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*Research Article*

# ROLE OF PROTEIN DENATURING AGENTS AND EDTA ON α-AMYLASE ACTIVITY FROM *BACILLUS SUBTILIS* KIBGE HAS

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**ABSTRACT**

Alpha amylase from *Bacillus subtilis* KIBGE HAS was partially purified by ultrafiltration and ammonium sulphate precipitation. After partial purification, the specific activity of protein increased to 2813 U/mg with 20.8 fold purification. The enzyme showed activity in the presence of chelating agent (EDTA) and protein denaturing agents like PMSF, Urea, dithiothreitol (DTT) and β-mercaptoethanol. The enzyme remained active in the presence of Ethylenediaminetetraacetate (EDTA) which was a chelating agent, although enzyme activity declined to 44% at 10 mM concentration. PMSF exerted stimulatory effect on α-amylase and its activity increased up to 3.8 fold at 10 mM concentration. Furthermore, β-mercaptoethanol stimulates catalytic activity up to 3.0 fold at 10 mM concentration. In addition, DTT at 1 mM concentration enhanced α-amylase activity. Similarly urea also caused stimulatory effect on α-amylase activity.

**Keywords:** α-amylase, protein denaturing agents, β-merceptoethanol, DTT.

## INTRODUCTION

In native proteins, all peptide bonds are in the trans-configuration. Denaturation is always accompanied by a loss of biological function. Partial or complete protein denaturation can be accomplished by a variety of agents. Organic solvents serve to break electrostatic linkage. Trichloroacetic acid (TCA) can rapidly denature and precipitate proteins. High concentration of urea-guanidine HCl may break hydrogen, especially hydrophobic interaction. On the other hand, physical agents which cause denaturation are heat irradiation and surface tension[1].

Dithiothreitol (DTT) and β-mercaptoethanol are most broadly used reducing agents[2]. The reductive cleavage of disulfide bonds involves the treatment of the protein with β-mercaptoethanol. Urea only unfolds protein without reacting with the protein and hence protein becomes destabilized by the addition of urea[3].

PMSF is a protease inhibitor that reacts with serine residues to inhibit trypsin, chymotrypsin, thrombin and papain. The purified enzyme from *B. licheniformis* and *Bifidobacterium adolescentis* was strongly inhibited by EDTA [4,5].

The purpose of present study is to investigate the effect of protein denaturing agents on α-amylase activity from *B. subtilis* KIBGE HAS for the first time in this study.

## MATERIALS AND METHODS

*B. subtilis* KIBGE HAS been grown in liquid medium containing (g l−1): starch, 20.0; bacto-peptone, 10.0; yeast extract, 4.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.2. The pH of the medium was adjusted to 7.0 before autoclaving. The 100.0 ml of inoculum was transferred into 900.0 ml of sterile starch broth medium and incubated for 35°C for 24 h. After 24 h incubation, culture broth was centrifuged (35,000×g for 10min) at 0°C to remove the cell and supernatant was stored at −20°C.

### Ultrafiltration

Crude enzyme solution was concentrated under nitrogen stream by using ultrafiltration with

100,000 MrCO (relative molecular weight cut off) membranes and filtrate was collected. This filtrate was concentrated again with 30,000 MrCO membranes and retenate was collected for further purification.

**Partial purification of α-amylase**

Solid Ammonium sulphate was added slowly to the concentrated enzyme to give a final concentration of 40 % saturation. The $(NH_4)_2 SO_4$ was added with constant stirring at 4°C and then kept for 18 hours at 4°C. The precipitated proteins were separated by centrifugation at 35,000×g for 15 minutes at 0°C and dissolved in minimal volume of 0.1 M phosphate buffer (pH 7.5). The enzyme solution was dialyzed (using Molecular porous membrane tubing-Spectrum Labs.) for 18 hours at 4°C. The dialyzed sample was used for further studies.

