PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF ASPARTIC PEPTIDASE PRODUCED BY NOVEL ISOLATE *MUCOR CIRCINELLOIDES* (VON TIEGHEM) USING SSF PROCESS

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ABSTRACT

Microbial peptidases are among the most important hydrolytic enzymes which have a potential application in a wide number of industrial processes. In this study, the milk-clotting enzyme of a newly isolated strain *Mucor circinelloides* (von Tieghem) MG603064 was produced by solid-state fermentation using wheat bran as the substrate. The crude extract exhibited a maximum milk-clotting activity of 1500 ± 50.94 SU/mL after 72 h of incubation at 25 °C. Purification of the enzyme using fractionation at 20–70% $(NH_4)_2SO_4$ followed by size exclusion chromatography on Sephadex G-100 allowed us to obtain a 20-fold purified peptidase with a recovery of 18.41%. The highest activity of the purified enzyme (30 kDa) was obtained in 25 mM $CaCl_2$, at pH 5.0 and temperature of 60 °C. The enzyme was stable between pH 3.0–4.5 for 24 h at 4 °C in 0.1 M citrate buffer and retained more than 80% of its maximum activity at 45 °C for 1 h with complete inactivation at 75 °C. The enzyme inhibition of 94.5 and 98.6% by 0.02 and 0.1 mM Pepstatin A, respectively, confirmed that the enzyme is an aspartyl peptidase. A partial inhibition of 78.17% was noted for EDTA at 14 mM. The enzyme activity was improved significantly by Mg^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} by 40.5, 59.5, 75.6 and 85%, respectively, and strongly inhibited by Al^{3+} (93.1%) and Hg^{2+} (94.4%), at a concentration of 10 mM. Activity stimulation of 120% was maximum in the presence of Ba^{2+} using the same concentration. The crude and pre-purified extracts were applied in a trial of semi-hard cheese making (type Edam) as possible rennet substitutes.

Keywords: Milk clotting activity, peptidases, *Mucor circinelloides*, purification, cheese making

INTRODUCTION

Most of the enzymes currently developed for industrial applications are obtained from microorganisms and might constitute 90% of the global market (**Hirose, 2012; Guerrand, 2017**). Food processing industries utilized nearly about 29% of total produced enzymes, and out of this 58% are obtained from fungi (**Ahlawat *et al.,* 2018**). More than one hundred enzymes from fungal origin have been commercialized from 25 genera including *Aspergillus, Rhizopus, Trichoderma, Penicillium,* and *Humicola*. Fungal hydrolases are the most important class of enzymes with applications in different fields like in food and beverage industries (**Money, 2016**).

Peptidases or "peptide hydrolases" represent one of the three major groups of industrial enzymes and occupy 60% of total worldwide enzyme sales, of which microbial peptidases, as the leaders of industrial enzymes, account for approximately 40% (**Ahlawat *et al.,* 2018; Mamo & Assefa, 2018**).

Milk coagulation is the first stage in a typical cheese manufacture, besides milk pasteurization and possibly the addition of starter bacteria. For most cheese varieties (≈75% of total cheese), this step is achieved by adding small amount of chymosin (EC 3.4.23.4) (also termed rennin), which represents the ideal and best-known dairy enzyme used in cheese production due to its high specificity for κ-casein (**Walsh, 2014; Nasr *et al.,* 2016**).

The reduced worldwide supply of calf rennin, its high price, and the ever-increasing cheese production in combination with other factors have encouraged the search for substitutes from alternative sources such as animals, plants, and microbial peptidases (**Shah *et al.,* 2014; Walsh, 2014; Nasr *et al.,* 2016**). Research on microbial coagulants has resulted in the production of fungal aspartic peptidases characterized by low heat tolerance and low levels of proteolytic activity (**Yegin and Dekker, 2013; Mamo and Assefa, 2018**).

All microbial coagulants are produced commercially by fungal fermentation and are secreted directly into the culture medium in a dilute form free of cell proteins. This greatly simplifies the downstream process, where few subsequent purification steps are required to concentrate these enzymes, remove contaminating enzymes (peptidases, lipases and amylases), and improve the curdling activity (**Harboe *et al.,* 2010; Yegin *et al.,* 2011**).

According to the literature, only affinity chromatography with N-acetylpepstatin and N-isobutyrylpepstatin has been effectively used for the purification of microbial aspartic peptidases (**Preetha and Boopathy, 1997**), but conventional gel filtration and ion-exchange chromatographic methods have also been successfully used for the purification of these enzymes with high purification level (**Kumar *et al.,* 2005; El-Bendary *et al.,* 2007**) that can reach to 220.9-fold (**Khalil Moghaddam *et al.,* 2008**).

Few works have been published on milk-clotting peptidases production by strains of *Mucor circinelloides*, study of their properties and possible applications specifically in cheese manufacture. The present study was carried out to purify and to identify the properties of an extracellular rennin-like enzyme produced by novel isolate *Mucor circinelloides* (von Tieghem). Semi-hard cheese (type Edam) trials from cow milk were done using the crude and pre-purified extracts produced by the strain, and their performance was compared to that obtained by the commercial rennin.

MATERIAL AND METHODS

Chemicals

Ammonium sulfate and EDTA were purchased from AnalaR NORMA PUR®, VWR International Prolabo (Leuven, Belgium). Bovine serum albumin, Folin Ciocalteu reagent, Pepstatin A, 2-mercaptoethanol and prestained molecular markers for electrophoresis were obtained from Sigma-Aldrich (St. Louis, USA). Sephadex G-100, PMSF and Coomassie Brillant Blue R-250 were from Pharmacia Fine Chemicals INC. (Uppsala, Sweden), Thermo Scientific (USA) and Panreac Quimica (Barcelona, Spain) respectively. All the other chemicals were of analytical grade.

Enzyme production by SSF

Mucor circinelloides 2095-2047 was isolated from agricultural soil (El-Hadjout, Tipaza, Alegria) and identified by the Belgian Co-Ordinated Collections of Micro-Organisms, Laboratory of Mycology, the Catholic University of Louvain-la-Neuve (BCCM-MUL, Belgium) using macroscopic and microscopic observations; and by molecular method (ITS sequence identification). It was successfully integrated into the GenBank database, and it has accession number of MG603064. The strain was maintained on potato dextrose agar (PDA) slants and sub-cultured once every two months.

The milk-clotting enzyme (MCE) was produced by the strain through solid-state fermentation (SSF) process under previously determined optimizing conditions as following: 10 g of wheat bran (purchased from a local market) containing 1% solid ammonium sulfate $(NH_4)_2SO_4$ were taken in 250 mL conical flasks moistened by the mineral solution M-9 (**Tunga *et al.,* 1998**) (pH 4.23) to achieve a moisture content of 56.1%. After autoclaving (115 °C for 15 min) and cooling, the medium was inoculated with the optimum spore count of 12.6×10^7 spores/mL (developed previously on PDA medium during 5–7 d, then recovered by a sterile solution of 0.1% Tween 80) and cultivated at 25 °C.

After every 24 h of incubation, over a period of 168 h, the MCE was extracted from the moldy bran (one entire SSF flask) with 50 mL of sterile distilled water by shaking at 160 rpm for 2 h in a rotary shaker (MaxQ 4000, Model 4331, Thermo Scientific™, Marietta, USA) at 30 °C, followed by centrifugation at 2504 × *g* for 15 min at 4 °C (HERMLE, refrigerated centrifuge model Z300K, Germany). The supernatant thus obtained represents the crude enzymatic extract.

