ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Milk-clotting properties and specific hydrolysis of caseins of the acid protease extracted from *Scolymus maculatus* flowers

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Manuscript Info

Manuscript History:

Received: 12 November 2013
Final Accepted: 22 November 2013
Published Online: January 2014

Key words:
Aspartic protease; *Scolymus maculatus*; Milk-clotting activity; Caseins hydrolysis.

Abstract

A milk-clotting acid protease was extracted from the flowers of an Asteraceae; a widespread plant traditionally used in Algeria, *Scolymus maculatus.* The enzyme was purified 40 fold using ammonium sulfate fractionation followed by exclusion chromatography. The estimation of the molecular mass of the protease by SDS-PAGE electrophoresis gave a weight of 45 kDa and Zymogram analysis revealed only one proteolytic band. The enzyme inhibition at 99% by pepstatin-A 5 mM proved that it is an aspartic proteinase; EDTA and iodoacetamide had no effect. The milk coagulation activity of this protease was optimal at pH 5 and 60°C, the rennet strength (RS) of the extract increased with calcium concentrations and was saturated at 50 mM. The enzyme exhibited hydrolytic activity toward κ-casein, α_s- and β-casein. These results indicated that *Scolymus maculatus* protease has technological effects similar to that of the chymosin; the enzyme could be used in cheese making as a substitute for rennet.

Introduction

Milk-clotting enzymes are the primary active agents in the manufacture of cheeses. They belong to aspartic proteinases group (EC 3.4.23) and are more active at acidic pH. Their action allows cleaving preferably peptide bonds between residues with hydrophobic side-chains (Davies, 1990). The chymosin, the primary active ingredient in rennet, is essential to the cheese manufacture. It is responsible for the hydrolysis of the $Phe_{105} - Met_{106}$ bond in κ-casein into para-κ-casein and glycolmacropeptid (Jollès et al., 1963). However, limited supply and high price of rennet, religious factors, diet or ban on recombinant calf rennet, have led to an increase in the demand for alternative sources of milk coagulants (Cavalcanti et al., 2004).

Our previous studies have led to the isolation of protease-producing fungal species which may be used as a substitute of rennet (Fazouane et al., 2010; Benlounissi et al., 2012). In the same context, many endemic plants are traditionally used for the preparation of cheese by artisanal methods both in Algeria than in many other countries. Thus, several traditional cheeses are manufactured for years in Portugal and Spain using several plant extracts, as the extracts of the flowers of *Cynara cardunculus* (Silva et al., 2002), the extracts of two Cynara species, *C. humilis* or/and *C. scolymus*, (Verissimo et al., 1998; Chazarra et al., 2007), *Silybum marianum* (Vairo Cavalli et al., 2005), *Centaurea calciprata*, *Onopordum turcicum* and *Onopordum acanthium* (Raposo & Domingos, 2008; Tamer, 1993; Brutti et al., 2012).

The evaluation of the proteases potential as rennet substitutes requires the study of the degradation patterns of caseins for their effects on yield, consistency and flavor of the final cheese (Fox, 1989). Rheological characteristics of cheese are complex during maturation and are influenced initially by hydrolysis of α_{s1}, α_{s2}, β and κ-casein and

subsequently by levels of peptides, amino acids, and free fatty acids resulting from more extensive proteolysis and lipolysis (Kim et al., 2004).
The enzymatic hydrolysis of milk proteins releases several bioactive peptides that show different biological activities, such as antioxidant, opioid, immunostimulating, antimicrobial (Haileselassie et al., 1999); this fact could be of great importance for the food industry.

This study aims to develop endemic plant species, traditionally used for the preparation of local foods. For this, the proteolytic activity present in flowers of *Scolymus maculatus* is characterized and their capacity to be used as milk clotting agent evaluated. For this, the effects of pH, coagulation temperature and calcium concentration on the milk-clotting activity of the purified protease are studied. Furthermore, the effects of the purified protease on different casein fractions are measured in order to understand the hydrolysis mechanism during the coagulation.

2. Material and methods

2.1. Plant material

Scolymus maculatus L. (spotted golden thistle or spotted oyster thistle), a flowering plant belonging to the Asteraceae family, was collected from roadsides in Constantine, Algeria, during flowering season (May).

