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Arctium minus (Hill) Bernh. (Asteraceae) aspartylendopeptidases with potential application in the formulation of nutraceutical products

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ABSTRACT

Food proteins encrypt bioactive peptides that can be released during gastrointestinal digestion or food processing by enzymatic proteolysis. Flowers of Carduae tribe, family Asteraceae, contain aspartic proteinases with milk clotting activity. Crude enzyme extracts with proteolytic activity were prepared from flowers of *Arctium minus* at pH 7.0, and were partially purified and characterized. Optimun bovine milk clotting activity was achieved with $CaCl_2$ 30 mM at 35 ºC. Inhibition of milk clotting activity was only promoted by pepstatin A, a highly selective inhibitor for aspartic peptidases. Analysis of crude extract by isoelectric focusing and zymogram showed an unique active band (pI 5.0). Molecular exclusion chromatography (Sephadex G-25 Fine) was employed to eliminate pigments and phenolic compounds, in order to obtain the partially purified extract (EE). Whey bovine hydrolyzates were performed with EE and analyzed by SDS-PAGE. Hydrolyzed whey was ultrafiltrated, and low molecular fractions (peptide mass ≤ 3000 Da) showed angiotensin-converting enzyme (ACE) inhibitory activity. Therefore, these peptides with antihypertensive activity could be potentially used in food industry for formulation of nutraceutical products.

Keywords: aspartic proteases, Asteraceae, whey hydrolyzates, ACE inhibitory activity

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Introduction

Biological active peptides are of particular interest in food science and nutrition, as they can play several physiological roles. Hidden (or at least inactive) within the amino acid sequence of dairy proteins, a class of such peptides can be released (or activated) *in vivo* during gastrointestinal digestion, or upstream during food processing via specific, enzyme mediated proteolysis (Silva & Malcata, 2005), and eventually can be absorbed into the bloodstream.

Among the bioactivities attributed to those peptides are angiotensin I –converting enzyme (ACE) – inhibitory activity, opioid activity, and the ability to sequester calcium and other minerals; thus acting as biocarriers (Addeo *et al*., 1992; Meisel *et al*., 1997; Smacchi and Gobbetti, 1998; Saito *et al*., 2000; Gómez Ruiz *et al*., 2002; Sforza et al., 2003). Raw and sterilized ovine and caprine cheeselike systems manufactured with proteases from *Cynara cardunculus*, are a source of peptides with ACE –inhibitory and antioxidant activities (Silva *et al.*, 2006).

Because of the presence of aspartic proteinases, aqueous crude extracts from flowers of *Cynara cardunculus* (Veríssimo *et al.* 1995, 1996), *Cynara humilis*, and/or *Cynara scolymus* are traditionally employed in the Iberian Peninsula as vegetable rennet for cheesemaking (Reis *et al.* 2000). Milk clotting activity was also proven in flowers of *Centaurea calcitrapa*, *Onopordum turcicum* & *Silybum marianum* (Tamer, 1993; Domingos *et al*., 1998; Vairo Cavalli *et al*., 2005). All these species are included within the family Asteraceae and furthermore in the same tribe: Cardueae Cass.=Cynareae Less. (Ariza Espinar and Delucchi, 1998). In this work novel aspartylendopeptidases from flowers of *Arctium minus* (Asteraceae) were employed in the digestion of bovine whey proteins to produce ACE inhibitory peptides.

Experimental

Vegetal source

Arctium minus (Hill) Bernh is a biennial thistle, also known as Burweed. This plant is native to Europe, but is now widespread throughout most of the Argentina as a common weed. Flowers are prickly and pink to lavender in color. Mature inflorescences were collected in the sorroundigs of La Plata (Buenos Aires, Argentina) during the spring season (october-november).

Enzyme extract preparation

Fresh flowers of *Arctium minus* were ground in a mortar under liquid nitrogen and homogenized in extraction buffer (1 g per 3 ml) to obtain a crude extract (CE). Three extraction buffers were proven for the optimization of peptidase isolation: a) 0.1 M citrate buffer (pH 3.0) (Faro *et al*., 1992), b) 0.1 M phosphate buffer (pH 7.0) containing 5 mM cysteine (Llorente *et al*., 1999) and c) 0.1 M phosphate buffer (pH 7.0) containing 5 mM cysteine and 1% PVP. In all cases 1.0 mM EDTA was added. The suspension was stirred for 30 min and centrifuged at 5000 *g* for 20 min at 4 °C.

