

Full Length Research Paper

Purification and characterization of a milk-clotting protease from *Mucor pusillus*: Method comparison

A. Nouani^{1,2*}, F. Moulti- Mati³, S. Belbraouet⁴ and MM. Bellal²

¹Laboratory of Food Technology, University of Boumerdes, Algeria.

²National Agronomic Institute, Algeria.

³Laboratoire de Biochimie Analytique et Biotechnologies (LABAB) Université M.Mammeri de Tizi Ouzou, Algeria.

⁴Ecole de nutrition et de Science des aliments- Université de Moncton, Canada.

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Crude enzymatic extract obtained from five fermentations (300 g of wheat bran) was characterized by a clotting activity of 0.34 ± 0.08 UP/ml with a strength ratio of 1/1: 200. The comparative study of the summaries from 2 purification protocols showed that it is possible to recover 6% of the initial proteins with a 44.54% activity after gel filtration (protocol I), which appeared more technically sound when compared to ion-exchange (1.80% of total proteins with a 23% performance) (protocol II). The protein homogeneity (a single electrophoretic band) of the monomeric protease was confirmed by both methods after precipitation with 80% saturated ammonium sulphate. Moreover, the fractional precipitation technique with this salt (40 and 80%) was useless in the experimental conditions employed and an important loss of activity was observed (28.53%) with a 3-fold purification. In another part of the study, without ammonium sulphate precipitation, the gel filtration enabled the elimination of almost 97% of the inactive proteins and improved the activity performance by 55.13%, while multiplying the specific activity of the coagulant by a factor of 20.88 against a 6.75-fold purification with ion-exchange and the appearance of a more or less 20 kDa peptide after electrophoresis. The proteolytic activity of the purified extracts had a similar appearance to a more pronounced kinetic when compared with the reference rennet. The purification protocols did not seem to have an impact on the isolated protease activity.

Key words: *Mucor pusillus*, protease, purification, enzymatic performance, electrophoresis, milk clotting, rennet.

INTRODUCTION

Algeria, which is dependent on foreign laboratories for its supply of milk clotting agents, imports these products to manufacture two types of cheese, mainly Camembert and Edam. From 1997 to 2001, the cheese industry used 4.324 kg of rennet with the cost of 16 million Algerian dinars (Anonymous, 2002). In 2005, Algeria was ranked as one of the largest milk and milk by-product consumer in Maghreb, with 110 L/year/inhabitant according to the Ministère du Commerce's statistical data for 2005 and an import volume of 1,106 kg in 2005 at an estimated value of 110,994 for rennet and its concentrates (Anonymous,

2005). In fact, the principal clotting agent of milk is rennet, the use of which is faced with the limitation caused by the slaughter of calves. Consequently, the cheese industry is going through a supply crisis regarding this coagulant. This situation has given rise to research on enzymes that can replace rennet (Ramet, 1997). Presently, industrial interest rests only on bovine and porcine pepsin. They have been shown to be acceptable and are thus, largely used to make cheese as a preparation with rennet (Cuvellier, 1999). Among the possible rennet substitutes, the production of milk-clotting enzymes from vegetal by-products (Lopes et al., 1998; Sidrach et al., 2005) and especially from microbial culture, are interesting for local cheese factories and throughout the world where many strains of clotting protease microorganisms are being produced industrially: *Mucor miehei*,

*Corresponding author. E-mail: nouani2002@yahoo.fr. Tel: +213771724252. Fax: +213 24911669.

Mucor pusillus, *Endothia parasitica*, *Irpex lacteus*, *Aspergillus niger*, *Kluyveromyces lactis* and *Escherichia coli* (Olson 1995; Channe and Shewale, 1998). Comparative studies of these clotting enzymes and of chymosin have shown great similarities in their milk-clotting mechanism and several varieties of cheese prepared with these extracts are similar to those obtained with traditional rennet (Goursand, 1999; Desmazeaud and Spinnler, 1997; Ramet, 1997; Kumar et al., 2005).

In order to improve production performances and to decrease the proteolytic activity that affects the quality of cheese, recent studies have taken on the search for new microbial sources (Cavalcanti et al., 2004; Alam et al., 2005; Esawy and Combet-blanc, 2006; Chwen-jen et al., 2009). The biochemical mechanisms through which these enzymes are produced, that is, the purification process, have been thoroughly studied. Moreover, aspartyl proteases have shown a tendency toward self-inactivation during long purification processes (Preetha and Boopathy, 1997). It is along this line of research that this study's objective sought to improve the knowledge of a *Mucor pusillus* through the comparative analysis of the enzymatic performances obtained from different purification, biochemical and electrophoretic methods. Furthermore, the work includes a partial analysis of the protease (Nouani et al., 2009).

MATERIALS AND METHODS

Obtaining clotting extract from *Mucor pusillus*

The lyophilized fungal strain *M. pusillus* (n°953771) used throughout the study was obtained from the Laboratory of Cryptogamy of the National Museum of Natural History, Paris. A bimonthly subculture was necessary in order to keep the strain healthy. As suggested by Levadoux et al. (1989), a solid-state culture condition (surface fermentation) was employed, composed of 60 g wheat bran and 100 ml of ammonium sulphate solution at 0.01%. After inoculating the Petri dish which contained the malt agar and incubating at 37°C for 5 days, the spores were liberated with the tween 80 solution at 0.1%. The spore suspension was filtered through a sterile glass tool. The exocellular enzyme was extracted with a phosphate buffer (0.02 M, pH 6.0).

Purification of the enzymatic extract

Purification methods

To purify the clotting enzymatic extract obtained from the *M. pusillus* culture, two purification protocols were compared to evaluate their performances. The first, according to Somkuti and Babel (1968), suggests the use of an ion-exchanger followed by gel filtration. The second, according to Fernandez-Lahore et al. (1998), reverses the purification process.

Ammonium sulphate precipitation

The crude enzymatic extract was subjected to precipitation with 80% saturated ammonium sulphate. Thus saturated, the enzymatic solution was decanted for one night at 4°C. After a 10,000 g

centrifugation at 30 mn and 4°C, the pellet was suspended in the phosphate buffer (0.02 M; pH 6).

Desalting the enzymatic extract

Eliminating the ammonium sulphate from the clotting extract was achieved by gel filtration through a Pharmacia column (20 cm x 1 cm) containing Sephadex G-25. The active fractions are gathered and concentrated with saccharose.

Ion-exchange chromatography

The QEAE (Quaternary-amino-ethyl)-Sephadex A-50 exchanger, a dextran gel on which the positively charged groups are fixed was used. The gel, swollen in a phosphate buffer (0.02 M, pH 6.0) was degassed and poured in a column (20 cm x 1 cm) previously equilibrated with the same buffer. After concentrating the extract, the proteins were eluted at 4°C with a linear gradient of 0 - 0.5 M NaCl prepared in the same buffer. The elution flow rate was kept at a constant 45 ml/h. Optimal density was measured at 280 nm. The fractions that showed a clotting activity were gathered and dialysed against the phosphate buffer (0.02 M; pH 6.0) for 16 h at 4°C.

