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Identification and characterization of milk-clotting proteases produced by two species of mold

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Two strains of fungi were isolated and identified as *Aspergillus tamarii* and *Penicillium pinophilum* which showed good enzymatic activity on casein, 1933.33U and 1822, 21U, respectively. The search for milk clotting enzymes by fermentation at acid pH on culture medium containing whey and, after purification by molecular exclusion chromatography, affinity chromatography and SDS-PAGE helped to find two enzymes having a coagulant activity and approximately molecular weight of 35 and 30 KDa for *Aspergillus tamarii* and *Penicillium pinophilum*, respectively. The optimum pH of activity was 5.5 for both strains and the optimum temperature was 50 and 35°C for *A. tamarii* and *P. pinophilum*, respectively.

Key words: *Aspergillus*, *Penicillium*, milk-clotting enzyme, extraction and purification.

INTRODUCTION

Several proteases of microbial origin are used in production of biological detergents (Lalitha Kumari et al., 2010) as agent for meat tenderization (Chekireb et al., 2009), as milk clotting agent in dairy industries (chymosine and pepsin) and in medical and clinical applications (Llorente et al., 2004), (Sangupta and Dasgupta, 2006). Milk coagulation is a basic step in cheese manufacture and recently, attention has been focused on the production of milk clotting enzymes. Enzymes are aspartic proteases (APs) (EC3.4.23.X) characterized by being more active at acid pH (Timotijević et al., 2004). The strains *Mucor* and *Penicillium* are used mainly as adjuncts in different type of cheese (Fox, 1982), and these recent years other strains like *Rhizopus* (Chun-Chang et al., 2009) and *Aspergillus* (Fezouane-Naimi et al., 2010) prove interesting as a producer of rennet-like enzyme. The goal of this investigation was to identify and characterized milk clotting enzymes produced by two species of mold isolated locally and identify.

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MATERIALS AND METHODS

Reagents

All reagents used were of analytical-reagent grade and solutions prepared using Milli-Q water (Purelab option Q, ELGA). Albumin from bovine serum, calcium chloride, D-tyrosine, ferrous sulfate, folin-Ciocalteux phenol reagent, glucose agar, lactose, magnesium sulfate, peptone, potato-dextrose agar, potassium phosphate monobasic, sodium citrate, trichloreacetic acid (4% (V/V)), Sabouraud 2% and yeast extract were obtained from Sigma-Aldrich (France). Bio-Rad protein assay reagents were purchased from Bio-Rad Laboratories GmbH (Germany).

Culture conditions

Strains of *Aspergillus tamarii* and *Penicillium pinophilum* were seeded on Potato-Dextrose-Agar (PDA) medium at 25°C and monthly transferred. Cultures were stocked as lyophilized spores. Inocula were prepared in 250 mL flask using 50 mL PDA medium. After six days of incubation at 25°C, 50 mL of milliQ water were added. Spores were suspended under agitation with a magnetic stirrer, counted in a cell of malassez and stored at 4°C.

Fermentation medium

The whey (the exploitation of Monique Perruset at Vaugneray, France) was filtered on Whatman No. 1 paper and diluted 1:1 with 0.1 M phosphate buffer, pH 4.0 to generate the basic culture medium. Then, different components were added to this medium: peptone (12.09 g/l), $CaCl_2$ (13.25 g/l) for *A. tamarii* and lactose (12.88 g/l), yeast extract (16.82 g/l), $CaCl_2$ (18.72 g/l) for *P. pinophilum.* Concentrations were previously selected by statistical designs used to optimize the enzymes production. All cultures were performed in 500 mL sterilized flasks (120°C for 20 min) containing 100 mL of culture medium in which 10^6 spores were added. Fermentation was carried out using a horizontal shaker (160 rpm or 100) at 25°C for seven days.

Obtaining of crude protein extract

After fermentation the culture medium is filtered on Whatman's paper to separate mycelium from the extracellular medium containing the desired proteins. Proteins were quantified using the Bradford method (1976), using bovine serum albumin (Sigma-Aldrich) as standard.

Enzyme activity essays

Enzyme activity was measured by the method of Folin-Ciocalteu. Briefly, 0.5 mL of enzyme solution (purified enzyme or culture extract) were added to 0.5 mL of phosphate buffer (0.1 M, pH 4) and 1.5 mL of casein 2.5% in sodium citrate 0.02 M (pH 4), and incubated at 40°C for 60 min. The reaction was then stopped by the addition of trichloro-acetic acid and the amount of released tyrosine was determined (Lenoir et al., 1979). One unit of enzyme activity corresponds to 1 µg of tyrosine released per mL and per hour.

