

Research Article

Characteristic Analysis of Crude and Purified α -amylase from *Bacillus licheniformis* ATCC 6346 and comparison with Commercial enzyme

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Abstract: Thermostable α -amylases are generally used for industrial applications. The objective of this study is to compare the kinetic properties of crude and purified α -amylase from *Bacillus licheniformis* ATCC 6346 with commercial (Termamyl^R, NOVO industries from Denmark) α -amylase from *Bacillus licheniformis*. Commercial and crude α -amylases showed zero order kinetics for 10 min while purified α -amylase showed 8 min at pH 7.0 and 85°C. The activities of crude, purified and commercial α -amylases were measured at different temperatures ranging from 40 to 95°C and the optimum temperature for the activities of crude and purified enzymes was 85°C while that for the commercial enzyme was 90°C. The optimum pH was 7.0 for the crude, purified and commercial enzymes at 85°C. When the crude enzyme was pre-incubated at 85°C and at pH 7.0, it lost 40% of its initial activity at 10 min while the purified enzyme lost 75% of its initial activity at 10 min and the commercial enzyme did not lose activity at 10 min. Half-life of crude and purified α -amylases were 13.9 and 4.7min respectively while that for commercial enzyme was 823.97 min at pH 7.0 and 85°C.

Keywords: *Bacillus licheniformis*, Zero-order kinetics, α -amylase, Half-life

INTRODUCTION

α -Amylase is an important industrial bulk enzyme for the food processing industry. (EC 3.2.1.1, 1,4- α -Dglucanohydrolase, endoamylase) hydrolyses starch, glycogen and related polysaccharides by randomly cleaving internal α -1,4-glycosidic linkages to produce different sizes of oligosaccharides. Thermostable α -amylase has been produced by different types of microorganisms such as *Bacillus thermoleovorans* [1, 2], *Bacillus licheniformis* [3], *Bacillus* sp. [4], *Bacillus stearothermophilus* [5], *Norcardiopsis* sp. [6], *Bacillus amyloliquefaciens* [7], etc.

The purification of α -amylase from the fermented broth is essential for stability and characterization [8]. Thermodynamics and activation parameters provide a detailed mechanism for many chemical and biological reactions [9]. It is necessary to have enzyme kinetic information for any enzymatic process. Poor enzyme stability under standard conditions of pH, temperature and pH inhibition affects the end product yield [10]. To obtain effective enzyme activity the kinetic properties of the enzyme has to be understood and should be used in the reactions. The kinetic properties of α -amylases obtained from different sources differ from one another. *Bacillus licheniformis* CUMC 305 produced thermostable α -amylase, which was optimally active at 90°C and pH 9.0 and 91% of this activity retained at 100°C. In the presence of substrate (starch), the enzyme was fully stable after

4hours at 100°C [11]. The binding of Ca^{2+} ions has been shown to increase the α -amylase stability [12]. The objective of this study is to compare the kinetic properties and stability of crude and purified α -amylase produced by *Bacillus licheniformis* ATCC 6346 with commercial enzyme.

MATERIALS AND METHODS

Strain of α -amylase producer and crude enzyme production

Bacillus licheniformis ATCC 6346 from Heriot-Watt University U.K was used in this study. The nutrient agar medium contained (gL⁻¹) nutrient agar, 25.0 and soluble starch, 3.0 and the activation medium contained (gL⁻¹) Nutrient broth, 25.0 and soluble starch 3.0 at pH 7.0. The fermentation medium contained (gL⁻¹) soluble starch, 4.0; (NH₄)₂SO₄, 5.0; peptone, 6.0; FeCl₃, 0.01; MgCl₂ .6H₂O, 0.01; CaCl₂ .2H₂O, 0.01; KH₂PO₄, 4.0 and K₂HPO₄, 7.5 at pH 7.0. A loopful of *Bacillus licheniformis* ATCC 6346 from nutrient agar slants with 0.3 % soluble starch (grown at 37 °C for 24 h) was transferred to 10 mL activation medium which was incubated at 42 °C in a rotary shaker (100 rpm) for 12 h and used as inoculum. The fermentation medium was inoculated with 20 % (v/v) inoculum and the inoculated flasks were incubated for 48 h at 42 °C with shaking at 100 rpm. The culture filtrate was used as crude α -amylase.

