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Biochemical Characterization of Raw-starch-digesting Alpha Amylase Purified from *Bacillus amyloliquefaciens*

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Abstract Alpha amylase (E.C. 3.2.1.1) of *Bacillus amyloliquefaciens* produced by submerged fermentation was purified to near homogeneity by ion exchange chromatography. Through the process 38.6-fold increase in purity with a specific activity of 72 U/mg proteins was obtained. The apparent molecular weight of the purified enzyme was found to be 58 kDa by SDS-PAGE. The enzyme was relatively stable between pH 5.0–8.0 and temperature between 50 and 60°C. The enzyme did not show any obligate requirement of metal ions but Ca^{2+} and Cu^{2+} enhanced the enzyme activity marginally and the thermostability was enhanced in the presence of Ca^{2+} ions. The purified enzyme exhibited maximal substrate specificity for amylose and efficiency in digesting various raw starches. The K_m and V_{max} of the enzyme was determined using both amylose and soluble starch as substrate. The analysis of the hydrolyzed products of soluble starch by thin layer chromatography showed the yield of maltosaccharides after 6 h of hydrolysis.

Keywords *Bacillus amyloliquefaciens* · Alpha amylase · Raw starch digestion · Ion exchange chromatography · Submerged fermentation

Introduction

α-Amylase (EC 3.2.1.1), hydrolyzes the internal α-1, 4 linkages in starch in a random fashion leading to the formation of soluble maltodextrins, maltose, and glucose. Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity, and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes [1]. Alpha amylase has been derived from several fungi, yeasts, bacteria, and actinomycetes. However, enzymes from fungal and bacterial sources have dominated in industrial applications [2]. The enzyme has a wide spectrum of applications in many fields such as starch saccharification, textile industry, food, brewing,

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and distilling industries. Even though crude preparations of the enzyme are generally preferred for commercial applications, the pharmaceutical and clinical applications demand a highly pure enzyme. An understanding about the biochemical properties of the purified enzyme is very essential for its proper application. Among the bacterial amylases, several species of bacillus culture such as *B. amyloliquefaciens*, *B. subtilis*, *B. stearothermophilus*, and *B. licheniformis* are known as potent producers. [3, 4, 5]

Gelatinization of starch is a high-energy-consuming process and the complete saccharification of raw starch requires liquefaction of the gelatinized starch. Glucoamylases of fungi such as *Aspergillus* spp. and *Rhizopus* spp. are widely reported to be efficient in raw starch digestion and assumed to have the C-terminal starch binding domain [6]. An enzyme capable of digesting raw starch granules would be of value to reduce the cost of gelatinization and to simplify the entire process of starch conversion. On the basis of raw-starch digestion, bacterial α-amylases are divided into two groups; raw-starch hydrolyzing and adsorbing and raw-starch hydrolyzing but non-adsorbing [7]. Hayashida et al. [8] reported that the alpha amylase of *Bacillus subtilis* 65 could hydrolyze raw starch although it showed no adsorption onto raw starch.

A raw-starch-degrading α-amylase of *Bacillus* sp. IMD434 was purified to homogeneity by acetone precipitation, ion exchange chromatography and hydrophobic interaction chromatography [9]. Similarly, Freer has reported the purification of a raw-starch-degrading extracellular alpha amylase from *Streptococcus bovis* JB1 by ion exchange chromatography [10].

In the present study, the alpha amylase produced by *B. amyloliquefaciens* ATCC 23842 from agro residual products such as wheat bran and ground nut oil cake was found to be quite capable of raw-starch digestion and was purified by ion exchange chromatography.

Materials and Methods

Microorganism

Bacillus amyloliquefaciens ATCC 23842 was used for the present study. The strain was grown on nutrient agar slants at 37°C for 24 h and sub-cultured every 2 weeks.

