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FULL COMMUNICATION

Partial Molecular Characterization of *Arctium minus* Aspartylendopeptidase and Preparation of Bioactive Peptides by Whey Protein Hydrolysis

Cecilia V. Cimino,* María Laura Colombo,* Constanza Liggieri, Mariela Bruno, and Sandra Vairo-Cavalli

Department of Biological Sciences, Faculty of Exact Sciences, Plant Proteins Research Centre (CIPROVE), National University of La Plata, La Plata, Argentina.

ABSTRACT In this article, we report the cloning of an aspartic protease (AP) from flowers of *Arctium minus* (Hill) Bernh. (Asteraceae) along with the use of depigmented aqueous flower extracts, as a source of APs, for the hydrolysis of whey proteins. The isolated cDNA encoded a protein product with 509 amino acids called *arctiumisin*, with the characteristic primary structure organization of typical plant APs. Bovine whey protein hydrolysates, obtained employing the enzyme extracts of *A. minus* flowers, displayed inhibitory angiotensin-converting enzyme (ACE) and antioxidant activities. Hydrolysates after 3 and 5 h of reaction (degree of hydrolysis 2.4 and 5.6, respectively) and the associated peptide fraction with molecular weight below 3 kDa were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, matrix-assisted laser desorption ionization/time of flight mass spectrometry, and reverse phase-high-performance liquid chromatography. The results obtained in this study demonstrate the viability of using proteases from *A. minus* to increase the antioxidant and inhibitory ACE capacity of whey proteins.

KEY WORDS: • ***angiotensin-converting enzyme inhibitory activity*** • ***antioxidant capacity*** • ***arctiumisin*** • ***cloning*** • ***milk protein*** • ***typical plant aspartic protease***

INTRODUCTION

ENZYMATIC HYDROLYSIS, FERMENTATION, or a combination of both have been employed with success for the production of bioactive peptides. The use of commercial enzymes—for example, pepsin, trypsin, and chymotrypsin—is still the most common method to produce bioactive peptides. Less conventional sources of proteolytic enzymes, which can cleave the whey protein chains at specific and less usual sites, have been tried.[1,2] An interesting alternative are aspartic proteases (APs) present in the flowers of Cardueae tribe Cass., Asteraceae family.

Among studied bioactive peptides derived from whey protein digest, angiotensin-converting enzyme (ACE)-inhibitory peptides deserve special mention since ACE is an effective target for antihypertensive medication in patients with hypertension—a disorder that constitutes a major public health problem worldwide.[2] Bovine whey protein concentrates, as well as purified proteins from whey after hydrolysis by the proteases from cardoon flowers (*Cynara cardunculus*), release highly potent ACE-inhibitory peptides.[2,3] Active fragments generated from α-lactalbumin and β-lactoglobulin were different from previously reported peptides obtained by enzymatic digestion of the same substrate with other peptidases.[2,4] The ACE-inhibitory activity was also reported by Cimino *et al.*[5] upon hydrolysis of whey protein with flower aqueous extracts of *Arctium minus*, another member of the Asteraceae family.

The enzymatic hydrolysis of whey protein was also employed for the generation of bioactive peptides with antioxidant properties.[4,6] The antioxidant activity of peptides comes from their abilities to inactivate reactive oxygen species, to scavenge free radicals, to chelate prooxidative transition metals, and to reduce hydroperoxides.[7] The activity is based on the inherent amino acid composition and sequence of antioxidant peptides, usually consisting of 5–11 amino acids, including amino acids with aromatic rings.[8]

In this work, we isolated the cDNA encoding a novel typical AP from flowers of *A. minus*. We employed depigmented aqueous enzyme extract (EE) from these flowers to obtain hydrolysates of whey proteins with antioxidant power and ACE-inhibitory activity. The herein reported activities were recovered in both ultrafiltrated whey digests and fractions separated by reverse phase-high-performance liquid chromatography (RP-HPLC).

*These two authors equally contributed to this work.

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Address correspondence to: Dr. Sandra Vairo-Cavalli, Department of Biological Sciences, Faculty of Exact Sciences, Plant Proteins Research Centre (CIPROVE), National University of La Plata, 47 and 115 S/N, La Plata B1900AJL, Argentina, E-mail: svairo@biol.unlp.edu.ar

MATERIALS AND METHODS

Plant material

Arctium minus (Hill) Bernh inflorescences were collected in the surroundings of La Plata (Buenos Aires, Argentina) during summer (voucher specimen LPE 1164).