Purification of the milk-clotting enzyme

Ammonium sulfate precipitation

In the first purification step, proteins from the crude extract were subjected to fractionation by adding solid ammonium sulfate as described by **Burgess (2009)** to determine the optimal precipitation conditions of the MCE. Precise quantities of $(NH_4)_2SO_4$, were added gradually and separately to 10 mL of the crude extract in five tubes, with slow stirring at 0 °C for 30–45 min to achieve 20%, 30%, 40%, 50%, and 60% saturations. The saturated solutions were centrifuged and solid $(NH_4)_2SO_4$ was again added to the recovered supernatants to raise each to a 10% higher level of saturation. After 16–18 h at 4 °C, five pellets of 20–30%, 30–40%, 40–50%, 50–60% and 60–70% saturation were collected by centrifugation (2504 × *g*, 30 min at 4 °C), then re-suspended in a small volume of citrate/sodium phosphate buffer (0.1 M, pH 5.2) and assayed for enzyme activities and total protein content.

Dialysis against buffer

The pellet resulting from the optimal $(NH_4)_2SO_4$ fractionation was introduced into a dialysis tubing membrane (Size 9-36/32, diameter 28.6 mm, MWCO 12-14 kDa, Medicell Membranes Ltd, London, UK) and dialyzed at 4 °C against several changes of citrate/sodium phosphate buffer (0.1 M, pH 5.2) over a 24 h period to remove residual salt. The dilute enzyme preparation was concentrated for 2 h against 50% sucrose solution and kept at 4 °C for further purification.

Gel filtration chromatography

A required amount of Sephadex G-100 was suspended in an excess volume of citrate/sodium phosphate buffer (0.1 M, pH 5.2). After swelling, the gel was washed twice by the same buffer to eliminate the fine particles and then heated at 90 °C for 3 h to degas the medium. Then, 1 mL of the dialyzed extract was applied to a 1.5 × 30 cm column (DWK Life Sciences Kimble™ Flex-Columns™, Fisher Scientific, USA) crammed with the gel and equilibrated with citrate/sodium phosphate buffer (0.1 M, pH 5.2). The column was linked to a Spectra/Chrom Fraction Collector CF-2 (Spectrum Labs™, Houston, USA) and **REGLO Digital MS-2/6** peristaltic pump (ISMATEC, Wertheim, Germany). Fractions of 2 mL were collected at a flow rate of 0.3 mL/min. The fractions with high milk-clotting activity were pooled and concentrated by dialyzing against a sucrose solution at 50% and ultra-filtration using an Amicon® Ultra-4 Centrifugal Filter Unit with a 10-kDa cut off (MILLIPORE Corporation, Massachusetts, USA), by centrifugation at 2504 × *g*.

Polyacrylamide gel electrophoresis

Protein profiles of the enzyme purification steps were analyzed by SDS-PAGE using 5% stacking and 10% resolving gels, and performed according to the method of **Laemmli, (1970)** under reducing conditions. Samples were mixed with SDS reducing buffer (ratio 1:2 v/v) containing 0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol, and then heated at 95 °C for 5 min. After loading the samples into the stacking gel, they were migrated at a constant current of 70 V for 30 min and at 120 V for about 2 h in the separating gel. Separated proteins were visualized by staining the resolving gel with 0.15% Coomassie Brilliant Blue R-250 in 10% acetic acid for 24 h, then destaining by a mixture of 40% methanol, 10% acetic acid and 50% distilled water. The molecular weight was determined using prestained molecular weight markers (SDS7B2): Triosphosphate isomerase from Rabbit Muscle (26.6 kDa), Lactic dehydrogenase from Rabbit Muscle (36.5 kDa), Fumarase from Porcine Heart (48.5 kDa), Pyruvate kinase from Rabbit Muscle (58 kDa), Lactoferrin from Human Milk (90 kDa), β-Galactosidase from *E. coli* (116 kDa) and α2-Macroglobulin from equine serum (subunit) (180 kDa).

Enzyme assays

Milk-Clotting Activity (MCA)

Assay of milk-clotting activity was done according to the method of **Arima e*t al.* (1970)** using skim milk powder prepared by 10 g dissolved in 100 mL of 0.01 M $CaCl_2$ (pH 6.40) as substrate. To 10 mL of substrate (S) pre-incubated at 35 °C for 15 min, 1 mL of the enzyme extract (E) was added. The MCA was expressed in Soxhlet Unit (SU). One Soxhlet unit is defined as the volume of milk, that one volume of enzyme preparation is able to clot in 40 min at 35 °C, and is calculated by the formula: MCA (SU/mL) = 2400 × S∕E × T
Coagulation time (T) is the time (s) elapsed between the addition of the enzyme extract to the substrate and the appearance of clots on the inner wall of test tube.

Protease Activity (PA)

The proteolytic activity was measured by a modified method of **Anson (1938)**. The substrate was prepared by 2.5 g of casein in 100 mL of 0.1 M citrate/sodium phosphate buffer (pH 5.2). The reaction mixture consisted of 2.5 mL of casein solution, 0.5 mL of enzyme preparation and 0.5 mL of the same buffer, was incubated at 40 °C for 30 min. The reaction was terminated by adding 5 mL of 4% pre-chilled trichloroacetic acid (TCA). The blank was prepared in the same conditions but TCA solution was added before the enzyme extract. After filtration through Whatman paper no.1 to eliminate the resultant precipitate, the concentration of the TCA-soluble products was measured by the change of the absorption at 750 nm (UV-1800 Shimadzu Corporation spectrophotometer, Kyoto, Japan) using Folin-Ciocalteu reagent. One unit (U) of activity represents the amount of enzyme required to liberate 1 μg of tyrosine from the substrate per min under assay conditions.

Protein content

Estimations of the protein concentration in the different enzymatic extracts were determined following the method of **Bradford (1976)** using bovine serum albumin as a standard. The protein content of the individual fractions obtained after gel filtration chromatography was evaluated by measuring the absorbance at 280 nm (UV-1800 Shimadzu Corporation spectrophotometer, Kyoto, Japan).

Characterization of the purified enzyme

Optimum pH and temperature

The effect of temperature on the MCA of the purified extract was investigated under the standard assay conditions. First, 2.5 mL of the substrate were pre-incubated at different temperatures varying from 25–75 °C. After 15 min, 0.25 mL of enzyme extract were added and the milk-clotting time was determined in each case.
The optimal pH of the MCE was determined by measuring the clotting time of the reaction mixture at milk pH in the range from 5.0–8.0 adjusted with HCl and/or NaOH (1N) solutions. The MCA was determined as previously described (10% skimmed milk in 0.01 M $CaCl_2$, 35 °C).
The curdling activity was expressed in percentage relative to the highest activity obtained (the shortest clotting time).

pH and thermal heat stability

The pH stability was determined by measuring the residual MCA of the enzyme solution maintained at 4 °C for 24 h in 0.1 M citrate buffer (pH 3–5) (v/v) and 0.1 M phosphate buffer (pH 6–8) (v/v).
Study of the thermal stability was carried out by incubating the enzyme extracts at temperatures ranging from 30–75 °C in citrate/sodium phosphate buffer (0.1 M; pH 5.2) for 1 h.
The residual MCA, expressed as a percentage relative to the initial MCA, was determined according to the standard conditions of measurement.