**Enzyme assay and Total protein**

The activity of α-amylase was assayed by incubating 0.1 ml enzyme with 1.0 ml soluble starch (1.0 w/v) prepared in 0.1 M phosphate buffer, pH 7.5. After incubation at 50°C for 5 minutes, the reaction was stopped and the reducing sugars released were assayed calorimetrically by the addition of 1.0 ml DNS reagent[6]. Total protein concentration of the enzyme samples were estimated using the method of Lowry *et al.* (1951)[7] with bovine serum albumin used as a standard.

**Effect of protein denaturing agents on α-amylase activity**

EDTA, DTT, β-mercaptoethanol, PMSF and urea, in a concentration of 5 mM and 10 mM were added to the enzyme solution and incubated at 30°C for 30 min. Relative enzyme activities were determined by taking control as 100%.

**RESULTS AND DISCUSSION**

**Partial purification**

A summary of purification steps for α-amylase is given in table 1. The partially purified α-amylase showed specific activity of 2813 U/mg of protein. The fold purification increase up to 20.8 fold.

**Effect of EDTA On α-Amylase Activity**

The α-amylase from *Bacillus subtilis* KIBGE HAS was active in the presence of EDTA and retained 96% of its activity at 1mM concentration. As the concentration of EDTA was increased up to 10 mM, the enzyme activity decreased to 44% (Figure 1). It has been reported that α-amylase (iso-enzyme a and b) from *Pseudomonas* sp. SPH4 was inhibited by chelating agent (EDTA) at 2 mM and 5 mM, which may be due to the fact that these iso-enzymes referred to metallo amylases in which the activity of enzyme is dependent on the some kinds of ions. Thus, addition of chelating agents to the reaction mixture results in the formation of complexes with the ions in the active site, which cause inhibition of enzyme activity[8]. Similarly, α-amylase produced by *Bacillus stearothermophilus* was inhibited by chelating agent (EDTA) at 10 mM concentration[9] (Nielson et al, 2001).

It has been also reported that saccharifying amylases from *Bacillus* sp. strain A-40-2, *Bacillus* sp. strain NRRL B-3881 and *Bacillus alkalothermophilus* A-3-8 are all stable in response to EDTA treatment. Whereas, liquefying enzymes from *Bacillus* strains require calcium ions for expression of enzyme activity[,10-13] (Horikoshi, 1971, Ozaki and Tanaka, 1990, Boyer & Ingle, 1972, Yamatoto, 1988).

**Effect of PMSF on α-Amylase Activity**

PMSF exerted stimulatory effect on amylase and its enzyme activity increased up to 3.8 fold at 10 mM concentration, reason is unknown (Fig 2). Therefore, it was concluded that no thiol or carboxylic residue are essential for its catalytic activity. The activity of α-amylase produced by *Bacillus* sp. did not affect in the presence of 10 mM of PMSF[14] (Lin et al, 1998). In addition, α-amylase from halophilic *Nesterenkonia* sp. strain F. was not inhibited by PMSF[15] (Shafiei et al, 2012).

**Effect of β-mercaptoethanol and DTT on α-amylase activity**

Both β-mercaptoethanol and DTT enhanced α-amylase activity up to 3.0 and 3.8 fold, respectively (Fig 4 & 5); because thiol compounds such as β-mercaptoethanol and DTT caused stimulatory effect on amylase activity indicating that cysteine residue (s) do not take part in catalysis. In addition, this activation is attributed to the reduction in aggregate size by destroying the intermolecular disulfide linkages and protection of thiol groups that stabilize the three dimensional structure of enzyme[16] (Khedher, et al, 2008).

**Effect of urea on α-amylase activity**

Urea slightly stimulated enzyme activity. Similar result has also been reported for α-amylase from *T. profundus* DT5432 [17]. It may be due to the fact that the concentration of urea that is needed to denature a protein depends on the type of protein. Lysozyme from hen egg white cannot in a practical

way be unfolded by urea at physiological pH and room temperature since very high urea concentrations are needed[18] .On the contrary, urea had an inhibitory effect on α-amylase activity from *Alicyclobacillus acidocaldarius* [19]. In addition, Urea is found to passively unfold proteins by decreasing the refolding rate of local parts of the protein.