Crude extract

Fresh flowers were ground in a mortar under liquid nitrogen and homogenized in 0.1 M phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid and 5 mM cysteine. The suspension was stirred for 30 min and centrifuged at 5000 *g* for 20 min at 4°C.[5]

Enzyme cDNA cloning and sequence analysis

Total RNA was isolated from 100 mg of immature flower of *A. minus* with the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality and relative amount of RNA were estimated by agarose gel electrophoresis. cDNA was synthesized with M-MuLV Reverse Transcriptase (Thermo Scientific, Vilnius, Lithuania) and primer poly-dT17. cDNA was used as template in PCR with primers specifically designed for highly conserved 5′and 3′ ends of AP coding sequences (forward primer: 5′-ATGGGT AACTCAATCAAAGCA-3′; reverse primer: 5′-TCAAGCT GCTTCTGCAAATCC-3′). PCR products were visualized on a 0.01 g/mL agarose gel and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid DNA from positive clones was analyzed by *Not*I/*Eco*RI double digestion and subjected to automated DNA sequencing. Sequences were analyzed with the Basic Local Alignment Search Tool from the National Centre for Biotechnology Information and conserved domains were identified using NCBI's CD-Search service.[9]

Peptidase partial purification

An EE was obtained by size exclusion chromatography.[5] Protein content was measured following the method of Bradford,[10] using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as standard.

Milk clotting activity

Enzyme preparations (100 μL) were added to 1 mL skim milk (0.1 g/mL skim milk powder; San Regim, SanCor, Sunchales, Santa Fe, Argentina, reconstituted in 30 mM $CaCl_2$, containing 15.4 mM sodium azide) and the clotting time was measured. Assays were performed at 35°C. Positive and negative controls were carried out with an extract of flowers of *Cynara scolymus*[11] and extracting buffer, respectively. One rennet unit is the amount of enzyme that coagulates 10 mL milk in 100 s in the assay conditions.[12]

Enzymatic hydrolysis of bovine whey

Substrate for hydrolysis consisted of a 0.01 g/mL whey suspension (LAC PRODAN 80; Arla Foods Ingredients S.A., Martinez, Buenos Aires, Argentina) with a protein concentration of 3.8 ± 0.2 mg/mL estimated by the micro-Kjeldahl technique.[13] Reaction was started by addition of 4.6 mL of substrate to 90 μL of the EE. The reactions were performed at 35°C and quenched at 3 and 5 h by 5 min of boiling. Controls containing whey suspension at the same concentration without enzyme addition were performed. Samples were analyzed by 0.2 g/mL discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[14] Gels were stained with Coomassie Blue G-250.

Fractionation, enrichment, and mass spectrometry profile of bioactive peptides

Hydrolyzed whey was ultrafiltrated in Amicon Ultra-15 centrifugal filter units with Ultracel-3 membrane (Merck Millipore, Billerica, MA, USA) in a swinging bucket rotor at 4000 *g*. Low molecular fractions ($\leq$ 3000 Da) were analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI/TOF MS), and the spectra were acquired on a Bruker ultrafleXtrem spectrometer equipped with a Smartbeam-II laser in reflectron-positive mode using a matrix of α-cyano-4-hydroxycinnamic acid.

Degree of hydrolysis determination

The sample (0.04 mL) was mixed in a test tube with 0.32 mL of 0.2125 M phosphate buffer (pH 8.2) containing 0.01 g/mL SDS and 0.32 mL of 1% v/v 2, 4, 6-trinitrobenzenesulfonic acid in water.[15] Mixtures were incubated at 50°C for 60 min protected from light. Reactions, quenched by addition of 0.64 mL of 0.1 N HCl, were incubated at room temperature for 60 min. Absorbance was measured at 340 nm. Controls were performed with water.

The concentration of free amino group liberated was expressed as leucine equivalent using a calibration curve constructed with standard leucine (0.225–2.25 mM). Degree of hydrolysis (DH) percentage was calculated employing the following equation:

$$DH\% = 100 \times (NH_{2t} - NH_{2t=0})/(NH_{2\infty} - NH_{2t=0})$$

$NH_{2\ t=0}$ and $NH_{2\ t}$ indicate the concentration of free amino groups in the nonhydrolyzed sample and at different hydrolysis time, respectively. Whereas $-NH_{2\ \infty}$, representing total hydrolysis, was estimated according to

$$NH_{2\infty} = (1/_{Maa}) \times (1 + f_{Lys}) \times C_{prot} \times 1000$$

Maa is the average molecular weight of amino acids present in whey proteins (130 g/mol), f_{Lys} is the proportion of lysine in whey proteins (1/15), and C_{prot} is the protein concentration (g/L).

HPLC analysis

The 3 kDa permeates of digested whey proteins were analyzed by RP-HPLC (ÄKTA Purifier; GE Healthcare, Uppsala, Sweden) using a LiChrospher 100 RP-C18 (5 μm, 250 × 4 mm; Merck, Darmstadt, Germany). Chromatographic conditions included solvent A, 0.1% v/v trifluoroacetic acid

in H_20; solvent B, 0.1% v/v trifluoroacetic acid in CH_3CN/H_20 50:50; flow rate, 0.8 mL/min; detection at 215 and 280 nm. A fraction below 3 kDa of control bovine whey was also analyzed under the same conditions.

