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Purification and Characterization of Milk Clotting Enzyme Produced by Rhizomucor Rmiehei

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ABSTRACT

Milk clotting enzyme (M CE) produced by: Rhizomucor miehei was purified and characterized. The enzyme was purified 220.29-fold with specific activity about 14444.2 U/mg protein by ultrafiltration, ammonium sulfate fractionation, Sephacryl S-300 chromatography. The maximum enzyme activity was at 65°C.

The milk clotting activity was decreased steadily as pH is increased and indicated maximum activity at pH 5.3.Ferthermore we did some computational methods for understanding the changes in energy.

Keywords: RhizoMucor miehei; Milk clotting enzyme (MCE); Purification, Langvin, Molecuar dynamics

INTRODUCTION

The growing demand of the cheese industry and the increasing scarcities of calf rennet have stimulated searches for clotting enzymes from alternate sources. Various animal, vegetable and microbial proteases have been suggested as milk coagulants (1). Microbial enzymes are especially favored because they can be mass produced and offer a variety of properties permitting selection of those most suitable in cheese production. Many species of filamentous fungi produce rennin type enzymes (2, 3, 4, and 5). Fermentation liquors from some are being offered on the market, under various trade names for making cheese.

The characteristic flavor of Italian-type cheeses has been attributed primarily to short chain fatty acids (6, 7, 8). These fatty acids are produced by lipolytic enzymes contained in "rennet paste", which is a crude extract of the abomasum of certain suckling mammals and has been used in the manufacture of these types of cheeses. Isolation and identifi- cation of the lipolytic activity of rennet paste has allowed use in cheese making of more highly purified pregastric esterases obtained from calves, kids, and lambs (9, 10, 11). The majority of cheeses produced around the world are manufactured using rennet, a milk coagulant traditionally

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extracted from the fourth stomach of milk-fed calves, lambs, and kids.

Commercial calf rennet consists mainly of two proeolytic enzymes chymosin (EC 3.4.23.4) and pepsin. The relative proportion of the two enzymes varies with the age of animal. The major, milk-clotting component of standard rennet is chymosin (88 to 94%), although mature animal rennet may contain up to 90 to 94% of pepsin and only 6 to 10% of chymosin. Rennet is used in cheese manufacturing primarily as a milk coagulant. Other enzymes present in rennet also play an important role in cheese production, especially in cheese ripening and may be a cause of the development of bitterness during storage.

Chymosin (EC3.4.23.4) is a milk clotting enzyme (MCE) obtained from the fourth stomach of the unweaned calf (12) .

Chymosin is an acid protease with Asp 32 and Asp 215 acting as the catalytic residues. Chymosin reacts specifically with κ-casein, cleaving the protein between the amino acids phenylalanine(105) and methionine (106), producing two fragments. The soluble fragment (residues 106-169), which becomes part of the whey, is known as glyco macro peptide and contains the glycosylation sites for κ-casein. The other component (residues 1-105) is insoluble, and in the presence of calcium ions causes the coagulation of the casein micelles to form a curd (13, 14, 15). Problems associated with animal slaughtering have necessitated finding other alternatives to calf chymosin (16,17).

In this regard, various alternatives are used for chymosin production; these sources are animals, plants and microorganisms.

Rhizomucor miehei aspartic protease is the most commonly used milk-clotting enzyme, due to its low cost and favorable performance (18, 19, 20 and21).

This enzyme has a high ratio of MCA/PA (milkclotting activity/proteolytic activity) (16, 19, 21) an important requirement to substitute calf rennet.

Therefore, in the present study an attempt was made to purify and characterize the milk clotting enzyme produced by Rhizomucor miehei PTCC 5145.

MATERIAL AND METHODS

2.1 Microorganism And Enzyme Production:

Mucor miehei PTCC 5145 was used in this study. The spore suspension (40,000,000 spores/mL, determinated by Neubauer Chamber) was transferred to 500 ml erlenmeyer flasks containing 100ml sterilized (121°C/20 min) growth medium. The composition of growth medium was Potato Dextrose Broth (PDB) pH 5. The flasks were incubated in orbital shaker at 40 °C and 120 rpm (1day). 7%(v/v) of micelliumes was also carried out in 500 ml erlenmeyer flasks containing 100ml sterilized (121°C/20 min) growth medium. The composition of growth medium was PDB (pH6). The flasks were incubated in orbital shaker at 37 °C and 180 rp (5days).

The samples were taken each 6 days of cultivation and separated from the mycelium through centrifugation (4,000 g) the filtrate was used for enzyme assay.

2.2 Purification of M CE:

in the first step pH of Culture supernatant was raised to 6.5 with 0.5 M disodium hydrogen phosphate,then was concentrated by ultrafiltration using a 10 kDa membrane.(Millipore Corporation, Bedford, Massachusetts, USA

As a second step, ultrafiltrate was fractioned by ammonium sulfate at 30, 50 and 80% (22, 23).