2.2. Enzyme purification

Crude extract was obtained by homogenization of dried flowers of *S. maculatus* in 50 mM sodium citrate buffer pH 5.5 for 30 min, then centrifugation at 10,000 g for 20 min. The supernatant was subjected to fractionated precipitation by ammonium sulfate $(NH_4)_2SO_4$ from 30% to 80% saturation. The precipitate was removed by centrifugation at 10,000 g for 30 min and suspended in 50 mM citrate buffer (pH 5.5) then dialyzed. The resulting extract was applied to size- exclusion chromatography on a Sephadex G-100 column (60 cm x 1.5 cm) pre-equilibrated with 50 mM citrate buffer. All steps of enzyme purification were carried out at 4°C.

2.3. Protein and protease assays

Quantitative estimation of proteins was determined according to Lowry et *al*. (1951) using bovine serum albumin as a standard (SAB, sigma chemical). Samples were incubated at room temperature and absorbance read at 700 nm. The protease activity assay was determined as described by Mechakra et *al*. (1999). One unit of protease activity was defined as the enzyme quantity which liberates amino acids and non-precipitated peptides equivalent to 1 µg/mL of tyrosine per hour under assay conditions.

2. 4. Determination of the Molecular Weight

The molecular mass of the purified protease was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (w/v) acrylamide, according to Laemmli (1970). The activity was detected by submerging the gel in 2% (w/v) casein in citrate buffer pH 5.5 for one hour at 55°C. After the incubation, the gel was 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.

2.5. Effect of inhibitors

Effect of pepstatin A, iodoacetamide and EDTA (1 and 10 mM) on protease activity was measured at 25°C for 120 min. The effect of each inhibitor was expressed as relative activity percentage compared to control.

2.6. Milk clotting activity

The milk clotting activity (MCA) of the *S. maculatus* 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×V / Tc × v, where RS = rennet strength, V one volume of milk (ml), v one volume of rennet (ml) and t the clotting time in seconds.

2.7. Effect of pH, and temperature on MCA

The optimal pH of milk coagulation of the S. *maculatus* protease is determined at the pH range of 5-7. The optimal temperature of the protease was tested between 30 to 60°C. The MCA was determined as described previously.

2.8. Effects of NaCl and $CaCl_2$ on MCA

The effect of salts on milk-clotting activity of the purified protease was studied in a skimmed milk at pH 6.5 with concentrations of 10 to 60 mM for NaCl and of 10 to 50 mM for $CaCl_2$.

2.9. Casein hydrolysis

The action of the S. *maculatus* protease was tested on whole commercial α, β and κ-casein caseinates (Sigma Aldrich Co.) 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) at 30°C. The reactions were started by addition of 450 µl of each substrate to 45 µl of *S. maculatus* protease at 40°C. The reactions were quenched by addition of 500 µl of 5% TCA (w/v) at 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. The precipitates were redissolved in 450 µl of sample buffers, 62.5 mM Tris–HCl 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, were also sampled. Samples were subjected to denaturing electrophoresis (SDS PAGE) in glycine gel as described by Laemmli (1970) at 4°C using a constant voltage (90–100 V) using a staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 blue in ethanol.

3. Results and discussion

3.1. Purification of the acid protease

The results of the partial purification of the protease extracted from *S. maculatus* are summarized in table 1. Fractionation precipitation of the crude extract by 30 to 80% ammonium sulfate leads to the separation of the enzyme at 30% saturation with 4.2 fold purification and 63.9% recovery.

Table 1: Purification table of the acid protease of *Scolymus maculatus*

	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	53,500	534	100	100	1
$(NH_4)_2SO_4$	34,166	81,3	420	63.9	4.2
Sephadex G 100 1st fraction	20,066	5	3,960	37.5	40
2nd fraction	1,800	1	1,421	11	6

The size-exclusion chromatography of the 30% fraction permitted the separation of two activity peaks; the major fraction reached 3,960 U/mg with 40 fold at a yield of 37.5%. Only the first fraction has a milk clotting activity. This high specific activity is desirable for rennet substitutes.

Our result is similar to that of ginger protease which was purified 10.23 fold with a yield of 34.9% and (Hashim et al., 2011) while fungal protease was purified with a higher degree ; 91 for *Rhizopus oryzae* enzyme (Kumar et al., 2005).

3.2. Determination of the Molecular Weight

Enzyme extract zymography analysis using casein as substrate showed a unique band which indicated the presence of a single proteolytic activity form (Fig. 1) with a molecular weight determined with respect to protein markers of 45 kDa.