Milk clotting activity (MCA) determination

The determination of milk clotting activity was done according the Berridge clotting time method (1952). To 10 ml of milk in the test tube were added to 0.5 ml enzymatic solution and put in water bath at 35°C. The test tube was submitted to a slight rotation until the flakes of milk appear inside of the wall of the tube test. The time obtained is the mean of two trials. The units of coagulant activity (UAC) or rennet unit (PU) is defined as the amount of enzyme per milliliter of enzyme extract causing flocculation of 10 ml substrate and is calculated as follows:

$$UP = 10 \times V / Tc \times Q$$

Where, UP= Unit Presure (rennet), V = volume of substrate used (ml), Tc = Clotting time (seconds) and Q = Volume coagulant extract (ml).

The coagulant activity can also be expressed as "force coagulant Soxhlet" (F), using the following equation: $F = \frac{UP}{0.0048}$ (Bourdier and Luquet, 1981).

Enzyme purification

Enzymes were first separated according to their size on analytical Sepharose G25 columns (AMERSHAM Biosciences) equilibrated with 25 ml of 50 mM acetate buffer, pH 3.8. After size exclusion chromatography, fractions exhibiting enzymatic activity at pH 4 were purified using affinity column of pepstatin A-agarose (Sigma-Aldrich, France). The column was washed and equilibrated with 50 mM acetate buffer, pH 3.8 and bound proteins eluted with 50 mM Tris/Hcl and 0.5 M NaCl, pH 7. The fractions showing proteolytic activity were pooled and applied to SDS-PAGE electrophoresis. Before any other experiments, the fractions from affinity chromatography purification were concentrated on Amicon ultra-0.5 centrifugal filter units at 14000 rpm for 10 min.

PAGE and molecular mass determination

SDS-polyacrylamide gel electrophoresis of enzyme samples was performed in a Miniprotean II cell (Bio-Rad) on 12% gels (Stacking 5%) according to the procedure of Laemmli (1970). Samples were treated in denaturing conditions with loading buffer and 1 M dithiothreitol (DTT) and boiled 5 min before electrophoresis. Electrophoresis was run at 120 V until the blue dye marker disappeared from the separating gel. Gels were stained with Coomassie Z-blue (Sigma-Aldrich) and un-stained by repeated washing in distillated water. Molecular weight markers used were Euromedex 06P-0111-250 µl (10 to 170 KDa).

Determination of pH and temperature optima

The optimal pH of aspartic proteases was determined by incubating the purified enzyme at 40°C for 1 h in different buffers: acetate buffer (20 mM, pH 3 to 5) and Tris/HCl (20 mM, pH 5 to 8). To determine the optimal temperature, the enzymes of molds were incubated in Tris/Hcl buffer (20 mM, pH 5.5) for 60 min at different temperature: from 25 to 75°C. To determine the thermostability of enzyme activity, the purified enzymes was incubated at different temperatures (45, 50 and 55°C) for *A. tamarii* and (30, 35 and 40°C) for *P. pinophilum*. After each incubation, the enzyme activity was determined as described above.

RESULTS AND DISCUSSION

Purification and characterization of enzymes

Two enzymes with the milk clotting activity were found in

Table 1. Purification data of milk-clotting proteases from two fungi species.

Strain	Purification	Total protein (µg)	Total activity (U.)	Specific activity (U/µg)	Purification fold	Yield (%)
A. tamarii	Crude extract	10309.79	141133.09	13.69	1	100
	Sephadex G -25M	5138.47	78475	15.27	1.11	55.60
	Pepstatine-A agarose	765.77	64931.31	84.79	6.19	46.01
P. pinophilum	Crude extract	10089.33	133021.33	13.18	1	100
	Sephadex G- 25M	9507.52	132860	13.97	1.06	99.87
	Pepstatine-A agarose	3244.12	131562.06	40.55	3.08	98.90