Effect of CaCl2 and NaCl on MCA

The optimal concentration of calcium chloride ($CaCl_2$) was determined by observing the coagulation time under standard assay conditions using substrates

prepared with precise concentrations of $CaCl_2$ (10% skim milk powder in 5 mM to 50 mM $CaCl_2$).

To evaluate the effect of sodium chloride (NaCl) addition on the MCA of the purified enzyme, the standard substrate was prepared with increasing concentrations of NaCl from 1–10% (w/v) (**Ahmed and Helmy, 2012**). The activity was expressed in percentage relative to that measured in the absence of the salt.

Inhibition assays

The effects of four inhibitors: Phenylmethylsulphonyl fluoride (PMSF), 2-mercaptoethanol, Pepstatin-A and Ethylenediaminetetraacetic acid (EDTA) at different concentrations on the MCA of the purified enzyme were tested after incubating with each inhibitor separately for 30 min at 35 °C (**Yegin et al., 2012**). The impact of different metal ions ($CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $HgSO_4$, KCl, $AlCl_3$, $BaCl_2$, NaCl and NH_4Cl) was tested under the same conditions at concentrations of 5 mM and 10 mM.

The residual MCA was measured following the standards conditions, and the activity calculated in the absence of inhibitor or metal ion was taken as 100%.

Effect of substrate concentration

The values of K_m and V_{max} of the purified enzyme were determined using casein as a substrate with concentrations in the range from 1.0–3.5% prepared in citrate/sodium phosphate buffer (0.1 M, pH 5.2). For each concentration of casein, a blank was prepared as previously described. The two kinetic parameters were determined graphically from the Linweaver-Burk plot.

Cheese making

Bovine raw milk was used in trials of semi-hard cheese making (Edam, continental cheese) in order to get an estimate on the quality of the fungal extracts and their potential for cheese production.

The milk (pH: 6.61, fat: 34 g/L, total solids: 118.08 g/L, with total absence of antibiotics) was heat treated at 90 °C for 15 s, and cooled to a constant temperature of 38 °C. Then 0.3 g/L $CaCl_2$, 0.3 g lactic starter cultures: FD-DVS R-707 (*Lactococcus lactis* subsp. *cremoris; Lactococcus lactis* subsp. *lactis*), FD-DVS ST-B01 (*Streptococcus thermophilus*) (Chr. Hansen, Denmark), and four to five drops of diluted colorant E160b at 1% (Annatto extract) were added to milk. After stirring during 10 min, the milk (pH 6.4) was divided into three equal portions and separately inoculated with 0.5 mL of commercial rennin CHY-MAX® (600 IMCU/mL, Chr. Hansen, Denmark) prepared in water (0.166 g/mL), 10.8 mL of the crude extract, or 1.33 mL of the dialyzed extract produced by *M. circinelloides.* The volume of each coagulant was standardized to start coagulation within 10 min for a known volume of milk, based on preliminary tests.

After complete coagulation and hardening (25–40 min), the cheese curds were cut into cubes (the size of corn kernel >0.5 cm ~1.0 cm) during 15–20 min. The cut curds were allowed to settle for 5 min and then washed with a precise volume of

water (40 °C) following the first step of whey elimination to reduce acidity. After 20 min, the second whey was removed and the curd grains were transferred into rounded molds with cheese cloths, pressed for 20–30 min (2–3 bars), then inverted and pressed again (4.5–6 bars) until the evacuated whey reached a pH of 5.40 and the cheeses reached their desired shape. The resulting cheese blocks were immerged in salt brine (33%, w/v) for 5 h at 10–14 °C. After drying for 24 h, they were transferred into a ripening room (85–95% humidity at l0–12 °C) for 3–4 weeks and inverted twice a week. For the last steps, the surface of cheeses was scarped and washed with water, recovered with melted red paraffin wax (110–115 °C) and vacuum-packed in cellophane foil to prevent drying, then stored at 6 °C.

The analyses of cheese were performed using standard methods: determination of pH by direct contact with grated cheese using a pH-meter (Hanna instruments, Romania), dry matter/moisture by drying at 102 °C to constant weight (IDF Standard 4A:1982, AOAC Method 926.08), and fat content determination was done according to the Gerber-van Gulik method (IDF Standard 5B:1986; AOAC Method 933.05) (**Ardö and Polychroniadou, 1999**).

Statistical Analysis

All assays were carried out in triplicate and each result is the mean value of three trials ± standard deviation. The confidence level for statistical significance was considered at $P<0.05$; and was calculated using Excel software version 7.0.

RESULTS AND DISCUSSION

Production of the crude extract

The crude extract produced by *M. circinelloides* under the optimal conditions exhibited the highest MCA of 1500 ± 50.943 SU/mL with MCA/PA ratio = 41.34 ± 0.20 after 72 h of incubation at 25 °C (Tab 1). The maximum proteolytic activity of 39.325 ± 0.55 U/mL was obtained after 96 h, which was probably caused by the production of other non-specific peptidase(s).

MCA expressed directly by the crude extract was higher than those produced by fungal strains: *Thermomucor indicae-seudaticae* N31 (**Merheb-Dini et al., 2010**), *Rhizopus microsporus* var. *rhizopodiformis* (**Sun et al., 2014**), and *Rhizomucor miehei* EMCC 841 (NRRL 3420) (**Aljammas et al., 2018**), as well as by the bacterial species: *Bacillus subtilis* natto (**Wu et al., 2011**), *B. amyloliquefaciens* SP1 (**Guleria et al., 2016**) and *B. methanolicus* LB-1 (**Li et al., 2019**) through solid or liquid fermentations as follows: 167.6 SU/mL, 1001 SU/mL, 600 SU/mL, 1043.5 SU/mL, 142 SU/mL, 578.4 SU/mL, respectively.

From the Table 1, a pH decline to a low value of 5.32 was observable (determined after addition of distilled water to the moldy bran) followed by an increase, which is in accordance with previous research, indicated that activation of the microbial coagulants required a pH reduction (**Harboe et al., 2010**). The decrease in MCA after 72 h was probably caused by the reduction of nutrients and deactivation by other metabolites.

Table 1 Evolution of pH and enzymatic activities of the crude extract produced by *M. circinelloides* as function of the fermentation time.

Incubation time (h)	MCA (SU/mL)	PA (U/mL)	MCA/PA ratio	pH of the crude extract
24	13.15 ± 0.18	15.65 ± 1.80	0.84 ± 0.09	6.13
48	679.36 ± 11.00	33.42 ± 0.17	20.33 ± 0.26	5.47
72	1500.00 ± 50.94	36.28 ± 0.18	41.34 ± 0.20	5.32
96	1359.48 ± 45.28	39.32 ± 0.55	34.57 ± 1.63	5.66
120	1125.54 ± 30.00	36.17 ± 1.03	31.12 ± 1.67	6.28
144	1014.5 ± 25.10	35.84 ± 0.66	28.30 ± 0.41	6.43
168	692.44 ± 11.64	34.67 ± 1.28	19.97 ± 1.09	6.61

MCA: milk-clotting activity. PA: proteolytic activity.
The results were shown as mean value ± standard deviation of three replicates.
One entire SSF flask was harvested for each sample point.