Scavenging of $ABTS^{+\bullet}$ radical

$ABTS^{+\bullet}$ cation radical [2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging ability was measured, as described by Kumaraswamy and Satish,[16] with slight modifications. $ABTS^{+\bullet}$ was produced by reaction of 7 mM ABTS and 2.45 mM potassium persulfate incubated at room temperature for 16 h in darkness. The $ABTS^{+\bullet}$ solution was diluted with 5.0 mM phosphate buffer (pH 7.4) to a final absorbance of 0.70 ± 0.02 at 734 nm. $ABTS^{+\bullet}$·dilution aliquots of 1 mL were mixed with 10 μL of hydrolysate samples or different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as antioxidant standard. Ten minutes later, the absorbance was measured at 734 nm against the corresponding blank. The percentage of inhibition was calculated from

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

AB_{0min} and AB_{10min} are the absorbance of reaction mixtures without sample at 0 and 10 min, respectively, whereas AS_{10min} is the absorbance of the mixtures with sample at 10 min.

Results were expressed in percentages of scavenging capacity (mean of triplicate determinations ± standard deviations) and in mg of Trolox equivalents per mL of sample.

β-*carotene-linoleic acid assay*

The antioxidant activity was determined employing the method of Tepe *et al.*[17] with modifications. First, 0.8 mg β-carotene was dissolved in 4 mL chloroform (HPLC grade), then 80 mg linoleic acid and 800 mg Tween 20 were added. Chloroform was completely evaporated at 40°C. Subsequently, 200 mL of distilled water saturated with oxygen was added with vigorous shaking; 2.5 mL of this reactive was dispensed to test tubes with the addition of 0.1 mL of samples, water (negative control), or ascorbic acid standard solution (positive control, concentration range: 0.1–0.00001 g/mL). The emulsion system was incubated for 120 min at 50°C. Absorbance was measured at 470 nm at 0, 30, 60, 90, and 120 min. The antioxidant activity percentage was determined as follows: absorbance (final time)/absorbance (initial time) × 100.

Inhibitory ACE activity

RP-HPLC selected peaks were tested for ACE *in vitro* inhibition. Kinetic assays were performed using fluorescence resonance energy transfer substrate Abz-PheArgLys(DNP)-Pro-OH[18] in a spectrofluorometer RF-1501 (Shimadzu Corporation, Kyoto, Japan), recording fluorescence emission (λ_{ex} 320 nm; λ_{em} 420 nm) during 3 min. Controls were performed employing captopril stock (217.29 μg/mL in water), EE, a 3 kDa whey permeate previously exposed to the same temperature treatment, and buffer.

Determinations were performed in triplicate and standard deviations were calculated. Data were expressed as inhibition percentage.

RESULTS AND DISCUSSION

cDNA cloning and sequence analysis

APs are present in a wide variety of plant species and have been characterized and purified from different tissues.[19] Because of their diversity, plant APs have been grouped into three classes—that is typical plant-aspartic, atypical plant-aspartic, and nucellin-like proteinases—depending on their putative domain organizations and their active-site-sequence motifs.[20] cDNA was synthesized using total RNA isolated from *A. minus* young flowers and used as PCR template with primers designed for conserved regions of the open reading frame 5′ and 3′ ends of plant APs. The amplified product of 1530 bp was cloned. The nucleotide and deduced amino acid sequences are shown in Figure 1 (GenBank accession number: KJ452158). According to the AP-naming conventions, we proposed the name arctiumsin for the aspartyl endopeptidase cloned in this research. This protein product encoded a preproenzyme of 509 residues with a putative hydrophobic signal peptide of 24 amino acids, a prosegment of 44 residues, and a 441 amino acid-long polypeptide interrupted by 105 residues corresponding to the plant-specific insert (PSI) domain. Neither PSI sequences nor PSI structures have homology with animal or microbial APs; however, the PSI domain is homologous with the precursor of mammalian saposins.[21] The preproenzyme has a predicted molecular mass of 55 kDa with a theoretical pI of 5.3. Protein sequence and conserved domain analysis[9] confirmed the nature of cDNA-deduced amino acid sequence as AP homologue.

When compared with other plant AP precursors (Fig. 2), the amino acid sequence of arctiumsin showed a high degree of similarity with cirsin (AFB73927) from *Cirsium vulgare* (identities: 96%, positives: 98%), cyprosin B (CAA57510) from *C. cardunculus* (identities: 96%, positives: 98%), cenprosin (CAA70340) from *Centaurea calcitrapa* (identities: 96%, positives: 98%), silpepsin 1 from *Silybum marianum* (AGE15494, identities: 95%, positives: 98%), the AP from *Helianthus annuus* (BAA76870, identities: 82%, positives: 91%), all peptidases belonging to A1 family, MEROPS-the Peptidase Database (http://merops.sanger.ac.uk/).

