ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Purification and characterization of Milk -Clotting Enzyme from the edible mushroom *Pleurotus albidus.*

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ABSTRACT

Milk clotting enzymes (MCE) produced by fungi have an important application in food and drug technology. In this study, the edible mushroom *Pleurotus albidus* was used as safe and efficient source of MCE. The produced MCE was purified by ammonium sulfate precipitation, dialysis, DEAE-Cellulose and Sephadex G-100 column chromatography. The full homogenized one band enzyme had an enzyme activity of 4727.27 (U/ml), protein content of 38.0 (mg), specific activity of 124.40 (U/mg), purification fold of 7.09 and recovery of 236.3 %. The SDS PAGE confirm the full purification of MCE as it appears as one band of apparent molecular weight of approximately 34 KDa. The study of different factors affected MCE activity showed that the optimum temperature and pH were 60°C and 6, respectively. The pure enzyme was stable up to 4 hours at 60°C and pH 6. The enzyme and substrate concentrations were found to be 88.4 mg/ml enzyme and 0.14 g/ml cow milk casein. The value of Km and Vmax determined by Line Weaver Burk plot were 0.2 g/ml and 5000 (U/ml), respectively. The enzyme found to be highly active on cow milk casein more than each of azo-casein and bovine serum albumin while it had no activity on gelatin. The enzyme inhibited by Zn^{+2}, Mg^{+2} and Na^{+} and activated by Ca^{+2}. It also inhibited by the serine inhibitor PMSF indicating that it a serine protease. The optimum concentration of Ca^{+2} for MCE activity was found to be 0.09 M.

Keywords: Milk-Clotting Enzymes, *Pleurotus albidus*, Enzyme Purification, Enzyme Characterization.

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ISSN: 0975-8585

INTRODUCTION

Clotting enzymes can be obtained from many sources such as Animal: calf rennet (1), Plant: Plant latex (2) and microorganisms: *Mucor pusillus* (3).Chymosin or rennin is the major milk-clotting component of natural rennet preparations (4).Calf rennet, which is a milk clotting enzyme derived from the mucosal lining of calf stomach has traditionally been used in cheese making industry. (5)

Plant proteases employed for cheese production in various areas of the world include papain, bromelin, ficin, oryzasin, cucumisin, sodom apple and *Jacaratia corumbensis* protein (6). Limited supply, high cost, and unspecific proteolytic activities of natural (animal and plant) rennet have simulated the search for alternative sources of milk-clotting enzymes of microbial origin (7).

Microbial milk clotting enzyme constitute about 33% of total protease, which account for 60% of the worldwide commercialization (8). It largely replace calf rennet for milk clotting in cheese industry as they possess high milk-clotting activity and low proteolytic activity, could be mass produced, production is cheaper, great biochemical diversity and easy genetic modification (9).

Researchers discovered and still discovering new sources that have milk clotting activity rather than enzymes extracted from traditional rennet (10). Because the production of cheese is increasing and the cost of rennet is high, scientists encouraged to find a new source for milk-clotting enzymes especially from microorganisms. Fungi are microorganisms that have many advantages because they generally regarded as Safe (GRAS) and their extracellular enzymes are easy to be recovered in bioprocess.

Edible mushrooms are macro fungi, they have fleshy and edible fruit bodies .They can appear either below ground (hypogenous) or above ground (epigeous).

Pleurotus is a genus of gilled mushrooms which includes one of the most widely eaten mushrooms, *P.osreatus*. Species of *Pleurotus* may be called oyster, abalone, or tree mushrooms, and are one of the most commonly cultivated edible mushrooms in the world. Using of edible mushrooms have been raising due to their medicinal and nutritional importance. Among 7000 mushrooms species, only 2000 species considered as safe.

In general, milk-clotting enzymes belong to the small group of aspartic acid proteinases which contain two aspartic acid residues at their active site, act at pH range 2.5–7 and are inhibited by pepstatin .The molecule of these enzymes is heterogeneous because of N- glycosylation, phosphorylation, deamination or partial proteolysis process. The aspartic proteinases from fungi are generally divided into two groups: pepsin-like enzymes derived from *Aspergillus, Penicillium, Rhizopous* and *Neurospora*. Chymosin like enzymes (formerly rennin)- derived from *Cryphonectria , Rhizomucor* spp and *Mucor spp*. (11).

R. pusillus proteinase has specificity towards κ-casein, with optimum pH around 4 .The active protease consists of a single polypeptide chain having 361 amino acid. Molecular weight is 49 kDa. It has high ratio of MCA/PA so it considered as best milk-clotting enzyme and this is an important criterion for the choice of a new strain or microorganism for producing new microbial coagulant.

Many results demonstrate that aspartic proteases, which usually have high milk clotting activity, are predominantly derived from fungal strains, and therefore fungal enzymes appear to be more suitable for use in the cheese industry (12). Technological Applications of Milk-Clotting Enzyme due to the growing demand for natural coagulants led to an increased necessity for rennet substitutes and promoting a search for new sources of proteases with coagulant properties (13). For milk-clotting enzymes with high non-specific action, several improved strategies have been developed to produce cheeses with sensory properties close to those of animal rennet (14). According to Martim *et al.* (10) *Pleurotus albidus* was the one that expressed the highest value of coagulant ratio (21.60). The milk-clotting enzymes showed maximum activity at 60°C and pH 6.0.

Mechanism of Enzymatic Coagulation include three: Primary Stage is the enzyme (rennet) cuts off a specific fragment of one of the caseins, namely k -casein. At the natural pH of milk, about 80% of -casein must be cleaved to permit aggregation of the micelles to proceed. Secondary Stage is the physical process of aggregation of casein particles (micelles) to form a gel. After losing its water soluble tail, -casein can no longer

keep the casein particles separated, so they begin to form chains and clusters. The clusters continue to grow until they form a continuous, three dimensional network which traps water inside, and forms a gel. The tertiary stage refers to an ongoing development of the gel network (15).