Purification of MCE

The first step of purification was fractionation with solid $(NH_4)_2SO_4$ which represents the most commonly used precipitant for salting out of proteins, due to its high solubility at any temperature, low cost, protection of proteins in solution from denaturation, and its stabilizing effect on most enzymes (**Hirose, 2012; Walsh, 2014**).

From all of the fractions resulting after precipitation (Tab 2), the fraction P4 was had the highest MCA, best MCA/PA ratio, and the highest degree of purification,

with a recovery in activity of 307.7% and a yield of 46.15%, followed by the fraction P5.

The pellet recovered after precipitation using $(NH_4)_2SO_4$ at 20% saturation and the supernatant of the fraction P5 exhibited a proteolytic activity of 8.70 ± 0.68 U/mL and 13.21 ± 0.30 U/mL, respectively with no clotting activity (clotting time was more than 120 min). Thus, the fraction P6 was recovered to be compared to the other five fractions. The recovery in activity of 300% ($P = 0.232 > 0.05$) and the MCA/PA ratio of 46.62 ($P = 0.082 > 0.05$) were very close to that obtained with the fraction P4 (no significant differences). In addition, we noted a yield of 60%

which was higher than those of the two fractions P4 and P5 were. Consequently, the fraction P6 was considered superior and was loaded onto the Sephadex G-100 column after dialysis.

Table 2 Precipitation of the milk-clotting enzyme of *M. circinelloides* with solid ammonium sulfate.

	Total (SU)	MCA	Total protein (mg)	PA (U/mL)	MCA/PA ratio	Recovered MCA (%)	Specific MCA (SU/mg)	Fold purity	Yield (%)
Crude extract*	5714.30 ± 142.87	±	4.75 ± 0.343	37.80 ± 0.717	15.12 ± 0.66	100.00	1203.01	1.00	100.0
P1 (20–30%)	111.87 ± 3.331		0.126 ± 0.007	15.91 ± 0.738	4.70 ± 0.145	13.05 ± 0.40	887.85	0.74	1.96
P2 (30–40%)	180.00 ± 1.566		0.242 ± 0.067	22.04 ± 0.414	5.44 ± 0.11	21.00 ± 0.183	743.80	0.62	3.15
P3 (40–50%)	233.76 ± 2.276		0.405 ± 0.035	23.72 ± 1.655	6.57 ± 0.43	27.27 ± 0.265	577.20	0.48	4.09
P4 (50–60%)	2637.36 ± 114.2		1.23 ± 0.05	35.62 ± 0.254	49.0 ± 2.465	307.70 ± 13.323	2144.20	1.78	46.15
P5 (60–70%)	1742.86 ± 49.50		0.855 ± 0.104	33.54 ± 0.147	34.64 ± 0.83	203.33 ± 5.773	2038.43	1.70	30.50
P6 (20–70%)	3428.60 ± 131.96		2.09 ± 0.063	36.77 ± 0.350	46.22 ± 1.852	300.00 ± 11.55	1640.48	1.36	60.00

*The crude extract used in the optimization of salting out fractionation by $(NH_4)_2SO_4$ differs from the one used in the purification process.

Analytical methods were applied directly without using dialysis to remove salt from the pellets. MCA: milk-clotting activity. PA: proteolytic activity.
The results were shown as mean value ± standard deviation of three replicates.

Recovered MCA $(\%) = \left(\frac{\text{Inial activity of fraction X}}{\text{Initial activity of the crude extract}} \right) \times 100$, Specific MCA$=$ $\frac{\text{Total activity (SU)}}{\text{Total protein (mg)}}$, Fold purity$= \frac{\text{Specific activity of fraction X}}{\text{Original specific activity}}$, Yield $(\%) = \left(\frac{\text{Total activity of fraction X}}{\text{Total original activity}} \right) \times 100$

The dialyzed extract was separated to three peaks of proteins using Sephadex G-100, where only one peak exhibited MCA, which was eluted from fraction no.10 to fraction no.22 (Fig 1). Similar findings have been reported by **El-Bendary *et al.* (2007)**, **Nouani *et al.* (2009)** and **El-Tanboly *et al.* (2013)**, using Sephadex G-100 to purify milk-clotting extracts produced by *B. sphaericus* NRC 24, *M. pusillus*, and *M. pusillus* QM 436 respectively. The results of the purification procedure were summarized in Table 3.
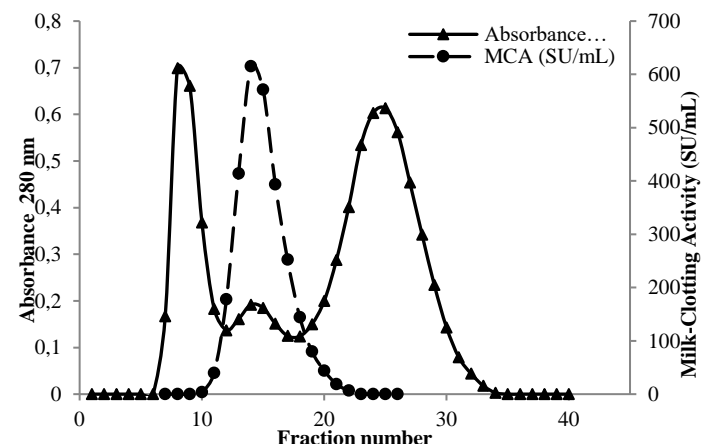
Fractionation with $(NH_4)_2SO_4$ at 20–70% of saturation followed by dialysis allowed the removal of 82.63% (37.35 mg) of the contaminating proteins from the crude extract and resulted in a 6.445-fold purification. According to the literature, as with any precipitation method, the salting out is not very selective and led to purification factors below 10, even under optimized conditions, which limits the use of $(NH_4)_2SO_4$ as a means of concentrating proteins (**Illanes, 2008**).
High recoveries of biological activity are usually recorded using $(NH_4)_2SO_4$ precipitation (**Walsh, 2014**). In this study, a yield of 111.9% was obtained after dialysis, used to remove the salt, resulted from inhibitors and/or heavy metal ions elimination. Similarly, a very close yield of 103.3% was reached after fractionation of the milk-clotting extract produced by *R. oryzae* with solid $(NH_4)_2SO_4$ (**Kumar *et al.*, 2005**).
After Sephadex G100-column, the peptidase of *M. circinelloides*, free of brown pigmentation (Fig 2), was purified 19.9-fold with a recovery of 18.41% and a high specific activity of 20202 SU/mg of protein. The decrease in proteolytic activity from 36.28 ± 0.82 U/mL to 23.37 ± 0.80 U/mL resulted from elimination of non-specific peptidases during the purification process and thus enhanced the quality of the purified extract as a possible rennin substitute.

Figure 1 Elution profile of fraction P6 on Sephadex G-100 (Column 30 cm × 1.5 cm) eluted with citrate/sodium phosphate buffer (0.1 M, pH 5.2), flow rate = 0.3 mL/min, fraction = 2 mL.

