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The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, *Anoxybacillus beppuensis* TSSC-1

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ABSTRACT

Anoxybacillus beppuensis TSSC-1 (GenBank Number, EU710556), a thermophilic bacterium isolated from a hot spring reservoir, was found to optimally secrete a monomeric α-amylase at 55 °C and pH 7. The enzyme was purified to homogeneity by a single-step purification on phenyl sepharose 6FF, achieving a 58% yield, 10,000 U/mg specific activity and 19.5 fold purification. The molecular weight, K_m and V_{max} were 43 kD, 0.5 mg ml^{-1} and 3571.42 μmol ml^{-1} m^{-1}, respectively. The enzymatic catalysis of soluble starch was optimum at 80 °C and pH 7. The thermodynamic parameters, K_d, $t_{1/2}$, ΔH^*, ΔS^*, E and ΔG^*, were consistent. The very compact structure of the enzyme and the transitional enzyme–substrate complex resisted denaturation at extreme temperatures and alkaline pH. The K_d and $t_{1/2}$ measurements were consistent with the high thermostability and pH tolerance observed. The structural stability of the enzyme was also reflected by the values of ΔH^*, ΔS^*, E and ΔG^*. While the enzyme did not exhibit metal ion dependency, it was resistant to chemical denaturation. The broad thermo- and pH-tolerance of this enzyme suggests potential commercial opportunities.

1. Introduction

The success of thermophiles and actinomycetes under extreme conditions is largely due to the stability of their macromolecules [1]. Thermophiles have high metabolic rates; typically, they have enzymes that are very stable, both physically and chemically, but, not surprisingly, they almost invariably exhibit lower growth rates than mesophiles. Importantly, they can generate exceptionally high end-product yields. Heat-resistant enzymes offer commercial opportunities. Higher temperatures can overcome the viscosity problems associated with some commercial transformations and may also accelerate endothermic reactions. Importantly, the general resistance of these unique thermostable enzymes to solvents, detergents and chaotrophic agents means that novel and more efficient industrial recovery processes can be designed and attempted [2,3]. Apart from the exciting applications existing only in concept, there are real and immediate opportunities for their use in current manufacturing processes, such as beer manufacture. Concerns over sustainability and cost pressures make barley grain a desirable alternative to malted grain. This in turn creates a need for a cheap, active and heat-stable (~70 °C) amylase, which the malt would otherwise provide.

Amylases hydrolyze starch and in beer manufacture, gelatinized starch to form diverse products including dextrins and progressively smaller polymers of glucose [4]. The α-amylase (E.C.3.2.1.1) family members all act on glucose residues linked via α-1-1, α-1-4 or α-1-6, linkages [5,6]. Endoamylases produce linear and branched oligosaccharides of various chain lengths, while exoamylases attack the non-reducing end of shorter fragments [7]. Amylases account for ~30% of the enzyme market [6] and are being used in the starch liquefaction process, in the manufacture of maltose, high-fructose syrups, oligosaccharides mixtures, maltotetrose syrups and high-molecular-weight branched dextrins, in the removal of starch from textiles, in the direct fermentation of starch to ethanol and in the treatment of starch processing waste water [8]. The studies concerning the strain selection, growth and enzyme yield optimization of thermophiles are relatively scarce compared to the studies accumulated over many years concerning mesophiles and even psychrophiles [9]. We have isolated a thermostable, Ca^{++} independent monomeric α-amylase from the thermophilic bacterium, *Anoxybacillus beppuensis* TSSC-1. A one-step purification of the enzyme is achievable. The optimization of cellulase production from *Anoxybacillus* sp. 527 has recently been reported [10]. However, to our knowledge, ours is the first report on the characterization of an extremozyme from any *Anoxybacillus* species.

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2. Materials and methods

2.1. Materials

The phenyl sepharose 6FF was purchased from Sigma (St. Louis, MO, USA). The starch and protein markers were purchased from Merck, Mumbai, India. The medium components were purchased from Hi Media Laboratories, Mumbai, India. All the other chemicals were obtained from Rankem Laboratories, Mumbai, India.