α-Amylase Production and Activity

The production medium was composed of 12.5% *w*/*v* of wheat bran and groundnut oil cake (1:1) supplemented with $MgSO_4$ 0.05 M, NH_4NO_3 0.2 M, KH_2PO_4 0.05 M, $CaCl_2$ 0.0275 M. The production was carried out in a 250-ml Erlenmeyer flask inoculated with 10^6 CFU/ml of an 18-h-old culture and incubated at 37°C at 180 rpm. The sample was withdrawn after 42 h of fermentation, centrifuged at 2,862×*g* for 20 min and the clear supernatant collected was used as crude enzyme.

α-Amylase activity was determined by the method of Okolo et al. [11]. The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.5 ml 0.1 M acetate buffer (pH 5.0), and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method [12]. The color developed was read at 575 nm using a Shimadzu UV-160A (Japan) spectrophotometer. Glucose was used as the standard. The blank contained 0.75 ml of 0.1 M acetate buffer (pH 5.0) and 1.25 ml 1% starch solution. One unit (IU) of α-amylase is defined as the amount of enzyme releasing 1 μmol of glucose equivalent per minute under the assay conditions. Total soluble proteins in the sample were estimated with

crystalline serum albumin (Sigma, USA) as per the standard protocol [13]. All other chemicals were purchased from Himedia, India unless otherwise specified.

Enzyme Purification

The crude enzyme collected after fermentation was concentrated approximately to fivefold by lyophilization. The concentrated enzyme was subjected to ammonium sulfate fractionation ranging from 30–90%. The precipitate was collected in each step by centrifuging at 2,862×*g* for 20 min. The pellet and supernatant were assayed for alpha amylase activity and the active fractions were pooled. The pooled fractions were dialyzed overnight against 0.1 M acetate buffer pH 5.0. The next step of purification of the enzyme was carried out by ion exchange chromatography using a Q-Sepharose Fast Flow (Sigma, USA) chromatographic column (1.6 cm×20 cm, Amersham Biosciences, UK) at 4°C and atmospheric pressure. Protein (20 mg) was loaded onto the column pre equilibrated with 500 mM Tris HCl buffer, pH 9.0. Stepwise elution was performed with 0.1–1.0 M NaCl in 50 mM Tris HCl buffer, pH 9.0 with a flow rate of 1.5 ml/min. Fractions (2.0 ml) were collected and the elution profile was determined by checking the absorbance at 280 nm. The alpha amylase active fractions were dialyzed against 20 mM acetate buffer pH 5.0. 12% SDS-PAGE was performed [14] to check the homogeneity of the purified enzyme and determined its molecular weight. The proteins were stained with Coomassie brilliant blue R-250.

Biochemical Characterization of the Purified Enzyme

pH and Temperature Studies

The relative enzyme activity was determined at various pHs (4.0–10.0). Different buffers (50 mM) such as citrate buffer, acetate buffer, phosphate buffer, and Tris buffer were used to adjust the pH of the reaction mixture at 4.0 and 5.0, 6.0 and 7.0, and 8.0–10.0, respectively.

The effect of temperature on enzyme activity and thermal stability of the enzyme was studied by determining the relative activity at 40–80°C for 30 min. Thermal inactivation of the enzyme was done by incubating the enzyme at 60 °C for 35 min. The effect of Ca^{2+} ions on thermal stability was checked in the presence of 5-mM Ca^{2+} ions.

Influence of Metal Ions and Inhibitors

The effect of metal ions as activators was studied in the presence of various cations (5 mM and 10 mM) such as K^{+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+}. The relative activity was determined in each case. The relative inhibitory activity of SDS, urea, and EDTA were also studied at 5 and 10 mM concentration.

Adsorption and Hydrolysis of Various Raw Starches

The efficiency of the enzyme to digest various substrates (soluble starch, amylose, raw starches such as potato, corn, wheat, and cassava) was studied by incubating 100 IU of the purified enzyme with 1% of each substrate for 5 h. The amount of reducing sugar released in each case was estimated. The digestibility of the different starches at various concentrations such as 5, 10, and 15% (*w*/*v*) over a period of 4, 8, and 12 h was also studied (data not shown).

Table 1 Summary of purification steps of *B. amyloliquefaciens* α-amylase.