APs from family A1 are bilobed molecules with two aspartic residues responsible for catalysis located on each side of a catalytic cleft. The deduced amino acid sequence presents two conserved catalytic triads, DTG 103–105 and DTG 290–292 corresponding to DTG 32–34 and DTG 215–217 of pepsin numbering, respectively. Additional conserved residues—found to participate in substrate alignment, specificity, and/or efficient catalysis[22]—are tyrosine, glycine, and threonine residues in positions 147, 148, and 149 (YGT 75–77 using pepsin numbering). Furthermore, two putative N-glycosylation sites conserved in several

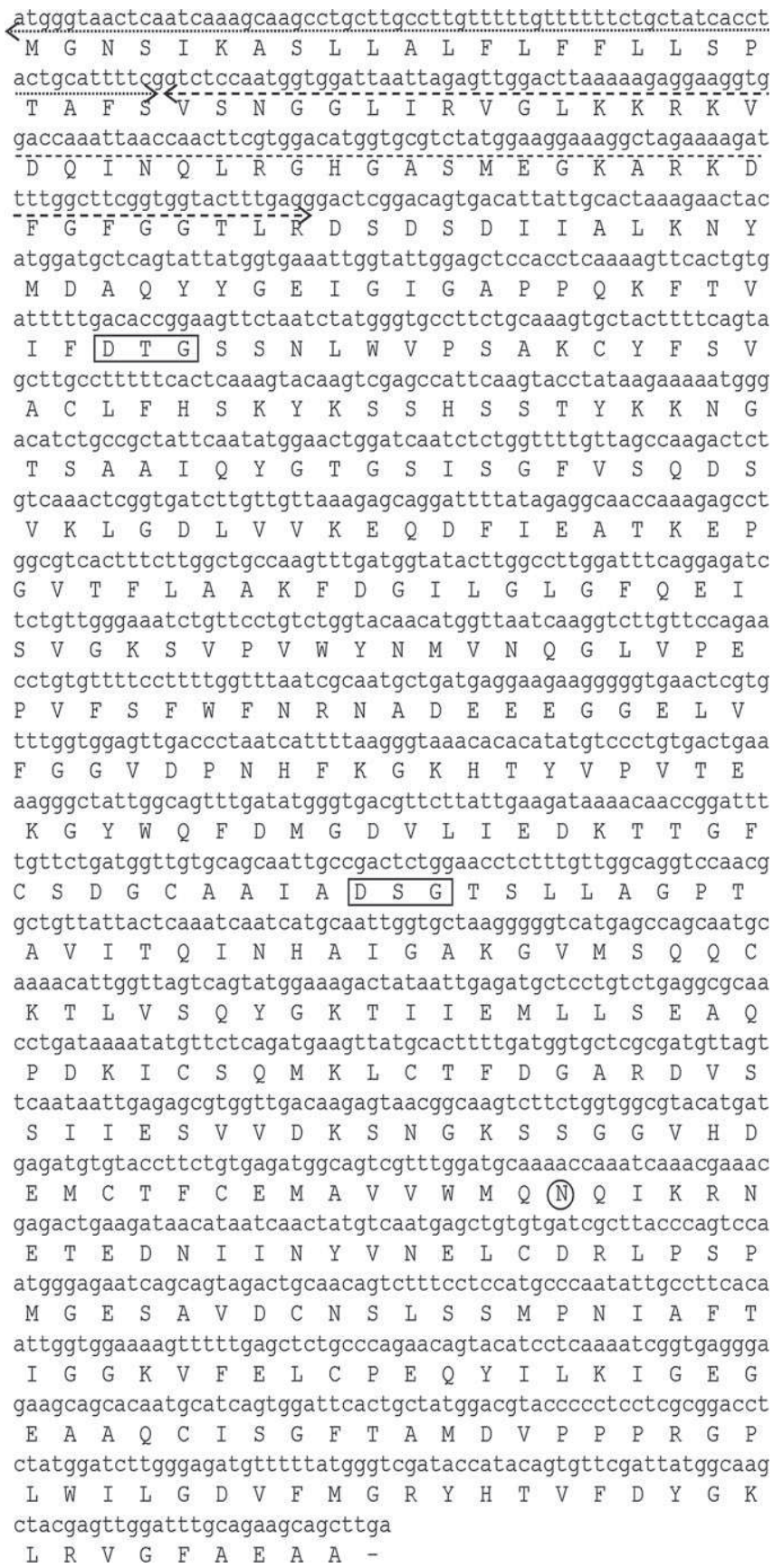

FIG. 1. Full-length cDNA and derived amino acid sequence of artiumsin. First strand cDNA was synthesized from total RNA isolated from young flower buds and used as a template in PCR amplification with primers specifically designed for the highly conserved 50 and 30 ends of aspartic proteases (APs) coding sequences. The regions corresponding to the N-terminal signal peptide (*dotted arrow*), the prosegment (*dashed arrow*), and the plant-specific insert (*italic*) are indicated. Potential N-linked glycosylation sites are circled and the catalytic triads are *boxed*. Artiumsin cDNA sequence has been submitted to the GenBank under the accession number KJ452158.