After standing in the ammonium sulfate solution for 2 h, the precipitate was collected by centrifugation at 4000× g for 10 min and dissolved in 50 mM phosphate buffer pH 6.

The active fraction with high milk clotting activity (MCA) was loaded on

to a Sephacryl S 300 column (0.8cm x 10cm) pre-washed with 0.05 M phosphate buffer at pH 6. . The enzyme was eluted with the same buffer at room temperature at a flow rate of 0.1ml/min (1.4 ml fraction) (24,25). The active enzyme fractions were stored at 4°C for further studies.

2.3 Enzyme Assay:

The enzyme source (0.1 ml) was added to 5 ml of substrate solution (10% skim milk powder in 0. 1M CaCl 2). The time necessary for the formation of curd fragment was measured. Milk

clotting activity is expressed in term of Soxhlet unit.

Soxhlet units were calculated using the following equation:

Where M is the volume of substrate (ml), E is the amount of enzyme (ml), t is the clotting time (sec) and T is the reaction temperature (°C) (25).

2.4 Protein Estimation:

Quantitative estimation of protein was done by the method of Lowry et al. (26).The protein content of individual fractions obtained after chromatography was monitored by measuring the extinction at 280 nm.

2.5 Biochemical Properties of Purified Enzyme:

2.5.1 pH Profiles:

Effect of pH values on the activities of purified enzyme carried out at pH range 5.3 -7 (27).

2.5.2 Temperature Profiles:

Activity of purified enzyme was determined at indicated temperatures (15-70°C) (27).

2.6 Theoretical study:

In this part we do some computational methods like molecular dynamic and langvin. All calculations have been performed using the Hyper 6 program. The structure of our molecule has been shown in figure 1.

RSULTS AND DISCUSSION

Culture supernatant produced by Mucor miehei PTCC 5145 was concentrated by ultrafiltration. This resulted in a 1.09 -fold higher specific activity in comparison to crude enzyme. After the ammonium sulphate precipitation (final concentration of 80% (w/v)) and subsequent centrifugation, the specific activity increased 3.35-fold with 60.6 % recovery of MCE activity as shown in Table 1. The enzyme was dissolved in buffer phosphate and loaded on a Sephacryl S-300 column chromatography (Figure2). The active fraction were exhibited 220.29-fold higher specific activity in comparison to the culture supernatant. A summary of the purification procedure is shown in Table 1.

The relationships between relative activity with temperature and pH have been studied separately. The temperatures have been varied between 23 to 70 degree of Celsius.

The most relative activity% is in 65°C, which is equal to 100.

The pH has been varied between 5.3 to 7.5.More pH increases the relative activity% decreases. Table2 and 3 and figures 3and 4 show this matter clearly.

Purified M CE from Mucor miehei exhibited the maximal rate of reaction at pH 5.3. On the other hand., (28) reported that MCE worked optimally close to pH 4 - 5.5 .

At lower pH,were not able to determine enzyme activity because in lower pH of 5.3 we would have acidic curd .

In computational part we calculate energy in 5 temperatures: 300,305,310,315 and 320 K. You see the changes in table 4 and figures 5 and 6.

Table1. Purification index of MCE from Rhizomucor miehei

Stage	Total Volume (ml)	Activity (u/ml)	Protein (mg/ml)	Specific activity (u/mg)	Purification fold	Recovery %
Crude Enzyme	250	1200	18.3	65.57	1	100
Ultra filtrate 10 kDa	50	6000	83.9	71.51	1.09	100
AmmoniumSulfae(80%)	7	26000	118.3	219.7	3.35	60.6
Gel chromatiography – continuously[1] (sephacryl S-300)	1.4	14545.4	1.007	14444.22	220.29	47.5

1- The amount of enzyme was added to gel chromatography procedure was 1 ml.

Table 2. The values of Temperature and Relative activity %.

Temperature(°C)	Relative activity %	Temperature(°C)	Relative activity %
23	5.8	50	50
30	12	55	60
35	20	61	66.66
40	24	65	100
45	40	70	46.15

Table 3. The values of pH and Relative activity %.

pH	Relative activity %	pH	Relative activity %
5.3	100	6.5	14.27
5.5	75	7.0	3.75
6.0	60	7.5	0.0

Table 4. The values of temperature and energy

T(K)	E(kcal) Molecular Dynamics	T(K)	E(kcal) Langvin
300	2621.975	300	2619.13
305	2847.251	305	2716.27
310	2691.501	310	2816.42
315	2814.523	315	2748.09
320	2619.132	320	2748.09