Milk clotting activity (MCA)

Skim milk powder, (San Regim, SanCor) was reconstituted by dissolving 10 g in 100 ml of 10 mM or 30 mM $CaCl_2$, containing 0.1% sodium azide. CE (100 μl) was added to 1 ml milk and the clotting time was measured. The assays were performed at 30 °C and 35 °C. Positive controls were performed with an extract of flowers of *Cynara scolymus* (Llorente *et al.*, 2004) while negative controls were carried out with extraction buffer.

One rennet unit (RU) is the amount of enzyme that coagulates 10 ml milk at in 100 s in the assay conditions (Barros *et al.* 2001).

Inhibition assay

To obtain data on the mechanistic grouping of this new proteolytic activity the effect of a set of inhibitors was tested (Dunn, 2001). The CE was preincubated for 15 min at 25 °C in the presence of the following inhibitors: 50 μM pepstatin A, 1.5 mM PMSF, and 0.1 mM E-64. The remaining MCA was determined as previously described. A control assay was done without inhibitors and the resulting activity was taken as 100%.

Isoelectric Focusing (IEF) and Zymogram

IEF was performed in a Mini IEF Cell (Model 111, Bio-Rad). The CE was concentrated and deionized by acetone precipitation and further centrifugation at 3000 *g* for 15 min; the precipitate was redissolved with deionized water and the treatment was repeated twice. Polyacrylamide gels containing broad pH range ampholytes (3.0–10.0) were used. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V for 15 min, 200 V for 15 min and 450 V for 60 min. Gel was fixed and then stained with Coomassie Brilliant Blue R-250. In order to ascertain which protein fractions showed proteolytic activity, unstained gel was contacted for 20 min at 56 °C with an agarose gel imbibed during 20 min in 2% hemoglobin suspension (pH

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4,0) and washed twice with distilled water. Agarose gel was stained with Coomassie Brilliant Blue R-250. Proteolytic activity became visible as clear bands on the stained agarose gels (Westergaard *et al*., 1980).

Peptidase partial purification

Pigments and other phenolic compounds were eliminated by size exclusion chromatography (SEC). 1.5 ml of CE was applied to a PD 10 Pharmacia column packed with Sephadex G-25 Fine (Amersham Biosciences) equilibrated with citric-disodium phosphate buffer (pH 6.2). Elution was performed with the same buffer at 0.45 ml/min flow rate and fractions collected were monitored at 260, 280 y 330 nm. The enzyme extract (EE) was obtained at the void volume.

Protein content was measured following the method of Bradford (1976), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO 63178, USA) as standard.

Electrophoresis (SDS-PAGE)

Samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 14% (w/v) polyacrylamide gels (Laemmli, 1970). Current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 min. Applied volumes varied between 10 and 15 µl. Gels were fixed and stained with Coomassie Blue G-250. The molecular weight standards used ranged from 14.4 kDa to 94 kDa (GE).

Enzymatic hydrolysis of bovine whey

The substrate for hydrolysis consisted on a 1% bovine whey suspension (LAC PRODAN 80). The reaction was started by addition of 4.6 ml of substrate to 90 µl of the EE (208 µg protein/ml). The assays were performed at 35 °C. The reaction was quenched at 3 and 5 h by 5 min boiling. Controls containing bovine whey suspension at the same concentrations without addition of enzyme, with the enzyme inhibited with pepstatin A and with enzyme thermally inactivated were also carried out. Samples were analyzed by SDS-PAGE in a 20% (w/v) polyacrylamide gel.

Hydrolyzed whey was ultrafiltrated in Amicon tubes at 4000 *g*, low molecular fractions (≤ 3000 Da) were stored for future assays.

Determination of antihypertensive activity

Protein hydrolyzates were tested as potential ACE inhibitors in kinetics assays by using the fluorogenic substrate abz-PheArgLys(DNP)Pro-OH. The activity was determined incubating the enzyme with substrate in the presence or absence of the hydrolyzates, using the commercial inhibitor captopril as control (Carmona *et al.*, 2006).