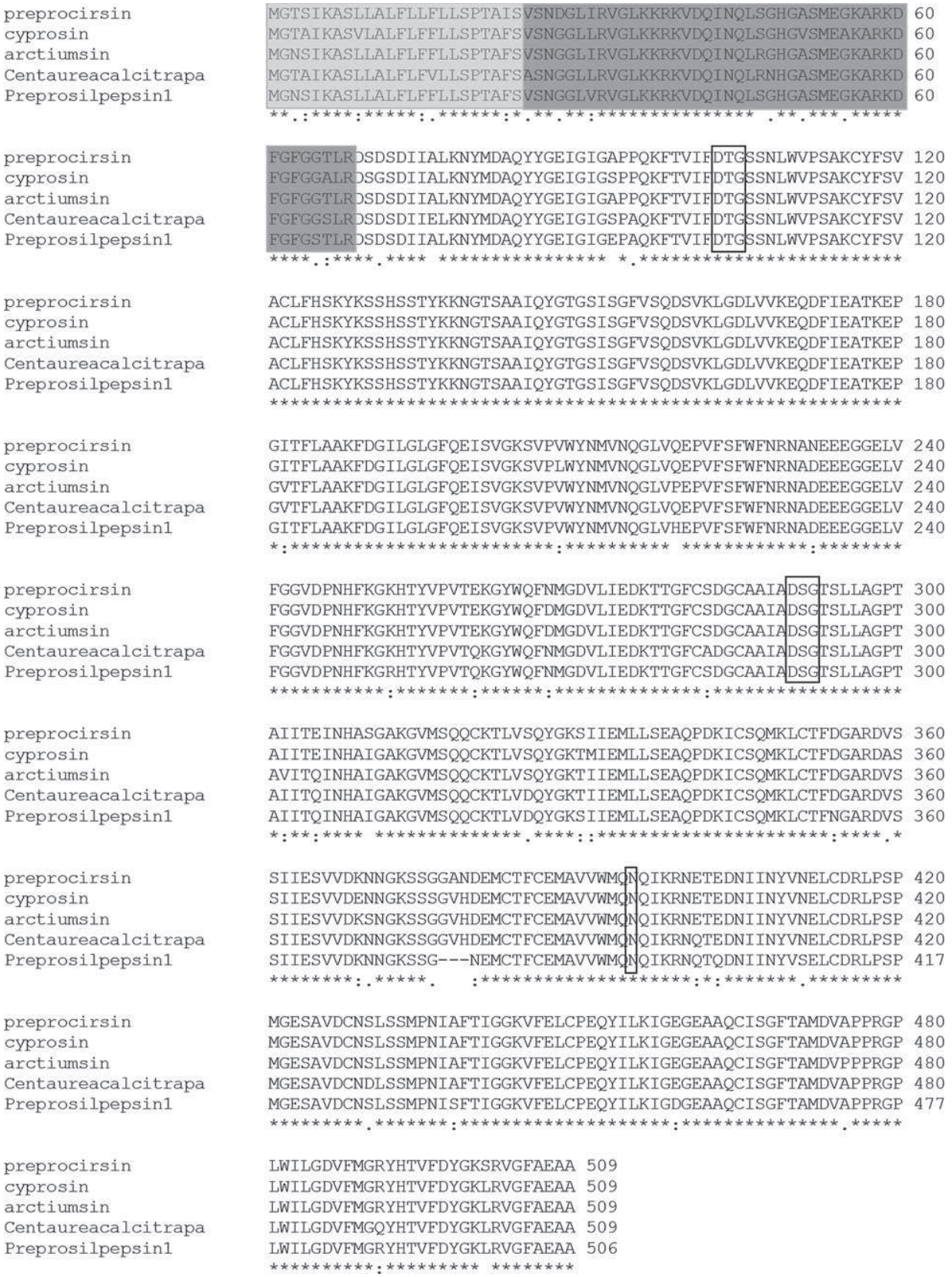

FIG. 2. Amino acid sequence alignment of thistle APs. The regions corresponding to the N-terminal signal peptide and the prosegment are indicated by *boxed light gray* and *dark gray*, respectively. Potential N-glycosylation sites are *grayed* out and the catalytic triads are *boxed*. Sequence of the enzymes used for the alignment are preprocirsin (*Cirsium vulgare*) AFB73927.2, cyprosin (*Cynara cardunculus*) CAA57510.1, cenprosin CAA70340.1 (*Centaurea calcitrapa*), and preprosilpepsin AGE15494.11 (*Silybum marianum*).