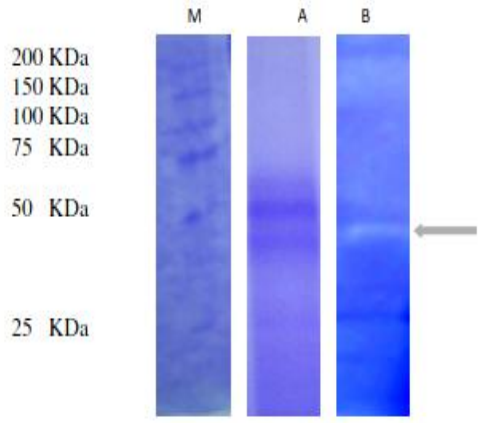

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the *S. maculatus* protease
M: standard protein markers; A: proteolytic extract; B: Gelatin zymogram performed with casein as substrate.

The molecular weight of *S. maculatus* protease was similar to that of the milk clotting protease from *Onopordum acanthium* that also gave a single band (Brutti et al., 2012). It was lower than that of a mature protease from *Centaurea calciprata* cell suspension cultures (50 kDa) (Raposo and Domingos, 2008) and higher than *C. calcitrapa* seeds (Salvador et al., 2006) and *R. oryzae* proteases (Kumar et al., 2005).

3.3. Effect of inhibitors on the *S. maculatus* protease activity

The inhibition results of the *S. maculatus* protease by pepstatin A, EDTA and iodoacetamide are summarized in table 2. The action of the pepstatin-A at 1 and 10 mM caused 98.5% and 99% inactivation of the protease respectively. These inhibition data indicate that the proteolytic activity is mainly due to the presence of an aspartyl residue.

Table 2: Inhibitors effects on the activity of the purified protease of *Scolymus maculatus*

	Inhibitors Concentration (mM)	Residual activity (%)	Inhibitor Class
Pepstatin-A	1 10	1.5 1	Aspartic
EDTA	1 10	98.5 99	Metallo
Iodoacetamide	1 10	95 97.5	Cysteyl

However EDTA and iodoacetamide have no influence on the activity of protease at 10 mM with 99% and 97.5% respectively; these results indicate that the *S. maculatus* protease does not belong to the metallo or cysteyl groups. Many others aspartyl proteases have been identified in plants like *Centauria calciprata* (92% of inhibition) (Raposo and Domingos, 2008) and *Onopordum acanthium* (99.5% of inhibition) (Brutti *et al.*, 2012) and in molds like *Thermomucor indicae-seudaticae* N31 (Merheb et al., 2010) and *R. oryzae* (93%) (Kumar et al., 2005).

3.3. Effect of pH on the milk clotting activity

The figure 2 shows the influence of pH on the milk-clotting activity of the *S. maculatus* protease. The increase of pH was accompanied by a loss of the MCA. The aspartic protease from *S. maculatus* exhibited the maximal rate of reaction at milk pH of 5. At a pH close to neutrality, the loss of activity is almost total (98%).

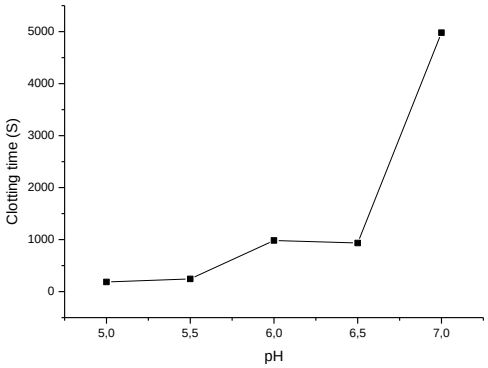

Figure 2: Changes in coagulation time with respect to pH on *S. maculatus* protease coagulating activity.

The same optimal pH have also been reported for the MCA with the proteases extracted from *R. oryzae* (Kumar et al., 2005), *Cynara scolymus* (Chazarra et al., 2007), *Thermomucor indicae-seudaticae* (Merheb et al., 2010) and *Brassica napus* (El-Sayed et al., 2013). The later authors found the same loss of activity as the *S. maculatus* protease at pH 7. Also, calf rennet acts strongly in the acidic range (Richardson et al., 1967).

3.4. Effect of temperature on the milk clotting activity

The influence of the temperature on the MCA was studied between 30 and 60°C. The figure 3 shows that the clotting time decreased when the temperature increased; the maximum milk clotting activity was reached at 60°C.

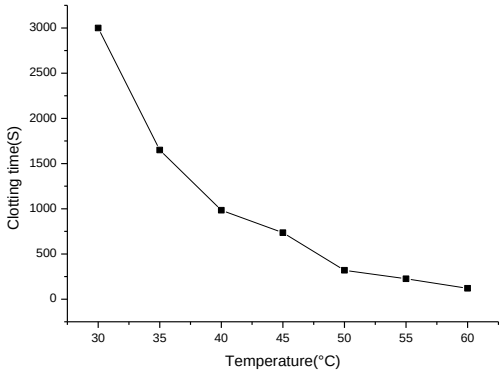

Figure 3: Changes in coagulation time with respect to temperature on *S. maculatus* protease rennet coagulating activity.