Purification steps	Total protein (mg)	Total activity (U/ml)	Specific activity	Yield (%)	Purification fold
Crude enzyme	250	1050	4.2	100	1.0
Ammonium sulphate precipitation	145	941	6.4	89.6	1.7
Q-Sepharose column	6.5	470	72	44.7	38.46

The adsorption rate on different starches was determined. A protein concentration of approximately 200 μg/ml in 50 mM sodium acetate buffer (pH 6.0) was mixed with 100 mg of starch to a final volume of 1 ml. The resulting samples were shaken at 4°C for 1 h and then centrifuged to sediment the insoluble substrate. The residual protein in the supernatants was measured. The adsorption rate (AR) was defined by the following equation: $\mathrm{AR}(\%) = [(B - A)/B] \times 100$, where A indicates the residual protein after adsorption and B represents the protein concentration in the original enzyme solution.

Enzyme Kinetics

To understand the basic kinetics of the enzyme, various concentrations of soluble starch and amylose (0.2–2.0%) were used. The kinetic constants K_m and V_{max} were determined by the Lineweaver Burk [15] method which was analyzed by Hyper 32 software, Department of Biochemistry, University of Liverpool, Liverpool, UK.

Analysis of the Hydrolyzed Products

The hydrolysis products of starch by alpha amylase were analyzed by thin layer chromatography using silica gel plates. One percent of starch was incubated with 100 U of the enzyme at 50°C for 12 h. Glucose and maltose were used as standards, and butanol, acetone, and water (4:5:1) was used as the solvent system. The spots were developed by diphenyl amine method [16].

Results and Discussions

Enzyme production was carried out as per the conditions standardized earlier [4]. Ammonium sulfate precipitation was employed for the partial purification of the enzyme. As shown in Table 1, maximum enzyme activity was obtained in the 70–80% fraction with a high yield of

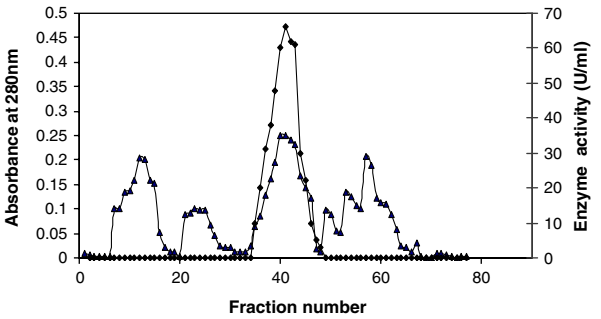

Fig. 1 Ion exchange chromatography of *B. amyloliquefaciens* α-amylase on Q-Sepharose column. **a** *filled upright triangle* absorbance at 280 nm **b** *filled diamond* alpha amylase activity

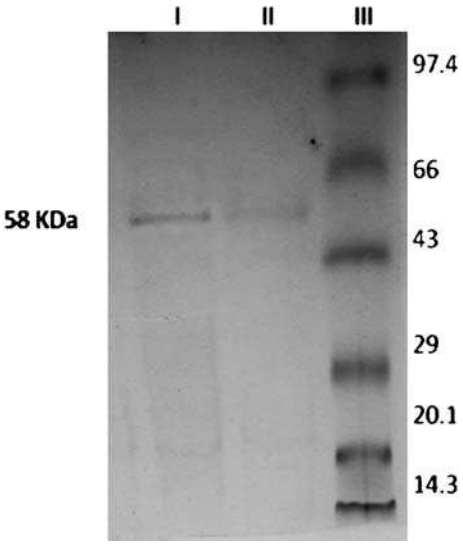

Fig. 2 SDS-PAGE of the purified alpha amylase of *B. amyloliquefaciens*. *Lanes I* and *II* fractions with amylase activity from Q-Sepharose column; *Lane III* molecular weight marker (kDa)

89.6% and specific activity of 6.44. The collected fraction was dialyzed against 50 mM acetate buffer pH 5.0 and subjected to ion exchange chromatography. Fractions 28–45 eluted between 0.3–0.5 M NaCl in 50 mM Tris HCl buffer had maximum specific activity (72) and enzyme yield of 44 (Fig. 1). The homogeneity of the purified enzyme was checked by running SDS-PAGE (Fig. 2). The apparent molecular weight of the purified enzyme was found to be 58 kDa. The molecular weight of *Bacillus* sp. YX-I capable of raw-starch digestion has been purified and the molecular weight characterized to be 56 kDa [17]. Higher molecular weight alpha amylase of *Bacillus* sp. IMD 435 of 63 kDa, a raw-starch digesting but non-raw-starch adsorbing was reported by Hamilton et al. [7].