Purified α -amylase

Purified α -amylase from *Bacillus licheniformis* ATCC 6346 was used [13].

Commercial α -amylase

Termamyl[®] (60L), activity 67.5 KNU.g⁻¹ was from NOVO industries, Denmark.

Kinetic studies on crude, purified and commercial α -amylases

Effect of time

Soluble starch was allowed to react with crude, purified and commercial α -amylases at 85°C and the amount of glucose produced was monitored. The time suitable for the incubation was optimized.

Effect of temperature

The effect of temperature on crude, purified and commercial α -amylases activities were determined by incubating the appropriately diluted enzymes for optimized time with soluble starch at different temperatures, varied from 40 to 95°C. Then activities of the different enzyme samples were measured [14] and relative activities were calculated.

Effect of pH

The effect of pH on activities of crude, purified and commercial α -amylases were measured by

preparing soluble starch in buffers of different pH values ranging from 3.0 to 10.0. Crude and purified enzymes were incubated at optimized temperatures for optimized period and commercial enzyme was incubated for optimized period at 85°C.

Stability of enzymes with temperature

Crude, purified and commercial α -amylases were pre-incubated at 85°C and at pH 7.0 and the activities of the enzymes were monitored.

RESULTS AND DISCUSSION

Determining the optimum time for α -amylase activity measurement

The influence of incubation time on the production of glucose from the reaction of α -amylase with starch (20gL⁻¹) was studied for 1h at pH 7.0 and at 85°C. Crude and commercial α -amylase preparations showed a linear relation ship between the time and production up to 10minutes (Figure 1) but purified enzyme showed up to 8 minutes (Figure 1). Hence, it was decided to fix the reaction time for 5 min.

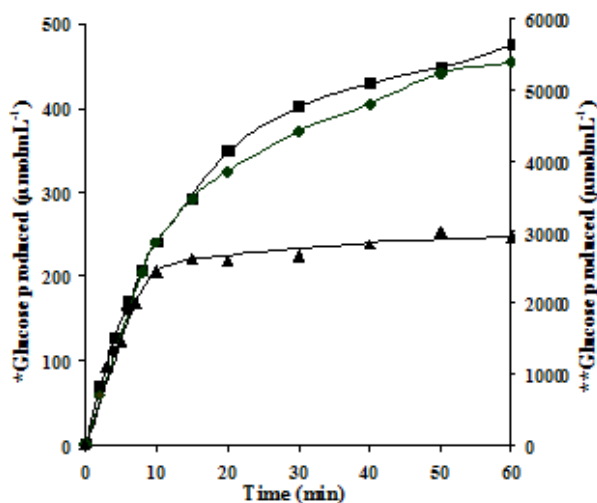


Figure 1: Production of glucose (●), crude; (▲), purified and (■), commercial α -amylase preparation on starch (20gL⁻¹)-0.01M phosphate buffer (pH 7.0) at 85°C.

* - Glucose produced by crude and purified α -amylase (μmolmL^{-1})

** - Glucose produced by commercial α -amylase (μmolmL^{-1})

Effect of temperature on the activity of crude, purified and commercial α -amylase

Relative activity of crude, purified and commercial α -amylases on starch at different temperatures (40, 50, 60, 70, 75, 80, 85, 90 and 95°C) and at pH 7.0 is shown in Figure 2. The initial relative activity of crude and purified α -amylase increased to 100% as the temperature increased up to 85°C. Maximum activity was obtained at 85°C and pH 7.0 for the substrate starch. Above 85°C, α -amylase activity was decreased sharply due to thermal denaturation of

the enzyme and lost the activity. Hence 85°C was chosen as the optimum temperature for the assay of crude and purified α -amylases. But commercial α -amylase showed maximum activity at 90°C and at pH 7.0 for the substrate starch.