The same optimal temperature has been reported with several other enzymes like proteases from *C. scolymus* (Chazarra et al., 2007), *R. oryzae* (Kumar et al., 2005) and the *Brassica napus* flowers of (El-Sayed et al., 2013). Additionally, the crude proteolytic extracts of *Thermomucor indicae-seudaticae* N31showed a maximum MCA at 65°C **(**Merheb et al., 2010).

3.5. Effect of calcium and sodium chloride on MCA

$CaCl_2$ at concentrations from 10 to 50 mM stimulated the milk clotting activity of the *S. maculatus* protease (Fig. 4A).

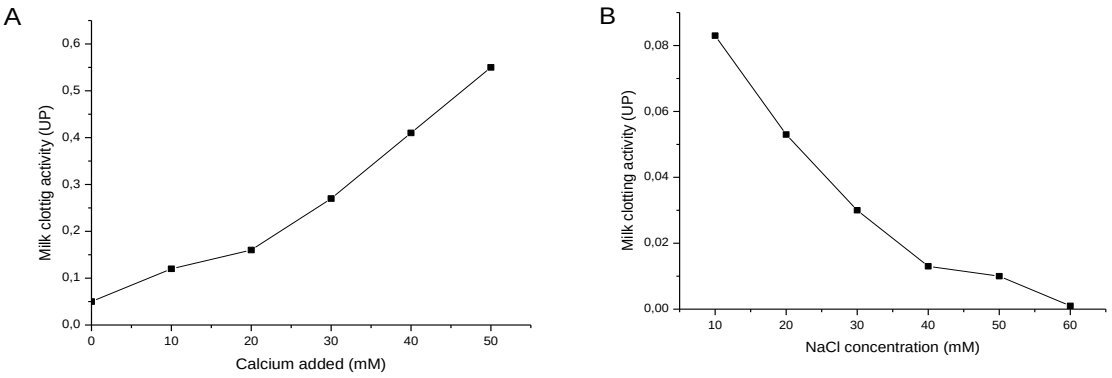

Figure 4: Effect of $CaCl_2$ (A) and NaCl (B) on milk clotting activity

An increase of $CaCl_2$ concentrations from 10 to 50 mM results in an increase of MCA (fig.4A). After the addition of calcium and the pH adjustment, the MCA variations with calcium concentrations are due to a direct effect on the aggregation and firming rates (Castillo et al., 2002). A similar behavior of $CaCl_2$ on MCA was reported by Vairo-Cavalli et al. (2005) for the *Sylibum marianum* protease (40 mM). This result is also consistent with that found for aspartic protease from *Brassica napus* (El-Sayed et al., 2013)

Additional increments of NaCl between 10 and 60 mM decreased the MCA reaching zero at 60 mM (Fig. 4B). Sodium chloride is added to milk in order to protect it against spoilage by various microorganisms. This salt promotes the dissociation of calcium and phosphate from within casein micelles and into solution (Gaucheron et al., 2000). This result is in agreement with previous data for other rennet solutions like in the case of milk clotting enzymes isolated from *Onopordum turcicum* (Tamer, 1993) and *Brassica napus* (El-Sayed et al., 2013).

3.7. Caseins hydrolysis

The caseins degradation patterns was investigated in order to evaluate the potential rennet substitute of the *S. maculatus1* protease. The degradation degree of the α_s, β and κ-casein caseins and their hydrolysis products were analyzed at 30, 60 and 120 min; the results are depicted in fig.5.