The effect of pH and temperature on the enzyme activity was studied and showed in Table 2. It was found that maximum activity was obtained at pH 5.0. From Table 2, it is clear that more than 60% activity was retained between pH 6–8. Most bacterial α-amylases are optimally active at slightly acidic to near neutral pH 9 [18]. These were similar values reported by Freer [10] and Morgan et al. [18]. Majority of the alpha amylases produced by non-halophilic microorganisms showed an optimum pH within the acidic range (5–7) [19]. The temperature profile was studied between the temperatures 40–80°C (Table 2). Maximum activity was obtained at 50°C. From Fig. 3, it is clear that at 50°C, over 90% activity was retained up to 30 min incubation. Similarly, the purified enzyme retained 75% of activity at 60°C, but an increase in temperature to 70°C showed a sharp decrease in

Table 2 Effect of pH and temperature on the activity of the purified enzyme.

pH	Relative activity (%)	Temperature (°C)	Relative activity (%)
4.0	62	40	78
5.0	100	50	100
6.0	82	60	80
7.0	78	70	40
8.0	65	80	18
9.0	35	–	–
10.0	20	–	–

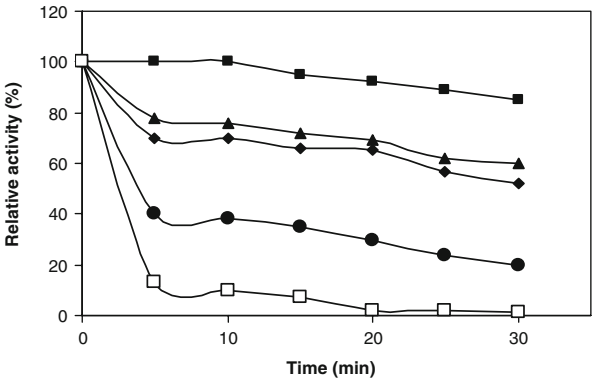

Fig. 3 Thermal stability of the purified α-amylase of *B. amyloliquefaciens*. **a** *filled upright triangle* 40°C; **b** *filled square* 50°C; **c** *filled diamond* 60°C; **d** *filled circle* 70°C; **e** *empty square* 80°C

relative activity of 20% (Fig. 3). This may cause limitation for the usage of the enzyme in industry which requires high temperatures but may be favorable in the baking industry that requires complete inactivation of the enzyme at high temperatures [20]. The thermal stability of the enzyme was studied at 60°C and it was found that in the absence of $CaCl_2$, the enzyme was stable up to 25 min retaining 65% of the activity. The presence of 5 mM $CaCl_2$ retained 90% activity up to 25 min (Fig. 4). Calcium has been shown to regulate the stability and reactivity of a wide variety of biological proteins. In particular, its binding to α-amylase is essential in activating and stabilizing the enzyme proteins [21].

The effect of metal ions as activators was studied in the presence of various cations (K^+, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Cu^{2+}) at two different concentrations (5 mM and 10 mM) The effect of metal ions on alpha amylases highly varied among the reported enzymes. K^+ and Mn^{2+} were found to inhibit enzyme activity while Ca^{2+} and Cu^{2+} were found to play a role in enhancing the enzyme stability (Table 3). Contradictory results were reported where K^+ activated the effect for *L. manihotivorans* LMG 18010 amylase [22], and 1 mM Mn^{2+} was found to be promoting for *Bacillus* sp.I-3 [23]. Zn^{2+} and Mg^{2+} did not show any effect on enzyme activity at 5 mM concentration but led to inhibition at 10 mM. As it is shown in Table 3, the enzyme was easily susceptible to SDS and urea. Eighty percent of the activity was lost when the enzyme was incubated with 10 mM EDTA, thus, indicating the enzyme was a metalloenzyme.