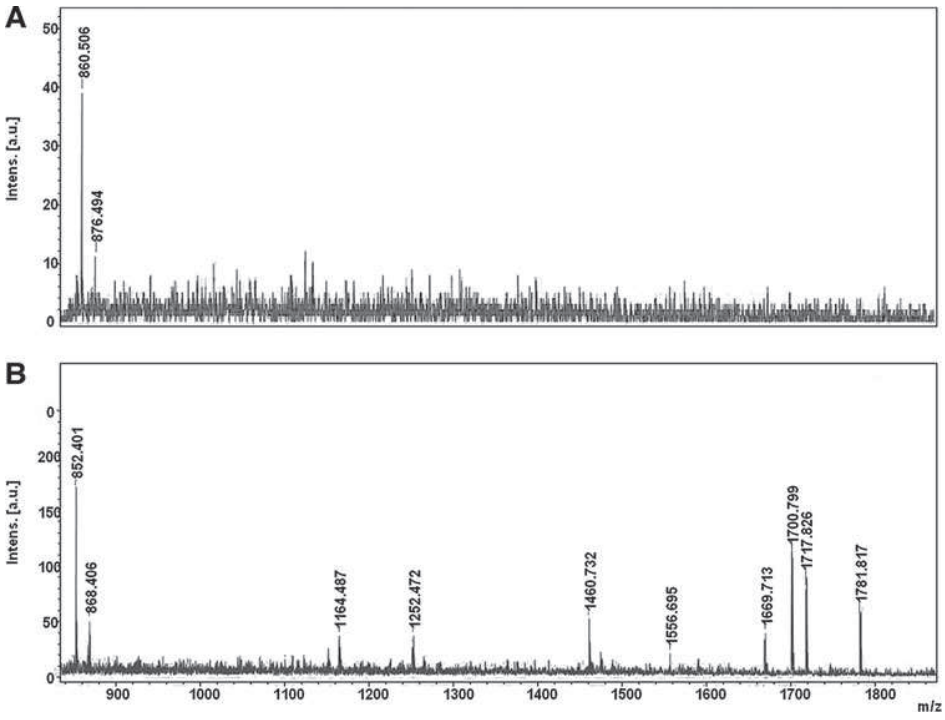

FIG. 3. Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry spectra of **(A)** low molecular fractions (≤3 kDa) of whey protein control and of **(B)** whey proteins hydrolyzed for 3 h with enzyme extracts of *Arctium minus* flowers.

typical plant APs[23] were also predicted at residues N139 and N400 (Asn-X-Ser/Thr motives).

Production of bioactive peptides

Aqueous crude extracts from flowers of genus *Cynara* are traditionally employed in the Iberian Peninsula as vegetable rennet for cheesemaking.[24] APs are responsible for the milk clotting activity (MCA) of these flower extracts; MCA was also proven in flower extracts on other members of the Cardueae tribe—for example, *A. minus.* In this article, a crude extract with 0.0167 RU/mL of MCA was partially purified by size exclusion chromatography. The resulting EE with 208 ± 4 μg protein/mL retained 0.001449 RU/mL.

Bioactive peptides are considered as prominent candidates for various health-promoting and disease risk reduction functional foods. The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules.[25] Whey protein is an abundant low-cost by-product of the dairy industry that has high nutritional, functional, and biological value.[6] Whey proteins were susceptible to proteolysis by EE from *A. minus* flowers. The extract employed is a source of APs, which are able to cleave whey proteins at more specific and unusual sites than those that commercial enzymes can do.[5] The degradation profile of whey hydrolysates were analyzed by SDS-PAGE (data not shown). Although bands corresponding to polypeptides produced by the hydrolysis were not observed by

Table 1. Analysis of Masses from Mass Spectrum with FindPept Tool

Bovine whey protein sequence analyzed	*Matched peptides*	*Δ mass (Da)*	*Position in protein*
α-Lactalbumin	Not found		
β-Lactoglobulin	(L)EILLQKWENGECAQ(K)	−0.004	71–84
	(T)DYKKYLLFCMENSA(E)	0.006	114–127
Serum albumin	(R)RPCFSALTPDETY(V)	0.009	508–520
	(L)VDEPQNLIKQNCDQ(F)	−0.008	404–417
	(K)DVCKNYQEAKDAFL(G)	−0.004	337–350
	(C)CAADDKEACFAVEGPKL(V)	0.001	582–598
Lactoferrin	(N)DTVWENTNGE(S)	−0.006	565–574
	(N)IPMGLIVNQTGSCA(F)	−0.008	488–501
	(L)AENRKSSKHSSLDCV(L)	0.002	431–445

http://www.expasy.org/tools/findpept.html.

Mass peaks ranging from 1000 to 2500 Da (m/z) present in the hydrolyzed sample mass spectrum and absent in the control were subjected to analysis with FindPept tool to identify possible peptides derived from whey proteins, which could be assigned to the peaks of the spectrum.

Analysis conditions were mass tolerance of 0.01 Da, cysteines treated with iodoacetamide (underlined), and unspecific cleavage. Amino acids in brackets are residues forming the putative scissile bonds.

