% of caseinolytic activity after 5 min boiling, being no detectable with thermal pretreatment of 10 min (Fig. 1). This behaviour is similar to observed for papain, which was inactivated by heating at 100 °C for 10 min (Sokol, Barton, Farr, & Medzhitov, 2008). Inactivation of peptidases is important to standardize assay conditions when preparing bioactive peptides, because if these enzymes remained active, formed peptides could be degraded.

3.2. Preparation and characterization of bovine whey hydrolysates

Hydrolysates properties are dependent on the type of enzyme used, the degree of hydrolysis, and the substrate pretreatment (Jeewanthi et al., 2014). In present work, whey hydrolysates of 5.894 ± 0.004 mg/ml of protein were prepared, employing peptidases from *M. pomifera* at 45 °C. Presence of aggregates and/or gels was observed when hydrolysates were heat-inactivated or later, when were defrosted. This effect, observed in both 3 kDa cut-off filtered and unfiltered hydrolysates, made these preparations difficult to manipulate. Consequently, to ensure the complete dissolution of gels, heating at temperatures above 50 °C and stirring with vortex were required. Zhang and Vardhanabhuti (2014) reported that thermal processing of whey protein leads to the formation of aggregates presenting various sizes and shapes. Physicochemical properties of formed aggregates depend on protein and salt concentrations, pH and heating temperature, and Rocha et al.

Table 1
Specific activity of extracts (Ucas/mg). Crude extract (CE) and ethanolic precipitate redissolved (EPR-1 to 6) obtained by precipitation with 1–6 ethanol volumes.

Proteolytic extract	Specific activity (Ucas/mg)
CE	4.4 ± 0.2
EPR-1	5.4 ± 0.4
EPR-2	5.0 ± 0.4
EPR-3	4.0 ± 0.1
EPR-4	3.8 ± 0.4
EPR-5	3.1 ± 0.2
EPR-6	2.7 ± 0.4

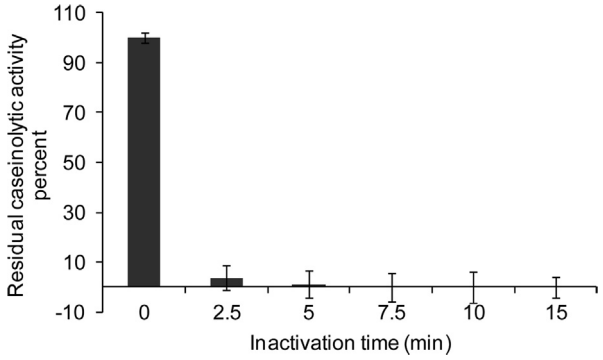

Fig. 1. Heat inactivation of EPP. Enzyme extracts were preincubated at 100 °C and then caseinolytic activity was determined. All assays were performed by triplicate; vertical bars correspond to standard deviation.

(Rocha, Teixeira, Hilliou, Sampaio, & Gonçalves, 2009) concluded that limited proteolysis can affect gelling capacity, by changing the balance between attractive and repulsive interactions. Limited treatment with proteases leads the exposure of buried hydrophobic and other interactive groups, being free to interact with neighbouring polypeptides, thus promoting protein aggregation and subsequent gel setting. Therefore, the proteolysis reaction must be carefully monitored and controlled in order to manufacture products with desired functionality. While some enzymes can induce gelation following whey protein hydrolysis; others impair gelling properties.

Degradation profile of major whey proteins was analysed by tricine SDS-PAGE. Densitograms of whey proteins (Fig. 2) show that after 180 min of hydrolysis practically all α-LA and β-LG appear degraded. On the other hand, protein fractions of high molecular masses remain almost unchanged at the same time. Effectiveness of hydrolysis process and type of peptide produced depend on the amino acid sequence and the three-dimensional protein structure. Whey proteins are globular, a fact that makes difficult the access by proteolytic enzymes (Benitez et al., 2008). Certain studies show that hydrolysis is effective after heat (Adjonu, Doran, Torley, & Agboola, 2013) or high-pressure pretreatment, which allow proteolytic attack when facilitating enzyme access to the protein sequence by partial unfolding of the whey proteins compact globular assemblies (Piccolomini, Iskandar, Lands, & Kubow, 2012). From the results presented in this paper, degradative behaviour of *M. pomifera* peptidases on whey at mild conditions would be promising for preparing hydrolysates from this source.

