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Physical and chemical properties of the acid protease from *Onopordum acanthium*: Comparison between electrophoresis and HPLC of degradation casein profiles

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A protease was extracted by grinding, precipitation and gel filtration from *Onopordum acanthium* flowers. The physicochemical study of the enzyme showed an optimum pH of 4, a temperature of 40°C and kinetic parameters of 12.25 mM^{-1} for K_M and 1329.6 UmL^{-1} for V_{max}. The inhibition by pepstatin indicated that it is an aspartyl-protease (APs). Zymogram showed that the protease has a monomeric structure and a molecular mass (MM) of 45 kDa. The hydrolysis of α, β and κ- and whole casein by the protease was evaluated using electrophoresis and HPLC; the profiles showed many similarities between the vegetal protease action and that of industrial chymosin. So, the properties of the protease studied and the quality of its action showed its effectiveness and relevance of its use as a milk clotting enzyme which leads to a better use of extract of flowers *O. acanthium* as a locally substitute for rennet.

Key words: Aspartic protease, *Onopordum acanthium*, purification, characterization, casein hydrolysis.

INTRODUCTION

Proteases are one of the industrially most important enzymes. They modify the chemical, physical and biological properties of proteins and, account for approximately 60% of all enzyme sales because of their varied applications in food, pharmaceutical and a number of other industries (Ikasari and Mitchell., 1996). Proteolysis is the principal set of biochemical changes during ripening of most cheeses. The demand for alternative sources of milk coagulants, to replace the expensive and limited natural rennet supplies, has increased (Esteves et al., 2001). In Portugal and bordering regions of Spain, crude aqueous extracts of thistle flowers (*Cynara*

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cardunculus, Cynara humilis and *Cynara scolymus*), have been used for centuries as coagulant of milk for the artisanal cheese making (Reis et al., 2000). The extracts of the flowers of *Cynara* species have been claimed to be effective as rennet (Silva and Malcata, 2000; Chazarra et al., 2007). The flower extracts from C. *cardunculus* possess two aspartic proteases, A and B cardosins, which are chymosin- and pepsin-like in activity and specificity, respectively (Heimgartner et al., 1990; Silva et al., 2006). All commercial enzymes used in milk coagulation are aspartic proteinases (E.C. 3.4.23); they are most active at acidic pH, specifically inhibited by pepstatin A and contain two aspartic residues for catalytic activity (Davies, 1990). In most studies of plant enzymes, other aspects take generally precedence over their characterization.

The main objectives of the present work were to characterize the physical and chemical properties of aspartic protease obtained from flowers of *Onopordum acanthium* (family *Asteraceae*) and to determine its milk coagulating ability by analysis of the products of casein hydrolysis with electrophoresis and reverse phase high performance liquid chromatography (RP-HPLC) techniques.

MATERIALS AND METHODS

Plant material

O. acanthium L., a flowering plant belonging to the *Asteraceae* family, was collected from roadsides in Constantine, Algeria, during flowering season (May). Flowers were dried in a desiccator using the $CaCl_2$.

Enzyme extraction and purification

The dried flowers were ground in a mortar under liquid nitrogen, solubilized in citrate/sodium 0.05 M buffer pH 5.5, stirred for 30 min and cleared by centrifugation at 15 000 *g* for 20 min at 4°C. The supernatant obtained was the crude extract.

Ammonium sulfate fractionation

Crude extract was precipitated between 30 and 80% saturation of $(NH_4)_2SO_4$. The precipitate obtained was centrifuged at 10000 *g* for 30 min at 4°C, suspended in 0.05 M citrate/sodium buffer pH 5.5 then dialyzed overnight against the same buffer. The salt concentration providing the highest protease recovery was chosen for further purification.

Size-exclusion chromatography

The concentrated enzyme dialyzed was subjected to gel filtration on a Sephadex G-100 column (60 cm × 1.5 cm) pre-equilibrated with 50 mM citrate/sodium buffer pH 5.5. Enzyme fractions of 2 ml were eluted at 12 ml/h flow rate with the same buffer and were analyzed for protease activity, milk-clotting activity and protein content. Active enzyme fractions were pooled, concentrated by lyophilization and used for molecular weight determination and for further assays.

Protein and proteolytic activity assays

Proteins content was assessed with the Lowry method (1951), using bovine serum albumin as the standard (BSA, sigma chemical). The proteolytic activity was measured according to Mechakra et al. (1999) method using casein as substrate (2% in 0.02 M citrate/sodium), the reaction solution containing 2.5 ml of casein plus 1.5 ml citrate/sodium buffer (pH 5.5). The reaction was started by adding 1 ml of the enzyme solution; the assay mixture was incubated at 40°C in water bath for 60 min, 5 ml of the 4% (w/v) trichloroacetic acid was added to stop the reaction. Blanks were prepared by adding TCA to the enzyme, then adding the substrate. The precipitates formed were removed by filtration through Whatman No. 1 filter paper. 2.5 ml of 2% of Na_2CO_3 dissolved in 0.1 N NaOH was added to 1 ml of the above clear filtrate then incubated at room temperature for 10 min then adding 0.25 ml of Folin-Ciocalteu reagent diluted to 50%. This was further incubated for 30 min at room temperature for colour development. The optical density at 750 nm expresses activity. One unit of protease activity was defined as the enzyme quantity which liberates 1 µg/mL of tyrosine per hour under assay conditions.