Bacillus licheniformis ATCC 6346 producing crude and purified α -amylases showed highest activity at 85°C and commercial α -amylase showed at 90°C. The proteins, other than enzyme proteins present in the crude enzyme have not influenced the activity of

enzyme at different temperatures. The purified α -amylase of *Bacillus licheniformis* CUMC 305 showed maximal activity at 90°C and pH 9.0 [11]. The purified α -amylase obtained from *Bacillus subtilis* was optimally active at 80°C and pH 5.6 [15]. Maximum

activity of α -amylase from *Bacillus licheniformis* BLM 1777 was obtained at 85°C and at pH 6.0 [16]. The optimum temperature for the activity of α -amylase from thermophilic *Bacillus* sp TS-23 was 70°C [17].

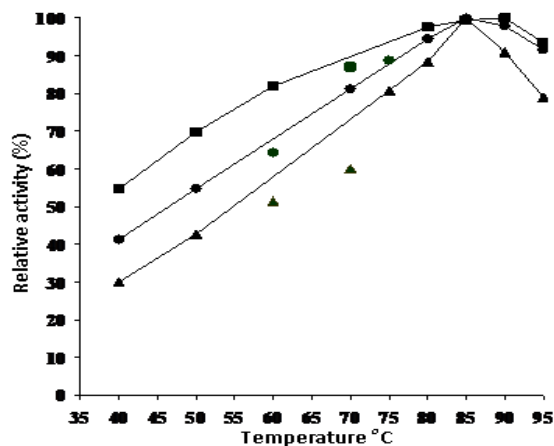


Figure 2: Effect of temperature on the activity of (●), crude; (▲), purified and (■), commercial α -amylases with starch (20gL⁻¹) at pH 7.0. α -Amylases activity were measured at different temperatures of 40, 50, 60, 70, 75, 80, 85, 90 and 95°C, using 20gL⁻¹ starch as substrate by incubating for 5 min at pH 7.0.

Effect of pH on the activity of crude, purified and commercial α -amylases

The influence of pH on crude, purified and commercial α -amylases activities with starch at different pH value were studied (Figure 3). When the pH was increased, the maximum activities of crude, purified and commercial α -amylases were obtained at pH 7.0. Increases in the activities were observed of crude and commercial enzyme at pH 9.0 but the activities were less than that obtained at pH 7.0. Therefore for further studies pH of 7.0 was selected.

Neutral pH was found to be optimal for amylase production by *B.thermooleovorans* NP54 as also reported in *B.coagulans* [18], *B.licheniformis* [11]

and [19]. The dependence of enzyme activity on pH is a consequence of the amphoteric properties of proteins [20]. The majority of thermostable α -amylases from *Bacillus spp*, heretofore purified, have shown maximal activity in the acidic to neutral pH range [11]. The optimum pH of activity of purified α -amylase from *Bacillus* sp. TS-23 and *Thermus sp* were 9.0 and 5.5-6.5 respectively [21]. *Streptococcus bovis* JB1 producing α -amylase activity on souble starch was optimal at pH 5.0 to 6.0. The enzyme was relatively stable between pH 5.5 and 8.5 and at temperature below 50°C [22]. *Aspergillus tamaris* producing α -amylase showed optimal of pH with starch as substrate was 4.5-6.5 [23].