Lower pH increases enzyme activity and neutralizes charge repulsion between micelles. Therefore, both primary and secondary stages of coagulation proceed more quickly at lower pH (16). Calcium is not required for the primary stage (i.e., enzyme hydrolysis of -casein) but is essential to aggregation of the casein micelles. At low levels of calcium the primary phase goes slowly to completion. Subsequently, consequent coagulation can be induced by adding sufficient calcium chloride (17). The optimum coagulation temperature for most cheese is 30-32C, the exception is Swiss cheese which is set at 37C. At temperature less than 30C the gel is weak and difficult to cut without excessive yield loss due to fines (18).

The aim of this study was planned to produce, purify and characterize the milk clotting enzymes from edible mushroom *Pleurotus albidus*

MATERIALS AND METHODS

Production and extraction of milk clotting enzyme

We will use the edible mushroom, *Pleurotus albidus*

For mentainance cultures were cultivated in Potato Dextrose Agar (PDA medium and 0.5% (w/v) yeast extract) in Petri dishes (19, 20|). The cultures were incubated at 25°C for 12 days (20, 21).

Fermentation Media and Culture Conditions

In 125 ml flasks containing 50 ml of autoclaved medium, medium composed of (g/L): glucose (20), yeast extract (5), peptone (5)and gelatin (5), pH (5.6) (19,20). After cooling, each flask was inoculated using 10 mycelial discs of 1 cm diameter.Then incubated at 30°C, on rotatory shaker 150 rpm for 72 h. The biomass was separated from the crude extract by centrifugation.

Then the cell free supernatant containing crude enzyme was collected. Crude extract was precipitated with ammonium sulphate (60–80% saturation) with gentle stirring and left overnight at 4ºC for complete precipitation. The precipitate was collected after centrifugation at 15,000 rpm for 30 min at 4 Celsius, air-dried and re-dissolved in a minimum amount of 100 mM sodium acetate buffer (pH 4.0) followed by overnight dialysis against 20 mM of the same buffer to rid get other components (21).

Protein estimation

Protein concentration was estimated according to the method described by Lowry *et al* (1951). (22)

Milk-clotting activity assay

The milk-clotting enzyme activity was assayed according to the standard procedure of Carlson et al. (1985) (23) with some modifications. The substrate used was dried skimmed milk (12%, w/v) dissolved in 0.01 M calcium chloride solution. The reaction mixture contained 0.5 ml enzyme and 1 ml of substrate was incubated for 1 h. The enzyme activity was calculated according to Otani et al. (1991) (24) as follows: Milk-clotting activity units = 2400/T X S/E, where: **T** is the time necessary for the crude fragment formation, **S** is the volume of milk, and **E** is the volume of enzyme.

Purification of *Pleurotus albidus* 's milk clotting enzyme

The crude enzyme precipitated at 80% $(NH_4)\ SO_4$ was estimated before and after dialysis.

The dialysed enzyme was applied to DEAE-Cellulose column chromatography. Elution was performed with a linear gradient of 0.1 M Sodium Phosphate buffer pH 6.5 containing NaCl 0.1 M. 0.2 M, 0.3 M, 0.4 M and 0.5 M. Fractions (5 ml) were collected at a flow rate of 15 ml / hour. Protein content and milk clotting enzyme

activity were assayed and the specific activity was calculated. The pooled fractions of highest milk clotting enzyme activity obtained from the previous column were applied in a small amount of phosphate buffer pH 8 to Sephadex G-100 column (1.3 x 90 cm), which had been previously equilibrated with the same buffer. Elution was performed at a flow rate of 12 ml / hour and 5 ml fractions were obtained. The protein content and milk clotting enzyme activity were determined. The fractions which showed highest milk clotting enzyme activity were pooled for further investigation.

Estimation of the molecular weight of the purified milk clotting enzyme by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method for rapidly assessing the purity of proteins, and is routinely used in the development and validation of a purification strategy (45). The method was also used for estimation of the molecular weight of the purified milk clotting enzyme using Thermo Scientific™ PageRuler™ Prestained Protein marker (25). The Laemmli SDS-PAGE discontinuous system (26) with homogenous 10 % gel was adopted. Equal amounts of proteins (15 µg/25 µl) were loaded in each well. Electrophoresis was carried out at about 100 volts. The gel was stained with Coomassie blue R-250.

The gel was placed between two sheets of cellophane membrane and dried on gel drier for 2 hours and photographed.

Characterization of milk clotting enzyme

Effect of temperature on activity and the stability of milk clotting enzymes

The optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 40 to 75°C. In thermal stability, the enzyme extracts were incubated at different temperatures ranging from 40 to 60°C for 4 hours before adding the substrate. The MCE activity and protein content were estimated.

Effect of pH on activity and the stability of milk clotting enzymes

To determine optimum pH, clotting activity was assayed at different pH values using the following 0.1 M buffer solutions , sodium acetate (4,5) and sodium phosphate (6,7 and8). For the pH stability, the enzyme extract was dispersed (1:1) in the following 0.1 M sodium acetate buffer solution (4,5) and sodium phosphate buffer (6,7,8) maintained at 25°C for 1,2,3 and 4 hour. After that the clotting activities were assayed.

Effect of enzyme concentration on milk clotting activity

Different concentrations of purified milk clotting enzyme 7.4, 41.1, 54.8, 88.2, 109.6 mg/ml were used to study their effect on the rate of milk clotting enzyme activity.

Effect of Substrate Concentration (cow milk casien) on milk clotting activity

The effect of substrate concentration on the milk-clotting activity of the purified enzyme was determined by increasing the concentration of the cow milk casein from 0.036 to 0.24 g/mL.. The Michaelis constant (Km) and the maximal velocity of proteolysis (Vmax) were calculated from Lineweaver-Burk plot.

