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Biophysical and Biochemical Characterization of a Hyperthermostable and Ca^{2+}-independent α-Amylase of an Extreme Thermophile *Geobacillus thermoleovorans*

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Abstract α-Amylases reported from various microbial sources have been shown to be moderately thermostable and Ca^{2+} dependent. The bacterial strain used in this investigation is an extremely thermophilic bacterium *Geobacillus thermoleovorans* that produces a novel α-amylase (26 kDa; α-amylase *gt*), which is hyperthermostable (T_{opt} 100 °C) and does not require Ca^{2+} for its activity/stability. These special features of α-amylase *gt* make it applicable in starch saccharification process. The structural aspects of α-amylase *gt* are, therefore, of significant interest to understand its structure–function relationship. The circular dichroism spectroscopic data revealed the native α-amylase *gt* to contain 25% α-helix, 21% β-sheet, and 54% random coils. The addition of urea, at high concentration (8 M), appeared to expose the buried Trp residues of the native α-amylase *gt* to the aqueous environment and thus showed low fluorophore. Fluorescence-quenching experiments using KI, CsCl, *N*-bromosuccinimide, and acrylamide revealed interesting features of the tryptophan microenvironment. Analysis of K_{sv} and f_a values of KI, CsCl, and acrylamide suggested the overall Trp microenvironment in α-amylase to be slightly electropositive. Fluorescence-quenching studies with acrylamide revealed the occurrence of both collisional as well as static quenching processes. There was no change in the α-helix content or the enzyme activity with an increase in temperature (60–100 °C) that suggested a critical role of the α-helix content in maintaining the catalytic activity.

Keywords Ca^{2+}-independent α-amylase · Quenchers · Tryptophan · Fluorescence · Circular dichroism

Introduction

A number of extremely thermophilic and hyperthermophilic bacteria and archaea are known to be capable of degrading starch and other biopolymers such as hemicellulose,

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cellulose, and proteins [1, 2]. In addition to the industrial relevance of heat-stable amylolytic enzymes, the existence of these enzymes in all kingdoms of life allows comparative studies on thermostability and folding of proteins from mesophilic and thermophilic microbes [3].

α-Amylases (endo-1,4 α-D-glucan glucohydrolase; E.C. 3.2.1.1) are among the most important enzymes in present-day biotechnology. This family of enzymes finds potential applications in a wide range of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries [4]. A great variety of α-amylases has been produced and characterized from various microbial sources [5–8]. The moderate thermostability and Ca^{2+} requirement, however, limit their industrial potential, as the added Ca^{2+} must be removed from the product stream by using ion exchangers that increases the processing cost. *Geobacillus thermoleovorans* is an extremely thermophilic bacterium, which grows optimally at 70 °C on complex media under aerobic conditions [9–12]. This extreme thermophile is equipped with a novel starch-degrading α-amylase (α-amylase *gt*), which is optimally active at 100 °C and independent of Ca^{2+} for its activity and/or stability [13].

Structural studies of enzymes are the main priorities of protein engineering. In spite of increasing knowledge and success attained in laboratories, convincing general rules for enzyme stabilization have not yet emerged, and the elucidation of molecular processes that occur in proteins when exposed to different environments remains a specific task [14]. Role of aromatic residues in maintaining the tertiary activity of proteins has been well documented [15]. A recent study on α-amylases highlighted the possible involvement of tryptophanyl (Trp) and tyrosyl residues in carbohydrate binding [16]. In this context, it is of particular interest to study the environment of Trp residues using fluorescence as a probe. Among the aromatic amino acids that are present in a protein, the fluorescence of phenylalanine is insignificant. In proteins containing tryptophan and tyrosine, the major contribution to the fluorescence spectrum is by tryptophan [17], in the absence of which the tyrosine fluorescence is detected in a very low yield with a band maximum at 304 nm. The analysis of the effects of different quenchers and denaturants suggested interesting features of the microenvironment of Trp.

Circular dichroism (CD) spectroscopy is a widely used technique for obtaining information about protein structure and conformation. The sensitivity of far UV protein CD spectra at different temperatures and chemical environments is of primary concern for studying the change in protein folding. This investigation is aimed at deducing the structure–function relationship for the Trp residues involved at the active site by correlating the effect of *N*-bromosuccinimide (NBS) on the fluorescence and quenching of fluorescence by various solute quenchers and denaturants and in understanding the stability and conformational dynamics of native and chemically modified forms of α-amylase of *G. thermoleovorans*.

Materials and Methods

Materials

NBS, acrylamide, CsCl, KI, guanidine hydrochloride (Gdn-HCl), and urea were purchased from Sigma Chemical, St. Louis, MO, USA. Buffer solutions were filtered through 0.22-μm filters and carefully degassed. All buffers and solutions were prepared with ultrahigh quality water (ELGA UGQ, UK). All other chemicals were of analytical grade.

