Purification and Characterization of Milk Clotting Enzyme Produced by Bacillus sphaericus

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Abstract: Milk clotting enzyme (MCE) produced by Egyptian Bacillus sphaericus NRC 24 was partially purified and characterized. MCE was obtained by fractional precipitation with acetone, followed by the chromatography of the most active fraction on DEAE-Sephadex A-25 and finally on Sephadex G-100 with 48 purification fold and specific activity about 648148 U/mg protein. The maximum enzyme activity was at a wide range of pHs (5.7-7.5) and 55ºC. The clotting activity of the purified enzyme was stimulated with increasing CaCl₂ concentration up to 0.25%. However, a gradual reduction of the activity was observed by increasing NaCl concentration between 5-20%. Zinc ions had a stimulating effect on the purified enzyme; while Ni and Hg ions had an inhibitory effect on the purified enzyme.

Key words: Bacillus sphaericus, Milk clotting enzyme, purification

INTRODUCTION

Milk coagulation is the basic step in cheese manufacturing. Milk clotting enzymes are the primary active agents in cheese making, which involves the enzyme-mediated cleavage of kaba-casein at the peptide bond Phe 105-Met 106 that renders the casein micelles unstable and eventually causes aggregation that yields a clot and a gel afterwards[1].

Chymosin (EC 3.4.23.4) is a milk clotting enzyme (MCE) obtained from the fourth stomach of the unweaned calf. Problems associated with animal slaughtering have necessitated finding other alternatives to calf chymosin. In this regard, various alternatives are used for chymosin production; these sources are animals, plants and microorganisms. These enzymes are purified and characterized by a number of authors[1-8].

During a study on rennin-like enzymes produced by Bacillus sphaericus, an Egyptian isolate, B. sphaericus NRC 24, showed a high milk clotting activity and was considered a novel and promising producer[9]. Therefore, in the present study an attempt was made to purify and characterize the milk clotting enzyme produced by B. sphaericus NRC 24.

MATERIALS AND METHODS

Microorganism And Enzyme Production: B. sphaericus NRC 24 was used in this study. Fodder yeast (4%) supplemented with NYSM salts[10] was inoculated with the test organism and incubated for three days at 30ºC on orbital shaker at 150 rpm. The cells were harvested by centrifugation and the supernatant (crude enzyme source) was used for purification experiments.

Purification of MCE: The crude enzyme solution was fractioned by acetone at 30, 50 and 70%. The active fraction with high milk clotting activity (MCA) was further purified by passing through a column (1.5 x 40cm) of DEAE-Sephadex A-25 pre-equilibrated with 0.02 M phosphate buffer at pH 6. Elution of protein was then carried out by batch wise addition of 40 ml portions of increasing molarities (0.0 –0.4 M) of NaCl in 0.02 M phosphate buffer at pH 6. Fractions of 5 ml each were collected at room temperature (25ºC) at a flow rate of about 20 ml/h and analyzed for MCA and protein content. The active fractions were dialedyzed against distilled water and concentrated via lypholization. The concentrated enzyme was loaded on to a Sephadex G-100 column (2cm x 46cm) pre-washed with 0.02M phosphate buffer at pH 6. Fractions of 5 ml each were collected at room temperature at a flow rate of about 30 ml/h. The active enzyme fractions were pooled and stored at 4ºC for further studies.

Enzyme Assay: The enzyme was assayed as described by Greenberg[11] with some modification. The enzyme source (0.2 ml) was added to 2 ml of substrate solution (12% skim milk powder in 0.01M CaCl₂). The time necessary for the formation of curd fragment was measured. Milk clotting activity is expressed in term of Soxhlet unit.

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Soxhlet units were calculated using the following equation:

\[
\text{Soxhlet units} = \frac{M \times 35 \times 2400}{E \times t \times T}
\]

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

**Protein Estimation:** Quantitative estimation of protein was done by the method of Lowry et al.\(^{[12]}\).

The protein content of individual fractions obtained after different steps of chromatography was monitored by measuring the extinction at 280 nm.

**Biochemical Properties of Purified Enzyme:**

**pH Profiles:** Effect of pH values on the activities of purified enzyme carried out at pH range 3.5-8 (by using appropriate buffer solutions).