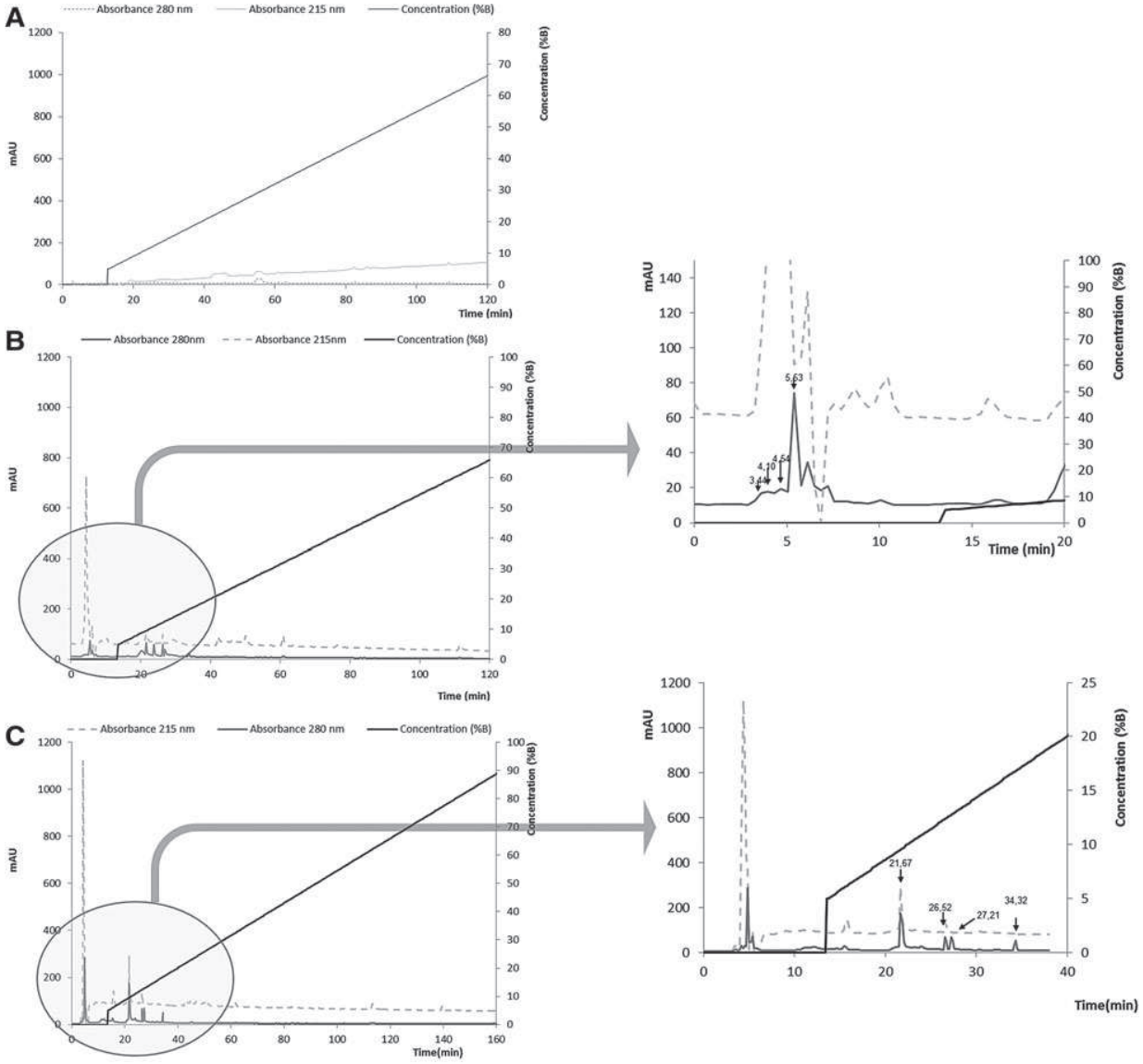

FIG. 4. **(A)** Typical chromatogram pertaining to fractionation by RP-HPLC of the ≤3 kDa-permeate obtained from bovine whey proteins (control chromatographic profile), **(B)** typical chromatogram pertaining to fractionation by RP-HPLC of the ≤3 kDa-permeate obtained from bovine whey proteins by enzyme extracts of *Arctium minus* after 5 h of hydrolysis, and **(C)** after 3 h of hydrolysis. RP-HPLC, reverse phase-high-performance liquid chromatography.

SDS-PAGE, main whey protein bands became less intense as reaction time elapsed. The hydrolysates after 3 or 5 h of reaction were characterized by DH of 2.4 ± 0.4% and 5.6 ± 0.5%, respectively.

Peptide mixtures with antioxidant and ACE inhibitory activities under optimal processing conditions (temperature, pH, and time) were obtained; that is, bioactive peptides encrypted in intact molecules were released from their encrypted position by *A. minus* APs during the reaction. Many studies have described protein hydrolysates from various sources that exhibit remarkable antioxidant properties. Tavares *et al.*[2] reported that peptides produced by digestion of whey protein concentrates and α-lactalbumin with APs from cardoon flowers exhibited a scarce antioxidant activity. On the contrary, Contreras *et al.*[6] have demonstrated a high antioxidant activity in thermolysin whey protein concentrate hydrolysates. The total *in vitro* antioxidant activity of raw whey hydrolysates after 3 and 5 h of reaction was calculated from the decolorization of $ABTS^{+\bullet}$. Hydrolysates of 3 h displayed 11.6 ± 6.5% of antioxidant activity with a Trolox equivalent antioxidant concentration of 0.06 ± 0.03 mg/mL, while hydrolysates of 5 h showed 19.5 ± 1.8% of antioxidant activity, which corresponds to 0.10 ± 0.01 mg/mL of Trolox. In view of these results, the 5-h hydrolysate was selected for subsequent antioxidant activity determinations.