Results and Discussion

Table 1 shows MCA of CE obtained with different buffers at different $CaCl_2$ concentration. As can be seen phosphate extraction buffer without PVP and milk prepared with 30 mM $CaCl_2$ were the best assay conditions with the highest UR values. Thus, buffer 2 was selected for further extractions.

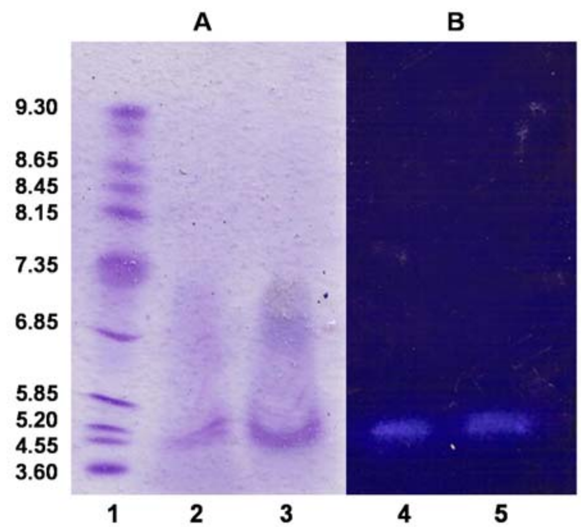

Figure 1. A: IEF. *Line 1*: isoelectric point markers: Amyloglucosidase, 3.6; Soybean trypsin inhibitor, 4.55; β-Lactoglobulin A, 5.20; Bovine carbonic anhydrase B, 5.85; Horse myoglobin-acetic band, 6.85; Horse myoglobin-basic band, 7.35; Lentil lectin-acidic band, 8.15; Lentil lectin-midle band, 8.45; Lentil lectin-basic band, 8.65; Trypsinogen, 9.30. *Line 2:* CE of *A.minus* with PVP. *Line 3:* CE of *A.minus* without PVP. **B**: zymogram. Line 4: CE of *A.minus* with PVP. *Line 5:* CE of *A.minus* without PVP.

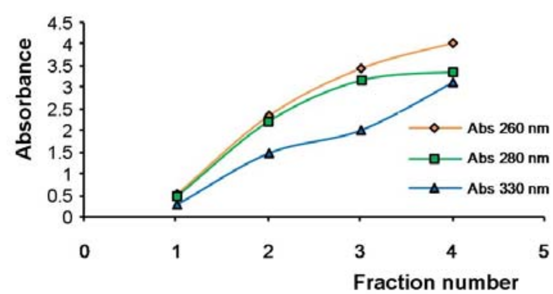

Figure 2. UV Absorbance of fractions eluted from size exclusion chromatography of *Arctium minus* crude extract.

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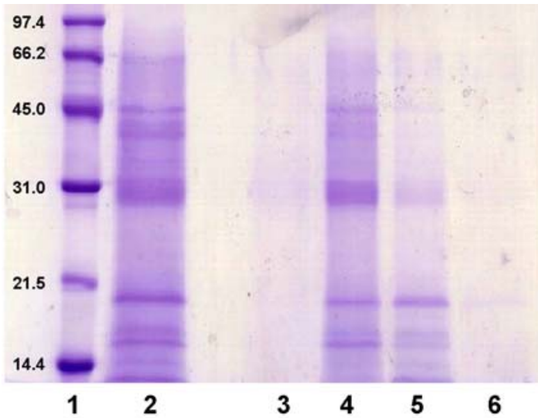

Figure 3. SDS-PAGE. *Line 1*: Low molecular weight protein markers: Phosphorylase b, 97.4 kDa; Serum albumin, 66.2 kDa; Ovalbumin, 45.0 kDa; Carbonic anhidrase, 31.0 kDa; Trypsin inhibitor, 21.5 kDa; Lysozyme, 14.4 kDa. BioRad; *Line 2*: Crude extract of *A. minus*; *Lines 3,4,5 and 6*: fractions eluted from size exclusion chromatography.

Table 1: *Arctium minus* crude extract milk clotting activity.