**Temperature Profiles and Thermal Stability:** Activity of purified enzyme was determined at indicated temperatures (40-80°C).

The thermal stability of purified enzyme was ascertained by measuring the activity of the residual enzyme exposed at various temperatures (40-70°C) for 10, 15, 20 and 30 min.

**Effect of Substrate Concentrations (Skim Milk):** The effect of increasing the substrate concentrations from 1% up to 15% (w/v) was investigated.

**Effect of CaCl\(_2\) and NaCl Concentrations:** Effect of calcium chloride (0.002-0.4%) and sodium chloride (3-20%) concentrations on MCA of purified enzyme were tested.

**Effect of Metal Ions and Inhibitors:** The effect of some metal ions (Ni\(^{2+}\), Hg\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\) and Fe\(^{2+}\)) at 5mM concentration and some inhibitors (2-mercaptoethanol, iodoacetamide, soy bean trypsin inhibitor, soy bean Ti), phenylmethylsulphonyl fluoride (PMSF), EDTA and pepstatin on purified enzyme activity was tested. The concentrations of inhibitors are listed in Table 3. The purified enzyme was incubated at room temperature for 30 minutes with metal ions and inhibitors separately. Then, the residual MCA was measured by the standard assay procedure relative to control.

**RESULTS AND DISCUSSIONS**

**Purification of MCE:** MCE produced by *B. sphaericus* NRC 24 was fractioned at 50% acetone with 2-fold purification as shown in Table 1. Passage from DEAE-Sephadex A-25 column purified the enzyme to about 13-fold purification with specific activity of 66163 U/mg. Finally the concentrated active fractions passed through Sephadex G-100 and the enzyme purified about 48-fold (specific activity 648148 U/mg). Figures 1 and 2 showed the elution diagrams of MCE during fractionation of the crude enzyme using DEAE-Sephadex A-25 and Sephadex G-100 columns, respectively. Purified MCE was separated into two

![Fig. 1: Elution diagram of MCE by using DEAE Sephadex A-25 column chromatography](image)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume of fraction (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>MCA (U/ml)</th>
<th>Total MCA</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>30</td>
<td>4.2</td>
<td>1212</td>
<td>36364</td>
<td>289</td>
<td>1</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>15</td>
<td>2.1</td>
<td>2545</td>
<td>38182</td>
<td>1212</td>
<td>2</td>
</tr>
<tr>
<td>DEAE Sephadex A-25</td>
<td>5</td>
<td>0.23</td>
<td>15217</td>
<td>76087</td>
<td>66163</td>
<td>13</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>5</td>
<td>0.09</td>
<td>58333</td>
<td>291667</td>
<td>648148</td>
<td>48</td>
</tr>
</tbody>
</table>
Fig. 2: Elution diagram of MCE using sephadex G-100 columns chromatography.

Fig. 3: Effect of reaction pH on MCA of purified enzyme.

peaks of proteins with only one peak having MCA. These two peaks of proteins appeared as two bands having molecular mass of 25 and 47 kDa on SDS-PAGE.

Chymosin was purified 29-fold using anion exchange and gel filtration chromatography and its molecular weight was 35.6 kDa\textsuperscript{[13]}. Buffalo chymosin was purified by affinity chromatography on gramicidin S-agarose followed by ion exchange chromatography on g-aminopropyl-silochrom and gave one band with molecular weight 36 kDa\textsuperscript{[3]}. MCE from Rhizopus oryza purified 91 times using ammonium sulphate fractionation, DEAE-Cellulose and Sephadex G-100\textsuperscript{[5]}. The enzyme has a molecular mass of 34 kDa. Partially purified MCE from Nocardiosis sp. was obtained by fractional precipitation with ammonium sulphate then the chromatography in DEAE-cellulose\textsuperscript{[14]}.

Biochemical Properties of Purified Enzyme:

Optimum pH: Purified MCE exhibited the maximal rate of reaction at a broad milk pHs, (6-7.5) as shown in Figure 3. At lower milk pH, it formed bad and non-firmed clots therefore, their results were excluded.

It was reported that gels made at low pH (6.0 and 6.3) appeared to have a denser or more interconnected structure than gels made at pH 6.7 by using plant coagulant and chymosin\textsuperscript{[15]}. In contrast, the optimum pH was 7.5 for purified MCE from B. polymyxa B-17 and Nocardiosis sp\textsuperscript{[14, 16]}. On the other hand,\textsuperscript{[5, 17, 18]} reported that MCE worked optimally close to pH 4-5.5.