Milk clotting activity

The milk clotting activity (MCA) of the plant protease was determined as described by Berridge (1945). One milk clotting unit is defined as the amount of enzyme present in 1 ml of extract clotting 10 ml substrate in 100 s at 30°C according to the calculation:

$$RS = 10 \times V / Tc \times v$$

Where, RS = rennet strength, V one volume of milk (ml), v one volume of rennet (ml) and t the clotting time in seconds.

Characterization of protease

Optimum temperature and thermal stability

The protease activity was measured using casein as a substrate at different temperatures ranging from 20 to 80°C at pH 5.5. Heat stability and half-life time of the purified protease were estimated by measuring the residual activity after incubation at 60°C for 10 to 60 min. The non-heated enzyme was used as 100% control. The experimental half-life for the characterized protease is the time at which loss of activity reached 50%; ($t_{1/2}$= ln 2/k), with k is the constant of inactivation enzyme.

Determination of optimum pH

The optimum pH was determined by measuring the effect of pH between 2.5 to 6.5 on the proteolytic activity using denatured hemoglobin as substrate instead of the casein which precipitates at acid pH.

Kinetic parameters determination

The kinetic parameters (V_{max} and K_M) of the pure protease were calculated from the graphical representations of the effect of substrate concentration on the activity according to the Michaelis and Lineweaver-Burk method.

Effect of inhibitors

Effects of pepstatin-A (1 mM and 10 mM), EDTA (10 mM) and iodoacetamide (10 mM) on the protease activity have been examined. The enzyme extract was incubated with each compound for 2 h at 20 °C. The residual activities were measured at pH 5.5 using casein as substrate. Activities were compared with the enzyme activity in absence of any inhibitor (100%).

Determination of the molecular mass

The molecular mass of the purified protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (w/v) acrylamide, according to Laemmli (1970). Zymogram was made to confirm proteolytic activity of the bands, according to the Westergaard's method (Westergaard et al., 1980) with minor modifications. The activity was detected by incubating the gel in 2% (w/v) casein in citrate/sodium 0.05 M buffer pH 5.5 for 1 h at 40°C. The gel was then washed three times in water and stained with Coomassie Brilliant Blue R-250. The development of a clear colorless area on the blue background of the gel indicated the presence of protease activity.

Enzymatic hydrolysis of caseins and electrophoresis

When a potential rennet substitute is studied, it is important to evaluate the degradation patterns of the caseins because of their effect on the yield, consistency, and flavor of the final cheese (Fox, 1989). The action of the *O. acanthium* protease was tested on whole commercial α, β and κ-casein. Caseinates (Sigma Aldrich Co.) were dissolved to a final concentration of 1% (w/v) in 100 mM sodium phosphate buffer (pH 6.5) containing 0.1% sodium azide (w/v) to prevent protein degradation by adventitious microflora. The reactions were started by addition of 450 µl of each substrate to 45 µl of commercial rennet and extract flower enzyme at 40°C. The reactions were stopped by addition of 500 µl of 5% TCA (w/v) at 30, 60, 90 and 120 min. The samples were left to precipitate overnight at 4°C, and then centrifuged at 10,000 *g* for 15 min. For the electrophoretic analysis, the precipitates were dissolved in 450 µl of 62.5 mM Tris–HCl buffer pH 6.8 containing 2% SDS (w/v), 0.5% 2-mercaptoethanol (v/v), 0.02‰ (w/v) bromophenol blue and 10% glycerol (w/v). The mixture was vortexed four times for 30 s, then heated at 100°C for 5 min. Controls containing Na-caseinate and NaN_3 at the same concentrations but without addition of enzyme was also sampled. Samples were subjected to denaturing electrophoresis (SDS PAGE) in glycine as described by Laemmli (1970) at 4°C using a constant voltage (90 to 100 V) and a staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol.

Enzymatic hydrolysis of caseins and RP-HPLC

Hydrolysis and RP-HPLC analyses were carried out according to Gallagher et al. (1994) with some modifications. Hydrolysis of caseins (Sigma Aldrich Co.) at 1% in 50 mM sodium citrate buffer pH 5.5 was realized at 40°C by addition of 0.1 mL of enzyme solution to 0.9 mL of substrate. Enzyme solutions (*O. acanthium* extract and commercial rennet enzyme) were standardized to equal milk-clotting activity by the method of Arima et al. (1970). The reaction was stopped by heating at boiling temperature for 6 min. An aliquot was filtered through 0.45 µm filters prior to RP-HPLC analyses to remove any particulate protein material in the hydrolysates. For RP-HPLC analyses, a Jasco PU-2089 HPLC Pump was fitted with a TRACER EXTRASIL ODS2 C18 5 µm reversed phase column (4.6 to 250 mm) and a UV detector at a wavelength of 220 nm. Each sample (20 µL) was injected and eluted with 0.06% trifluoroacetic acid (TFA)/HPLC grade water as a mobile phase, at a flow rate of 0.7 mL/min. The concentration of the mobile phase modifier (0.056% TFA/HPLC grade methanol) was increased linearly from 0 to 91%.