Effect of Substrate specificity on milk clotting activity

Different substrates was evaluated by a modified method of Arima et al. (27). One milliliter of (0.1 mg/ml) purified *Pleurotus albidus* MCE was added to 2 ml 10% caw milk casein, azo-casein ,bovine albumin ,and gelatin as substrates . Then the clotting activity was determined.

Effect of inhibitors and metallic ions on milk-clotting activity:

The effect of inhibitors on milk-clotting activity was investigated by using 0.1 ml of 1 mM of pepstatin A and 10 mM of phenyl methyl sulfonyl fluoride (PMSF), ethylene-diaminetetraacetic acid (EDTA),and some

metals at 10 ppm $CaCl_2$, $CuSO_4$, KCl, $FeSO_4$, $MgSO_4$, , NaCl and $ZnSO_4$ in the reaction mixture . Samples were incubated at 40°C for 30 min and the milk-clotting activity was determined. Relative enzyme activities were determined as compared to the control, without inhibitors and metallic ions and corresponds to 100% of activity.

Effect of $CaCl_2$ concentration on milk clotting activity

The effect of the concentration of $CaCl_2$ on the milk-clotting activity of the purified enzyme was determined by adding 0.1 ml of different $CaCl_2$ concentrations (0.09, 0.2, 0.45, 0.9 and 1.35 M). Samples were incubated at 60°C and the milk-clotting activity was determined.

RESULT AND DISCUSSION

The purification protocol of MCE from *P.albidus* is presented in Table 1. The crud preparation of MMCE showed protein content of 114 mg, milk clotting activity and specific activity of 2000 U/ml, 17.54 U/mg, respectively. Ammonium sulfate fractionation at 80 % saturation achieved a specific activity equals to 0.444 (U/mg). The amount of total protein 900 (mg) as well as total milk clotting activity as 400 (U/ml), purification fold of 0.025 and recovery of 20%. When the dialysed extract applied to DEAE- Cellulose column, one major peak and four minor peaks of activity were detected. The main peak include fractions from 17 to 29, while the minor peak appeared in fractions 5-7; 9-12; 30-31 and 32-35. Figure (1) in the major peak the protein, enzyme activity, specific activity, purification fold and recovery were 37.9 mg, 4444.44 (U/ml), 117.3 (U/mg), 6.66 and 222.22%, respectively.

The active peaks from DEAE-Cellulose were pooled and applied to Sephadex G-100 column. One major active peak was appeared indicating the full homogeneity of the purified enzyme. The pure enzyme had clotting activity of 4727 (U/ml), protein content of 38.0 (mg), specific activity of 124.40 (U/mg), purification fold of 7.09 and recovery of 236.3%. (Table 1 and Figure 1 and 2)

Recovery (%) = MCE units as % from total MCA in crude extract.

Purification step	Protein (mg)	Milk clotting activity (U/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	114	2000	17.54	1.00	100
Ammonium sulfate	900	400	0.444	0.025	20
Dialysate extract	2100	3057.32	1.46	0.083	152.86
DEAE –Sephadex (major peak)	37.9	4444.44	117.3	6.66	222.22
Sephadex G-100 (major peak)	38	4727.27	124.40	7.0	236.3

Table 1: A summary of the purification steps of MCE, starting with crude extract to DEAE sephadex column and Sephadex G-100.

ISSN: 0975-8585

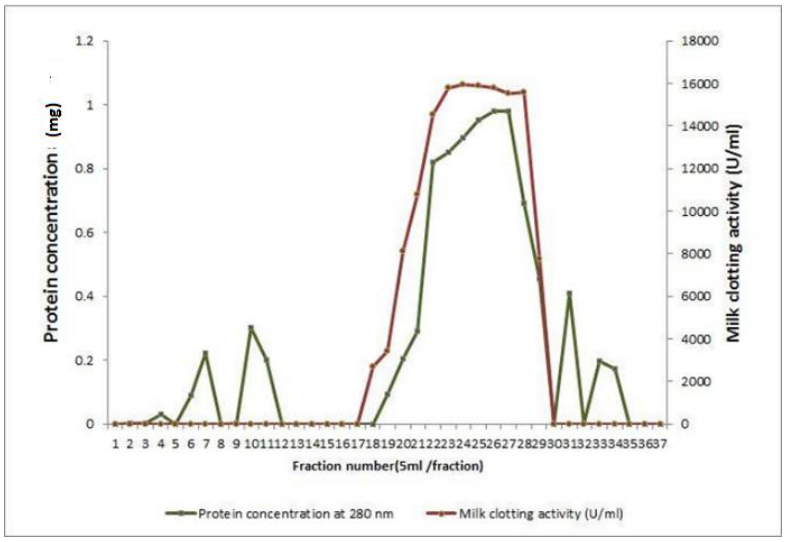

Figure 1: Typical elution profile of *P.albidus* MCE on DEAE-Cellulose column .

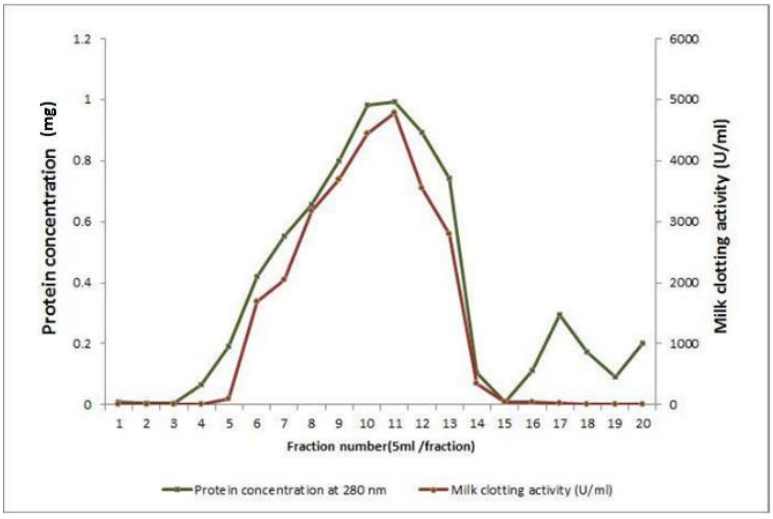

Figure 2: illustration of the elution profile of *P.albidus* MCE on Sephadex G-100 column .