		Extraction buffer		
		Buffer 1[a]	**Buffer 2[b]**	**Buffer 3[c]**
10% milk, 10 mM $CaCl_2$	**30°C**	Not coagulated	0.0034 UR/ml	0.00048 UR/ml
	35°C	-	0.0019 UR/ml	Not coagulated
10% milk, 30 mM $CaCl_2$	**30°C**	Not coagulated	0.0024 UR/ml	Not coagulated
	35°C	-	0.0167 UR/ml	Not coagulated

[a] 0.1 M citrate buffer (pH 3.0), 1mM EDTA.
[b] 0.1 M phosphate buffer (pH 7.0), 5 mM cysteine, 1.0 mM EDTA.
[c] 0.1 M phosphate buffer (pH 7.0), 5 mM cysteine, 1.0 mM EDTA and 1% PVP.
(-): Assay not done

Table 2. Inhibitory assays on milk clotting activity of crude extract from *A. minus*

Inhibitor	UR/ml
Negative control	0.0009259
Pepstatin A	Not coagulated
E-64	0.0009259
PMSF	0.0009259

Table 3. Milk clotting activity of fractions collected from size exclusion chromatography of the crude extract of *A. minus*

Fraction	UR/ml
1	0
2	0.001449
3	0.001449
4	0.000412

To elucidate the nature of the proteolytic activity involved in MCA the effect of a set of specific inhibitors was tested, though inhibition was only promoted (100% of inhibition) by pepstatin A. As pepstatin is one of the most specific inhibitors known in enzymology and highly selective for the aspartic peptidases (Dunn, 2001) CE MCA was due to the presence of aspartic peptidases. Inhibitory assays are shown in Table 2.

As can be observed in Fig. 1 proteins present in *A. minus* CE have mostly isoelectric points below 7. This extract presents a single active protein band corresponding to a pI below 5.20, as shown in Figure 1B. The great majority of aspartic peptidases are most active at acid pH, this acid nature had been previously observed in proteases isolated from others members of the family *Asteraceae* (Veríssimo *et al.* 1995, 1996).

The first four fractions collected from SEC showed significant absorbance values at 260, 280 and 330 nm as seen in Figure 2. MCA of these fractions was determined with milk reconstituted in 30 mM $CaCl_2$ at 35 °C. Fractions 2, 3 and 4 retained the clotting activity (Table 3). Fractions with clotting activity were pooled and constituted the partially purified enzymatic extract (EE). EE protein content determined by Branford's method was 208 µg / ml. Electrophoresis of CE and EE (Figure 3) shows the protein profile. These profiles are consistent with the results of MCA obtained for each fraction eluted from SEC (Table 3).

Degradation profile of bovine whey hydrolyzates were analyzed by SDS-PAGE, but no peptides were visualized in samples filtered with membrane with cut-off of 3 kDa (data not shown). This may be related to the electrophoretic technique used and the small size of the peptides obtained or the lack of fixation of small polypeptides.

A 17% of ACE inhibition was detected with whey and 22% with 3h hydrolyzate (Table 4). It is likely that the highest value can be attributed to

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the presence of peptides released during hydrolysis. 5 h hydrolyzate presented a considerably lower activity, which may result from the degradation of inhibitory peptides. Captopril (0.025 µg/ml) causes 30% of inhibition. Concentration of captopril of 0.25 µg/ml or greater caused 100% ACE inhibition. Because EE from flowers of *A. minus* release peptides from whey proteins with antihypertensive *in vitro* activity in controlled conditions, these extracts could be potentially used in food industry for formulation of nutraceutical products.

able 4. ACE inhibitory activity of whey hydrolysate

Simple	**ACE inhibitory activity of whey hydrolyzates**
Whey	17%
Hydrolyzed Whey 3h	22%
Hydrolyzed Whey 5 h	7%
Captopril (0.025µg/ml)	30%

Conclusions

A new proteolitically active extract was obtained from fresh flowers of *Arctium minus* containing aspartic proteases (pI 5.0). This extract provoked milk clotting in the presence of 30 mM $CaCl_2$ at 35 ºC. Hydrolyzed whey showed angiotensin-converting enzyme (ACE) inhibitory activity. Therefore, these peptides with antihypertensive activity could be potentially used in food industry for formulation of nutraceutical products.

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