Molecular exclusion chromatography

Separation was achieved in a decreasing order of molecular weight on a Sephadex G-100 gel through a Pharmacia column (60 cm x 1 cm) previously calibrated with a phosphate buffer solution (0.02 M; pH 6.0). An aliquot quantity of 1.5 ml sample was eluted with the same buffer at a flow rate of 2 ml/h. Optimal density was measured at 280 nm. The fractions that showed clotting activity were gathered, concentrated and conserved.

Analysis methods

The main objective of this study was to propose a comparative purification protocol. To this end, the crude enzymatic extract from *M. pusillus* was subjected to many purification steps. For this purpose, after each step, the total protease activity, the total protein quantity, the specific activity, the activity performance and the purification factor were determined.

Clotting activity measure

The clotting activity was measured according to the method of Berridge (1945), as modified by Collin et al. (1977). This method makes it possible to express enzymatic extract activity in rennet units (U), which corresponds with the quantity of enzymes necessary to clot 10 ml of standard substrate in 100 s at 35°C.

Proteolytic activity measure

The proteolytic activity of the purified fungal extract (recovered from protocols 1 and 2) was measured according to the method of Green and Stackpole (1975). This activity was determined by measuring the casein hydrolysis product (casein from bovine milk, Sigma) concentration, soluble in trichloroacetic acid (12% TCA). Soluble peptide dosage was achieved by following the method of Lowry et al. (1951).

Protein dosage

The dosage for the total proteins from clotting extracts was attained

Table 1. Purification of milk clotting extract of *M. pusillus* (Protocol I).

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
C.E	153	988	0.154	100	1
↓↓ (NH ₄) ₂ SO ₄ (80% saturation)	79.68	161.28	0.494	52.07	3.19
Gel filtration	68.16	57.6	1.183	44.54	7.68
Ion-exchange chromatography	28.8	10.8	2.666	18.82	17.31

↓↓, Precipitation; C.E, crude extract.

according to the method of Lowry et al. (1951). This concentration was determined with a calibrating curve set by using bovine serum albumin (200 µg/ml BSA).

Polyacrylamide gel electrophoresis (SDS-PAGE)

At each step of the purification process, a sample is either frozen or lyophilized for electrophoresis, in order to control the homogeneity and the purity of the enzyme. Samples were analysed by SDS-Polyacrylamide gel electrophoresis according to the method of Laemmli (1970) (separating gel: T=12%, pH 8.8; stacking gel: T=5%, pH 6.8) achieved by a "Max Fill Bioblock Scientific" electrophoresis system. The molecular weight markers were: α-lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovin serum albumin (67 kDa) and phosphorylase b (97 kDa). Revealing proteins that were thus separated was done by colouring the gel in Coomassie blue (R-250) for 2 h and then, removing the colour until the bands appeared clearly.

RESULTS AND DISCUSSION

Purification of the clotting extract of *M. Pusillus*

The crude enzymatic extract obtained from five fermentations (300 g of wheat bran) was characterized on average by a coagulating activity of 0.34 ± 0.08 UP/ml, a protein quantity of 2.47 ± 0.13 mg/ml and a clotting strength ratio of 1/1,200.

Method I (Somkuti and Babel, 1968)

The ammonium sulphate precipitation (80% saturation) of the crude enzymatic extract gave a 52.07% activity when compared with the initial activity and multiplied the specific activity of the coagulant by a factor of 3.19 (Table 1). The molecular exclusion chromatographic profile on Sephadex G-100 of a desalted and concentrated precipitate (161.28 mg) revealed two elution peaks (Figure 1), of which the first showed clotting activity. Moreover, a slight increase in specific activity and purification factor was detected with a 44.54% performance. The gel filtration eliminated almost 94.2% of the inactive proteins. The results obtained by Somkuti and Babel (1968) showed four elution peaks (of which only one was active), after gel filtration with the elimination of an important quantity

of color pigments in the crude enzymatic extract and a purification factor twice superior to the one observed in this study. In addition, Lenoir et al. (1979) were able to eliminate 98% of the inactive proteins with a 50% activity performance. The ion-exchange chromatographic profile of a QEAE-Sephadex A-50 of the active fraction obtained from molecular filtration shown in Figure 2, reveals three peaks of which only one was active. The enzyme was eluted with the linear gradient of 0.35 M NaCl. At this stage, a 17.31 increase in the purification factor was recorded. However, performance activity fell to 18.82%. After an ion exchanger, Somkuti and Babel (1968) isolated a principle enzymatic fraction of 0.3 to 0.4 M NaCl with a 55% performance and a 34-fold purification.

In a similar study on the purification of *Penicillium caseicolum* protease, Lenoir et al. (1979) obtained a purification performance superior to 40%, while also multiplying the specific activity of this coagulant by a factor of 29. However, "lettucine" purification, as achieved by LoPiero et Petrone (1999), with an ammonium sulphate precipitation, a gel filtration, an ion exchange and a second gel filtration, led to a purification summary that yielded a 60% performance and a 5-fold purification.

In order to determine the homogeneity of the purified protein, a polyacrylamide gel electrophoresis with SDS was carried out. To this end, several samples were used; crude extract, precipitated extract, active fraction obtained from gel filtration and active fraction from the ion-exchanger.

The electrophoretic pattern, shown in Figure 3, confirms the protein homogeneity of the purified fungal extract. In fact, a succession of bands in the crude supernatant (A) as well as in the ammonium sulphate precipitate (B) and a single band in the active fraction on Sephadex G-100 (C) and in the clotting fraction on QEAE-Sephadex A-50 (D) was observed.

The same bands were revealed in the precipitate and in the crude extract. This explained that the 80% saturated ammonium sulphate precipitation sought to concentrate the extract. In addition, gel filtration after precipitation yielded a homogenous band, which led to the conclusion that ion-exchange was not necessary in this case. Moreover, the results are in agreement with those obtained by other authors (Somkuti and Babel, 1968; Lenoir et al. 1979) who have observed a homogenous band at the end of the purification.