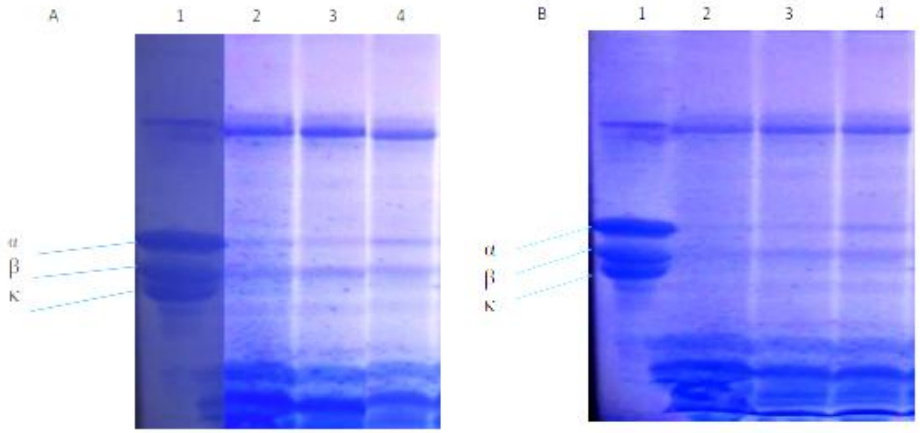

Figure 5: Electrophoretic analysis showing the degradation patterns of α, β and κ casein with *Scolymus maculatus* protease (A) and with commercial rennet (B).
Lane 1: intact casein. Lanes 2–4: whole casein after 30, 60, and 120 min of digestion.

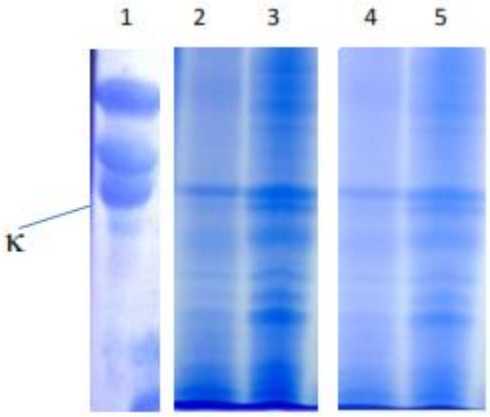

Figure 6: Degradation of κ casein by *Scolymus maculatus* protease and commercial rennet
Lane 1: intact casein; Lane 2- 3: casein hydrolysis by *Scolymus maculatus* protease for 5 and 120 min ; Lane 2- 3: casein hydrolysis by commercial rennet for 5 and 120 min.

The electropherograms show a highest mobility for the κ--casein followed by the α-casein and then by the β-casein. As can be seen, the content of casein components decreased in both types of protease while new bands with higher mobility appeared. The enzyme showed their capability of digestion for all three types of casein with complete degradation of the κ fraction (involved in milk coagulation). The α andκ caseins were degraded after 30 min, while βcasein was degraded only after 60 min; also in this electrophoresis the intensity of the hydrolysis bands increased up to 120 min of incubation.
The electrophoretic profile of the degradation of κ-casein (fig.6) by *S. maculatus* indicated a similar degradation behavior compared with that of calf rennet. This fraction began to be degraded after 5 min of reaction and it appears that it was cleaved at the same peptide bonds.
The κ fraction began to be degraded at the first time of reaction, while the degradation of the other casein fractions proceeded slowly enough as to guarantee the production and stable clots in short time; a behavior that could be of interest in the cheese industry.
These results are concordant with those obtained by other authors who reported that β-casein was less susceptible than α-casein to proteolysis by extracts of *Centaurea calcitrapa* (Tavaria et al., 1997) and *Cynara cardunculus* (Silva et al., 2002). A similar behavior was shown by stem bromelain (Bruno, et al. 2010), *Onopordum acanthium* L. (Brutti et al., 2012) and *Cynara scolymus* (Chazarra et al., 2007). The hydrolytic behavior of enzymatic extract from *S. maculatus* is also similar as the one observed for *T. aurantiacus*, studied by Merheb et al. (2010) who reported that at 60 min of hydrolysis, casein fractions had already been extensively degraded.
Several of these rennet plants were used in the production of traditional cheeses for the similarity of their degradation pattern to that of calf rennet. Extracts of *Cynara* are used chiefly in the making of various Spanish cheeses and Portuguese cheeses from sheep's milk (Roseiro et al., 2003).

4. Conclusion

A partially purified protease was obtained from *Scolymus maculatus* L. flowers. The main active component is an aspartic protease; its molecular weight was estimated to be 45 kDa by SDS–PAGE. The enzyme shows an optimal activity at pH 5 and at 60ºC; its action is highly dependent on calcium ions. This protease has a high capability to hydrolyze different caseins, the same behavior as commercial calf rennet. It could be useful in the dairy industry as a rennet substitute. Furthermore, this new enzyme preparation could as well be applied for the production of milk protein hydrolysates that would be used to obtain potentially bioactive peptides. Like for our precedent studies on isolated fungus proteases, these results emphasize the value of exploiting local biological resources in our country. *Scolymus maculatus* can be exploited for local cheese production as in Algerian traditional methods.

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