RESULTS AND DISCUSSION

Purification of protease

Table 1 shows the steps of purification of protease: Ammonium sulphate precipitated (30% saturation) and dialyzed, and partially purified protease was further purified by the size-exclusion chromatography. The extent of purification was up to 26.65-fold with 30% recovery (specific activity 5145.51 U/mg), having high specific caseinolytic activity desirable for rennet substitutes. These results are higher than those reported about microbial acid proteases as from *Synergistes* sp. and *Aspergillus niger* that were purified with 15.8 and 9.14 fold-purification and 2.4 and 20.4% recovery successively (Ganesh Kumar et al., 2008; Fazouane-Naimi et al., 2010). Effectively, the same protocol was followed in both work compared with ours, except for the concentration of ammonium sulfate used during the precipitation (80% instead of 30%). Indeed, a study conducted by Phanuphong et al. (2010) on the extraction and separation of proteases from papaya peels showed the highest protease activity recovery and the highest purification fold when concentration of $(NH_4)_2SO_4$, decreased. Narayan et al. (2008) reported that the increased concentration of $(NH_4)_2SO_4$, decreased the degree of purification significantly.

Milk clotting activity

The ability of crude extract and purified enzyme from *O. acanthium* flowers for milk clotting was measured compared with the commercial rennet at different concentrations (Table 3).

According to the results, the milk clotting activity obtained with the commercial rennet increases with the concentration of the enzyme as reported by the literature (Chitipinityol and Crabbe, 1998). For the crude extract (193,07U/mg total protein), the clotting activity was $46.3x10^{-4}$ corresponding at 6 h of clotting time. This value is better compared to that achieved for the crude extract from *C. calcitrapa* seeds (180 U/mg total protein) which noted $37.03x10^{-4}$ of clotting activity so 7.5 h of clotting time (Matos Salvador et al., 2006).

On the other hand, the purified enzyme showed higher milk clotting activity than commercial rennet. Similar data was noted by Hashim et al. (2011) using enzyme fractions of ginger rhizomes and by Su et al. (2009) which also reported that ginger proteases could be a choice for cheese making as well as a milk-clotting enzyme source.

These results confirm the effectiveness of *O. acanthium*

Table 1. Purification data of acid protease from *O. acanthium* flowers.

Step of purification	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	55	28681.01	148.55	193.07	1	100
(NH4)2SO4 30%	5	16456.32	42.27	389.31	2.01	57.37
dialyse	6.5	23226.92	35.55	656.45	3.4	80.98
Sephadex G100	16	8541.55	1.66	5145.51	26.65	29.78

proteases in the coagulation of milk as many others plant proteases as the fig tree latex (*ficus carica*) and thistles extracts (*Cynara cardunculus* L. and *Cynara scolymus*) used for a long time in Algeria in the traditional cheesemaking preparations (Androuet, 2002).

The high milk coagulating activity associated with its ability to hydrolyze casein showed that the *O. acanthium* purified enzyme could be useful in the dairy industry for milk coagulation and for the enhancement of cheese ripening process in order to save time and costs of storage for maturation of cheeses.

Temperature optima and thermal stability

Figure 1a shows that the purified acid protease acted at an optimum temperature of 40°C; it loosed 35% of activity after 60 min of incubation at 50°C and it was completely inactivated at 70°C as observed by Sushil Kumar et al. (2005) and Sumantha et al. (2006) for proteases from *Rhizopus oryzae* and seeds of *Centaurea Calcitrapa*, respectively. A progressive reduction in rennet coagulation times as temperature increases from 20 to 40°C has been reported by Najera et al. (2003). Figure 1b shows the thermal stability profiles at 60°C. Loss of activity of 50% is observed after 20 min of incubation and of 90% after 60 min. The *O. acanthium* protease is more stable than microbial like acid protease from *Mucor sp* which retained only 13% of activity at 60°C after 30 min incubation time (Fernandez-Lahor et al., 1999). The experimental half-life for the characterized protease was 19 min at 60°C (Figure 1c) indicated the better stability than the acid protease from *Penicillium expansum* which noted 17 min at 50°C (Umar Dahot, 2001).

Determination of the optimal pH

The effect of pH on the activity of purified protease was determined with denatured hemoglobin over the pH range of 2.5 to 6.5 using citrate sodium buffer (0.05 M). The maximum reaction product was at pH 4 (Figure 2), as observed for other aspartic proteinases such as calf, pig and lamb chymosins (Foltmann and Szecsi, 1998). This is similar to that of aspartyl proteases from *Silybum Marianum* (Vairo-Cavalli et al., 2005) and flowers of *C. calcitrapa* (Domingos et al., 1998) while higher peak activity in Australian cardoon (*Centaurea Cardunculus*) was reached at pH 6.0 (Chen et al., 2003), but in this case casein (not hemoglobin) was used as substrate crude extracts of dried flowers of artichoke obtained at different pH values tested for their clotting activity showed a maximum activity at pH of around 4. Extraction pH 4 was therefore used for rennet preparation (Chazarra et al., 2007).