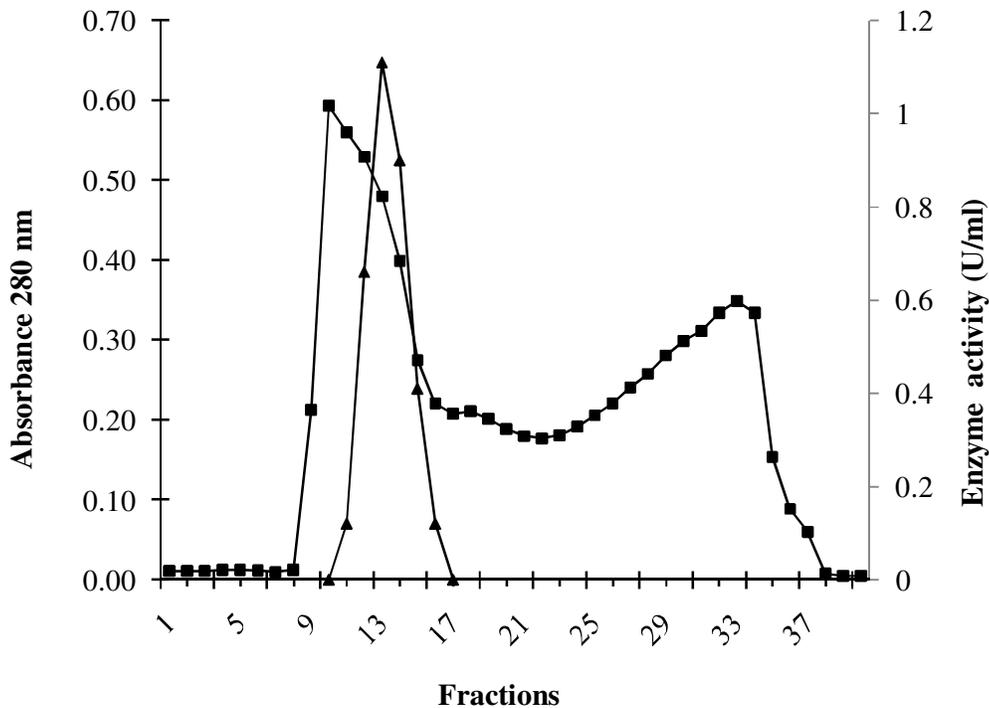


Figure 1. Elution profile of the enzymatic extract of *M. pusillus* on Sephadex G-100 after precipitation with 80% saturated ammonium sulphate (Pharmacia column (60 cm x 1 cm), phosphate elution buffer (0.02 M, pH 6.0), flow rate of 6 ml/h, fraction of 1 ml). Absorbance, ■; enzyme activity, ▲.

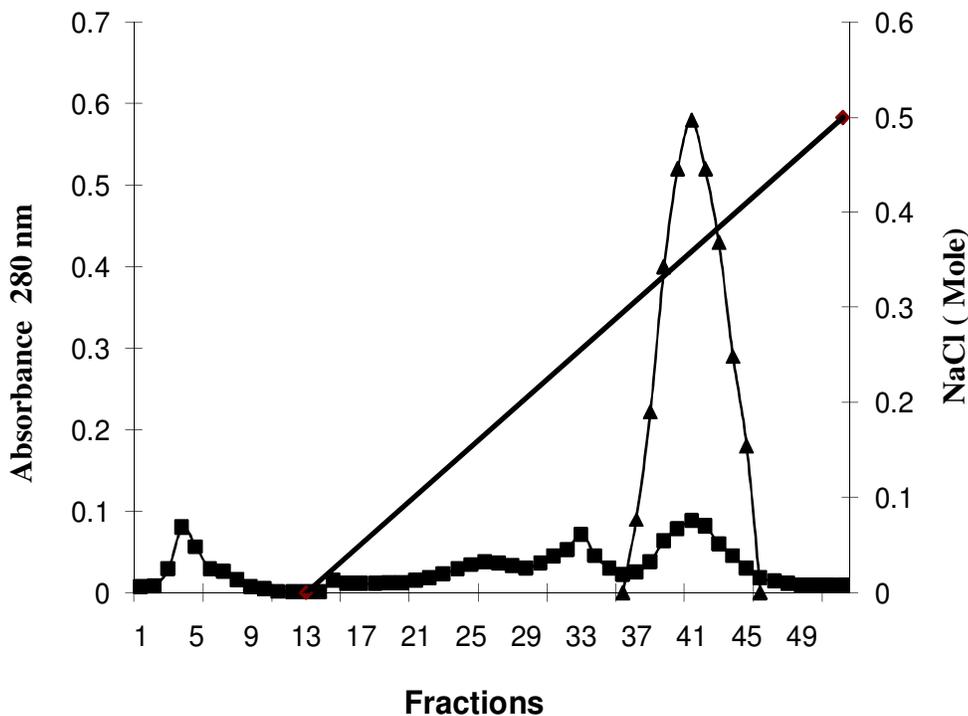


Figure 2. Elution profile on QAE-Sephadex A-50 of *M. pusillus* extract active fraction obtained from gel filtration (Column Pharmacia (60 cm x 1 cm), phosphate elution buffer (0.02 M, pH 6.0), linear gradient of 0 - 0.5 M NaCl, flow rate of 45 ml/h, and fraction of 2 ml). Absorbance, ■; enzyme activity, ▲; -, NaCl gradient.

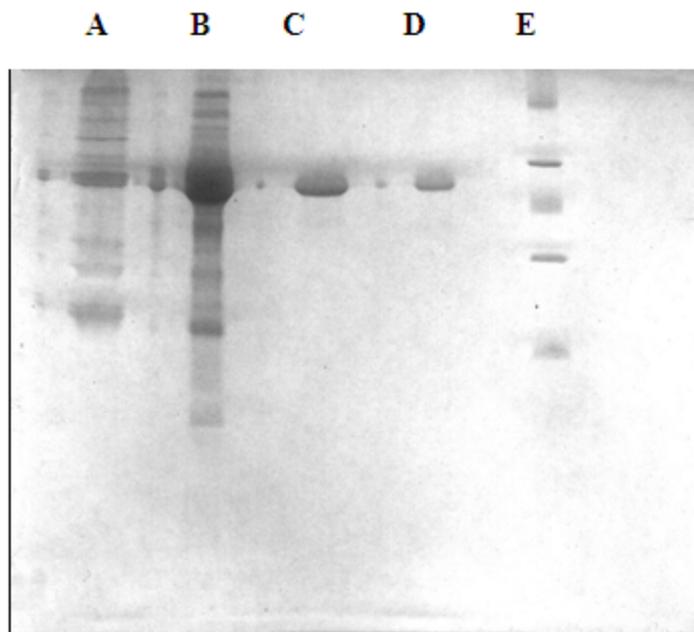


Figure 3. SDS- PAGE electrophoretic pattern of *M. pusillus* extracts (protocol I). Lane A, crude enzymatic extract; Lane B, extract precipitated with 80% saturated ammonium sulphate; Lane C, fraction obtained from gel filtration after ammonium sulphate precipitation; Lane D, clotting fraction from ion exchange after gel filtration; Line E, protein markers.