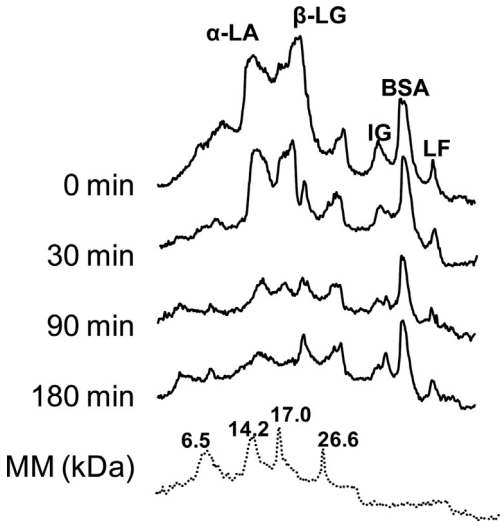

Fig. 2. Densitogram of tricine SDS-PAGE. Each plot corresponds to lanes of whey hydrolysates obtained from 0 (hydrolysis blank) to 180 min reaction and Molecular Markers (MM, Ultra Low Range Molecular Weight Marker, range 1060–26,600, Sigma); α-LA: alpha-lactalbumin; β-LG: beta-lactoglobulin; BSA: bovine serum albumin; IG immunoglobulins; LF: lactoferrin.

Complementarily with SDS-PAGE densitograms patterns, DH of whey hydrolysates was determined, exhibiting an increase with reaction time (Fig. 3). Values were sharply incremented within the first 90 min and then more gradually, reaching 31.3 ± 1.7% at 180 min.

Hydrophobic and hydrophilic amino acids are randomly distributed in whey proteins (Benitez et al., 2008), thus the hydrolysis process also produces peptides that possess a random distribution of hydrophobic and hydrophilic amino acids. Fig. 4 shows analysis of small peptides in filtered hydrolysates (3 kDa cut-off) by RP-HPLC. In absence of hydrolysis (Fig. 4A), only two hydrophilic peaks were eluted (time < 15 min). After 30 min of hydrolysis (Fig. 4B) an unretained fraction was observed and three hydrophobic peaks were detected (19.8, 21.3 and 25.2% B). Intensity of these retained peaks was increasing with hydrolysis time (Fig. 4C and D), while peaks observed in the chromatographic profile of blank sample were degraded (Fig. 4A and B). The 90 min hydrolysate profile shows four hydrophilic fractions that appeared at short times (10.4, 11.1, 17.9, and 17.7% B) and a last more hydrophobic (27.6% B). Two chromatographic fractions displayed after 90 min of hydrolysis (10.4 and 11.1% B) disappeared when the reaction continued for 180 min (Fig. 4D), and the intensity of the peak eluted on 17.9% B decreased. Additionally, three new peaks appeared (8.1, 8.8, and 13.9% B). This change in profile demostrates that hydrolysis continues slowly between 90 and 180 min, which is consistent with DH behaviour (Fig. 3).

3.3. Bioactive peptides screening

Data corresponding to ACE-inhibitory activity of 5-fold concentrated ultrafiltered hydrolysates showed significantly difference with whey blank ($P < 0.0001$). Tukey's test shows no significant difference between different hydrolysis times values (Table 2). EPP presented undetectable activity.