To obtain a more uniform product and taking into account that bioactive peptides usually contain 3–20 amino

Table 2. Antioxidant Activity Percentage and Its Equivalents in g/mL of Ascorbic Acid

RP-HPLC peak[a]	*Antioxidant activity %*	*Ascorbic acid (g/mL)*
3.44	50.7 ± 0.1	0.52 ± 0.01
4.10	74.1 ± 0.5	0.87 ± 0.01
4.54	79.3 ± 1.0	0.94 ± 0.01
5.53	26.7 ± 1.3	0.16 ± 0.02
20.15	9.7 ± 0.2	Undetectable
21.6	15.6 ± 0.4	Undetectable
23.67	12.8 ± 0.5	Undetectable
26.23	15.0 ± 0.4	Undetectable
26.98	4.4 ± 1.1	Undetectable

The activity of RP-HPLC fractions from ultrafiltration permeate after 5 h of hydrolysis was measured by β-carotene linoleic acid assay.

[a]Retention time (min).

RP-HPLC, reverse phase-high-performance liquid chromatography.

acids,[26] the crude protein hydrolysate was processed by passage through ultrafiltration 3 kDa molecular weight cutoff membrane. Resulting permeate was analyzed by MALDI/TOF MS (Fig. 3). Several mass peaks ranging from 1000 to 2500 Da (m/z)—absent in the control—were observed in the hydrolyzed sample spectra. As the specificity of the proteolytic cleavage of the protease/s of EE is not yet known, FindPept[27] (http://expasy.org/tools/findpept.html) was used to identify possible peptides derived from whey proteins, which could be assigned to the peaks of the spectrum with a mass tolerance of 0.01 Da (Table 1).

Antioxidant activity of the ultrafiltration permeate of the 5-h hydrolysate was evaluated by β-carotene linoleic acid assay. Bleaching inhibition of β-carotene was 12.6 ± 1.4%. Permeated fraction of the 5-h degradation time was fractionated by RP-HPLC (Fig. 4B). When comparing with the control chromatographic profile (Fig. 4A), nine peaks with low retention times were observed (Fig. 4B). The antioxidant capacity of RP-HPLC fractions of 5-h hydrolysates was analyzed by the method of β-carotene linoleic acid. As shown in Table 2, peaks with 3.44, 4.1, 4.54, and 5.53 retention time retained the antioxidant activity with values in the range of 0.16–0.94 g/mL of ascorbic acid.

ACE inhibitors interfere with the renin–angiotensin system and the kinin–kallikrein system, two metabolic pathways associated with the control of blood pressure. The ability to inhibit ACE *in vitro* is indicative of the potential of a given protein-derived peptide to act as a hypotensive agent *in vivo*.[28] Hydrolysates of whey proteins and fractions enriched in individual milk proteins are potentially good sources of ACE inhibitory peptides. Classical approaches involve the *in vitro* determination of the ACE inhibitory activity of milk protein hydrolysates, obtained by enzymatic digestion or microbial fermentation.[29] Cimino *et al.*[5] reported that a 3 kDa permeate of digested whey proteins hydrolyzed for 3 h with EE of *A. minus* flowers showed 22.0 ± 3.4% of ACE inhibitory activity, the obtained value for Captopril standard (5.43 ng/mL) was 29.6 ± 2.6%, while 16.8 ± 0.5% of ACE inhibition was detected for control whey. Authors also noted that the fraction below 3 kDa of a 5-h hydrolysate presented a considerably lower activity. According to these results, permeated fraction of 3 h was fractionated by RP-HPLC. When comparing with the control chromatographic profile (Fig. 4A), 12 peaks with low retention times were observed (Fig. 4C). Major peaks obtained through RP-HPLC were selected for ACE inhibitory activity analysis. 10.1 ± 3.6%, 22.4 ± 3.2%, and 4.1 ± 2.6% of ACE inhibition were recovered for the peaks with 26.5, 27.26, and 34.27 min of retention time, respectively. ACE inhibitory peptides are not as potent as the drugs commonly used for hypertension treatment, but if protein hydrolysates exhibiting this activity are included in the formulation of functional foods, they can easily be included in the daily diet.

Conclusions

cDNA encoding a typical aspartic proteinase precursor, preproarctiumsin, was isolated from *A. minus* immature flowers. Arctiumsin shows significant similarity in the amino acid sequence to other peptidases that belong to family A1 (MEROPS Database). Results obtained in this study demonstrate the viability of using the proteases from *A. minus* flowers to increase the antioxidant and ACE inhibitory capacity of whey proteins. After 3 h of digestion (DH = 2.4 ± 0.4%), the whey protein hydrolysate displayed a higher ACE inhibitory activity than the 5-h hydrolysate (DH = 5.6 ± 0.5%). By contrast, the 5-h protein digest exhibited a higher antioxidant activity than the lesser degraded protein mixture. Both the crude protein hydrolysates were processed by passage through ultrafiltration 3 kDa molecular weight cutoff membrane and the bioactivities were recovered in the permeate fractions. Ultrafiltration was followed by RP-HPLC and several peaks maintained their corresponding activities.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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