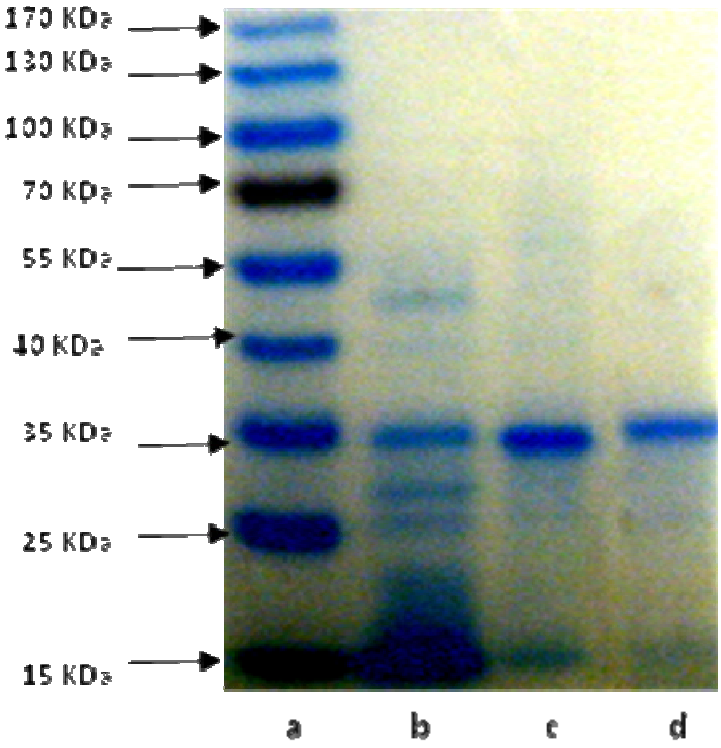

Figure 1. SDS-PAGE protein analysis during the purification of the milk-clotting protease from *Aspergillus tamarii*: a- molecular weight marker, b- crude extract, c- Sephadex G-25M fraction, d- Pepstatin A-agarose fraction.

the crude extract from fungi: *A. tamarii* and *P. pinophilum.* The yields after steps of purification of each strain were: 46.01 and 98.90% for *A. tamarii* and *P. pinophilum*, respectively, with purification folds of 6.19 and 3.08, respectively. The samples treatment with pepstatin A-agarose has eliminated up to 92.57 and 68% of inactive proteins for *A. tamarii* and *P. pinophilum*, respectively (Table 1). The purified enzymes were visualized on gel electrophoresis under denaturing conditions (SDS-PAGE), their subunits have molecular weight of about 35 and 30 KDa for *A. tamarii* and *P. pinophilum*, respectively. The results of gel filtration of the enzymes are shown in Figures 1 and 2.

Optimum pH and temperature

The purified enzymes showed an optimal pH around 5.5 on Tris/HCl and a maximum activity at 50 and 35°C for *A. tamarii and P. pinophilum*, respectively, which indicate that these enzymes might be a acid proteases. Zevaco et al. (1974) in the study of acid protease of *Penicillium roqueforti* observed a high activity of the enzyme on casein at pH 3.5 at 40°C with an optimum at 50°C and stability at pH 3.5 to 5.5.

Hashem (2000) indicates that the clotting enzyme produced by *Penicillium oxalicum* had maximal activity at 65°C and pH 4.0.

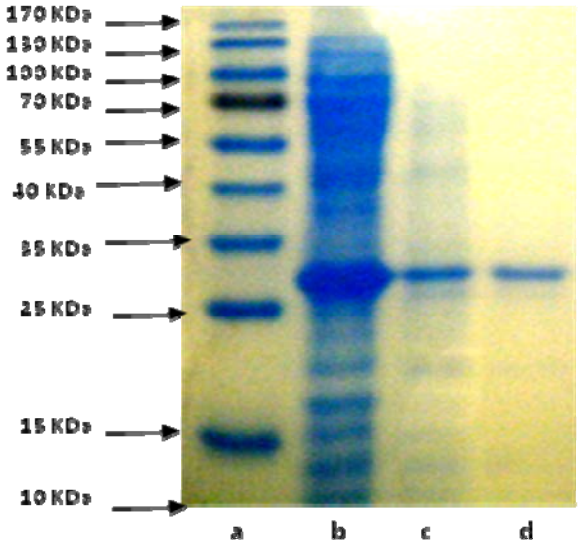

Figure 2. SDS-PAGE protein analysis during the purification of the milk-clotting protease from *Penicillium pinophilum*: a- molecular weight marker, b- crude extract, c- Sephadex G-25M fraction, d- Pepstatin A-agarose fraction.