In this connection, Li.Yang *et al*.(28) purified a MCE from *B.subtilis* .The enzyme was purified by culture filtration followed by ethanol 30%-60% precipitation and gel filtration , purified folds were (1, 3.22 ,9.5) respectively , while the highest specific activity (after gel filtration) was (8897.36 ± 360.21). Lebedeva and Proskuryakov. (29) Purified three extracellular milk clotting enzymes from a *Pleurotus ostreatus* isolate. The enzymes were isolated by Ammonium sulfate precipitation and gel chromatography. Two components had milk-clotting activity. Major proteolytic activity was detected in component II. It equals to 93% of the milk-clotting activity and 91% of total proteolytic activity of the solution applied to the column, and the degree of purification fold in component 1 and 2 were (0.81, 2.06) respectively.

Molecular mass determination

The SDS PAGE analysis confirm the full purification of MCE as it appears as one band of apparent molecular weight of approximately 34 KDa. Li et al. (2012) found that purified MCE from *Bacillus subtilis* (YB-3) gave a molecular mass of 42 KDa by SDS-PAGE and MAIDI-TOF-Ms analysis. It also have M.wt of 65. (30) Using BLUltra Prestained Protein Ladder. Lebedeva and Proskuryakor (2008) reported that MCE purified from *pleurotus ostereatus* (Fr.) kumm) was belong to serine protease class with isoelecteric points of 4.2, 6.7 and 8.8 (29).

ISSN: 0975-8585

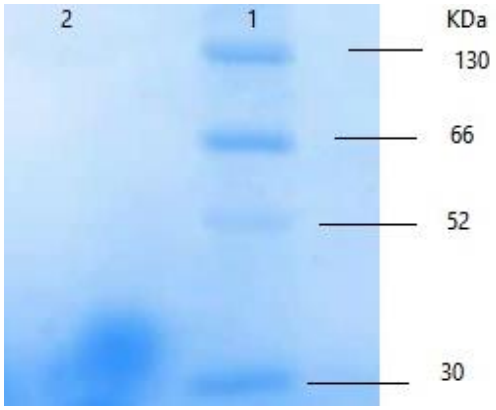

Figure 3: SDS-polyacrylamide gel electrophoresis of MCE from *Pleurotus albidus.* Lane 1: Protein marker (BLUltra Prestained Protein Ladder), Lane 2: Purified MCE from *Pleurotus albidus*

Effect of temperature on activity and stability of milk clotting enzymes

The temperature had great influence on the milk-clotting enzymes activity (Figure 4). The optimum temperature was achieved at 60°C. Similarly this temperature was the optimum for milk-clotting enzymes from other fungi such as *Rhizopus oryzae* (31), *Penicillium oxalicum* (32) and *Rhizopus microsporus* var. *rhizopodiformis* (33). While the temperature 37°C was the optimum for the milk-coltting enzyme from *Alternaria alternata* (34). The temperature 40°C was the optimum for MCE from *Aspergillus flavo furcatis* strains (35) and *Mucor mucedo* (36). , However 55°C was the optimum for the MCE from *Rhizomucor miehei* (37).

According to Dybowska and Fujio (1996) (38) at high degree of temperature, the process of coagulation is decreased. The increase in milk temperature affects the protein aggregation; increase the velocity of gel formation (39) and cause shortening of the protein matrix due the raise of hydrophobic interactions (40).

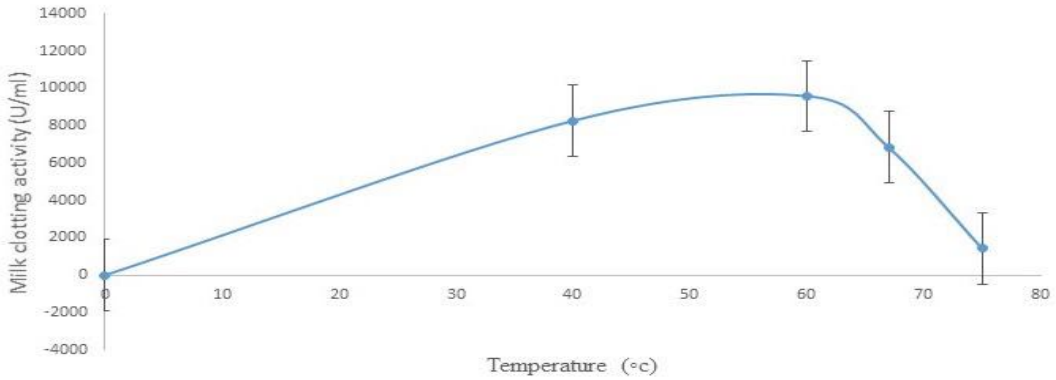

Figure 4: Effect of temperature on the purified milk-clotting enzyme activity from *P. albidus.*

The thermal stability of the purified milk clotting vary according the protein origin (41). Figure (5) indicated that the maximum thermal stability of the enzyme was at 40°C. As the temperature increased the thermal stability was decreased. The highest activity was recorded after one hour of incubation then the activity attenuated as the incubation period was increased to reach minimum after 4 hours incubation. At 40 °C the decrease in activity was observed after 4 hours incubation. *Termitomyces clypeatus* was stable between 35 and 50°C, retaining more than 80% of the activity, with a fast decrease according to the increase of temperature (21). The enzymes from *Aspergillus flavo furcatis* presented thermal stability higher than 70% between 40 and 60°C, however at the highest temperature a decrease of 28% in activity was observed (5).