Optimum Temperature and Thermostability:

Purified enzyme acted optimally at 55°C (Figure 4). Thermal inactivation experiments indicated that the enzyme was quite stable at 40°C for more than 30 min. While it lost 35% and 70% of its activity after 10 and 20 min incubation at 60°C, respectively (Figure 5). After 10min, the purified enzyme completely lost its activity at 70°C.

The maximum MCA was recorded at a milk temperature of 65°C for B. subtilis and B. megaterium\textsuperscript{[19]}. The optimal temperature of MCE produced by B. thuringiensis kurstaki W.P G-013 was at 70°C followed by complete loss of its activity at higher incubation temperature\textsuperscript{[20]}. The maximum MCA of purified enzyme produced by Rhizopus oryza was at 60°C\textsuperscript{[5]}. The partially purified enzyme from Nocardiosis sp. showed the maximum activity at 55°C and was inactivated at 65°C after 30 min\textsuperscript{[14]}. 

Fig. 4: Effect of incubating temperature on MCA of purified enzyme.

Fig. 5: Thermal stability of purified enzyme.
Effect of Substrate Concentration: The enzyme showed an increment in its activity with increasing skim milk concentration up to 9% (Figure 6). Michaelis constant as determined by double reciprocal plot was found to be 5 mg/ml. The $k_{max}$ value is similar to that of extracellular protease of *Rhizopus oryzae*.[5]

Effect of Calcium Chloride Concentration: Calcium chloride accelerated MCA at all the tested concentrations (Figure 7). It is known that Ca$^{2+}$ combines with para casein to form firm clot during second phase of clotting process. Ca$^{2+}$ was found to be potent activator with 250% increase in MCA compared to control, in absence of metal ion.[5]

**Effect of Sodium Chloride Concentration:** Milk is sometimes salted with sodium chloride for protection against spoilage by various microorganisms. Sodium chloride is usually used during the process of Domiati cheese manufactured in Egypt. As shown in Figure 8, a gradual reduction in MCA was observed by increasing the NaCl concentration. MCA decreased 13%, 20% and 28% at 5%, 10% and 20% NaCl respectively.

The amount of salt required to reduce water activity to prevent microbial growth is 4-5%. The sensitivity of MCE from various sources to sodium chloride is not the same. Bovine pepsin appears less sensitive to sodium chloride than calf rennet, particularly at high salt concentrations.[18]

**Effect of Metal Ions and Inhibitors:** As shown in Table 2, Zn$^{2+}$ ion activated the purified enzyme by 42%, whereas Ni$^{2+}$ and Hg$^{2+}$ ions inhibited MCA by 22% and 42% respectively. However, Co$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Cu$^{2+}$ and Fe$^{2+}$ ions have no effect on the enzyme activity.

The enzyme retained 88-100% of its activity in the presence of 2-mercaptoethanol, iodoacetamide, soy bean Ti, ethylenediaminetetraacetic acid (EDTA) and pepstatin (Table 3). However, complete loss of MCA was observed by phenylmethylsulphonyl fluoride (PMSF) pointing to the presence of serine at the active site of tested MCE.

**Table 2: Effect of metal ions on MCA of purified enzyme.**

<table>
<thead>
<tr>
<th>Metal ions (5mM)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni$^{2+}$</td>
<td>78</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>58</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>100</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>142</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>102</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>102</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>102</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 3: Effect of inhibitors on MCA of purified enzyme.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-mercaptoethanol</td>
<td>10 mM</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>10 mM</td>
<td>88</td>
</tr>
<tr>
<td>Soy bean Ti</td>
<td>100 mg/ml</td>
<td>96</td>
</tr>
<tr>
<td>PMSF</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
<td>100</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>0.02 mM</td>
<td>94</td>
</tr>
</tbody>
</table>

Fig. 6: Effect of skim milk concentration on purified enzyme.

Fig. 7: Effect of CaCl$_2$ concentration on activity of purified enzyme.

Fig. 8: Effect of NaCl concentration on purified enzyme activity.
REFERENCES