Method II (Fernandez – Lahore et al., 1998)

This protocol was consisted of an ammonium sulphate precipitation at 80% saturation, an ion-exchange chromatography and a gel filtration. The chromatographic profile on QAE-Sephadex A-50 of the 80% saturated ammonium sulphate precipitate (161.28 mg) revealed three distinct peaks (Figure 4). The clotting activity observed in the third peak was eluted at 0.35 M NaCl. This stage yielded a relatively elevated purification of 13-fold, however, the performance fell to 23.21% (Table 2). According to the study on *M. pusillus* coagulant carried out by Khan et al. (1979), pH 8.0 ion exchange revealed two peaks that corresponded with two proteases, one with a very significant clotting activity and the other with a very weak clotting activity with 29% performance.

The molecular exclusion chromatography of the active fraction revealed two elution peaks, of which the first was active (Figure 5). Moreover, a good purification fold (23) was obtained with a specific activity of 3.55. However, the activity performance remained very weak (10%) and only 0.43% of the total proteins were recovered. In fact, the gel filtration of the active fraction, according to Fernandez-Lahore et al. (1999), revealed only one active peak with 76.2% activity when compared with the initial report. Furthermore, purification of the *M. bacilliformis* protease by an ultrafiltration followed by an ion exchange yielded 64% performance and a 15.5-fold purification (Fernandez-

Lahore et al., 1998).

The electrophoretic profile, shown in Figure 10, revealed many bands in the crude enzymatic extract (A) and the precipitate (B), a band in the active fraction obtained from the ion exchange (C) and a band in the fraction obtained from molecular filtration.

Comparative summary of methods I and II

When comparing the protocols and using the electrophoresis results as a basis, it was concluded that a single chromatographic technique, after ammonium sulphate precipitation at 80% saturation, yielded a homogenous band. In addition, according to the purification summaries, it was more interesting to recover 6% of the initial proteins with 44.54% activity after gel filtration (protocol I), which seemed more technically sound when compared with ion exchange (1.80% total proteins and 23% performance) (protocol II). Moreover, optimising a purification method was sought after for aspartyl proteases that show quick autolysis and self-inactivation (Preetha and Boopathy, 1997).

Fractional precipitation with ammonium sulphate

Clearly, much loss of enzyme activity was recorded

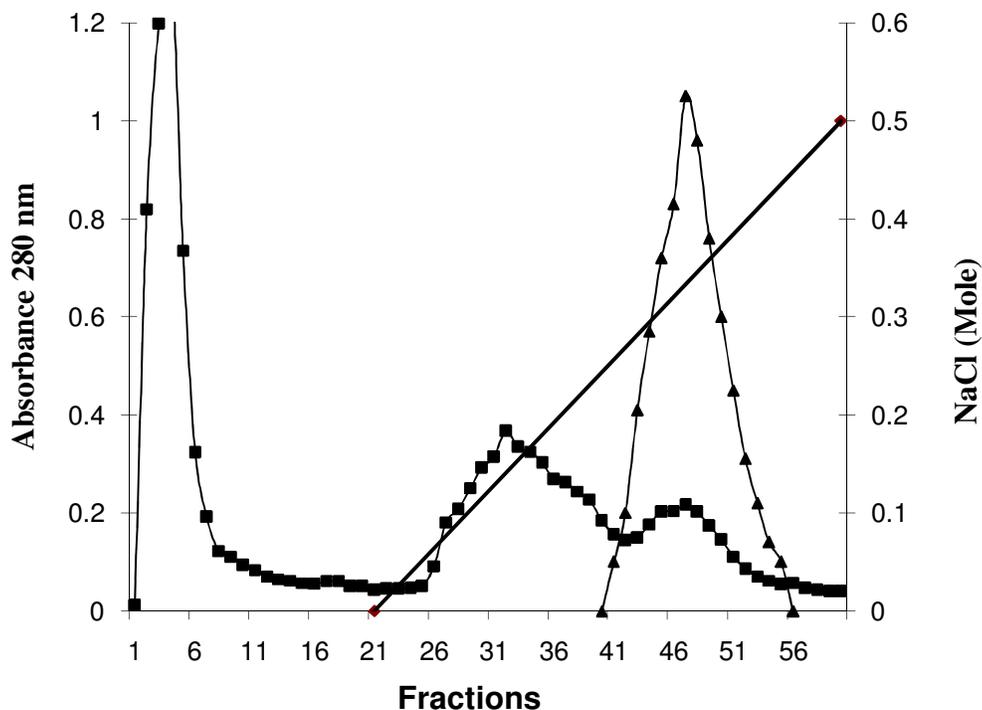


Figure 4. Elution profile on a QEAE- Sephadex A-50 of the clotting extract of *M. pusillus* after 80% saturated ammonium sulphate precipitation (Column Pharmacia (20 cm x 1 cm), phosphate elution buffer (0.02 M, pH 6.0), linear gradient of 0 to 0.5 M NaCl, flow rate of 45 ml/h, fraction: phase 1 = 3 ml, phase 2 = 2 ml). Absorbance, ■; enzyme activity, ▲; - NaCl gradient.

Table 2: Purification of milk clotting extract of *M. pusillus* (Protocol II).

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
C.E	153	988	0.154	100	1
↓↓ (NH ₄) ₂ SO ₄ (80% saturation)	79.68	161.28	0.494	52.07	3.19
Ion-exchange chromatography	35.52	17.76	2	23.21	12.98
Gel filtration	15.36	4.32	3.55	10.03	23.08

throughout the purification process (Belyauskaite et al., 1980). To this purpose and in accordance with other authors, an electrophoretic analysis of the precipitate of ammonium sulphate extracts was carried out. The results seemed to indicate that the fractional precipitation technique with salt (40 to 80%) was useless in the experimental conditions. Although, this protocol greatly improved the purification factor, it did not improve the activity performance of the purified extract. Indeed, the results shown in Table 3 reveal an important fall in activity by 28.53% when compared with the initial activity's 3-fold purification.

This observation does not agree with what was found by other authors who used the salt fractionation principle. In fact, in a similar study on *Bacillus subtilis* protease, Matoub (2000) noted a 93.75% performance activity. Moreover, Cavalcanti et al. (2004) recorded a 55%

performance and a 7-fold purification by fractioning the crude extract of fungal coagulant from *Nocardopsis sp.* Precipitation of *Rhizopus oryzae* extract lead to 103% performance. According to Kumar et al. (2005), this is due to the elimination of inhibiting substances present in the crude enzymatic preparation. The chromatographic profile on Sephadex G-100 of the fractional ammonium sulphate precipitate, shown in Figure 7, revealed a single peak with clotting activity. This step made it possible to multiply the specific activity of the coagulant by a factor of 18.30 (Table 3). However, the activity performance remained weak at 20.51%.