ISSN: 0975-8585

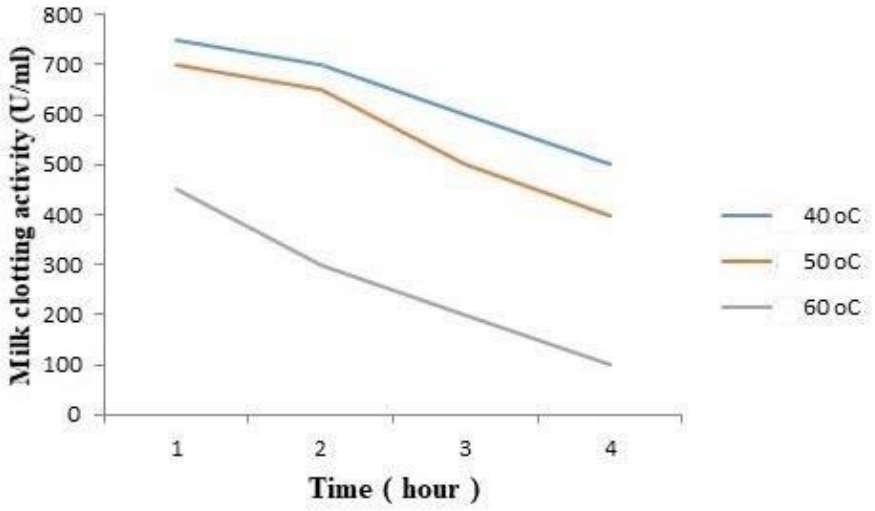

Figure 5: Thermal stability of the purified milk-clotting enzyme activity in *P.albidus* .

Effect of pH on activity of milk clotting enzymes

Studying on the optimum conditions of pH, temperature and stability can be used as indicative in enzymes industrial application (19).The effect of pH on the activity of *P. albidus* milk-clotting enzymes is shown in figure (6). The raising of pH value promoted the loss of milk-clotting activity. The enzymes exhibited highest activity at pH (6) (1371.4 U/ml) while at pH 8.0 the activity was highly reduced to 266.67 (U/ml). According to Vasconcelos *et al.* (42) the pH can influence the yield of milk-clotting enzymes. at pH ranges from 5.7 to 6.2, *Pediococcus acidilactici* showed high milk-clotting activity with optimum at pH 6.0, however, it was observed that, at pH 7.0 and 8.0 the activity decreased to 54 and21%, respectively, than the optimum (43). Vishwanatha *et al.* (2010) (44) reported high milk-clotting activity value at pH 6.3 from the enzymes produced by *Aspergillus oryzae* followed by reduction of the activity in conditions out of the optimum pH. The loss of enzymatic activity may be due to conformation changes in the protein structure caused by charges repulsion. The disruption enzymes cannot be associated with the substrate correctly.

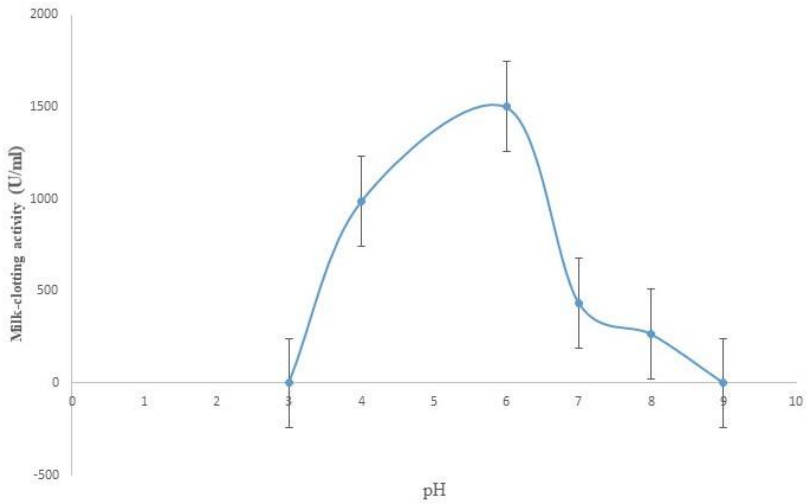

Figure 6: Effect of pH on the purified milk clotting enzyme activity in *P.albidus*.

pH stability of milk-clotting enzymes

Figure (7) shows the stability of milk-clotting enzymes from *P. albidus* when incubated at different pH values before assay. The enzymes showed stability at all studied pHs, however, a reduced stability was observed with the increase of pH values. The highest milk-clotting enzyme stability were observed at pH 5.8 and 6.0. Loss of activity was observed at pH 8.0. Merheb-Dini *et al.* (2010) (45) reported similar results using milk-clotting

enzymes from *T. indicae-seudaticae*. Where, the highest milk clotting stability were determined at pH 6.3.Yegin *et al.* (2012) (36) reported enzymatic stability of *Mucor mucedo* in the pH range from 5.0 to 5.5 with loss of milk-clotting activity at pH higher than 6.0.

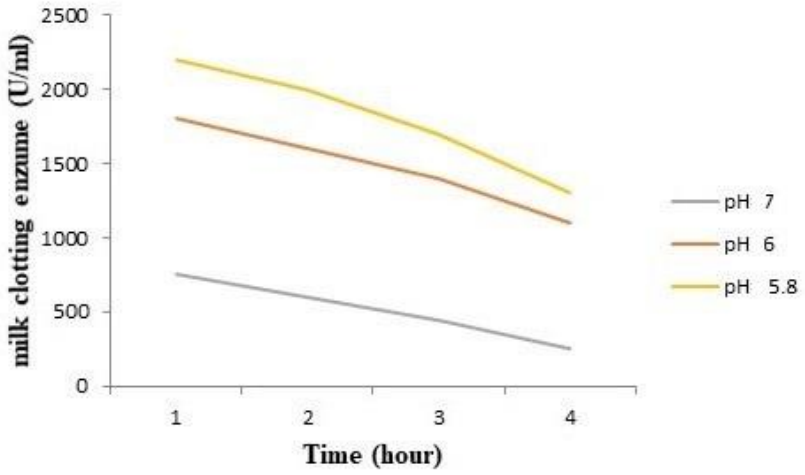

Figure 7: pH stability of purified milk clotting enzyme activity from *P.albidus*.