**Table 1: Partial purification of α-amylase from *Bacillus subtilis* HAS**

| Purification steps | Enzyme Activity (Units) | Total Protein (mg) | Specific Activity (U/mg) | Fold Purification |
|---|---|---|---|---|
| **Crude Enzyme** | 684000 | 5063 | 135.0 | 1.0 |
| **Ultrafiltration** | 141000 | 308 | 457 | 3.4 |
| **Ammonium Sulphate Precipitation** | 47405 | 18.3 | 2590 | 19.1 |
| **Dialysis** | 41080 | 14.6 | 2813 | 20.8 |

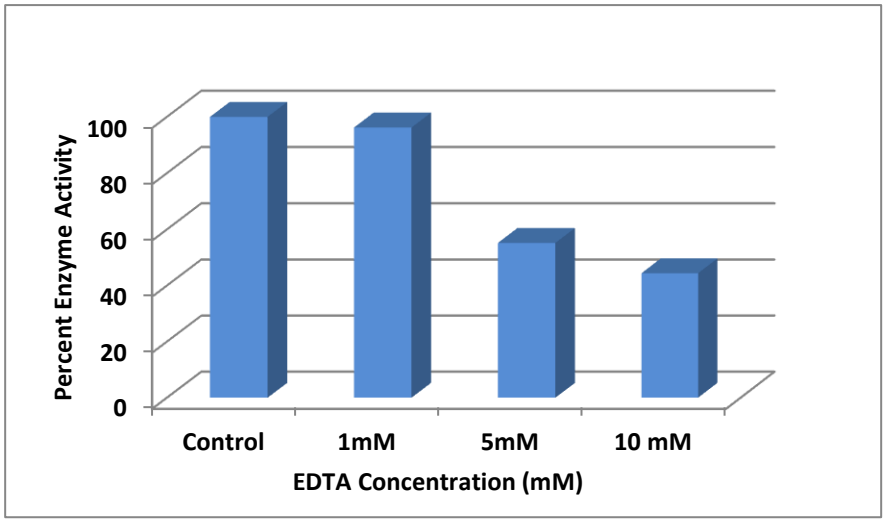