Table 3 Summary of purification procedure of the MCE produced by *M. circinelloides*

Purification steps	Total MCA (SU)	Total protein (mg)	PA (U/mL)	Specific MCA (SU/mg)	Fold purity	Yield (%)
Crude extract	45875.00 ± 1025.26	45.20 ± 1.78	36.28 ± 0.82	1014.93	1.00	100.00
$(NH_4)_2SO_4$ fractionation at 20–70%	36000.00 ± 1484.50	23.89 ± 1.033	39.60 ± 0.91	1507.06	1.48	78.47
Dialyzed extract	51333.33 ± 8082.90	7.85 ± 0.434	34.10 ± 0.60	6541.78	6.44	111.90
Sephadex G-100[a]	8444.44 ± 88.63	0.42 ± 0.13	23.37 ± 0.80	20202.00	19.90	18.41

[a]: fractions from no.11 to no.21 were pooled then concentrated by dialysis against sucrose.
MCA: milk-clotting activity. PA: proteolytic activity.
The results were shown as mean value ± standard deviation of three replicates.

Figure 2 Coagulation of skimmed milk by the crude extract and purified enzyme of *M. circinelloides* (**1**: control, **2**: crude extract, **3**: precipitated pellet with 20-70% (NH$_4$)$_2$SO$_4$, **4**: dialyzed extract, **5**: purified enzyme).

The purified peptidase of *M. circinelloides*, which migrated as a single band of 30 kDa in SDS-PAGE under reducing and non-reducing conditions, suggested that the purified protein is monomeric (Fig 3). The MCE was expressed as the main protein in the crude extract, like in most cases of *Mucor* spp., produced only one main MCE in the culture supernatants (**Yegin et al., 2011**).

Our results are in agreement with several cases of fungal aspartic peptidases presenting a monomeric structure with low molecular weights between 30–35 kDa (**Kumar et al., 2005; Vishwanatha et al., 2010; Yegin et al., 2012; Benlounissi et al., 2014**).
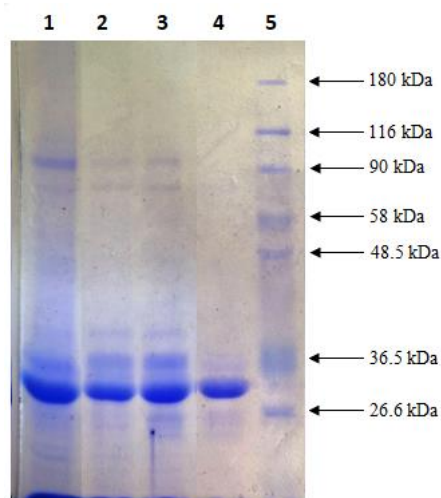

Figure 3 Electrophoretic profile of enzymatic extracts of *M. circinelloides* during purification steps on SDS-PAGE (lane **1**: crude extract, lane **2**: precipitated fraction with 20-70% (NH$_4$)$_2$SO$_4$, lane **3**: dialyzed extract, lane **4**: purified enzyme, lane **5**: Molecular weight markers).

pH optima and pH stability

It is known that pH affects the rate of renneting, curd firmness, and syneresis, as well as fat recovery, Ca^{2+} retention, and the final texture of cheese (**Maciel et al., 2015**).

The effect of pH on the MCA of *M. circinelloides* peptidase is illustrated in Figure 4a. The results show a maximum activity at pH 5.0, then a gradual decrease with increasing milk pH, and a complete inactivation at pH 7.5. According to **Yegin and Dekker (2013)**, the optimum pH for the hydrolysis of κ-casein has been reported as from 5.1–5.3. The chymosin acted optimally on κ-casein at a pH very close to 5.5 (**Kumar et al., 2010**), while rennet was more active at pH 5.0 (**Nouani et al., 2009; Zikiou and Zidoune, 2018**).

The MCA cannot be processed at pH values below 5.0, because the caseins in milk are insoluble at their isoelectric points (pH ~4.6) at temperatures >~8 °C (**Guinee and O'Brien, 2010**) and clot without adding enzymatic solutions.

Similar finding were noted by **Nouani et al. (2009), Yegin et al. (2012)** and **Celebi et al. (2016)**. Characterization of coagulant enzymes from *B. amyloliquefaciens* D4 (**He et al., 2011**), *M. pusillus* QM 436 (**El-Tanboly et al., 2013**), *T. indicae-*

seudaticae N31 (**Silva et al. 2013**), and *R. microsporus* var. *rhizopodiformis* (**Sun et al., 2014**), revealed that all are active at an acidic pH, with an optimum for MCA between 5.2–5.5.

The purified MCE of *M. circinelloides* retained its maximum clotting activity (about 80%) in the pH range from 3.0–4.5 after incubation for 24 h at 4 °C in 0.1 M citrate buffer (Fig 4b). The same results were obtained with 0.1 M citrate/sodium phosphate buffer. Raising the pH induced a rapid decrease in the clotting activity until it was closely deactivated at pH 8.0. A good stability in the same range of pH was noted for the milk-clotting peptidases of *A. niger* FFB1 (**Fazouane-Naimi et al., 2010**) and *T. indicae-seudaticae* N31 (**Merheb-Dini et al., 2010; Silva et al., 2013**).
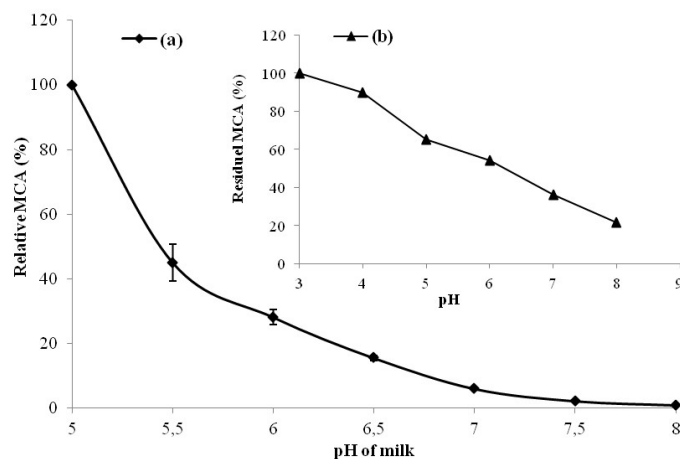

Figure 4 Effect of milk pH (**a**) on MCA of the purified enzyme (milk temperature 35 °C, 0.01 M of CaCl$_2$) and its pH stability (**b**) after 24 h of incubation at 4 °C. Error bars represent the standard deviation of three replicates.

Temperature optima and thermostability

According to results presented in Figure 5a**,** the maximum activity was reached at 60 °C, which is similar to that detected for milk-clotting peptidases produced by *R. oryzae* (**Kumar et al., 2005**), *M. pusillus* QM 436 (**El-Tanboly et al., 2013**), *B. subtilis* natto (**Wu et al. 2013**) and *R. microsporus* var. *rhizopodiformis* (**Sun et al., 2014**). A lower activity was obtained with the other temperatures tested, and it was abolished at 70 °C due to the instability of the conformational structure of the enzyme.

The optimal temperature for the MCA of microbial coagulants varied from one species to another. It was 40 °C and 45 °C for *M. mucedo* DSM 809 (**Yegin et al., 2012**) and *A. niger* FFB1 (**Fazouane-Naimi et al., 2010**), respectively; 50 °C in the case of *R. miehei* (**Celebi et al., 2016**) and *B. methanolicus* LB-1 (**Li et al., 2019**); and 55 °C for peptidase produced by *A. oryzae* MTCC 5431 (**Vishwanatha et al., 2010**). However, others coagulants were more active at highest temperatures of 65–70 °C (**Merheb-Dini et al., 2010; He et al., 2011; Ding et al., 2012**).

