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RESEARCH ARTICLE

Partial characterization of acid protease from *Galactites tomentosa* flowers

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Abstract

A plant aspartic protease from *Galactites tomentosa* Moench was purified 15,47 fold by salt precipitation then by size-exclusion chromatography.. The enzyme is an acid protease with optimum pH of 4. The optimal temperature for enzyme activity was 40°C and the half-life at 60°C was 21,6 min. The activity versus of the substrate concentration gave a hyperbolic curve, indicating a Michaelis kinetics. The K_M calculated from Lineweaver–Burk plot is 3,47g/l when casein was used as substrate. The enzyme inhibition of 100% by pepstatin and on non-inhibition by EDTA and iodoacetamide proved it to be an aspartate protease. SDS–PAGE plus casein showed a single active zone and indicated an estimated molecular mass of 45 kDa.

INTRODUCTION

The wide distribution of proteases among plants, animals, and microorganisms demonstrates that they are necessary for livings organisms and they are playing important physiological roles in quite diverse biological processes **(Rao *et al.*, 1998).** Proteases are the most commercially important enzymes due to their multiple applications including food science, technology, pharmaceutical industries and detergent manufacturing **(Doran, 2002).** Based on their acid-base behavior, proteases are classified in to three groups; acid, neutral and alkaline proteases. Acid proteases are active between pH 2 to 5.

Plant enzymes industrially used is still small, but it is growing fast in the last years. Indeed, they are the object of renewed attention from the pharmaceutical industry and biotechnology, not only because of their proteolytic activity on a wide variety of proteins but also because they are often active over a large range of temperatures and pHs. **(Dubey *et al.*, 2007).** All these have stimulated the research and increased the number of works on plant proteases.

Several plant proteinases are able to coagulate milk. Thus, the aqueous extracts of *Cynara cardunculus* flowers have been used for years in the manufacture of several traditional Portuguese and Spanish cheeses **(Silva et al., 2002),** the properties of these enzymes have been well characterized **(Silva and Malcata, 2004).**

This study fits into this context; it aims to partial purification and characterization of the aspartic protease present in *Galactites tomontosa* flowers.

Material and methods

Plant material

Galactites tomontosa, a plant belonging to the *Asteraceae* family, was collected from roadsides in Constantine, Algeria, during flowering season (May). Flowers were separated and dried using the $CaCl_2$.

Protease extraction and purification

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

Ammonium sulfate fractionation

Crude extract was precipitated between saturation 30 and 80% of $(NH_4)_2SO_4$. After centrifugation at 10000g for 30 min at 4°C, the precipitate was suspended in 0.05 M citrate/sodium buffer pH 5.5 and dialyzed overnight against the same buffer with repeated changes.

Size-exclusion chromatography

The concentrated enzyme dialyzed was submitted to a gel filtration on Sephadex G-100 column (60 cm x1.5 cm) pre-equilibrated with 50 mM citrate/sodium buffer pH 5.5. 2 ml enzyme fractions were eluted at 12 ml/h flow rate with the same buffer and were analyzed for enzyme 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 estimated with **Lowry** method **(1951)** using bovine serum albumin as standard (BSA, sigma chemical). The proteolytic activity was measured according to **Anson** method modified by **Mechakra et al.** method **(1999)**. One unit of protease activity was defined as the enzyme quantity which liberates 1 µg/mL of tyrosine per hour under assay conditions.

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 (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 3 to 6.5 on the proteolytic activity using denatured hemoglobin as substrate instead of the casein which precipitates at low pH values.

Kinetic parameters determination

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

Effect of inhibitors

Effects of pepstatin-A (1mM and 10mM); EDTA (10mM) and iodoacetamide (10mM) on the protease activity have been examined. The enzyme extract was incubated with each compound for 2h at 20°C. The residual activities were measured 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)**. The activity was detected by incubating the gel in 2% (w/v) casein in 0.05 M citrate/sodium buffer pH 5.5 for one hour 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.

Results and discussion

Purification of protease

The purification data are shown in Table 1. The enzyme was fractionated between 30 and 80% $(NH_4)_2SO_4$ saturation. 30% saturation fraction presented the highest specific activity (267,66) over the crude enzyme solution with 5,49 fold purification and 50,32% recovery. Chromatography on Sephadex G-100 column of the 30% dialyzed fraction permitted the separation of a single activity peak fraction with 15,47-fold and 23,09% recovery. The same fold purification (15.8) was reported about microbial acid proteases from *Synergistes sp* **(Ganesh.Kumar *et al.*, 2008)** while *Aspergillus niger* protease presented 9.14 fold with a yield of 20.4% recovery **(Fazouane-Naimi *et al.*, 2010). Hashim et al. (2011)** purified ginger protease and obtained 10.23 fold with a yield of 34.9%.

