

ISOLATION, PURIFICATION, AND CHARACTERIZATION OF HIGHLY ACTIVE PROTEASE FROM *Bacillus subtilis*-150

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UDC 577.152.3

Many research groups [1–4] are currently focusing on microbial proteases because they are used in the pharmaceutical, food, and cosmetic industries and in the production of protein hydrolysates [5]. Highly purified protease preparations are used in medicine to prepare diagnostic kits, therapeutic sera, and vaccines and in enzymodiagnosics and enzymotherapy [6]. We have previously selected the local bacterial strain *B. subtilis*-150, which produces active protease, and studied the conditions for active enzyme formation during cultivation of the strain on the optimum nutrient medium [7, 8].

We used fractional salting-out by 20–80% $(\text{NH}_4)_2\text{SO}_4$ in order to isolate *B. subtilis*-150 protease enzyme preparation from culture liquid (CL). This produced different protein fractions with protease activity. Proteins containing up to 65% (2856 U) of the total (4365 U) protease activity were precipitated simultaneously from CL by 60% saturated $(\text{NH}_4)_2\text{SO}_4$. The specific activity of the resulting preparation was 24.60 U/mg whereas the activity was 6.55, 12.34, and 5.60 U/mg for 20, 40, and 80% saturated $(\text{NH}_4)_2\text{SO}_4$, respectively.

The results showed that proteins should be precipitated by 60% saturated $(\text{NH}_4)_2\text{SO}_4$ in order to obtain active *B. subtilis*-150 protease. This fraction was used for subsequent purification.

Ion-exchange chromatography (IEC) over a column (10 × 250 mm) packed with CM Sephadex C-50F sorbent (Pharmacia, Sweden) was used to purify the protease. Elution of proteins by phosphate buffer (0.05 M) gave five protein fractions. The first protein fraction had amylase activity. Protease was eluted in the fifth fraction using a gradient (0.5 M KCl).

The purified protease had specific activity 48 U/mg and 6.1% yield of protein (Table 1).

The homogeneity and molecular weight of the purified protease, 39–40 kDa, was determined by electrophoresis in PAAG (12%) in the presence of SDS.

The physicochemical and catalytic properties such as temperature and pH optima and the stability and effect of various metal ions on the enzyme activity were studied in order to characterize the purified protease preparation.

The effect of pH on the activity and stability of the enzyme was studied in the range 3.5–11.5. The results indicated that the enzyme was most active at pH 8.5 whereas 40–45% of the activity was retained at pH 10.5–11.5.

The effect of temperature on the activity of *B. subtilis*-150 protease was studied in the range 10–80°C. The enzyme was highly active at 25–30°C for 30 min and retained 50–60% of the activity at 40°C and 25–30% at 50°C. At 70–80°C, the enzyme retained only 3–6% of the maximum activity. The maximum stability was observed at low (10–20°C) temperatures, where the protease retained 100% of its activity.

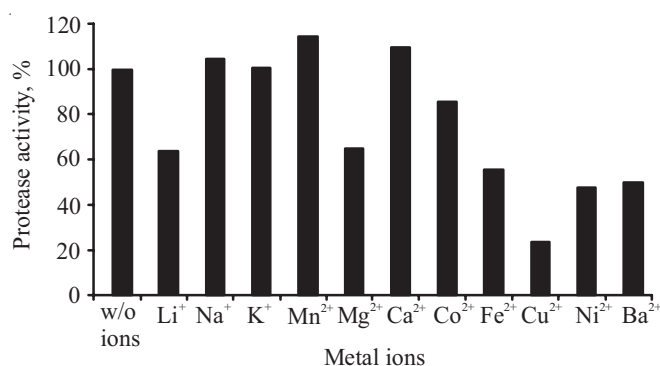
Several researchers have noted that proteolytic enzymes are metalloenzymes, the active centers of which contains Ca^{2+} and Mn^{2+} ions [9]. The effect of various metal ions on the activity of *B. subtilis*-150 protease was studied in order to confirm this hypothesis. For this, we prepared solutions (1 mM) of the buffer salts and an enzyme solution (1 mg/mL). Figure 1 shows the effect of various metal ions on the enzyme activity. Mn^{2+} and Ca^{2+} ions increased the enzyme activity by 15 and 10%; Na^+ , 5%; K^+ , 1%. On the other hand, Co^{2+} , Mg^{2+} , Li^+ , Fe^{2+} , Ba^{2+} , Ni^{2+} , and Cu^{2+} inhibited the enzyme activity from 24 to 76%.

Thus, precipitation of protease by 60% $(\text{NH}_4)_2\text{SO}_4$ and purification of the enzyme by IEC over CM-Sephadex C-50F sorbent were used to obtain a purified enzyme preparation of *B. subtilis*-150 protease. The optimum temperature and pH and the effect of various metal ions on the activity and stability of the enzyme were found.

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TABLE 1. Preparation of Purified Protease from *Bacillus subtilis*-150

Purification stage	Protein, mg	Protease activity		
		spec., U/mg	total, U	yield, %
CL supernatant	500	5.7	2850	100
Precipitation by (NH ₄) ₂ SO ₄ (60%)	71	22	1796	63
IEC over SM-Sephadex C-50	3.6	48	173	6.1

Fig. 1. Effect of metal ions on activity of *B. subtilis*-150 protease activity.

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