The enzyme hydrolyzed all substrates (1% *w*/*v*) tested with maximal activity on amylose followed by soluble starch, potato, corn, wheat, and rice starch with degradation rates of 120, 100, 95, 62, 56.5, and 43%, respectively, after 5 h treatment with 100 IU of purified enzyme (Table 4). Amylose, soluble starch, and potato starch were chosen for further

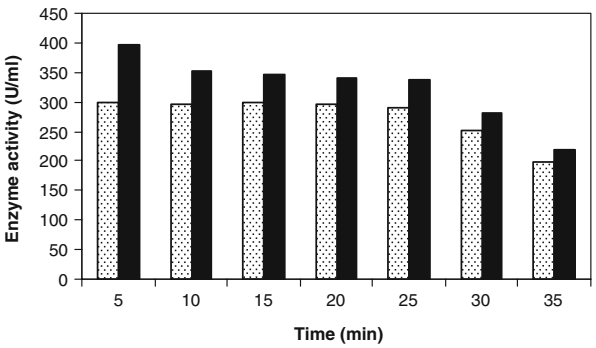

Fig. 4 Thermal inactivation study at 60°C in the presence and absence of Ca^{2+} ions on the purified enzyme of *B. amyloliquefaciens*. **a** *gray bar* absence of Ca^{2+} ions; **b** *black bar* presence of Ca^{2+} ions

Table 3 Influence of various effectors on the purified α-amylase of *B. amyloliquefaciens.*

Effectors	Concentration (mM)	Relative activity (%)
Control		100
K^+	5	60
	10	42
Zn^{2+}	5	90
	10	56
Mn^{2+}	5	50
	10	21
Mg^{2+}	5	92
	10	48
Ca^{2+}	5	120
	10	96
Cu^{2+}	5	120
	10	87
EDTA	5	22
	10	–
SDS	5	30
	10	12
Urea	5	58
	10	16

Table 4 Rate of hydrolysis of raw starches (1%) with 100 IU of purified enzyme for 5 h.

Substrate	Relative activity (%)
Control	100
Amylose	120
Potato starch	95
Corn starch	62
Wheat starch	56
Rice starch	43