Effect of enzyme concentration on milk clotting activity

A positive linear correlation was observed between clotting activity and enzyme concentration up till 88.2 mg/ml. The enzyme activity was almost constant above this concentration (figure 8). Furthermore, the milk clotting time decreased with increasing enzyme concentration. That was similar to the data where MCA increased with increasing enzyme concentration in enzymes extracted from the plants *Solanum dubium* (46), *Balanites aegyptiaca* (47) ,*Cynara scolymus* flowers (48). A new clotting enzyme from *Bacillus* sp, which was used in cream cheese development, had an MCA dependent on enzyme concentration and achieved highest activity at concentration 30 mg/ml.(49)

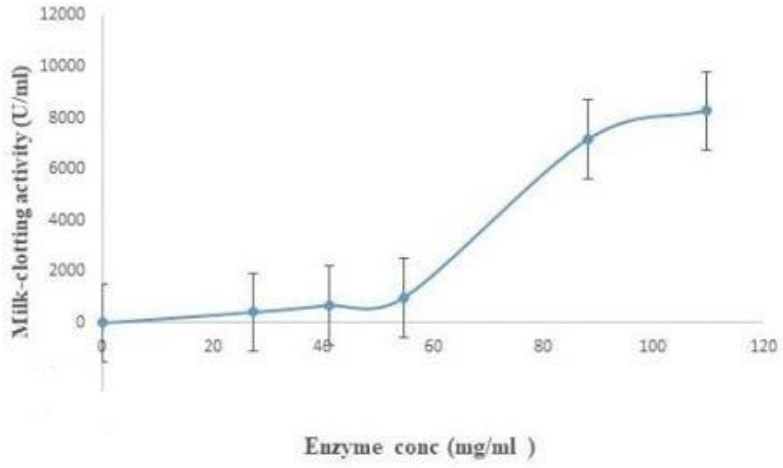

Figure 8: effect of different enzyme concentrations on the purified milk clotting enzyme activity in *P.albidus*.

Effect of Substrate Concentration (Cow Milk Casein)

The effect of the concentration of cow milk casein on the activity of the purified enzyme is shown in Figure (9). The milk-clotting activity reached a maximum when the cow milk casein concentration was increased to 0.14 g/mL. Substrate inhibition achieved on higher substrate concentration. The Line Weaver-Burk plot is shown in figure (10) the km value was determined to be 0.2 g/ml and Vmax 5000 U/ml. The Km is the concentration of substrate required to produce 50% of the maximum velocity value [50]. Each enzyme has a characteristic Km for a given substrate. The Km value provides information about the affinity of the enzyme and

substrate. A high Km indicates a low affinity. The Km value obtained in this study revealed that this milk clotting enzyme has a high affinity toward cow milk casein as substrate. The Km of 1.02 mM and a Vmax of 2.2 µmol/min of milk clotting enzyme were produced by *Aspergillus niger* (51) .The Km value is about 0.0796 and Vmax is around 0.4 (O.D. 660) of milk clotting enzyme produced from *Basidiomycetes* (52). The Km and the Vmax were found as 0.059 mg/ml and 10.3 mmol/ml/sec, respectively, of milk clotting enzyme produced from *Aspergillus candidus* (53). The Km was found to be 5 mg/ml of milk clotting enzyme produced by *Bacillus sphaericus* (54).

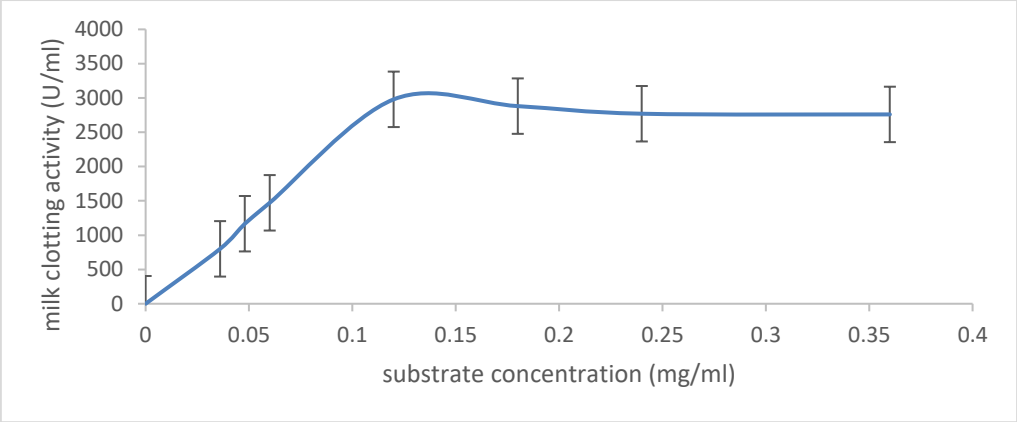

Figure 9: effect of different substrate concentrations on the purified milk clotting enzymes activity from *P.albidus*.