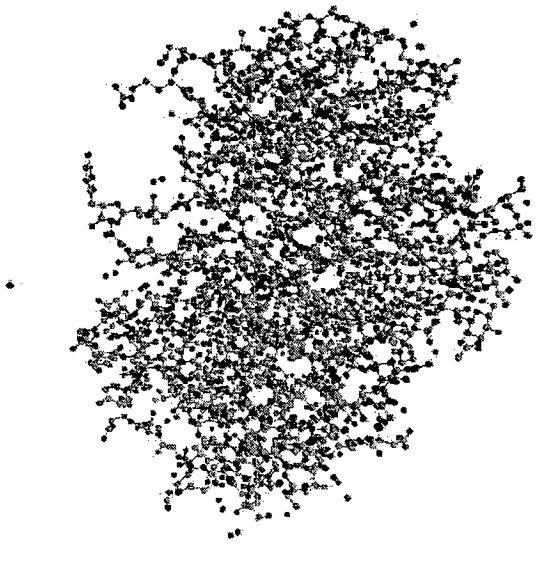

Figure 1. The structure of enzyme.

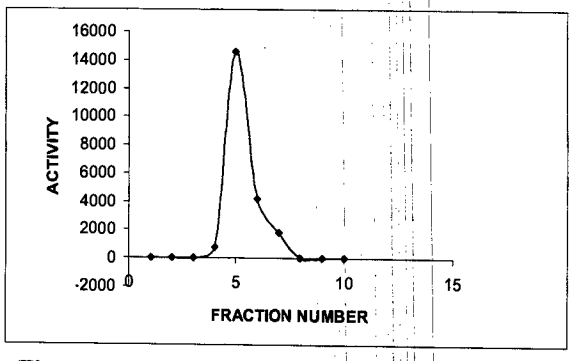

Figure 2. Elution diagram of M CE using sephacryl S-300 columns chromatography

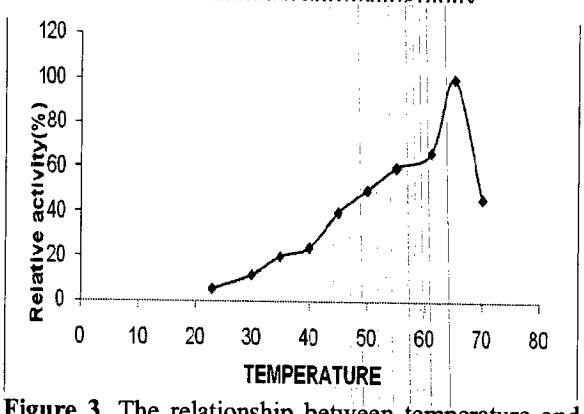

Figure 3. The relationship between temperature and relative activity %.

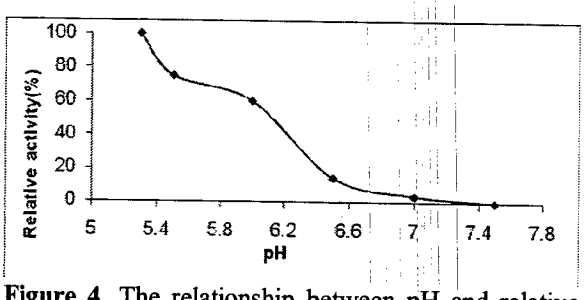

Figure 4. The relationship between pH and relative activity %

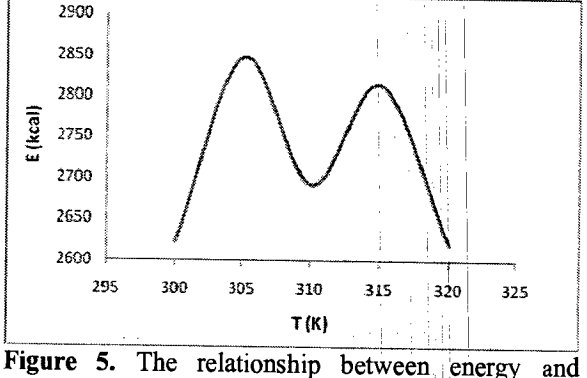

Figure 5. The relationship between energy and temperature (Molecular Dynamics).

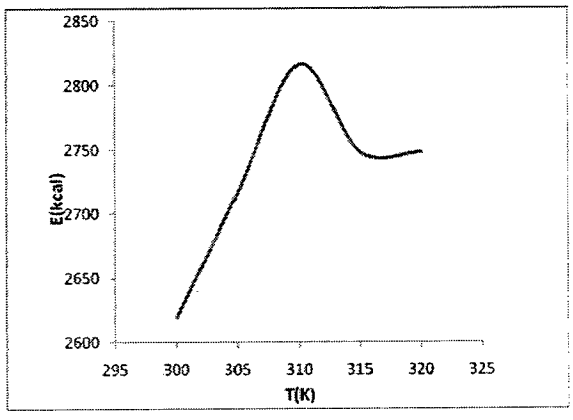

Figure 6. The relationship between energy and temperature (Langvin).

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