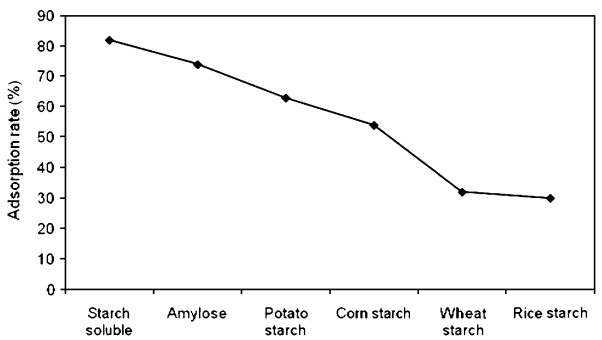

Fig. 5 Adsorption rate (%) on various starches

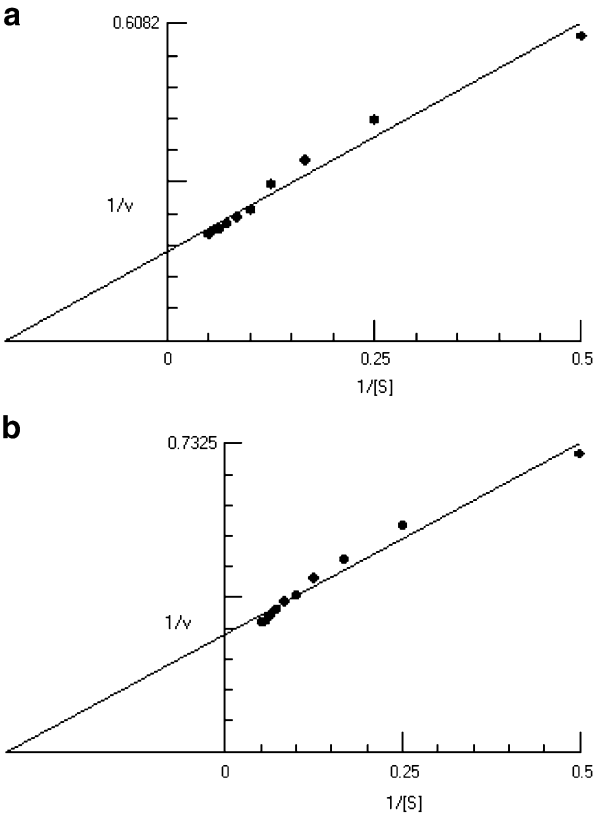

Fig. 6 **a** Lineweaver Burk plot for amylase; **b** Lineweaver Burk plot for soluble starch

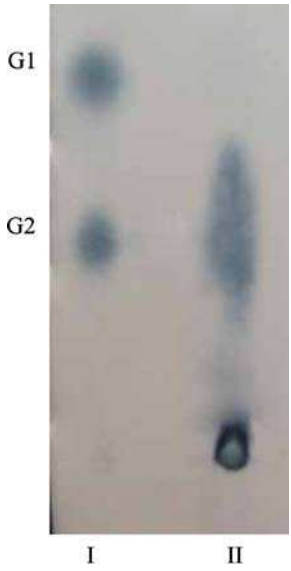

Fig. 7 Thin layer chromatogram: *Lane I* standard sugars maltose (*G1*) and glucose (*G2*) *Lane II* maltosaccharides formed by starch (1%) hydrolysis for 6 h at 50°C

studies of hydrolysis. The rate of hydrolysis was found to be constant up to 10% starch. The amount of reducing sugars decreased to 20, 15, and 12% for amylose, soluble starch, and potato starch, respectively, as the percentage of starch was increased to 15%. Enzymes that are capable of digesting raw potato starch are economically attractive for they can increase the range of starch sources for direct saccharification [8]. Alpha amylase from *Bacillus* sp. YX-1 which had a strong digesting ability towards various raw starches and efficiently hydrolyzes raw corn starch at a concentration of 20% and pH 5.0 in a period of 12 h has been reported [17]. Therefore, enzymes that are capable of digesting raw potato starch are economically attractive for they can increase the range of starch sources for direct saccharification [8]. Among the raw starches, the adsorption rate was highest in the case of potato starch followed by corn starch while adsorption was very low in the case of wheat and rice starch (Fig. 5). This indicates a strong correlation between the adsorption and rate of hydrolysis.

The enzyme followed the Michealis Menten kinetics of catalysis. The V_{max} and K_m of the enzyme was derived from the Lineweaver Burk plot and found to be 3.047 mg/min and 2.34 mg, respectively, (Fig. 6a) for amylose. The V_{max} and K_m values for soluble starch was found to be 4.11 mg/min and 3.076 mg, respectively, for starch (Fig. 6b). Low values of K_m indicate high affinity of the enzyme for the substrate [7]. Comparison of K_m values clearly indicates higher affinity of the enzyme towards amylose than soluble starch. The K_m and V_{max} values of different enzymes are difficult to compare as they depend on the substrate used and the reaction conditions. A similar report of K_m and V_{max} values was 3.44 mg/ml and 0.45 mg hydrolyzed starch/ml/min at 55°C, respectively, for *Lactobacillus manihotivorans* [24]. The hydrolysis of starch yielded maltosaccharides as end product (Fig. 7). The liquefying amylase from *B. amyloliquefaciens* has been reported to yield maltosaccharides predominantly while the saccharifying enzyme from *B. subtilis* produces largely glucose and maltose from starch [25]. The properties of the purified enzyme proved its efficacy for digesting diverse raw starches and, hence, its potential commercial value to use as an industrial enzyme. Moreover, the enzyme is produced from cheaply available agroresidual substrates like wheat bran and ground nut oil cake, hence, the process can be economically viable also.

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