When TE values of $ABTS^{+}$ radical scavenging capacity, corresponding to unfiltered hydrolysates, were compared (Table 3) ANOVA revealed significant differences ($P < 0.0001$). Pair of values analysed by Tukey test allowed to observe significant differences among hydrolysates and blanks ($P < 0.05$). This fact would correspond with the production of antioxidant peptides during

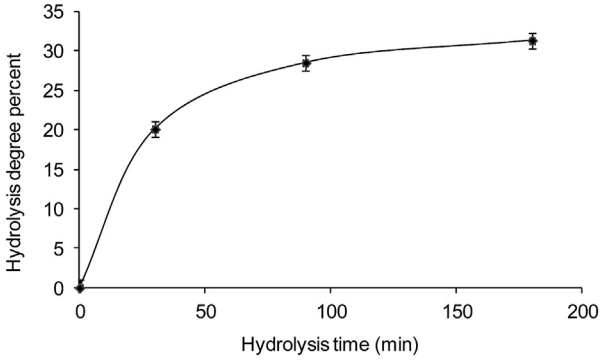

Fig. 3. Hydrolysis degree of whey hydrolysates. All determinations were carried out by triplicate; vertical bars correspond to standard deviation.

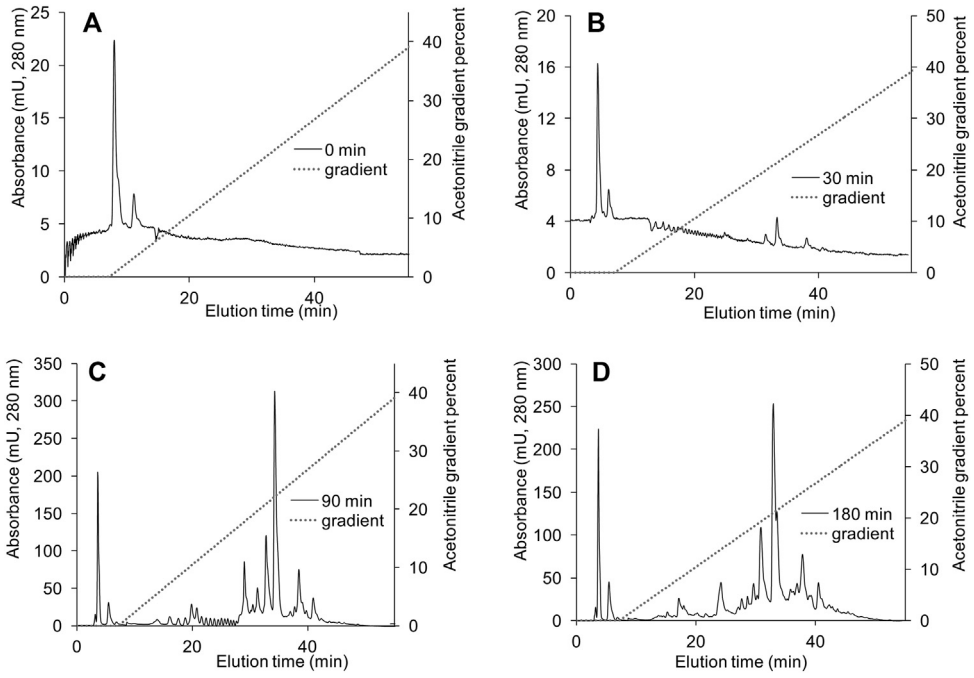

Fig. 4. RP-HPLC of 3 kDa filtered hydrolysates. Column: Bondclone 10 C-18 Phenomenex (3.5 mL). Flow rate: 1 ml min^{-1}. Elution conditions: linear gradient from 100 percent solvent A (0.50 mL TFA + 99.5 mL water) to 100 percent solvent B (0.25 mL TFA + 99.75 mL acetonitrile). At times >60 min no peaks were detected (data not shown). 4A: 0 min of hydrolysis (blank); 4B, 4C and 4D: 30, 90 and 180 min hydrolysis, respectively.

Table 2
ACE-inhibitory activity percent of 5-fold concentrated ultrafiltered hydrolysates (30, 60 and 90 min hydrolysis). Substrate blank value was subtracted and EPP blank value was not detected. All assays were performed by triplicate and results were expressed as mean ± SD. Means with the same superscript letter are not significantly different ($P > 0.05$).

Ultrafiltered hydrolysate (expressed in hydrolysis time)	ACE inhibitory activity (%)
30 min	50.8 ± 5.8[a]
90 min	54.4 ± 2.5[a]
180 min	55.6 ± 0.6[a]

Table 3
Antioxidant activity expressed in trolox equivalents (mg/ml). Displayed samples are whey hydrolysates of 30, 90 and 180 min, enzyme blank (EB) and substrate blank (SB). Values represent average of three determinations ± SD. Means with the same superscript letter are not significantly different ($P > 0.05$).