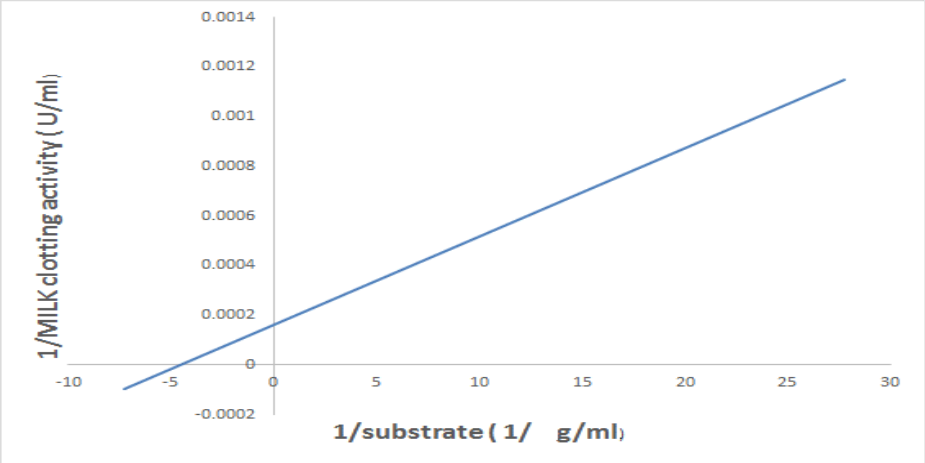

Figure 10: Line Weaver-Burk plot indicates the Km and the Vmax values.

Substrate specificity of milk clotting enzyme

In the study of substrate specificity, Figure (11) shows that the highest enzymatic activity of *P. albidus* was determined when cow milk casein was used as substrate, its activity was 800 (U/ml), then azo-casein caused, as its activity was 342.86 (U/ml), while the activity on bovine serum albumin was 72.07 (U/ml). No enzymatic activity was achieved when gelatin was used as protein substrate.

MCE purified from *B. subtilis* has high substrate specificity toward β-casein followed by yak milk casein, then κ-casein, followed by cow milk casein, while α-casein caused low enzymatic activity when used as substrate (55). *E. faecalis* MCE show enzymatic activity for various substrates. The order of substrate specificity of this enzyme decreasingly was: κ-casein, β-casein, α-casein, casein (Hammarsten), Gelatin (from bovine bone), Hemoglobin (from bovine), Egg albumin, and Soy bean flower. MCE however, were not show any enzymatic activity on elastin (10).

ISSN: 0975-8585

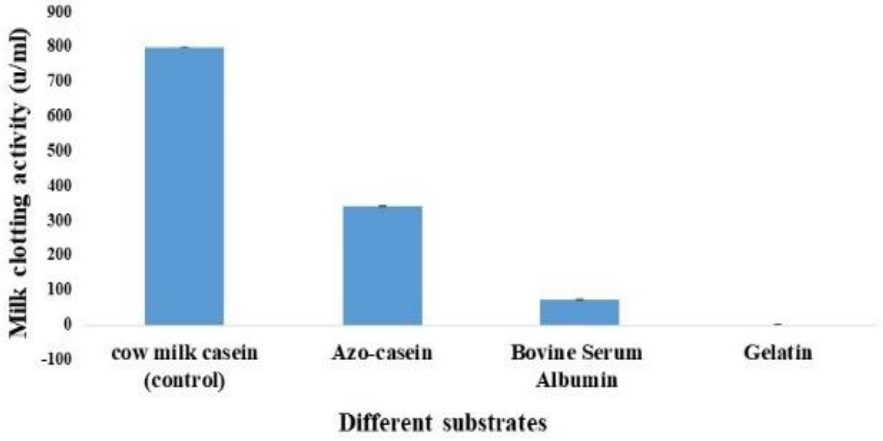

Figure 11: Effect of the purified milk clotting enzyme on different substrate (cow milk casein, azo-casein, bovine serum albumin, and gelatin).

Effect of metallic ions on milk-clotting activity

The effect of metallic ions on the milk clotting enzymes activity are shown in Figure (12). The ion Ca^{2+} raised the milk-clotting activity in 113.2%. Whereas ions Fe^{2+}, K^{+}, Zn^{+2}, Cu^{+2}, Mg^{+2}, Na^{+} caused a reduction of relative activity of 63.16 , 82.42 , 86.33, 84.5 , 94.34 and 79.68 %, respectively, of control. Martim *et al.* (2017) (58) showed that Zn^{+2} ion raised the milk-clotting activity in *Pleurotus albidus* 78%. The ions Mn^{+2}, Fe^{+2} and Cu^{+2} caused a reduction of 97.60, 78.75 and 35%, respectively. Mg^{+2}, $Na^{+}$and K^{+} induced a complete inhibition of the milk-clotting enzymes. Sun *et al.* (2014) (33) showed that Zn^{+2} ions raised the milk-clotting enzyme activity in *Rhizopus Microsporus* var. *Rhizopodiformis* . Ahmed and Helmy (2012) (40) showed that there was a raise of activity in the presence of Zn^{+2} ions (67.7%) in the study on *Bacillus licheniformis* 5A5 . Hashem (2000) (32) showed that Ca^{+2} and Mg^{+2} ions stimulated milk-clotting enzymes from *Pencillium. Oxalicum* . Alecrim *et al.* (2015) (35) showed that the milk-clotting activities from *Aspergillus flavo* furcates were inhibited to 42.88%, 47.36%, 49.36%, 52.08% and 52.56% by Zn^{+2}, Co^{+2}, $Ca^{+2}$and Na^{+} respectively . He *et al.* (2010) (56) showed that Na^{+} and K^{+} had a slight inhibitory effect, whereas Cu^{+2} and Zn^{+2} inhibited the milk-clotting activity significantly. In contrast, $Mn2^{+}$ had a significant stimulatory effect on milk clotting activity from *Bacillus amyloliquefaciens* . So SATO *et al.* (2003) (57) showed that the enzyme from *Enterococcus faecalis* TUA2495Lwas inhibited by the heavy metal ions Fe^{+2}, Cd^{+2}, Ni^{+2}, Cu^{+2} and Al^{+2}. Reactivation occurred to the enzyme activity with Ca^{+2}, Mn^{+2} or Zn^{+2}.