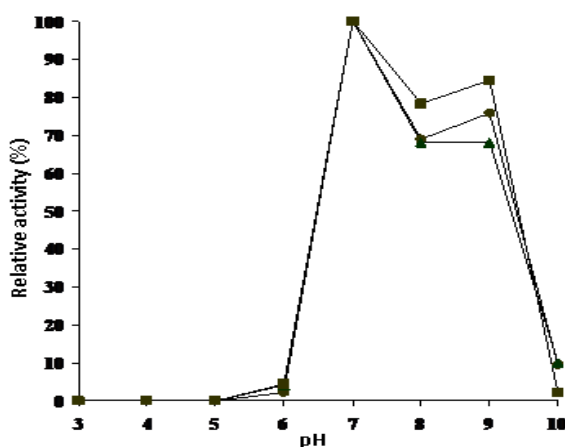


Figure 3: Effect of pH on the activity of (●), crude; (▲), purified and (■), commercial α -amylases with starch (20gL⁻¹) at pH 85°C. Activities were measured at different pH, using 20gL⁻¹ starch as substrate by incubating for 5 min at 85°C.

Stability of crude, purified and commercial α -amylases at 85°C

The thermostability of crude and purified α -amylase from *Bacillus licheniformis* ATCC 6346 was studied. When the crude α -amylase was pre-incubated at 85°C and pH 7.0 for 30 min it retained 31.2% of its initial activity and when it was pre-incubated at 85°C for 60min, lost all its activity (Figure 4). The purified α -amylase retained 10.69 and 9.57% of its initial activity at 30 and 60 min respectively at 85°C and pH 7.0. Half-life of crude and purified α -amylases were 13.9 and 4.7min respectively. Therefore in this case purification has reduced the α -amylase stability at 85°C. Presence of some proteins other than enzyme protein could support the stability of crude α -amylase. Commercial α -amylase showed 100% of its initial

activity at 60 min and 80 % of activity at 210 min and at pH 7.0 (Figure 4) and half-life was 823.97 min.

The extra thermostability of the thermophilic α -amylase was found to be mainly due to additional salt bridges involving a few specific lysine residues (Lys-385 and Lys-88 and/or Lys-253). These stabilizing electrostatic interactions reduce the extent of unfolding of the enzyme molecule at high temperatures, consequently making it less prone to forming incorrect (scrambled) structures and thus decreasing the overall rate of irreversible thermo-inactivation [24]. α -Amylase from *Bacillus* sp. WN11 retained 50% of its initial activity at 4h when the enzyme was incubated at 80°C [25]. Half-life of *Bacillus thermooleovorans* NP54 producing α -amylase was 3h at 100°C and pH 8.0 [1].

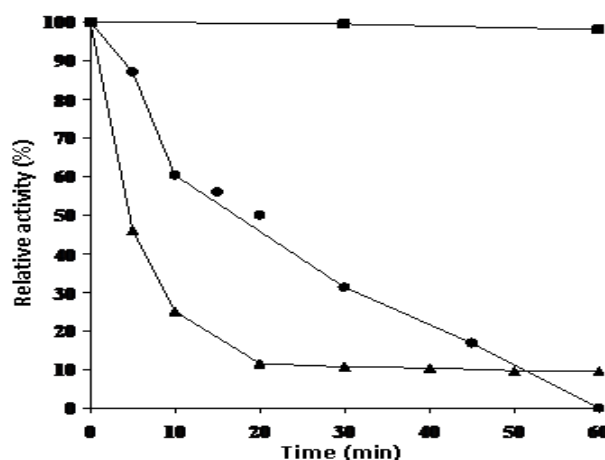


Figure 4: Stability of (●), crude; (▲), purified and (■), commercial α -amylase. α -Amylase activity was measured at 85°C using 20gL⁻¹ starch as substrate by incubating for 5 minutes at pH 7.0.

CONCLUSION

Kinetic properties of crude and purified α -amylases were compared with commercial α -amylase. The optimum time for first order kinetics was optimized as 5min for all type enzyme preparations at 85°C and pH 7.0. The optimum pH for the activity of crude, purified and commercial α -amylases at 85°C was 7.0. Crude and purified enzymes showed the highest activities at 85°C while the commercial enzyme showed at 90°C at pH 7.0. Commercial enzyme was more stable than crude and purified enzymes. Crude enzyme was more stable than purified enzyme.

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