2.2. Isolation and identification of the bacterial strain

The clay type soil sample was collected from the hot water reservoir at Tulsi Shyam, Gujarat, India. The pure culture was isolated from the soil sample and cultivated in the modified thermophilic medium as described earlier [11].

2.3. 16S rRNA gene sequencing

Genomic DNA was isolated from the pure culture pellet and used to amplify the ~1.5 kb 16S rDNA fragment using consensus primers and high-fidelity PCR Polymerase. The PCR product was bidirectionally sequenced using a forward, reverse and internal primer; the sequence was subsequently aligned and analyzed to determine the closest homolog to the microbe.

2.4. Amylase activity

The amylase activity was assayed by measuring the amount of reducing sugar released from the starch substrate according to Kikani and Singh, 2011 [11]. One unit of amylase is the amount of enzyme that liberates 1 μmol of maltose per minute under the assay conditions.

2.5. The effect of temperature pH of the medium on amylase production

The optimum temperature for amylase production was determined by inoculating *A. beppuensis* TSSC-1 in starch broth at pH 7 containing 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.2% (w/v) ammonium sulfate and 1% (w/v) soluble starch. The inoculated cultures were incubated within the temperature range between 45 °C and 60 °C. The amylase activity of the cell-free samples was measured at regular 24 h intervals for 120 h.

The optimum pH for amylase production was determined by inoculating the organism into starch broth as above at pH values between 7 and 9 and at a constant 55 °C. The activity was monitored as mentioned above. The experimental sets were performed in triplicate.

2.6. The effect of medium ingredients on amylase production

The effect of the medium components and their concentrations were monitored at a given pH and incubation temperature. The effects of the carbon and nitrogen sources and trace metal ions were measured. Each set was performed in triplicate.

2.7. Partial purification by ammonium sulfate fractionation

The crude amylase solution or the cell-free culture medium was adjusted to 50% (w/v) ammonium sulfate saturation. The recovered precipitate was suspended in a minimum volume of 20 mM phosphate buffer at pH 7. The remaining supernatant was adjusted to 70% ammonium sulfate. This 50–70% fraction was dissolved in a small volume of the same buffer and dialyzed against the same buffer for 24 h.

2.8. Single-step purification by hydrophobic interaction chromatography

Single-step purification was achieved by loading cell-free supernatant containing amylase in 1 M ammonium sulfate directly onto a phenyl sepharose 6FF column (Sigma; 1 cm × 6.5 cm) equilibrated with 0.1 M phosphate buffer at pH 7.5 containing 1 M ammonium sulfate. The fractions were eluted in the same buffer using a decreasing gradient of ammonium sulfate (1–0 M) at a flow of 0.7 ml m^{-1}. The active fraction was pooled and further analyzed. The purified amylase, partially purified precipitate and the dialyzed and the crude samples were stored at 4 °C for further characterization. The protein content was measured by the Bradford method using bovine serum albumin (10–100 μg/ml) as a standard.

2.9. The molecular weight determination and determination of the sub-unit composition

The purified enzyme was subjected to size exclusion chromatography using a sephadex G-100 column (Merck, Mumbai, India; 1.5 cm × 7 cm). The purified enzyme was analyzed by SDS-PAGE using the Laemmli method and 12% polyacrylamide gels [12]. The subunit composition was confirmed by Native PAGE. The protein bands were visualized by Coomassie brilliant blue R-250 staining.

2.10. The amylase characterization

2.10.1. The temperature and pH profile

The amylase activity was determined at various temperatures from 37 to 100 °C. The activity was also measured in the following buffers: 20 mM acetate buffer at pH 5, 20 mM phosphate buffer pH at 6–7, 20 mM Tris–HCl buffer at pH 8, 20 mM glycine–NaOH buffer at pH 9 and 20 mM NaOH–Borax buffer at pH 10.