Effect of some inhibitors on MCE activity

The milk-clotting activity retained 100 and 95.24 % respectively, of activity in presence of Pepstatin A and EDTA figure (12). The serine protease inhibitor (PMSF) caused the highest inhibition of milk-clotting activity (relative activity 12%). So it suggested that milk-clotting enzymes from *P. albidus,* in this study, belong to serine protease family. He *et al.* (2010) (56) stated that Protease inhibitors were used to identify the group at the active site of the enzyme. Inhibition studies showed the sensitivity of the purified enzyme to a serine protease inhibitor (PMSF), a cysteine protease inhibitor (iodoacetamide), a metalloprotease inhibitor (EDTA) and an aspartic protease inhibitor (pepstatin A). Machado *et al* (2017) (58) showed that the proteases from *P. ostreatoroseus* were inhibited at 95, 94 and 87% by iodine acetic acid, PMSF and EDTA, respectively. These results suggest that the proteases be classified as cysteine, metalo and serine proteases. Zhang *et al*. (2010) (59) observed inhibition in the proteases activity of *Hypsizigus marmoreus*, using PMSF.

Milk-clotting proteases from *Pleurotus albidus* maintained stability with EDTA (33).They retained 93.7% of milk-clotting activity. Pepstatin A and PMSF caused inhibition of 35 and 49.5%, respectively. Iodoacetic acid caused the highest inhibition of milk-clotting proteases (37.7%). These results demonstrated that SH groups probably can be involved in the catalytic mechanism and that milk-clotting enzymes from *P. albidus* belong to cysteine protease family. Milk clotting enzymes retained approximately 66.48%, 76.96% and 82.80% in EDTA,

PMSF and iodoacetic acid, respectively (35). The strong inhibition, 98.05% in presence of EDTA, showed that the enzyme from *Bacillus amyloliquefaciens* belongs to the metallo-protease group

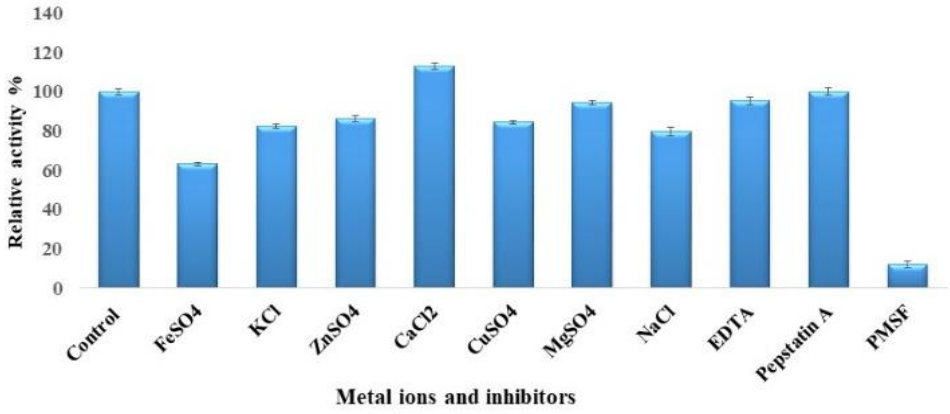

Figure 12: Effect of metal ions and some inhibitors on the purified milk-clotting enzyme activity of *P. albidus*.

Effect of $CaCl_2$ concentration

The milk-clotting activity, in this study, was highest at 0.09M $CaCl_2$ then decreased with increasing its concentrations (figure 13). Anema *et al.* (2007) (60) stated that calcium had a positive effect on the activity of the milk-clotting Enzyme. Calcium has been described as important in milk clot formation, when its concentration is high enough. The milk-clotting activity from *Bacillus amyloliquefaciens* D4 was highest at 25 mM $CaCl_2$. In the range 0-20 mM $CaCl_2$, the coagulation rate increased with increasing concentration of Ca^{+2}. Milk-clotting activity decreased at concentrations of $CaCl_2$ higher than 25 mM (56). Maximum activity of the enzyme from *Enterococcus faecalis* TUA2495L was obtained when 50 mM $CaCl_2$ was added to the skim milk .(57)

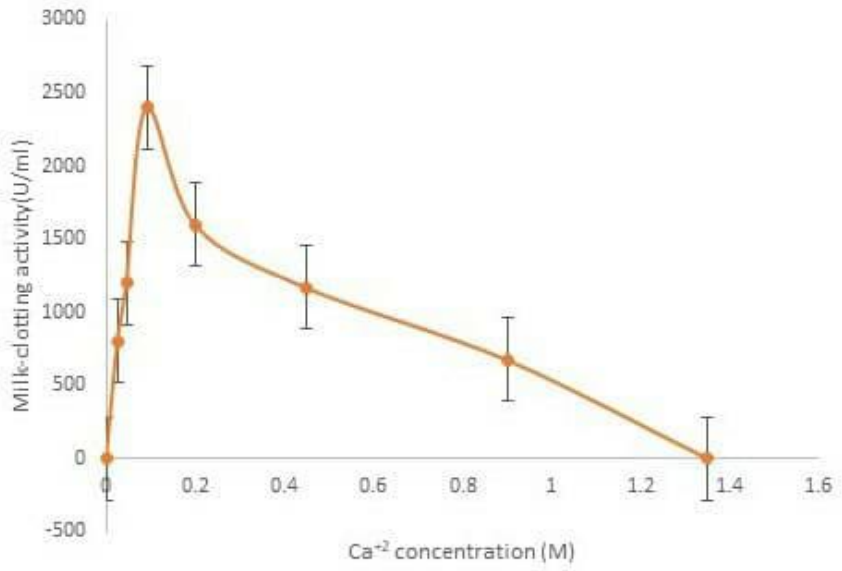

Figure 13: effect of $CaCl_2$ concentrations in milk-clotting activity of purified enzyme from *P. albidus*.

ACKNOWLEDGMENT

We would like to express our deep appreciation to biotechnology and bio molecular chemistry program. Grateful thanks are also intended to stuff members in Botany and Microbiology Departments-Faculty of science, Cairo University.

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