The works of Khan et al. (1979) concluded that, fractional ammonium sulphate precipitation (50 to 80% saturation), followed by an ion exchange at pH 6.0, separated the clotting protease from the non specific protease with 64% performance and a 29-fold purification.

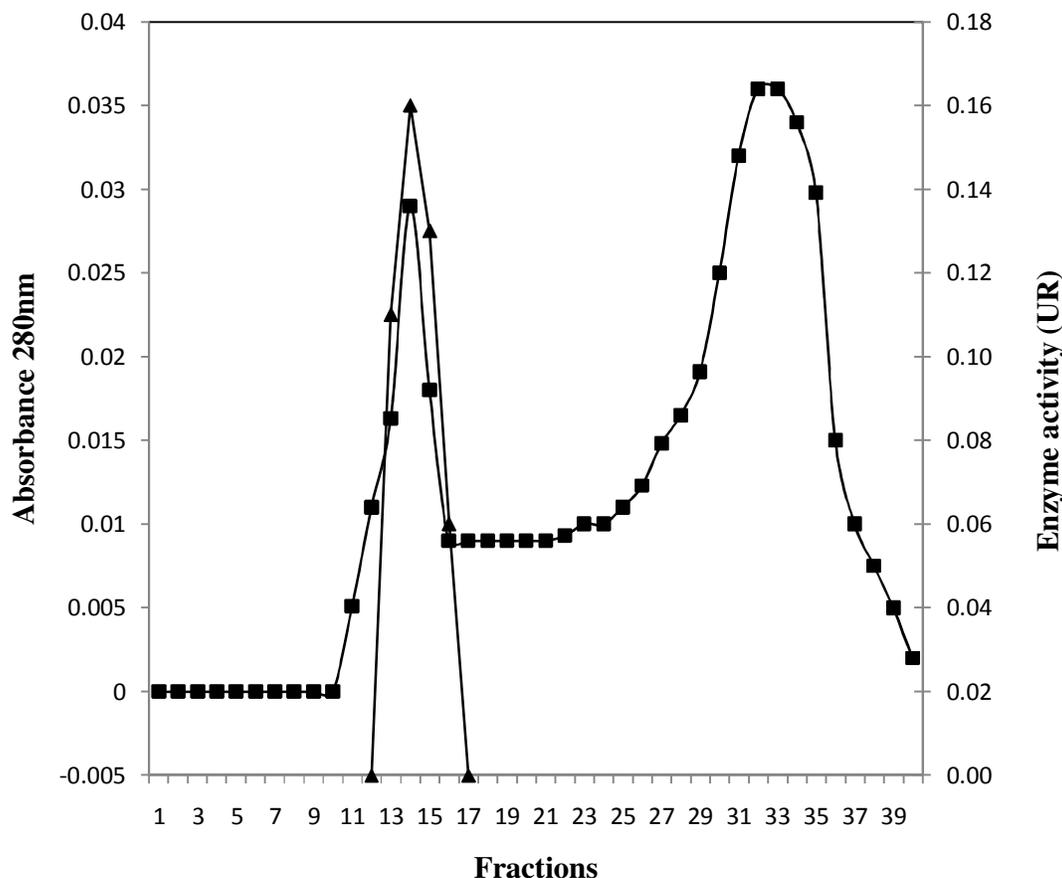


Figure 5. Elution profile on Sephadex G-100 of the active fraction of *M. pusillus*, obtained from the ion exchange (Column pharmacia (60 cm x 1 cm), phosphate elution buffer (0.02 M, pH 6.0), flow rate: 6 ml/h, fraction: 1 ml.). Absorbance, ■; enzyme activity, ▲.

Table 3. Purification of milk clotting extract from *M. pusillus* after ammonium sulphate fractional precipitation (40 to 80%).

Purification step	Total activity (RU)	Total protein (mg)	Specific activity (RU/mg)	Recovery (%)	Purification fold
C.E.	117	1012.5	0.115	100	1
↓↓ (NH ₄) ₂ SO ₄ (40 to 80% saturation)	39.96	140.04	0.341	28.53	2.96
Gel filtration	24	11.4	2.105	20.51	18.30

These authors did not carry out this separation by precipitating the crude extract at 80% (33% performance) followed by an ion exchange at pH 8.0 (29% performance).

Figure 8 show that the active fraction obtained from the molecular exclusion chromatography (after ammonium sulphate fractioning) was homogenous. This result was noted by many authors (Khan et al., 1979; Cavalcanti et al., 2004; Kumar et al., 2005). Moreover, the electrophoretic profile of the crude enzymatic extract, of the precipitate obtained by fractioning (40 to 80%) and of the precipitate at 80% did not indicate any difference between these three samples (Figure 9).

In order to justify the observations on the performance of protocol I (Method I) and the interest of gel filtration, the crude enzymatic extracts without the use of ammonium sulphate precipitation were lyophilized and respectively subjected to an exclusion chromatography and an ion exchange. The results in Table 4 show that, gel filtration enabled the elimination of approximately 97% of the inactive proteins and improved the activity performance by 55.13%, while also multiplying the specific activity of the coagulant by a factor of 20.88 against a 6.75-fold purification with ion exchange accompanied by the appearance of about 20 kDa after electrophoresis (Figure 10).

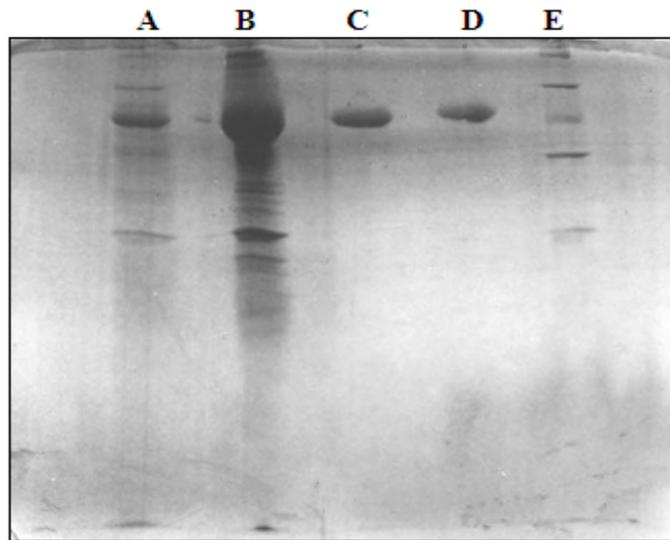


Figure 6. SDS-Page electrophoretic pattern of *M. pusillus* extract (protocol II). Lane A, Crude extract; Lane B, extract precipitated with 80% saturated ammonium sulphate; Lane C, clotting fraction obtained from ion exchange after ammonium sulphate precipitation; Lane D, clotting fraction obtained from gel filtration after ion exchange; E, protein markers.