2.10.2. The determination of K_m and V_{max}

The enzyme was assayed at substrate concentrations ranging between 0.1 and 3% (w/v) starch. The K_m and V_{max} were estimated from the reciprocal plot of the substrate concentration vs. the velocity.

2.10.3. The effect of cations, chelators and surfactants

The effect of mono- and divalent cations, including 10 mM $CaCl_2$, $MgCl_2$, KCl, NaCl, $FeCl_2$, $CuCl_2$, $NaNO_3$ and K_2HPO_4, tested in the 20 mM phosphate buffer at pH 7. To test the Ca^{++} dependency of the enzyme, the reaction mixture was incubated with the 10 mM chelating agents, *viz*., EDTA and EGTA, for 1 h. To investigate the effect of the surfactants, the enzyme was incubated for 1 h with an ionic surfactant, 20 mM sodium dodecyl sulfate (SDS) and non-ionic surfactants, *viz*., Tween-20, Tween-80 and Triton X-100 at 5% (v/v). The activities were calculated as a percentage of the activity of the control.

2.10.4. The thermostability of amylase

The α-amylase was incubated at various temperatures between 50 °C and 90 °C for 24 h. Aliquots were withdrawn for assay during the incubations. These tests were also performed with the cations described in the preceding section in the incubation mixtures. The enzyme was also incubated with 5–20 mM $CaCl_2$. The residual activity was expressed as a percentage of the activity at time 0.

2.10.5. The pH stability of amylase

The pH stability was assessed by incubating the α-amylase in various buffers, 20 mM phosphate buffer pH at 6–7, 20 mM Tris–HCl buffer at pH 8 and 20 mM glycine–NaOH buffer at pH 9, at various temperatures between 50 °C and 90 °C. The amylase activity was monitored for 24 h and the residual activities were then estimated.

2.10.6. The estimation of deactivation rate constant

The residual activity is directly related to the deactivation rate. The deactivation rate was calculated by the first-order expression [13]

$$\frac{dE}{dt} = -K_d E \tag{1}$$

which can also be expressed as

$$\mathrm{Ln}\left[\frac{E_t}{E_0}\right] = -K_d t \tag{2}$$

K_d is the deactivation rate constant, which is calculated from the plots of $\mathrm{Ln}[E_t/E_0]$ vs. t. The half-life ($t_{1/2}$) of the enzyme is defined as the time required for the enzyme to lose half its initial activity. It can be expressed as follows:

$$t_{1/2} = \frac{\ln 2}{K_d} \tag{3}$$

2.10.7. The changes in the energy, entropy and enthalpy values of amylase deactivation

Standard reaction theory was applied to obtain the changes in the energy, entropy and enthalpy values of amylase deactivation [14]. The thermodynamic data were calculated by rearranging the Eyring absolute rate equation as follows [15,16]:

$$K_d = \left(\frac{K_b T}{h}\right) e^{(\Delta S^*/R)} e^{(-\Delta H^*/RT)} \tag{4}$$

where h is the Planck constant, which is 6.63×10^{-34}; R is the gas constant, which is 8.314 J/K mol; T is the temperature in K; ΔS^* is the change in the entropy; ΔH^* is the change in the enthalpy; K_b is the Boltzmann constant, i.e., R/N, which is 1.38×10^{-23} J/K and N is the Avogadro's number = 6.02×10^{-23} mol^{-1}.

The ΔH^* and ΔS^* values can be calculated from the slope and intercept, respectively, of $\mathrm{Ln}[K_d/T]$ vs. $1/T$ as follows:

$$\Delta H^* = -R[\text{slope}] \tag{5}$$

$$\Delta S^* = R\left[\text{intercept} - \mathrm{Ln}\left(\frac{K_b}{h}\right)\right] \tag{6}$$

The Gibb's free energy change (ΔG^*) of the amylase was calculated as follows:

$$\Delta G^* = -RT\left[\mathrm{Ln}\left(\frac{K_d T}{K_b h}\right)\right] \tag{7}$$

ΔG^* can also be found from the following relationship:

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{8}$$