Sample	Trolox equivalents (mg/ml)
EB	0.081 ± 0.012[a]
SB	0.142 ± 0.001[b]
30 min	0.433 ± 0.000[c]
90 min	0.414 ± 0.046[c]
180 min	0.492 ± 0.010[d]

Table 4
IC_{50} values of antioxidant activity. Determinations were carried out by ABTS cation radical method. All assays were performed by triplicate and results were expressed as mean ± SD.

Sample	IC_{50} (mg/ml)
Trolox	0.31 ± 0.02
BHT	0.18 ± 0.04
Unfiltered hydrolysate of 180 min	4.44 ± 0.44

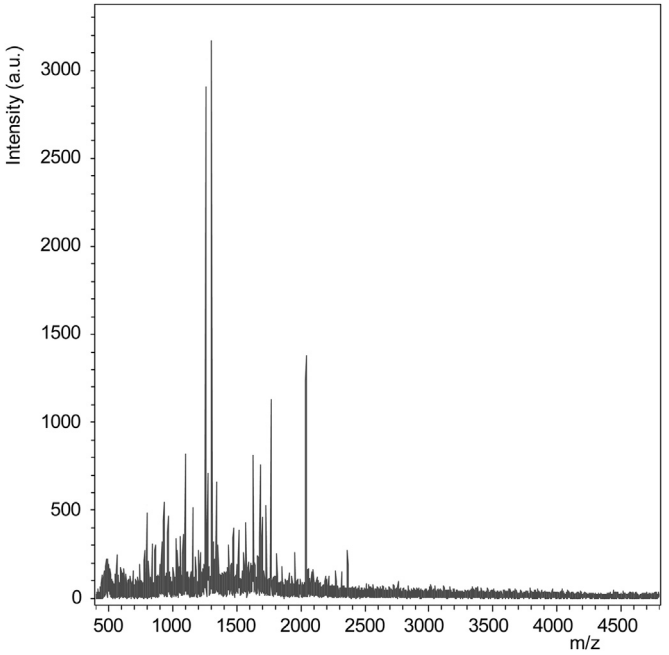

Fig. 5. Mass spectrometry of filtered hydrolysate of 180 min. Samples were prepared by mixing equal volumes of matrix solution (sinapinic acid dissolved in 1 g/100 mL TFA).

Table 5
Theoretical analysis of peptide sequences. Theoretical sequences of peptides present in unfiltered hydrolysate of 180 min into precursor sequence of α-LA (accession number: P00711; f(1–19): signal peptide) and β-LG (accession number: P02754; f(1–16): signal peptide). Numbering in the "peptide" column corresponds to the mature protein. For experimental and theoretical mass Cys-CAM occurring amino acid residues were considered (cysteines in samples have been treated with iodoacetamide to form carbamidomethyl-cysteine). The experimental and theoretical mass values matches were selected with a mass tolerance of ±0.1 Da.

5A

Experimental mass	Theoretical mass	Δ mass	Peptide	Position in α-LA sequence	Known modifications
780.355	780.316	−0.039	(F)HTSGYDT(Q)	32-38	
780.355	780.425	0.069	(D)LKGYGGVS(L)	15-22	
780.355	780.425	0.069	(L)KGYGGVSL(P)	16-23	
780.355	780.425	0.069	(L)KDLKGYG(G)	13-19	
808.210	808.284	0.074	(L)DDDLTDD(I)	82-88	
1080.630	1080.551	−0.079	(T)KCEVFREL(K)	5-12	CYS_CAM
1080.630	1080.551	−0.079	(K)CEVFRELK(D)	6-13	CYS_CAM
1214.661	1214.599	−0.062	(Y)WLAHKALCSE(K)	104-113	CYS_CAM
2039.944	2039.967	0.022	(Q)AIVQNNDSTEYGLFQINN(K)	40-57	