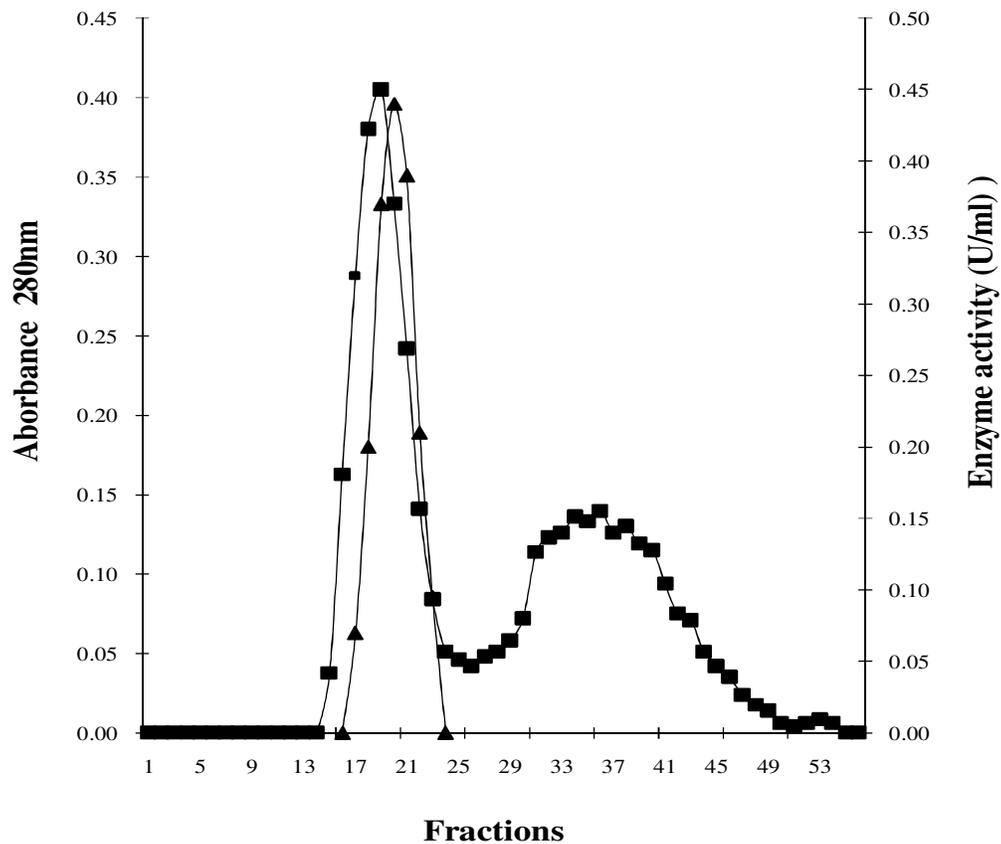


Figure 7. Elution profile on Sephadex G-100 of the clotting extract of *M. pusillus*, precipitated by ammonium sulphate fractioning (40 to 80% saturation) (Pharmacia column (60 cm x 1 cm), phosphate elution buffer (0.02 M, pH 6.0), flow rate: 6 ml/h, fraction: 1 ml.). Absorbance, ■; enzyme activity, ▲.

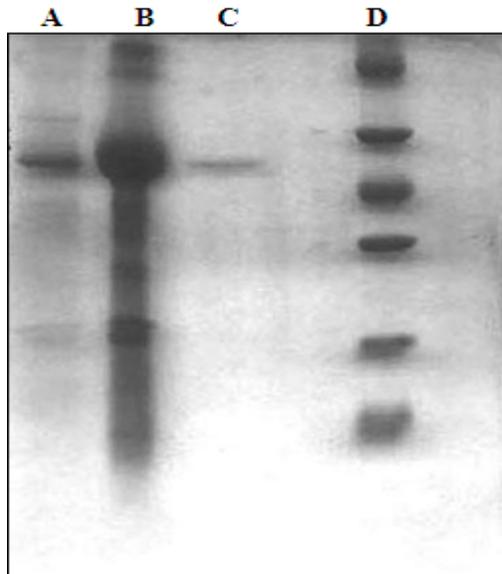


Figure 8. SDS-PAGE electrophoretogram of *M. pusillus* extracts. Line A, crude extract, Line B, crude extract precipitated 40 to 80 %; Line C, purified extract on size- exclusion after precipitation (40 to 80%); Line D, protein markers.

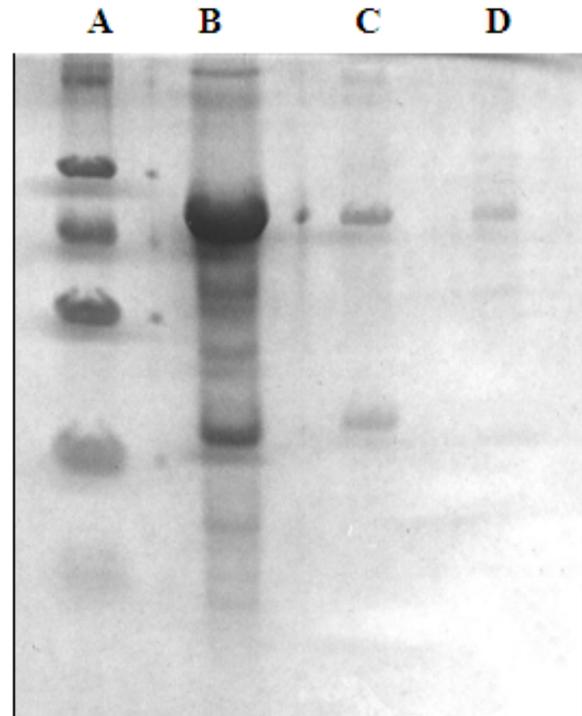


Figure 10. SDS-PAGE electrophoretogram of *M. pusillus* extracts. Line B, crude extract lyophilised; Line C, purified extract on QAE-Sephadex A-50; Line D, purified extract on Sephadex G-100.

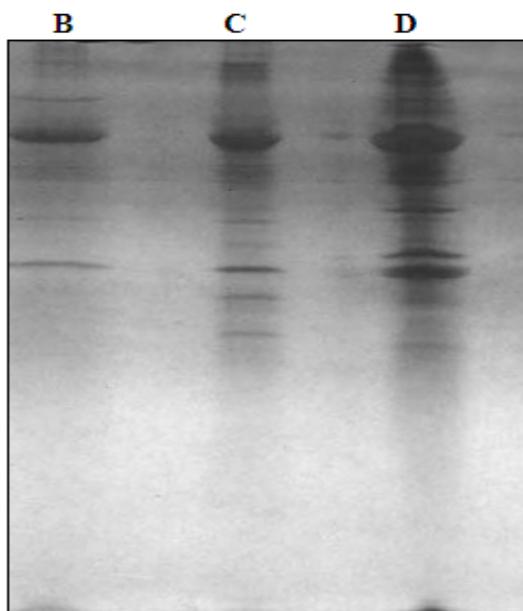


Figure 9. SDS-PAGE electrophoretogram of *M. pusillus* extracts. Line B, Crude extract; Line C, crude extract precipitated 40 to 80%; Line D, crude extract precipitated 80%.