**Fig. 1: Effect of EDTA on α-amylase activity**

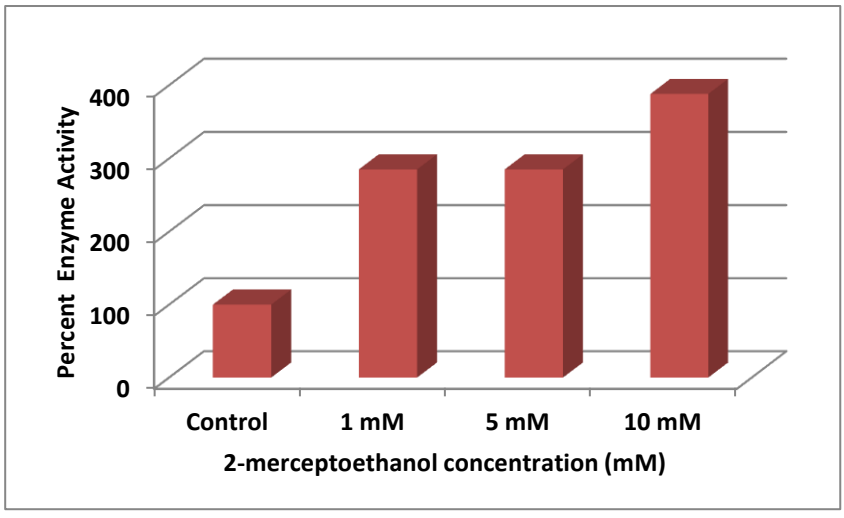


**Fig. 2: Effect of 2-merceptoethanol on α-amylase activity**

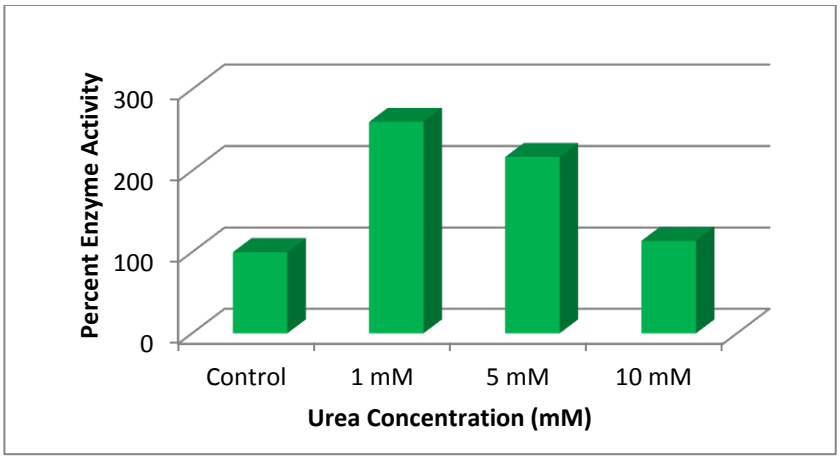


**Fig. 3: Effect of urea on α-amylase activity**

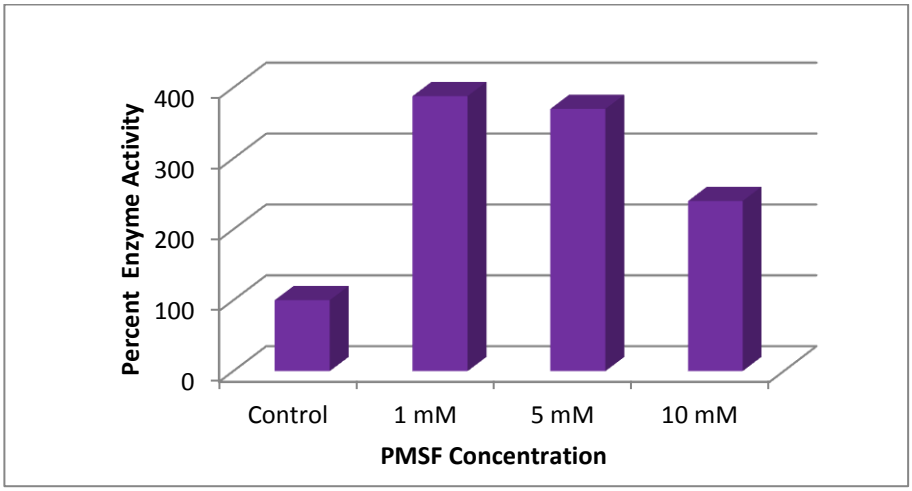


**Fig. 4: Effect of PMSF on α-amylase activity**

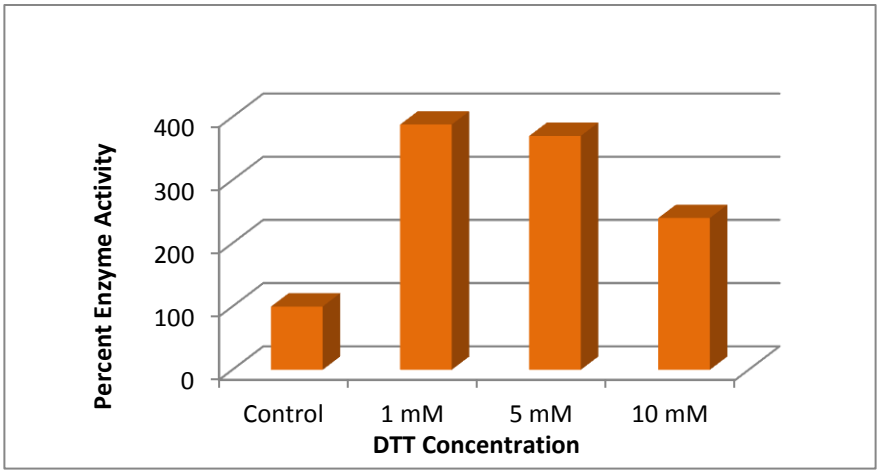


**Fig. 5: Effect of DTT on α-amylase activity**

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