The clotting activity of the purified enzyme was stable up to 45 °C, with more than 80% of maximum activity (Fig 5b). The activity decreased to 13% after incubation at 60 °C for 1 h, and it was completely inactive at 70–75 °C because of the denaturation of the enzyme.

The same range of stability (30–45 °C) has been reported for the coagulant enzymes of *R. oryzae* (**Kumar et al., 2005**), *A. niger* FFB1 (**Fazouane-Naimi et al., 2010**), *T. indicae-seudaticae* N31 (**Merheb-Dini et al., 2010**) and more than 70% of activity was retained up to 45 °C in the case of peptidase produced by *R. microsporus* var. *rhizopodiformis* (**Sun et al., 2014**),

Generally, microbial coagulants have a higher thermal stability than calf rennet (**Celebi et al., 2016**), in which aspartic peptidases from *Mucor* spp. are more thermolabile than those that were obtained from *Rhizomucor* spp. currently used in cheese making (**Yegin and Dekker, 2013**).

The purified enzyme of *M. circinelloides* was more sensitive to heat treatment and retained only 55% of its activity at 55 °C, compared to peptidases produced by *M. pusillus* (**Nouani et al., 2009**), *T. indicae-seudaticae* N31 (**Silva et al., 2013**), and *Rhizomucor pusillus* (**Yegin and Dekker, 2013**) that preserved more than 70% of activity at the same temperature. While under similar conditions, calf chymosin (**Merhe-Dini et al., 2010**) and coagulant enzymes produced by *M. mucedo* DSM 809 (**Yegin et al., 2012**) and *R. microsporus* var. *rhizopodiformis* (**Sun et al., 2014**) were completely inactivated.

In cheese manufacture, the high thermal stability of a coagulant prevents the usage of whey and results in the persistence of activities particularly the proteolytic one (0–15%) after the cooking of the curd, causing off-flavors, bitterness, and texture defects in the cheese during long maturation periods (**Merheb-Dini et al., 2010; Celebi et al., 2016**). Therefore, the ability of the purified enzyme to be rendered closely inactive by moderate heating, favors its application in the cheese manufacture.
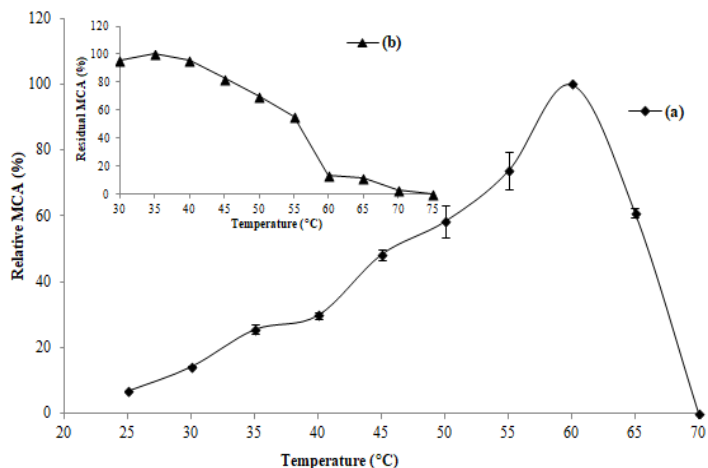
Figure 5 Influence of temperature **(a)** on MCA of the purified enzyme (milk pH 6.40; 0.01 M of CaCl$_2$), and its thermal stability **(b)** after 60 min of incubation. Error bars represent the standard deviation of three replicates.

The optimum concentration of CaCl$_2$

According to Figure 6, the MCA is optimal at a low CaCl$_2$ concentration of 25 mM. The decrease in activity at higher concentrations than this could be explained by the progressive saturation of negative residues (phosphoserine and carboxylic groups) of the casein micelles or the increase of ionic force (**Merheb-Dini *et al.*, 2010; He *et al.*, 2011**). Similar behavior has been reported by **He *et al.* (2011)** and **Li *et al.* (2019).**
The optimal activity of rennet was found at 20 mM CaCl$_2$ in the substrate (**Nouani *et al.*, 2009; Zikiou and Zidoune, 2018; Li *et al.*, 2019**), while for other peptidases, the MCA reached its maximum with 40 mM (**Merheb-Dini *et al.*, 2010; Silva *et al.*, 2013; Sun *et al.*, 2014**) and 60 mM CaCl$_2$ (**Ding *et al.*, 2012**).
Addition of calcium chloride to cheese milk presents several advantages: it speed up the curd formation, improves curd firmness, and the slight drop in pH promotes the action of the coagulant and increases the rate of casein micelles aggregation during the second phase (non-enzymatic) of coagulation (**Harboe *et al.*, 2010; Merheb-Dini *et al.*, 2010; Vishwanatha *et al.*, 2010; El-Tanboly *et al.*, 2013**). However, according to **Silva *et al.* (2013)**, the excess of CaCl$_2$ may result in the formation of a bitter taste in cheese, thus it is more useful to reach the maximum activity of coagulant at low concentrations of CaCl$_2$.
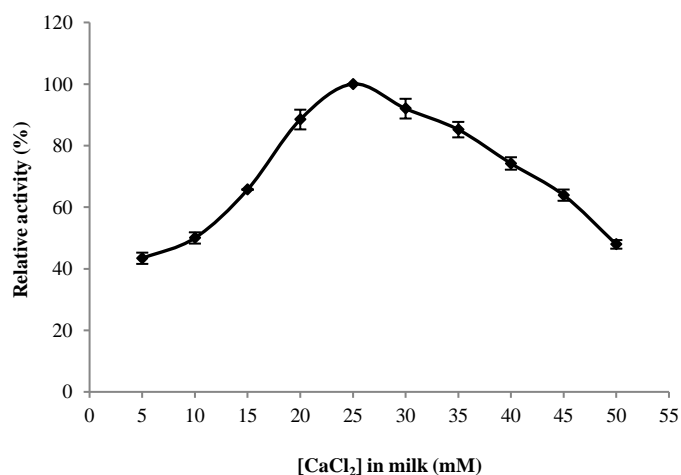

Figure 6 Influence of CaCl$_2$ concentration on MCA of the purified enzyme of *M. circinelloides* (temperature and pH of milk: 35 °C, 6.40). Error bars represent the standard deviation of three replicates.