Kinetic parameters determination (V_{max} and K_M)

The rate of aspartic protease catalyzed reactions was obtained at different concentrations of casein as substrate. A plot was drawn between the rates of acid protease catalyzed reaction (V) versus the casein concentration (S), varying substrate concentration gave an hyperbolic response (Figure 3a). The same data was obtained by Sushil Kumar et al. (2005). V_{max} and K_M evaluated from Lineweaver and Burk plot (Figure 3b) were 2.34 g/L and 1329.6 UmL^{-1}, respectively, with R^2 of 0.91. The low K_M and high V_{max} values inferred that the high affinity and efficient catalytic role of the enzyme.

Effect of inhibitors

The inhibitors (pepstatin A, iodoacetamide and EDTA) were tested to identify the active site groups of the studied enzyme (Table 2). The total inhibition in the presence of 1 mM of pepstatin A indicates that the protease belongs to the class of aspartic proteases (acid proteases), while the little inhibition by iodoacetamide (2%) and EDTA (2.5%) shows that the enzyme is not a cysteyl protease or a metalloprotease. Similar results were observed for acid protease from latex of *Ficus racemosa* which has been inhibited at 100% by pepstatin A (1 mM) and no affected with EDTA (residual activity of 99%) (Devaraj et al., 2008). Brutti et al. (2012) showed 99.5% of inhibition of acid protease from the same plant by the pepstatin- A and Benchiheb et al. (2013) obtained similar data concerning the inhibition of the acid protease from *Scolymus maculatus* flowers.

Determination of the molecular weight of *O. acanthium* protease

The SDS-PAGE and the zymogram analysis of *O.*

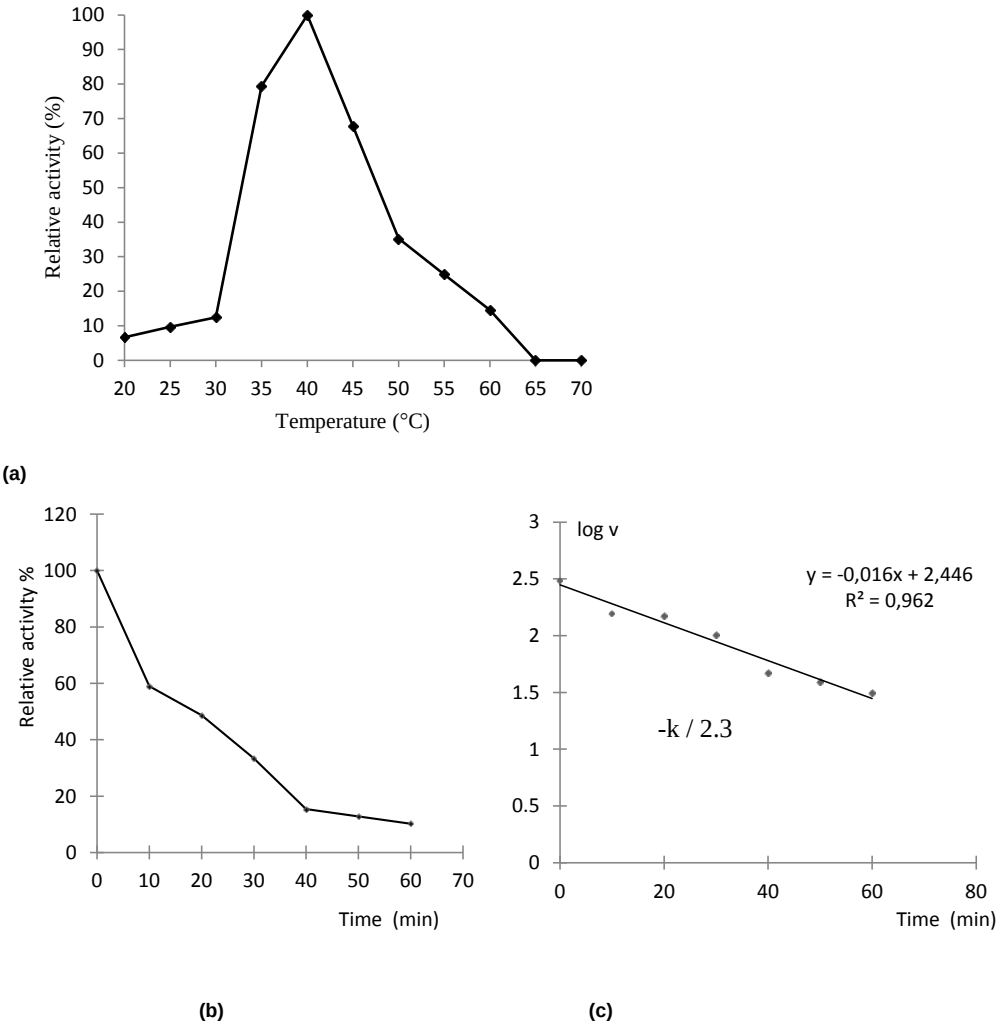

Figure 1. Effect of temperature (a) Activity in the temperature range of 20-70°C (b) Stability of the purified acid protease at 60°C (c) Half-life of acid protease at 60°C.