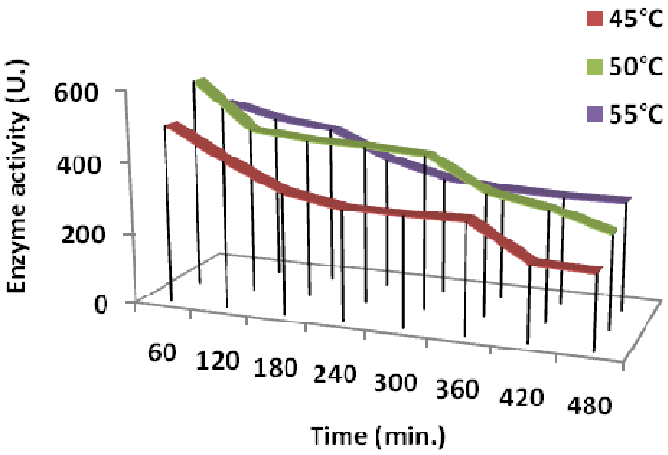

Figure 3. Effect of temperature on the stability of a protease purified from *Aspergillus tamarii*.

Thermal stability

The purified enzyme of *A. tamarii* was studied at various temperatures from 45 to 55°C, the enzyme activity drops significantly in these temperatures (Figure 3), which shows that the enzyme is unstable and its activity decreases from the first hour of incubation, and lost 60, 56 and 40% of the activity at the end of incubation at 45, 50 and 55°C, respectively.

In the case of *P. pinophilum,* the activity of the enzyme at 30°C, increased by 1% during the second hour of incubation and then lost about 13% of activity, every hour, until the end of the incubation, where the enzyme loses more than 57% of activity (Figure 4). At 35°C the enzyme was stable at 2 h, and then lost up to 45% of activity at the end of incubation, at 40°C the enzyme lost activity in the first hour of incubation and showed a $t_{1/2}$ of 4 h.

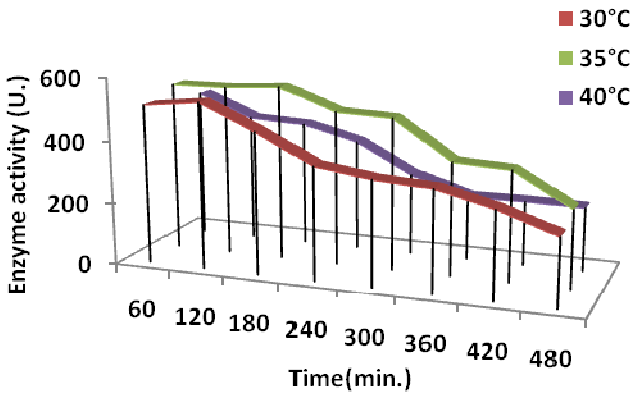

Figure 4. Effect of temperature on the stability of a protease purified from *Penicillium pinophilum.*

In the case of the acid protease of *Penicillium duponti*, Shigenori et al. (1976) showed that the enzyme was stable at pH 3.5 and 6.5 for 24 h of incubation at 30°C, 1 h at 60°C and 1 h at 75°C, pH 3.7.

Milk clotting activity

The crude extract of *A. tamarii* coagulates fresh cow milk at 35°C in 5 min. The enzyme produced coagulates milk in 2 min at 35°C with 184.44 of coagulating force (Table 2). The curd formed appeared clear yellowish serum (Figure 5). The crude extract of *P. pinophilum* coagulates fresh cow's milk in 35 min at 35°C (Figure 6), but the enzyme after purification coagulates milk in 5 min. The curd appears, white to yellowish less firm than that of *A. tamari* with a yellowish, clear and bright serum.

Conclusion

The isolation of fungi producing clotting proteases is important, not only because of the economic interests that represent these molecules but also the medium on which they are grow these strains. The enzymes show a very low apparent coagulating force, despite clotting time; the milk well is below that of rennet, which is explained by the dilution of enzyme solutions obtained after purification.

Conflict of interests

The author(s) have not declared any conflict of interests.

Table 2. Milk-clotting properties of proteases produced by two strains of molds.

Strain	Purification step	Clotting time (min.)	Coagulant activity (UP/ml)	Coagulating force
	Crude extract	5	0.33	73.33
A. tamarii	*Sephadex G -25M*	5	0.33	73.33
	Pepstatine-A agarose	2	0.83	184.44
	Crude extract	35	0.048	10.67
P. pinophilum	*Sephadex G -25M*	10	0.17	37.78
	Pepstatine-A agarose	5	0.33	73.33

Figure 5. Curd obtained by milk coagulation with protease from *A. tamarii*.

Figure 6. Curd obtained by milk coagulation with protease from *P. pinophilum*.

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