Effect of sodium chloride

Milk is sometimes salted with NaCl for protection against spoilage by various microorganisms. The amount of salt required to reduce water activity to prevent microbial growth is 4-5% (**Ahmed and Helmy, 2012**).
The MCA of the purified enzyme was rapidly decreased by increasing the NaCl concentration (Fig 7). The low concentration of NaCl at 1% induced a significant decrease in the activity to reach only 41.4%. The findings are in agreement with the results previously obtained (**El-Bendary *et al.*, 2007; El-Tanboly *et al.*, 2013**). It was suggested that NaCl such as CaCl$_2$ affected the hydrolysis of casein through some conformational changes in casein rather than the enzyme (**Salehi *et al.*, 2017**), where it promotes dissociation of calcium and phosphate from within casein

micelles and into solution, which reduce the rennet coagulability of milk (**Awad, 2007**).
In order to avoid the loss of MCA, it is more suitable to apply the purified enzyme in the cheese manufacture using unsalted milk.
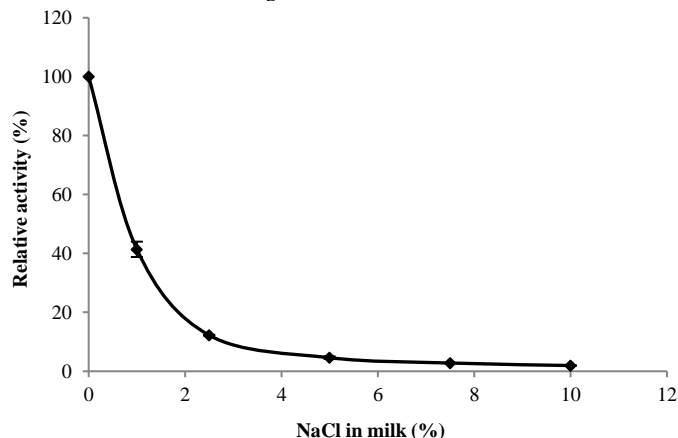

Figure 7 Effect of NaCl concentration on MCA of the purified enzyme of *M. circinelloides* (temperature and pH of milk: 35 °C, 6.40; 0.01 M of CaCl$_2$). Error bars represent the standard deviation of three replicates.

Protease inhibitors

The effects of various inhibitors on the MCA of the purified enzyme were investigated to identify the peptidase class (Tab 4). A very low inhibition was observed with the highest concentrations tested of PMSF (serine peptidases inhibitor) and 2-mercaptoethanol (cysteine peptidases inhibitor) where the enzyme conserved more than 87% and 93% of its original activity, respectively.
The enzyme retained 74.2% of its activity in 5 mM EDTA (metallopeptidases inhibitor), which decreased to approximately 22% with a concentration of 14 mM. These results are in agreement with those reported by **Hashem (2000)** and **Sun *et al.* (2014)**, but most of the milk-clotting peptidases previously studied have been insensitive to the action of EDTA (**Kumar *et al.*, 2005; El-Bendary *et al.*, 2007; Vishwanatha *et al.*, 2010**), or their activity was enhanced using low concentrations (**Merheb-Dini *et al.*, 2010; Yegin *et al.*, 2012**).
Theron and Divol (2014) as well as **Mamo and Assefa (2018)** reported the role of Ca^{2+} ions as cofactor for aspartic peptidases. Thus, the partial inhibition induced by EDTA can be explained by part of the Ca^{2+} ions essential for enzyme activity and the formation of milk-clot during the non-enzymatic phase of coagulation having been complexed with the chelating agent, and hence clotting was retarded (**Hashem, 2000; Salehi *et al.*, 2017**).
Incubation of the purified enzyme with 0.02 mM pepstatin A, as a specific reversible inhibitor of aspartic peptidases, induced a strong inhibition of 94.5% compared to the control and reached 98.6% at 0.1 mM.
The MCE of *M. circinelloides* was more sensitive to pepstatin A inhibition than to aspartic peptidases from *T. indicae-seudaticae* N31 (**Merheb-Dini *et al.*, 2010**) and *M. mucedo* DSM 809 (**Yegin *et al.*, 2012**), where 75% and 81% of activity were suppressed at 0.02 mM and 0.1 mM of the same agent, respectively.
The results suggest the presence of aspartate residue at the active site of the enzyme and allow the inclusion of the peptidase produced by *M. circinelloides* in the aspartyl-peptidases family.
Salehi *et al.* (2017) reported that the very weak inhibition of aspartic peptidases by some inhibitors (E-64 and PMSF) was probably due to a small non-specific effect from the carrier solvents and/or the high concentrations of the inhibitors themselves.

Table 4 Effect of different inhibitors on MCA of the purified enzyme

Inhibitor	Concentration (mM)[a]	Residual MCA (%)
Control	0	100
PMSF	1.0	89.87 ± 0.80
	2.0	87.65 ± 1.46
2-mercaptoethanol	5.0	98.50 ± 1.00
	10.0	93.70 ± 0.92
EDTA	5.0	74.20 ± 2.62
	7.0	72.73 ± 1.81
	10.0	34.78 ± 1.71
	14.0	21.82 ± 2.17
Pepstatin-A	0.02	5.44 ± 0.03
	0.06	2.88 ± 0.07
	0.10	1.40 ± 0.06

The inhibitors were prepared in ethanol. [a] is the final concentration of the inhibitor in the reaction mixture. MCA: milk-clotting activity.
The results were shown as mean value ± standard deviation of three replicates.

Effect of metal ions

Metallic ions such as Fe^{2+}, NH_4^{1+}, Zn^{2+} and Ba^{2+} enhanced significantly the MCA of the purified enzyme by 11, 13.3, 24.5 and 34.8%, respectively at a concentration of 5 mM (Fig 8). The MCA was strongly increased when using a concentration of 10 mM of Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Ba^{2+} where for the two last salts the percentage of activation reached 85 and 120% respectively.

The positive effects of Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} on the MCA of microbial coagulants have been reported by several studies (**El-Bendary** *et al.,* **2007; Ahmed and Helmy, 2012; Ding** *et al.,* **2012; Sun** *et al.,* **2014; Li** *et al.,* **2019**). Only **Mabrouk** *et al.* (**1976**) have reported the activating effect of $BaCl_2$ for the coagulant of *Penicillium expansum,* whereas for others, it has an inhibitory effect (**Sun** *et al.,* **2014**).

The K^{1+} has no effect on the enzyme activity at 5 mM. After incubation with sodium, potassium and ammonium chloride at 10 mM, the purified enzyme exhibited residual MCA raging from 70–90%. However, the other ions induced a significant inhibition specifically by 25.16, 57.66 and 85.5% with Cu^{2+}, Al^{3+} and Hg^{2+}, respectively, using a concentration of 5 mM. Similar findings have been obtained by **He** *et al.* (**2011**) and **Ding** *et al.* (**2012**). Increasing the concentration of Al^{3+} and Hg^{2+} inhibited strongly the enzyme activity to the extent of 93.1 and 94.4% respectively.
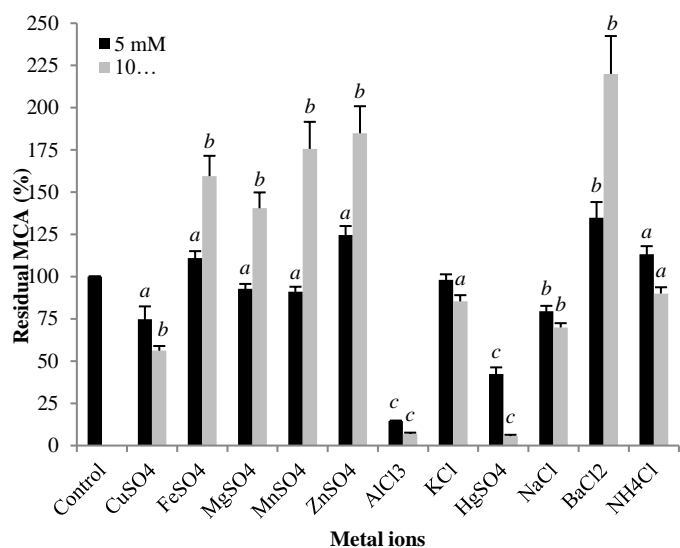

Figure 8 Influence of metallic salts on MCA of the purified enzyme. Error bars represent the standard deviation of three replicates. (*a*: $P < 0.05$; *b*: $P < 0.01$; *c*: $P < 0.001$).