Proteolytic activity

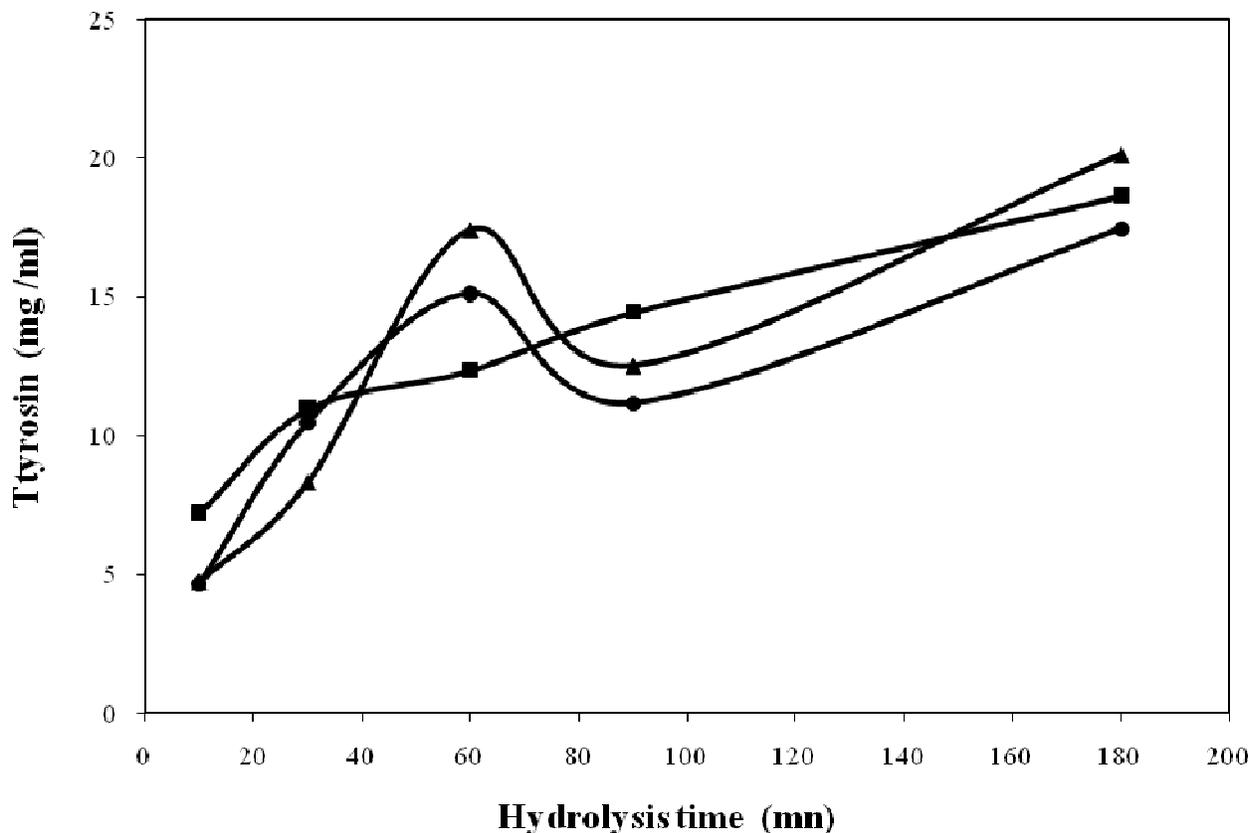
The evolution of non protein nitrogen during enzymatic hydrolysis is illustrated in Figure 11. The results obtained

showed that, the proteolytic activity of the purified extracts possessed a similar appearance with a more pronounced kinetic when compared with the reference rennet. Two phases were identified during which an important increase was observed in the rate of non protein nitrogen (between 10 and 70 mn). This result supposes that the proteins were hydrolysed and that free amino acids and peptides of weak molecular weight were released. The excessive activity of the crude clotting extract from *M. pusillus*, when compared with rennet, was probably due to the non specific action of fungal protease toward other milk caseins (α_s , β , δ caseins) during the primary reaction. The second phase corresponds to a level at which the general proteolysis was relatively stable (incubation of 90 to 180 mn). The purification protocols did not seem to have any impact on the activity of the isolated proteases.

The microbial and vegetal enzymes expressed a strong proteolytic activity when compared with the proteases of animal origin. Indeed, according to certain authors (Alais, 1984; Preetha and Boopathy, 1997; Krause et al. 1998), fungal enzymes express more activity than rennet and pepsin. In general, the coagulases must develop a general proteolysis activity that is weak and able to express itself on all of the milk proteins throughout the aging process, in order to preserve the organoleptic properties of cheeses.

Table 4. Compared purification of milk-clotting extract from *M. pusillus*.

Parameter	Crude extract lyophilised	Gel filtration on Sephadex G-100	Ion -exchange on QEAE Sephadex A-50
Total activity (U)	27.75	15.30	16.5
Proteins (mg)	125	3.30	11
Specific activity (U/mg protein)	0.22	4.63	1.5
Recovery (%)	100	55.13	59.45
Purification fold	1	20.88	6.75

**Figure 11.** Proteolytic activity of purified extract. Protocol I, ▲; purified extracts (Protocol II) ● and rennet ■

Conclusion

The aim of this study was to seek a better understanding of a milk-clotting protease obtained from the culture of the *M. pusillus* strain. The results showed that the crude enzymatic extract, obtained from the surface culture of *M. pusillus*, was characterized by a clotting strength ratio of 1/1,200, an activity interpreted as very acceptable for use in cheese-making technology. Furthermore, across the different purification protocols applied, it was concluded that fractioning with 40 to 80% saturated ammonium sulphate, the crude enzymatic extract of the fungal strain studied was not encouraged. In fact, an important fall in clotting activity was noted (28.53%) with a 3-fold

purification. In addition, the electrophoretic study on SDS-PAGE revealed no difference between the precipitated extract obtained by fractionation and the extract precipitated directly with 80% saturation. Moreover, based on the purification summaries it was more interesting to recuperate 6% of the initial proteins with a 44.54% activity after gel filtration (Method I), which seemed more technically sound according to Method II. The use of the exclusion chromatography technique in the purification process seemed more interesting. Indeed, on crude lyophilized extracts, it was possible to obtain a better performance (55.13), while also multiplying the specific activity of the protease by a factor of 20.88, when compared with the ion exchanger that yielded a 6.75-fold

purification for a similar activity.