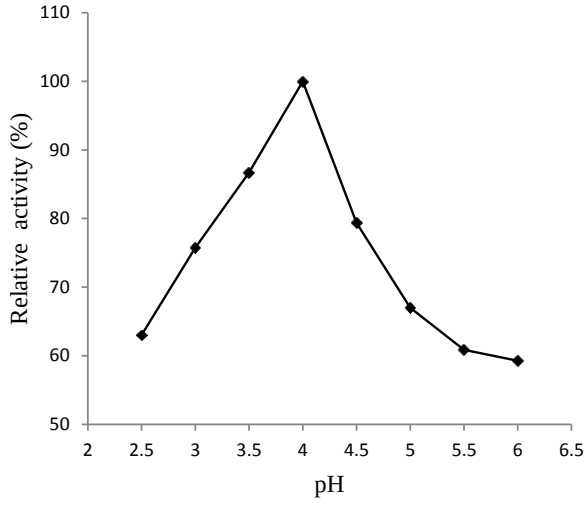

Figure 2. Effect of pH on protease activity purified from *Onopordum acanthium*.

acanthium protease obtained after molecular filtration showed a single band of proteolytic activity on the gel, so suggesting the monomeric nature of enzyme (Figure 4). The marker calibration curve allowed calculating a molecular weight of 45 kDa as obtained by Brutti et al. (2012). Many others aspartyl proteases have similar MM to our results; both for those obtained from plant sources and those from fungal sources. This is the case for the acid protease of *S. maculatus* Benchiheb et al. (2013) and of moulds Nouani et al. (2009) and Fazouane-Naimi et al. (2010).

Hydrolysis profile of caseins by *O. acanthium* protease

Electrophoresis profile of casein degradation

The extent of degradation of major caseins and the

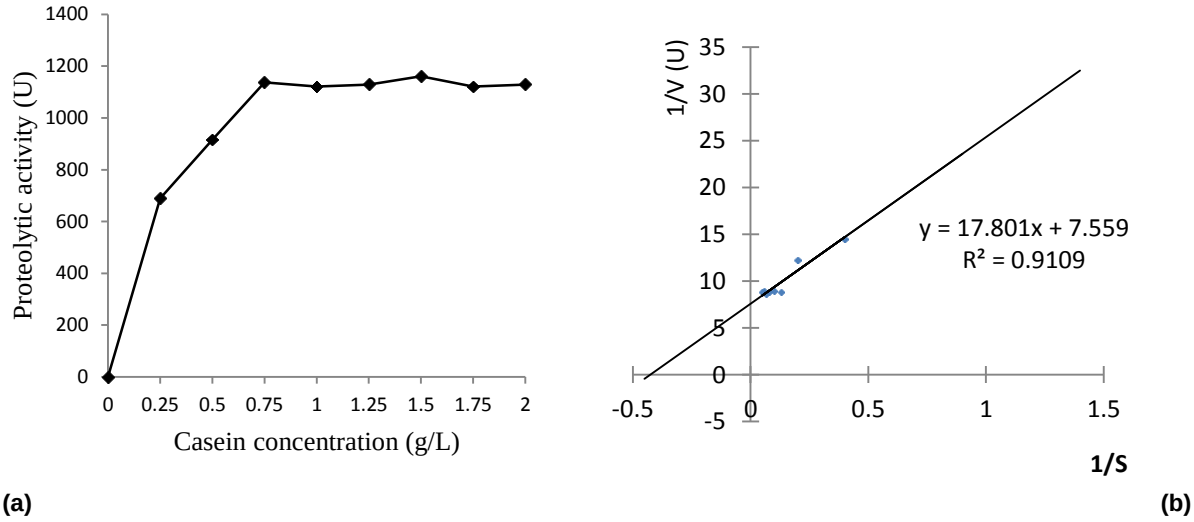

Figure 3. Kinetic parameters determination. (a) Michaelis-Menten plot V vs. (S); (b) Lineweaver-Burk plot 1/V vs. 1/(S).

Table 2. Effect of inhibitors on *Onopordum acanthium* acid protease.

Inhibitors	Concentrations (mM)	Residual activity (%)
None	-	100
EDTA	10	97.43
Pepstatin- A	1	0
Pepstatin- A	10	0
Iodoacetamide	10	98%

Table 3. Milk-clotting activity of *O. acanthium* crude extract and purified protease and of commercial rennet.

Enzyme	Concentration (mg /ml)	Milk-cloting activity
O. acanthium crude extract	2.7	$46.3x10^{-4}$
O. acanthium purified protease	0.10	$139\ x10^{-4}$
Commercial rennet	2	$10,73x10^{-3}$
Commercial rennet	4	$200x10^{-3}$
Commercial rennet	6	$220x10^{-3}$
Commercial rennet	8	$224x10^{-3}$
Commercial rennet	10	$330x10^{-3}$

hydrolysis products by protease from *O. acanthium* and by commercial rennet are presented in Figure 5. The κ -casein was the fraction with highest mobility than β-casein and α-casein as observed by Pardo and Natalucci (2001). After incubation with enzyme extract, casein bands tended to disappear; with higher mobilities appeared after 30 min, showing the formation of casein fractions with lower molecular mass. As can be seen in Figure 5, the content of casein components decreased in both types of enzymes. After 120 min, the degradation of κ-casein was lower with commercial rennet than those obtained with plant protease. Otherwise, both enzymes showed a similar behavior on α-casein, but the β-casein disappeared immediately in the case of *Onopordum* protease, and not completely degraded at 120 min in the case of the commercial rennet. The comparable data was obtained by many others such as Brutti et al. (2012); Vairo-Cavalli et al. (2005); Chazarra et al. (2007) and Egito et al. (2007).