***K_m* and *V_max* determination**

The values of K_m and V_{max} of the purified enzyme were calculated from the Lineweaver-Burk plot (Fig 9) as 125 mg/mL and 250 U/mL, respectively using 1–3.5% of casein as the substrate at pH 5.2. Saturation of the enzyme activity was attained at a casein concentration of 3.5% (w/v). The K_m value was superior to K_m values of coagulant enzymes that have been previously characterized (**Kumar** *et al.,* **2005; El-Tanboly** *et al.,* **2013; Salehi** *et al.,* **2017; Li** *et al.,* **2019**).

According to **Luo** *et al.* (**2018**), a lower K_m indicates a high enzyme affinity to the substrate when the substrate concentration is low, whereas a higher K_m indicates a high enzyme affinity to the substrate only when the substrate concentration is higher. Thus, this difference can be attributed to the conditions followed during the proteolytic activity assay and the selected substrate concentration range.
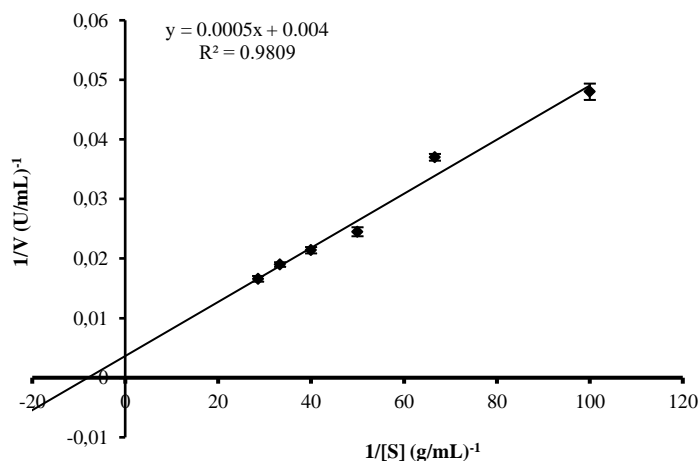

Figure 9 K_m and V_{max} determination using Lineweaver-Burk plot. Error bars represent the standard deviation of three replicates.

Semi-hard cheese-making

The dialyzed extract characterized by a high MCA = 7333.33 SU/mL and MCA/PA ratio of 215.05, was selected for cheese making trials in comparison to the crude extract and commercial rennin CHY-MAX®.

The cheese produced was Edam (ready for consumption), where the ripening procedure to develop flavor and body characteristics is normally lasts from 3 weeks at 10–18 °C, depending on the extent of maturity required (**FAO and OMS, 2018**). According to the results (Tab 5), a small difference was observed between the chemical compositions of the two cheeses produced by CHY-MAX® chymosin and the dialyzed extract of *M. circinelloides,* which were compatible with the standards. Only the fat levels of cheeses made with the fungal extracts were higher than norms. The chemical properties of cheese produced by the crude extract were of low quality, because of its contamination by other constituents, such as non-specific peptidases and possible lipases.

Cheese yield is important in cheese manufacture because of its direct impact on the process cost. The lowest yield (72.7 g/L) was obtained for the crude extract, which was enhanced to reach 79 g/L using the pre-purified extract from *M. circinelloides*, but it was still inferior to that of the commercial rennet. This difference can be explained by an excessive activity on proteins and fat content of milk (loss of proteins and fat in whey). The improvement of the cheese yield with the dialyzed extract indicated that the application of the two steps, $(NH_4)_2SO_4$ fractionation and dialysis, was able to reduce the degree of contamination by interfering enzymes, and thus we can predict that the purified extract, characterized by a low proteolytic activity, can achieve results very close to commercial chymosin.

Table 5 Characteristics of Edam cheeses produced by the fungal extracts of *M. circinelloides* or by CHY-MAX® rennin

Characteristics	Cheese made with			Typical values
	CHY-MAX® rennin	Crude extract of *M. circinelloides*	Dialyzed extract of *M. circinelloides*	
pH	5.50	4.90	5.49	5.1–5.5[a]
Moisture (%)	43.90	37.30	40.90	38–53[b]
Dry matter (%)	56.10	62.70	59.10	47– 62[b]
Fat (%)	23.00	25.00	25.00	20 ± 1[a]
FDM (%)	41.00	39.87	42.30	40–50[b]
Yield (g/L)	84.12	72.70	79.00	/

FDM: fat in dry matter. [a]: (standards of the production unit); [b]: (**FAO and WHO, 2018**).

The purified aspartic peptidase from *M. circinelloides* was used successfully in cheddar cheese making from fresh cows' milk with only a 5% difference in the cheese yield compared to commercial rennet (**Yegin** *et al.,* **2011**). In this study, a difference of 6.086% was noted between the cheese yields attained by the dialyzed extract and CHY-MAX® rennin.

The detection and enumeration of *Staphylococcus aureus* (Baird Parker Agar at 37 °C for 48 h, ISO 6888-1:1999 A1:2003) and *Salmonella* spp. pathogens (Selenite-F Broth for selective enrichment and Hektoen Enteric Agar for isolation at 37 °C for 72 h) in the cheeses produced revealed the total absence of contaminations, which indicated that the hygienic conditions were maintained during the cheese manufacture and the absence of germs in the fungal extracts.

For the physical properties, we have noted two remarkable defects: (1) unsuitable flavor (musty and pungent) only for cheese produced by the crude extract and (2) an uneven surface (internal appearance) for Edam given by the fungal extracts compared to a smooth texture for cheese made by CHY-MAX® rennin. No

difference was apparent for the color (buttery yellow) and the external appearance of all cheeses, but the consistency of cheese developed by the commercial rennin was softer and more flexible when compared to that resulting from the pre-purified extract.

CONCLUSION

The aspartic peptidase produced by *M. cicrcinelloides* (von Tieghem) using SSF system and purified by gel filtration on Sephadex G-100 was characterized by a high specific MCA of 20202 SU/mg, low proteolytic activity, thermostability up to 45 °C; pH stability in the acidic range with an optimum at pH 5.0, lower $CaCl_2$ sensitivity with maximum activity at a concentration of 25 mM, and were very close to the properties of calf rennet. Incorporation of NaCl in the milk even at 1% (w/v) affected strongly the curdling activity of the enzyme. The MCA of the purified enzyme was significantly enhanced by metallic ions Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Ba^{2+}, but appreciably inhibited by Al^{3+} and Hg^{2+}.

The results obtained in cheese making trials are promising, but in order to achieve the aim of the study, the purification process needs to be improved to get a purified enzyme that can be considered as a possible substitute for rennin based upon the most important properties for cheese making. Moreover, it is essential to complete our work by studying the proteolytic properties of the enzyme and their influence on the quality of the fungal coagulant, which allows the choice of cheese varieties to be produced. *M. circinelloides* is not known to produce mycotoxins, but it will be necessary to make toxicity studies for the microorganism and for the produced enzyme solution to be safely applicable in cheese production. Finally, cheese-manufacturing tests using the purified enzyme separately or in combination with commercial rennet to reduce the amount of this coagulant and production costs could be very interesting.

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