Temperature optima and thermal stability

Figure 1a showed that the purified acid protease acted at an optimum temperature of 40°C; it loosed 58% of activity after 60 min of incubation at 55 ° C. The activity rapidly decreased as the reaction temperature increased higher than 60°C due to thermal denaturation of the protein. The activity of purified aspartic proteases fraction from *Centaurea calcitrapa* cell suspension cultures decreased 60% after 60 min of incubation at 52°C **(Raposo *et al.*, 2008).**

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Figure 1b showed the thermal stability profiles. Loss of activity of 50% is observed after 21 min of incubation at 60°C. The *Galactite tomentosa* acid protease is more stable than microbial like acid protease from *Mucor sp* which retained only 13% of activity at 60°C after 30 min of incubation time **(Fernandez-Lahor *et al.*, 1999).** The biochemical properties of the plant enzyme such as the low thermostability encourage future cheese production experiments to check its potential as a plant rennin.

The experimental half-life for the characterized protease was 21,6 min at 60°C (Figure 1c). The acid protease from *Penicillium expansum* 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 3 to 6.5 using citrate sodium buffer (0.05M). The maximum reaction product was at pH 4 (Figure 2)**.** This is similar to that of aspartyl proteases from *Silybum Marianum* (**Vairo *et al.*, 2005)** and flowers of *Centaurea calcitrapa* (**Domingos *et al.*, 1998)** while higher peak activity in Australian cardoon (*C. Cardunculus*) was reached at pH 6.0 **(Chen *et al.*, 2003)**, but in this case casein (not hemoglobin) was used as substrate. **Chitpinitoyl and Crabbe. (1998)** reported that the plant aspartic protease active at acidic pH. 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). V_{max} and K_M evaluated from Lineweaver - Burk plot (Figure 3b) were 523.834U and 3,47g/l respectively. The low K_M and high V_{max} values inferred that the high affinity and efficient catalytic role of the enzyme. The Michaelis constant as determined by Substrate saturation

profile of an aspartate protease from *R. oryzae* found to be 5 g/l, when skim milk was used as substrate and varying substrate concentration gave also an hyperbolic response (**Sushil Kumar *et al.*, 2005)**.

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 action of the pepstatin-A at 1 and 10 mM caused total inactivation of the protease indicates that the protease belongs to the class of aspartic proteases (acid proteases), while the little inhibition by iodoacetamide (3%) and EDTA (2%) shows that the enzyme is not a cysteyl protease or a metalloprotease. Similar results were observed for acid protease from *Onopordum Acanthium* flowers (**Brutti *et al.*, 2012**). The same enzyme of latex of *Ficus racemosa* has been also inhibited at 100% by pepstatin A (1 mM) and no affected with EDTA (residual activity of 99%) (**Devaraj *et al.*, 2008**). **Matos et al. (2005)** showed 92%, 35% and 8% of inhibition by pepstatin A, iodoacetamide and EDTA respectively of acid protease from seeds of *Centaurea calcitrpa*.

Determination of the Molecular Weight

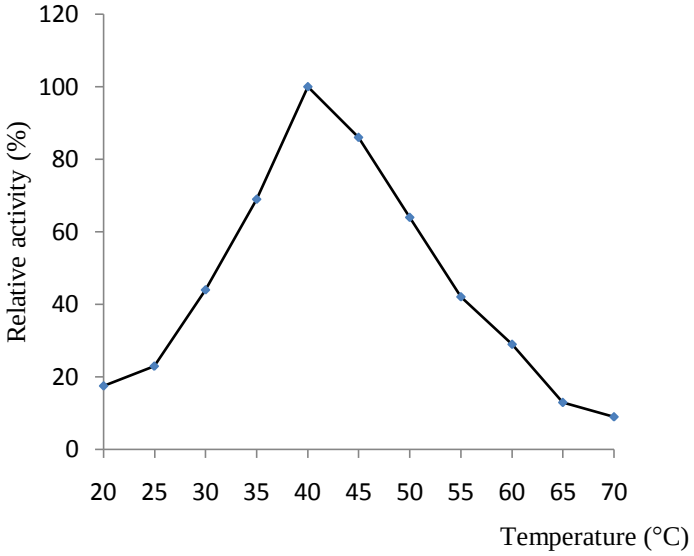
The zymogram analysis after column purification showed a single band of proteolytic activity on the gel suggested the monomeric nature of enzyme (Figure 4). The molecular weight of the enzyme was about 45KDa, as esteemed by SDS-PAGE. The same results were obtained concerning acid protease from flowers from *Scolymus maculatus* (**Benchiheub *et al.*, 2014)** and from *Mucor pusillus* and *Aspergillus niger* (**Nouani *et al.*, 2009; *Fazouane-Naimi et al.*, 2010**). **Mutlu and Gal. (1999)** reported that reduced number of plant aspartic proteinase are monomeric proteins with molecular mass of 36–65 kDa.