Electrophoresis profile of κ casein hydrolysis

After 30 min of incubation, the similar profile of the

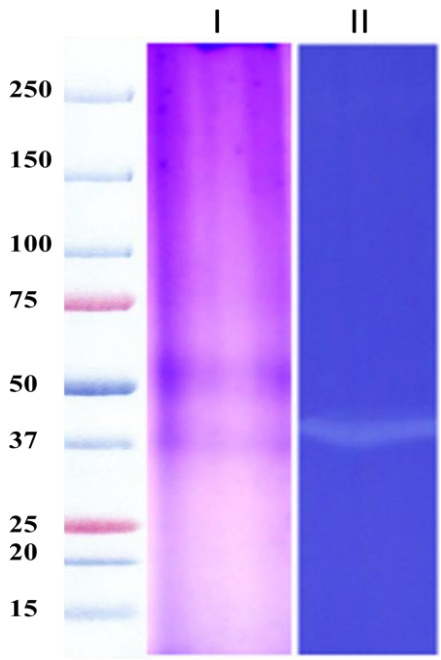

Figure 4: SDS-PAGE gel electrophoresis of the purified *Onopordum acanthium* protease. Lane I: purified protease; Lane II: zymogram of protease with casein as substrate.

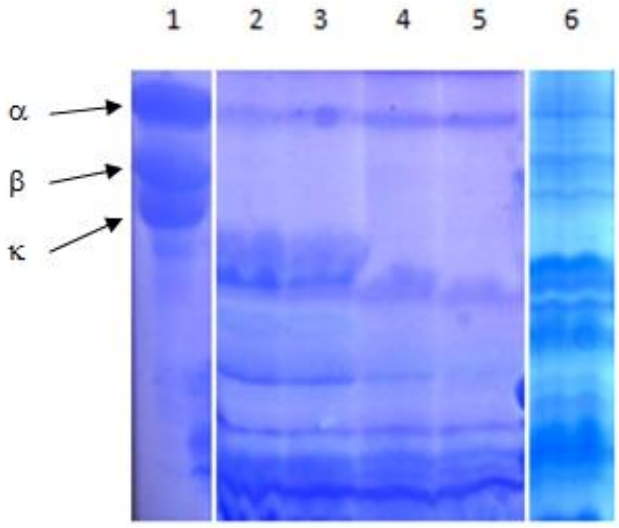

Figure 5. Degradation of of α, β and κ-casein by *Onopordum acanthium* protease and commercial rennet. Lane 1: intact caseins; Lanes 2-5: caseins hydrolysis by *O. acanthium* protease for 30, 60, 90 and 120 min; Lane 6: caseins hydrolysis by commercial rennet for 120 min.

degradation of κ-casein was observed by *O. acanthium* proteases and by commercial rennet (Figure 6), the band of κ-casein disappeared immediately in the cases 2 and

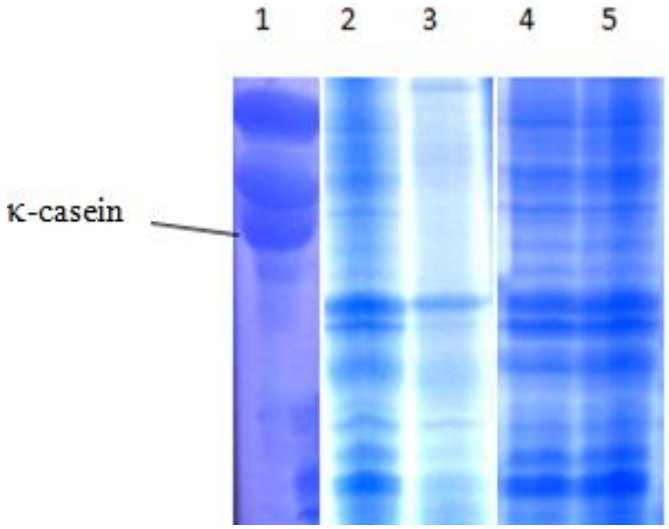

Figure 6. Degradation of κ casein by *Onopordum acanthium* protease and commercial rennet. Lane 1: intact casein; Lanes 2- 3: casein hydrolysis by *Onopordum acanthium* protease for 30min and 120 min; Lanes 4- 5: casein hydrolysis by commercial rennet for 30min and 120 min.

4. However, after 120 min, the degradation of k-casein was lower by commercial rennet than with *Onopordum* protease, so that confirm the last result obtained with degradation of different caseins (Figure 5). Both enzymes present the similar profile of degradation of the κ-casein, but with a faster migration of the plant protease activity. Similar electrophoretic patterns was obtained with the cynarases A, B and C extracted from *C. scolymus* and also, with calf rennet (Chazarra et al., 2007).