5B

Experimental mass	Theoretical mass	Δ mass	Peptide	Position in β-LG sequence	Known modifications
780.355	780.414	0.058	(V)YVEELK(P)	42-47	
929.622	929.530	−0.091	(N)ENKVLVLD(T)	89-96	
929.622	929.545	−0.076	(L)EILLQKW(E)	55-61	
929.622	929.545	−0.076	(E)ILLQKWE(N)	56-62	
1214.661	1214.678	0.016	(E)GDLEILLQKW(E)	52-61	
1214.661	1214.751	0.089	(T)KIPAVFKIDAL(N)	77-87	
1352.489	1352.579	0.089	(L)SFNPTQLEEQC(H)	150-160	CYS_CAM
1515.623	1515.690	0.066	(S)FNPTQLEEQCHI	151-162	CYS_CAM
2039.944	2039.945	0.001	(V)LDTDYKKYLLFCMENS(A)	95-110	CYS_CAM
2039.944	2040.011	0.066	(G)LDIQKVAGTWYSLAMAASD(I)	10-28	
2039.944	2040.011	0.066	(L)DIQKVAGTWYSLAMAASDI(S)	11-29	

hydrolysis process. Hydrolysate of 180 min presented the highest TE (0.492 ± 0.010 mg/mL; $P < 0.05$) and hydrolysates of 30 and 90 min showed no significant differences (0.433 ± 0.000 and 0.414 ± 0.046 mg/mL, respectively; $P > 0.05$).

Net TE antioxidant capacity of 180 min hydrolysate, calculated by resting both blanks, was of 0.269 ± 0.016 mg/mL.

Hydrolysate of 180 min was prepared in large quantity and the IC_{50} value was determined for both activities. In the case of ACE inhibitory activity, IC_{50} was 0.53 ± 0.02 mg/ml, a value of the same order as those obtained from Silvestre et al. (2012) for whey hydrolysates prepared in 5 h employing papain. IC_{50} of captopril standard was 0.54 ± 0.03 ng/ml (2.49 ± 0.15 nM), a suitable value for this expected reference compound.

Antioxidant activity IC_{50} values obtained for Trolox and BHT are higher than the corresponding to the 180 min unfiltered hydrolysate (Table 4). However it is necessary to consider that the hydrolysate is not composed of a single peptide but constitutes a complex sample, which may have some components with antioxidant effect or, in the worst case, may have prooxidant effect. Therefore it is necessary to plan the future purification work of these antioxidant peptides.

In Fig. 5, masses from MALDI-TOF of peptides lower than 3 kDa produced after 180 min hydrolysis can be observed. About thirty four masses were detected on the background and Table 5 A and B show possible sequences of peptides present. These sequences were obtained by comparing those of α-LA and β-LG (UniProtKB/Swiss-Prot, protein sequence database; accession numbers: P00711 and P02754, respectively) with experimental masses employing FindPept software tool for unspecific cleavage (Δmass tolerance: ±0.1 Da).

Only one matching peptide was found when Biopep database of bioactive peptides and other bibliography sources were consulted. Tavares et al. (Tavares et al., 2011) reported that fraction with molecular weight below 3 kDa of whey protein hydrolysate prepared with proteases from *Cynara cardunculus* presented ACE-inhibitory effect, exhibiting an IC_{50} value of 23.6 μg/mL. After purification steps, 14 peptides were identified, among them the peptide KGYGGVSL with an IC_{50} value of 234.8 μg/mL. This peptide derived from α-LA and constitutes one of those found as potential peptides in Table 5A.

4. Conclusion

Partially purified extracts from *M. pomifera* containing proteolytic enzymes, prepared with a simple procedure, can be used in preparation of sweet whey hydrolysates. The carried out hydrolysis method requires no previous adjustment of whey pH and no addition of compounds, as enzyme activators. Hydrolysis treatment is efficient at 45 °C and 180 min and boiling inactivation of enzymes can be reached in about 10 min.

This whey protein product yields bioactive peptides with antioxidant and ACE-inhibitory activities, further studies are required to elucidate if they could provoke physiological effects in the human body. This preparation would have health-promoting features and could be raw material for designing low-cost functional food that nowadays constitutes an excellent alternative for consumers.

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