Table 1: Purification data of acid protease from *Galactite tomentosa* flowers

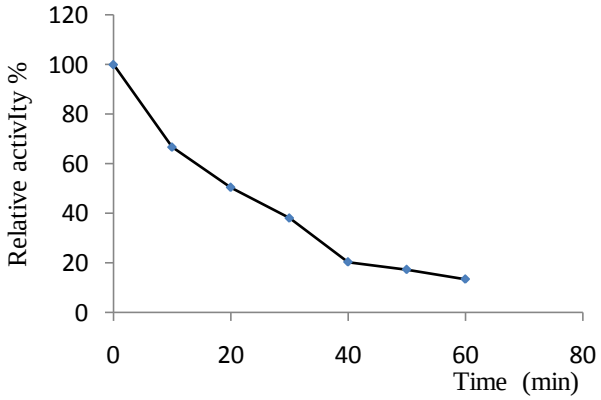
	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	35	19584,25	402	48,71	1	100
$(NH_4)_2SO_4$ 30%	2,5	7466,28	41,61	179,43	3,68	38,12
Dialysis	3,25	9855,48	36,83	267,66	5,49	50,32
Sephadex G100	25	4523,8	06	753,96	15,47	23,09

Table 2: Effect of inhibitors on *Galactite tomentosa* acid protease

Inhibitors	Concentrations (mM)	Residual activity (%)
None	-	100
EDTA	10	98
Pepstatin- A	1	0
Pepstatin- A	10	0
Iodoacetamide	10	97



(a)



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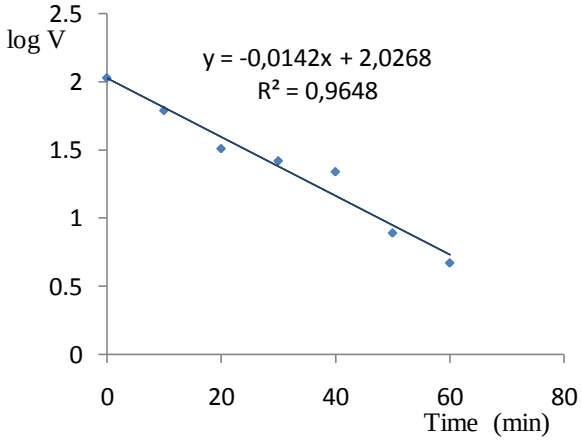

(c)

Fig. 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.

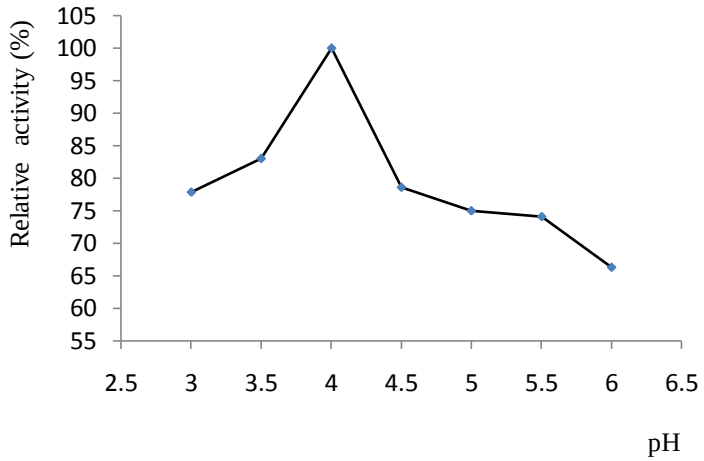

Fig. 2: Effect of pH on protease activity purified from *Galactite tomentosa*.