RP-HPLC profiles

The potential of hydrolysis of the casein fractions are important to characterize the viability of an enzyme's industrial application. The chromatograms of peptides from intact caseins and from the hydrolysates formed by the action of the commercial rennet and enzymatic extract from *O. acanthium* are shown successively in Figures 7 and 8. The order of hydrolysis obtained with the plant enzyme was show in the following: κ-CN, β-CN, and α-CN; it corroborates the data obtained with electrophoresis. The κ-CN and β-CN components disappeared in 30 min of hydrolysis whereas α-CN was still present after 24 h (Figure 8d, 8e and 8f). This order of hydrolysis was similar to the order obtained by commercial rennet (Figures 8a, 8b and 8c). The pattern of peptide fragments formed by the action of the enzymatic extract from O. acanthium flowers and by commercial rennet is not identical but present similarities especially within the range 0 to 15 min. The three main casein components, α-CN, β-CN and κ-CN, were more

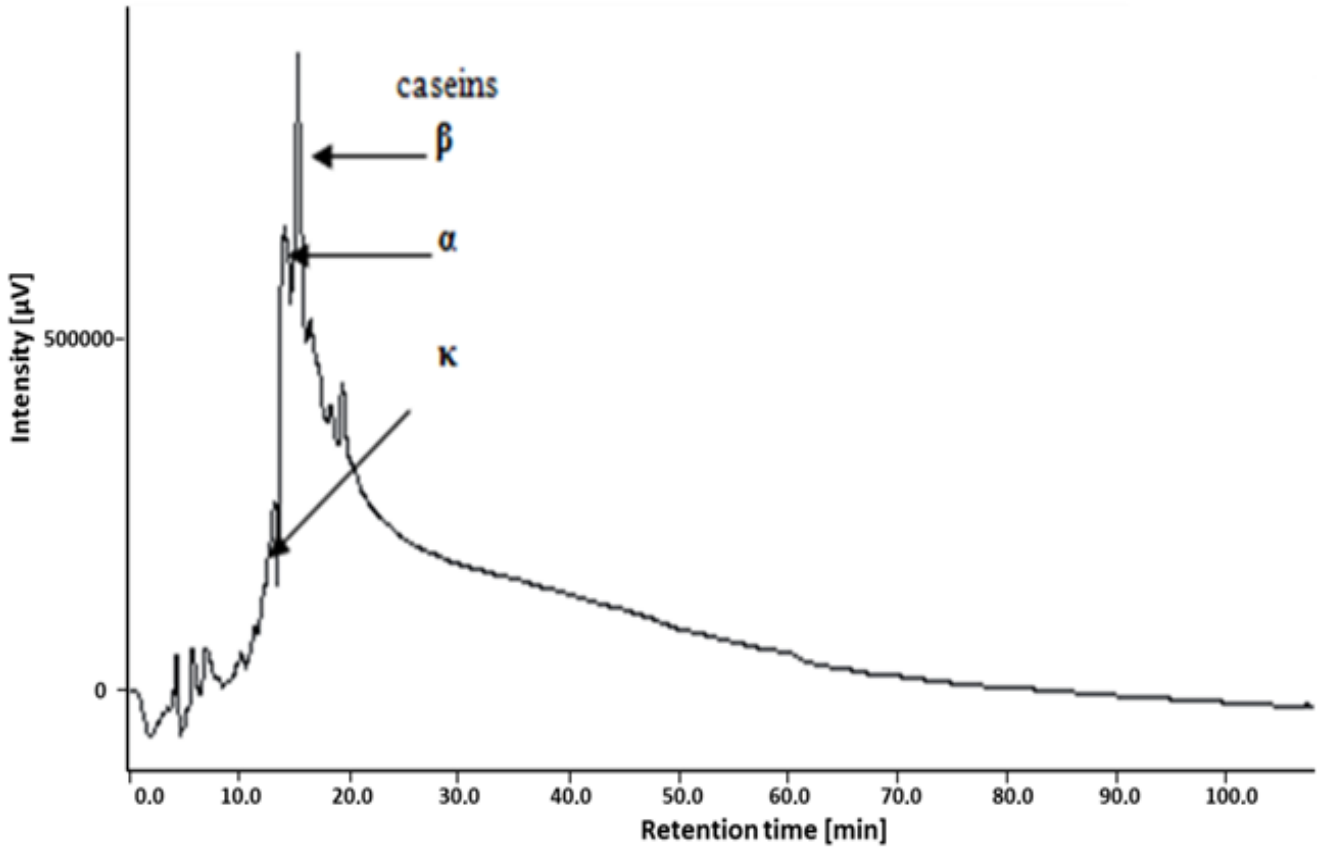

Figure 7. Reversed-phase high performance liquid chromatography (C18 column) of intact caseins (α, β and κ-casein).

sensitive to the action of the *O. acanthium* protease than toward the action of the commercial rennet (Figure 8). The same behavior was observed by Egito et al. (2007) where *Albizia* and sunflower seeds protein extracts were more proteolytic activity than chymosin. The three casein fractions were also degraded by the protease extracted from *Thermomucor indicae-seudaticae* N31 and the *Rhizomucor miehei* (Merheb-Dini et al., 2010). In contrast, the plant rennet from *C. cardunculus* was less proteolytic β-CN and α-CN than the animal rennet (Sousa and Malcata, 1997).

The study of the hydrolysis of caseins by *O. acanthium* acid protease by both methods, electrophoresis and HPLC, indicated the efficiency of the enzyme action on casein and their specificity on kappa-fraction. This action showed an hydrolysis of phe105-met106 responsible of the casein micelles aggregation that provokes the formation of calcium paracaseinate, which results in the coagulation of milk, as produced by commercial rennet (Gaucheron, 2008).