REFERENCES

- Anonymous (2002). Statistiques du Ministère du commerce (Alger).
- Anonymous (2005). Statistiques du Ministère du commerce (Alger).
- Alam SI, Dube S, Reddy GSN, Bhattacharya BK, Shivaji S, Singh L (2005). Purification and characterization of extracellular protease produced by *Clostridium* sp. from Schirmacher oasis, Antarctica. *Enzyme Microb. Technol.* 36:824–831.
- Alais C (1984). Sciences du lait. Principe des techniques laitières. Ed. Sepaic, Paris, 4^{ème} Ed., 68.
- Belyauskaite IP, Palubinskas VJ, Ancheuko OE, Vesa VS, Glemzha AA (1980). Purification and some properties of the extracellular acid proteases from *Mucor renninus*. *Enzyme Microb. Technol.*, 2 : 37- 44.
- Berridge NJ (1952). An improved method of observing the clotting of milk containing rennin. *J. Dairy Res.*, 9: 328–329.
- Cavalcanti MTH, Teixeira MFS, Lima Filho JL, Porto ALF (2004). Partial purification of new milk-clotting enzyme produced by *Nocardioopsis* sp. *Bioresource Technol.*, 93: 29-35.
- Channe PS, Shewale JG (1998). Influence of culture conditions on the formation of milk-clotting protease by *Aspergillus niger* MC4. *World J. Microbiol. Biotechnol.* 14: 11-15.
- Chwen-jen shieha, Lan-anh phan thib, Ing-lung shih (2009). - milk-clotting enzymes produced by culture of *Bacillus subtilis* natto. *Biochem. Eng. J.*, 43: 85–91.
- Collin JC, Grappin R, Legraet Y (1977). Étude de la méthode de mesure, selon Berridge, du temps de coagulation du lait additionné d'une solution enzymatique. *Rev. Lait. Franç.*, 355: 389-394.
- Cuvellier GF (1999). Production des enzymes. In : *Biotechnologie*. Goor. Scriban R, 5^e ed., pp: 345–363.
- Desmazeaud M, Spinnler E (1997). Laits et produits laitiers. In « *Enzymes en agroalimentaires* ». Ed. V. Larreta- Garde. Tech. et Doc. Lavoisier.
- Esawy Mona A, Combet-blanc Yannick (2006). Immobilization of *Bacillus licheniformis* 5A1 milk-clotting enzyme and characterization of its enzyme properties. *World J. Microbiol. Biotechnol.*, 22:197–200.
- Fernandez-Lahore HM, Andery RM, Fraile ER, Biscoglio de Jimenez M, Pirpignani L, Machalinsh C, Casone O (1999). Purification and characterization of an acid proteinase from mesophilic *Mucor* sp. solid – state cultures. *J. Dairy Sci.*, 83: 584-602.
- Fernandez-Lahore HM, Fraile ER, Cascone O (1998). Acid protease recovery from a solid – state fermentation system. *J. Biotech.*, 62: 83–93.
- Goursand J (1999). Réacteurs traditionnels à enzymes libres : cas de l'industrie laitière. In: *Biotechnologies*. Coord. Scriban R., 5^{ème} ed.: 365–401
- Green ML, Stackpole A (1975). The preparation and assessment of a suitable *Mucor pusillus* Lindt proteinase- swine pepsine mixture for Cheddar cheese-making. *J. Dairy res.*, 42: 297–312.
- Khan MR, Blain JA, Patterson JDE (1979). Extracellular protease of *Mucor pusillus*. *Appl. Envir. Microbiol.*, 37(4) : 719–724.
- Krause W, Patzsh M, Hassan ZMR, Haufe T (1998). Substrate and binding specificity of aspartic protease with milk-clotting properties. *Nahrung*, 42(3/4): 162-165.
- Kumar S, Sharma NS, Saharan NR, Singh R (2005). Extracellular acid protease from *Rhizopus oryzae* : purification and characterization. *Process Biochemistry*, 40: 1701-1705.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
- Lenoir J, Auberger B, Gripon JC (1979) : Les caractères du système protéolytique de *Penicillium caseicolum*. III. Caractérisation d'une protéase acide. *Le lait* 585–586, 244–268.
- Levadoux W, Johnson M, Ander G (1989). Monitoring the degradation of commercial microbial rennet preparations. *J. Dairy Sci.*, 72(3):627–634.
- Lopes A, Teixeira G, Liberato MC, Pais MS and Clemente A (1998). New vegetal sources of milk clotting enzymes. *J. Molecular Catalysis B: Enzymatic*, 83, p.181.
- Lo Piero AR, Petrone G (1999). Purification and partial characterization of an ATP-hydrolysing serine protease from lettuce leaves. *Phytochem.*, 51: 349–356.
- Lowry OH, Rosenbrough NJ, Farr AI, Randall RJ (1951). Protein measurement with the Follin phenol reagent. *J. Biol. Chem.*, 193: 265- 275.
- Matoub L (2000). Essai de purification et de caractérisation d'une coagulase produite par la souche locale *Bacillus subtilis* sélectionnée (Lc₃₃). Mémoire de Magister. Sciences alimentaires. I.N.A. El-Harrach.
- Nouani A, Belhamiche N, Slamani R, Fazouane F, Belbraouet S, Bellal MM (2009). Purification et caractérisation électrophorétique d'une protéase coagulant le lait de *Mucor pusillus*: comparaison de méthodes. *Eur. J. Sci. Res.*, 35(4): 512-521.
- Olson NF (1995). Cheese. In *Enzymes, Biomass, Food & Feed, Biotechnology*, Vol. 9 eds Rehm HJ & Reed G pp: 353 ± 384. Weinheim. VCH.
- Preetha S, Boopathy R (1997). Purification and Characterization of a milk-clotting protease from *Rhizomucor miehei*. *World J. Microb. Biotechnol.* 13: 673-678.
- Ramet JP (1997). Les agents de transformation du lait. In « *Le fromage* ». Paris, Ed. Tech. and Doc. Lavoisier (3^e édition), pp. 165-172.
- Sidrach L, Garcia-Canovas F, Tudela J, Neptuno Rodriguez-Lopez J (2005). Purification of cynarases from artichoke (*Cynara scolymus*): enzymatic properties of cynarase A. *Phytochemistry*, 66: 41-49.
- Somkuti GA, Babel FJ (1968). Purification and properties of *Mucor pusillus* acid protease. *J. Bact.*, 95(4): 1407–1414.
- Wynne MG, Yada RY (1991). Isolation of *Mucor miehei* and *Mucor pusillus* aspartic proteinases from partially purified sources using preparative isoelectric focussing. *J. Food Biochem*, 15: 347-366.