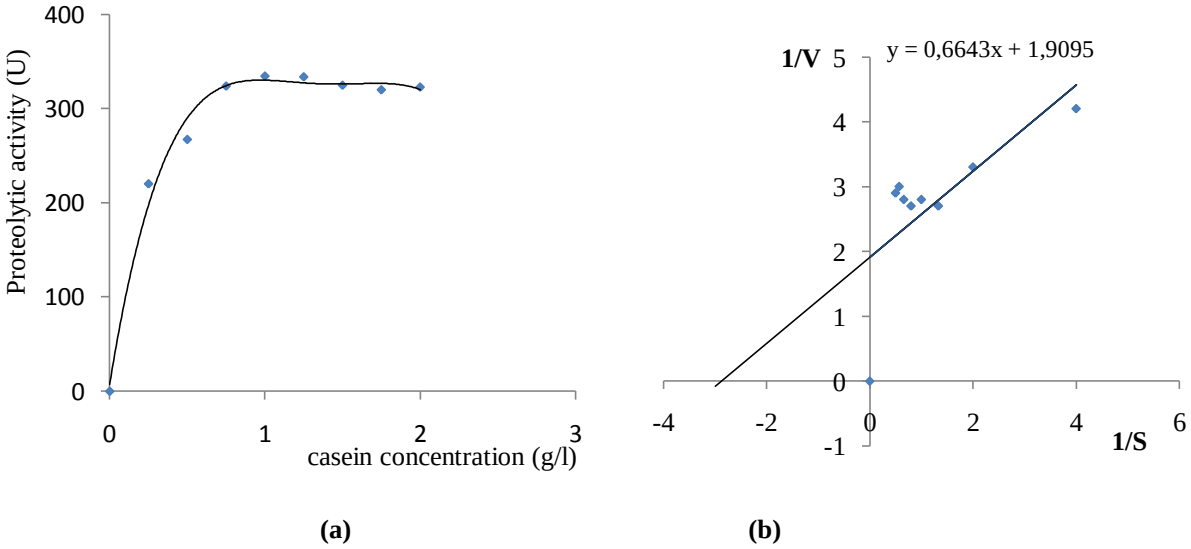

Fig. 3: Kinetic parameters determination. (a) Michaelis-Menten plot V vs. (S); (b) Lineweaver-Burk plot 1/V vs. 1/(S)

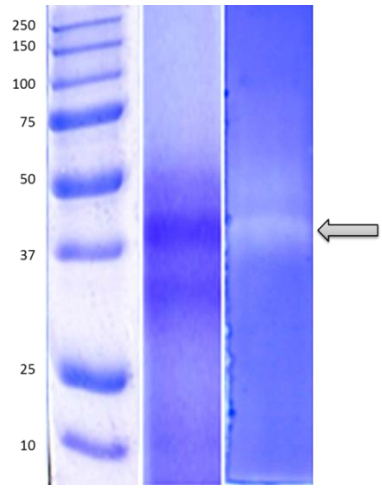

Figure 4: SDS-PAGE gel electrophoresis of the purified *Galactite tomentosa* protease enzyme. Lane I shows the molecular weight marker; LaneII shows the partial purified protease; Lane III represents zymogram of protease with casein as substrate.

Conclusion

Acid protease from *Galactites tomentosa* flowers was purified up to 15,47-fold with 23,09% yield. The main active component is an aspartic protease ; its molecular weight was estimated to be 45 kDa by SDS–PAGE. The enzyme showed an optimal activity at pH 4 and at 40ºC. The K_M and V_M were calculated to be 3,47g/l and 523.834U respectively. Besides the involvement of aspartic proteases in fundamental processes, this class of enzymes is very interesting because of the ability of some of them to clot milk.

References

Benchiheub, M., Benkahoul, M., Bellil, I., Mechakra_Maza, A. (2014) : A Milk-clotting properties and specific hydrolysis of caseins of the acid protease extracted from *Scolymus maculatus* flowers. *International Journal of Advanced Research*, 2:357-365.

Brutti, C. B., Marcelo, F.P., Nestor, O.C. and Claudia, L.N. (2012) : *Onopordum acanthium* (Asteraceae) flowers coagulating agent for cheesemaking. *Food Science and Technology*, 45: 172-179.

Chazarra, S., Sidrach, L., Lo´pez-Molina, D., Jose´ Neptuno Rodrı´guez-Lo´pez. (2007): Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus, L.*) flowers. *International Dairy Journal*, 17: 1393-1400

Chen, S., Zhao, J., Agboola, S. (2003) : Isolation and partial characterization of rennet-like proteases from Australian cardoon (*Cynara cardunculus* L.). *Journal of Agriulture and. Food Chemistry*, 51: 3127-3134.

Chitpinitoyl, S., Crabbe, M. J. C. (1998). Chymosin and aspartic proteinases. *Food Chemistry*, 61, 395-418.

Devaraj, K.B., Latitha, R., Gowda, A. and Prakash, V. (2008): An anusual thermostable aspartic protease from the latex of *ficus racemosa. Phytochemistry*, 69 : 647-660.