Conclusion

Acid protease from *O. acanthuim* flowers was purified up to 26.65 fold with 29.78% yield. The enzyme shows an optimal activity at pH 4 and an optimum temperature of 40°C. The molecular weight was estimated to 45 kDa and the K_M and V_M were calculated to be 2.34 g/L and 1329.6 UmL^{-1}, respectively. This protease has a capability to hydrolyze caseins and shows the same behavior as commercial rennet. The biochemical properties of the plant enzyme such as the low thermostability and the peptide profile encourage future cheese production experiments to check its potential as a plant rennin. Besides of the involvement of APs in fundamental processes, this protease enzyme is very interesting because of its ability to clot milk. It could be useful in the dairy industry as a rennet substitute. The peptide profile obtained with the enzymatic extract encourages further study of the plant enzymes. The *O. acanthium* protease represents a source of locally-available milk-clotting protease at low cost. Its use will enhance the artisanal production of traditional cheeses in Algeria.

Conflict of interests

The authors did not declare any conflict of interest.

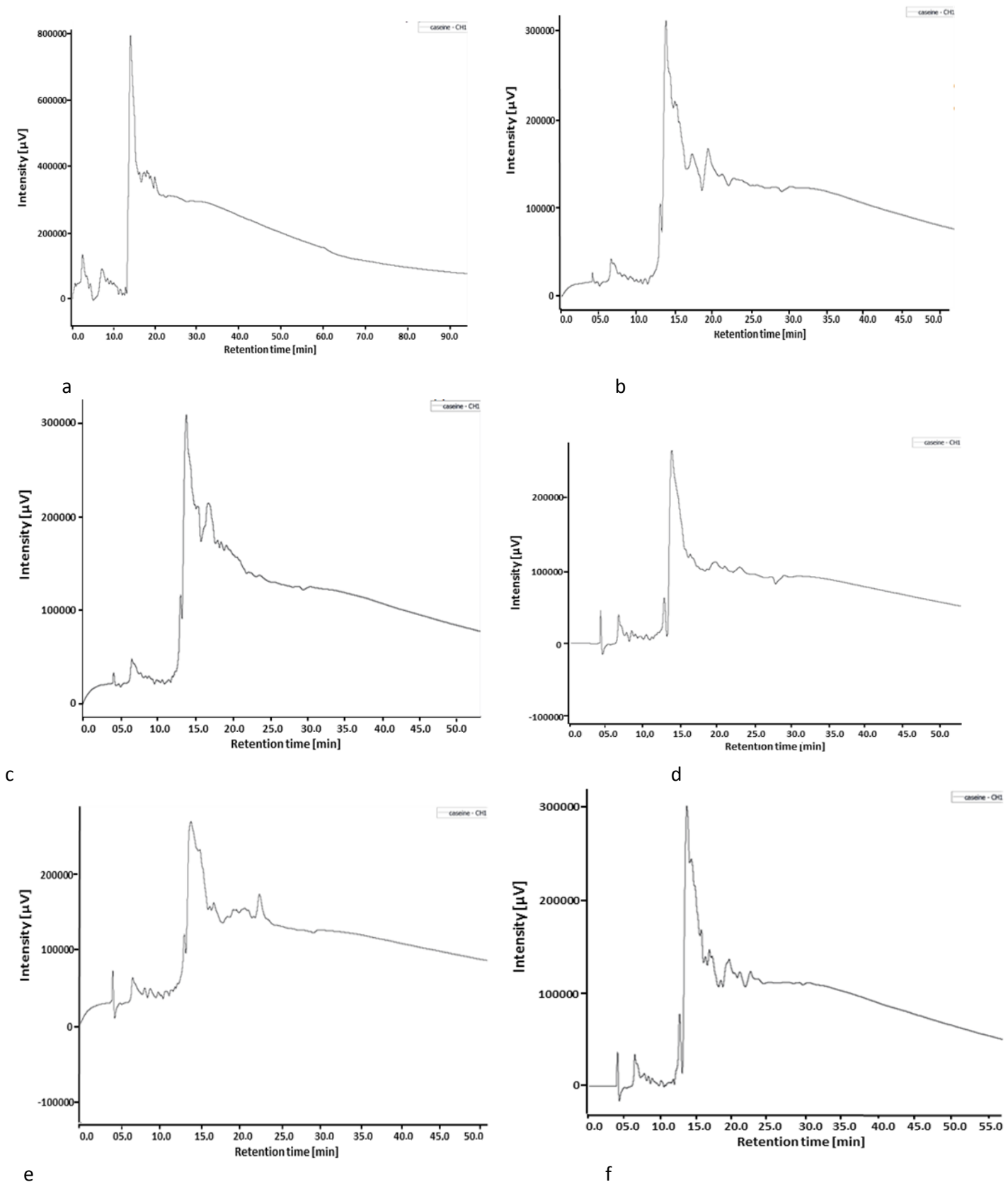

Figure 8. Reversed-phase high performance liquid chromatography (C18 column) of casein hydrolysates generated by *Onopordum acanthium* flowers protease extract and commercial rennet at pH 6.5, 40°C for different times. Commercial rennet: (a) 30 min, (b) 120 min (c) 24 ; Plant protease: (d) 30 min, (e) 120 min (f) 24 h.

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