Domingos, A., Xue, Z T., Guruprasad, K., Clemente, A., Blundell, T., Pais, M. S. (1998) : An aspartic proteinase from flowers of *Centaure calcitrapa.* Purification, caracterization, molecular cloning and modelling of its three-dimentional structure. *Advences in Experimental Medicine and Biology*, 436: 465-472.

Doran, P.M. (2002). Properties and applications of hairy-root cultures. In: Oksman-Caldenty K-M BarzWH, editors. Plant biotechnology and transgenic plants. NewYork:Mercel Dekker Inc.; p. 143-62.

Dubey, V. K., Pande, M., Singh, B. K. and Jagannadham, M. V. (2007) : Papain-like proteases: applications of their inhibitors. *African Journal of Biotechnology*, 6: 1077-1086.

Fazouane-Naimi F., Mechakra, A., Abdellaoui, R., Nouani, A., Magagi Daga, S., Marou Alzouma, A., Gais, S., Penninckx, M.J. (2010): Characterization and cheese making properties of Rennet like enzyme produced by a local Algerian isolate of *Aspergillus niger. Food Biotechnology, 24: 279-302.*

Fernandez-Lahore, H. M., Auday, R. M., Fraile, E. R., Biscoglio de Jimenez, M., Bonino Pirpignani, L., Machalinski, C., Cascone, O. (1999) : Purification and characterization of an acid proteinase from mesophilic *Mucor sp*. solid-state cultures. *Journal of Peptide Research*, 53: 599-605.

Ganesh Kumar, A., Nagesh, N., Prabhakar, T. G., Sekaran, G. (2008): Purification of extracellular acid protease and analysis of fermentation metabolites by *Synergistes sp*. utilizing proteinaceous solid waste from tanneries. *Bioresources Techn*ology, 99: 2364-2372.

Hashim, M. M., Mingsheng, D., Iqbal, M. F., Xiaohong, C. (2011) : Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochemistry*, 72: 458-464.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall RJ. (1951) : Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193: 265-75.

Matos, S., Novo, C. and Domingos, A. (2006): Evaluation of the presence of aspartic proteases from *Centaurea calcitrapa* during seed germination. *Enzyme and Microbial Technology*, 38 : 893-898.

Mechakra A., Auberger B., Remeuf F. and Lenoir J. (1999): Optimisation d'un milieu de culture pour la production d'enzymes proteolytiques acides par *Penicillium camemberti*. *Sciences des Aliments*, 19: 663–75.

Mutlu, A., Gal, S. (1999): Plant aspartic proteinases: enzymes on the way to a function. *Physiolgia Plantarum*, 105: 569-576.

Najera, A I., Renobales, M., Barron, L. J .R. (2003): Effects of pH, temperature, CaCl2 and nzyme concentrations on the rennet-clotting properties of milk: A multifactorial study. *Food Chemistry*, 80: 345-352.

Nouani, A., Belhamiche, N., Slamani, R., Fazouane, F., Belbraouet, S. and Bellal, M. M. (2009) : Purification et caractérisation électrophoretique d'une protéase coagulant le lait de *Mucor pusillus*: Comparaison de Méthodes. *European Journal of Science Research*, 35 : 512-521.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., and Deshpande, V. V. (1998): Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, 62(3): 597-635.

Raposo, S., Domingos, A. (2008): Purification and characterization milk-clotting aspartic proteinases from Centaurea calcitrapa cell suspension cultures. *Process Biochemistry*, 43: 139-144.

Silva, S. V., Barros, R. M., Malcata, F. X. (2002): Hydrolysis of caseins by extracts of Cynara cardunculus precipitated by ammonium sulfate. *Journal of Food Science*, 67: 1746-1751.

Silva, S. V., Malcata, F. X. (2004) : Influence of the coagulant level on early proteolysis in ovine cheese-like systems made with sterilized milk and Cynara cardunculus. *Journal of Food Science*, 69: 579-584.

Sushil, K., Sharma N.S., Saharan M.R. and Singh R. (2005): Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. *Process Biochemistry*, 40: 1701-1705.

Umar Dahot, M. (2001): Purification and some properties of Acid protease from *Penicillium Expansum*. *Pakistan Journal of Applied Science*, 1: 405-408.

Vairo Cavalli, S., Claver, S., Priolo, N. and Natalucci, C. L. (2005): Extraction and partial characterization of a coagulant preparation from *Silybum marianum* flowers. Its action on bovine caseinate. *Journal of Dairy Research*, 72 : 271-275.