Source of Culture

G. thermoleovorans was isolated from a water sample of a hot water spring of the Waimangu volcanic valley (New Zealand), characterized, and identified as described earlier [9–12]. The culture (MTCC 4220) is deposited at the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

Inoculum Preparation

The inoculum was prepared by transferring several loopfuls from a fresh culture of *G. thermoleovorans* into a 250-ml Erlenmeyer flask containing 50 ml starch–yeast extract–tryptone (SYT) broth (g/l: soluble starch 20.0, yeast extract 3.0, tryptone 3.0, sodium dodecyl sulfate [SDS] 0.3, polyethylene glycol 5.0, $MgSO_4$ $7H_2O$ 0.2, K_2HPO_4 1.0, NaCl 1.0; pH 7), incubated for 5 h in an incubator shaker at 70 °C and at 200 rpm. In order to obtain cells for inoculation, the culture fluid was aseptically centrifuged at 8,000×*g* at 4 °C for 20 min (Sorvall RC 5C plus, Kendro labs, USA), and the cells thus sedimented were washed twice with sterile distilled water and used for α-amylase production.

Production and Purification of α-Amylase *gt*

Erlenmeyer flasks (250 ml) containing 50 ml of SYT medium were inoculated with 5-h-old bacterial culture (1.8×10^8 colony-forming units per milliliter) and incubated in an incubator shaker at 70 °C for 12 h and agitated at 200 rpm. The cultures were harvested by centrifuging at 8,000×*g* for 15 min at 4 °C, and the cell-free supernatant was used as the source of extracellular α-amylase.

α-Amylase *gt* was extracted from native polyacrylamide gel (without SDS) with the help of GeBAflex-tube system (Gene Bio-Application). This universal technique allowed electro-elution of macromolecules from polyacrylamide or agarose gels and dialysis of samples with high performance in both extraction and dialysis. A support tray maintains one to four GeBAflex tubes in the correct position during electro-elution and holds the tubes fully immersed in the Tris–Cl buffer. The tray was positioned such that the tube membranes were perpendicular to the electric field inside a standard horizontal electrophoresis unit. Electric current at 100 V for 60 min was used for extraction of the desirable protein. After completion of electro-elution, the electric field was inverted for 1–2 min to release the macromolecules from the inner surface of the cellulose membrane, and percent recovery was calculated. The purity of α-amylase *gt* was conformed by silver staining of native polyacrylamide gel electrophoresis (PAGE).

Secondary Structure of α-Amylase *gt*

The purified α-amylase *gt* (0.1 mg/ ml) was thermal activated at 100 °C for 15 min, and the secondary structure was determined by CD spectroscopy carried out on a JASCO-810 Spectropolarimeter equipped with in-built Peltier controlled thermostat cell holder (PTC-423S). The path length of cuvettes used was 0.2 cm. Temperature, pH, denaturants, and quenchers scans were performed by exposing the α-amylase *gt* to different temperatures (60 to 90 °C), different pH (glycine–HCl, pH 4 to 5; phosphate, K_2HPO_4–KH_2PO_4, pH 6 to 8; glycine–NaOH, 9 to 11), different denaturants (urea, Gdn-HCl with/without starch [0.1%], and NBS) and different quenchers (KI, acrylamide, and CsCl); the changes in the structural conformation was recorded at a scanning rate of 20 nm/min from 200 to

320 nm, followed by the enzyme assay. Each CD spectrum was always an average of six scans, and data acquisition and analysis were performed on a computer, which is interfaced to the Spectropolarimeter. Nitrogen was flushed continuously through the machine at the rate of 5.0 ml/min. The CD instrument was routinely calibrated with D-10-camphor-sulfonic acid.

Fluorescence Measurements

Steady-state fluorescence titrations were performed in a Hitachi F-4500 fluorescence spectrofluorometer at room temperature using a slit width of 5/5 nm (exc/emi) with a slow scan of 15 nm/min and quartz cells of 1 cm path length. Fluorescence emission spectra were recorded between 300 to 400 nm using two different excitation wavelengths of 280 and 305 nm, respectively. Fluorescence spectrum of α-amylase *gt* was determined by excited at 305 nm reflected five times higher sensitivity as compared to that at 280 nm, followed by the enzyme assay. All the spectra were corrected for light scattering by subtracting the data for an appropriate control. All the fluorescence spectra reported were an average of six scans, and the error in the fluorescence intensity was always less than 5%.

Quenching of Fluorescence

Relative fluorescence intensity of thermal activated α-amylase *gt* (74 μg/ml) in 50 mM Tris–Cl buffer (pH 8.0) was recorded in the absence and presence of different concentrations of anionic/nonionic/cationic quenchers KI, acrylamide, and CsCl, respectively.

Chemical Modification with NBS

The effect of NBS on the fluorescence of thermal activated α-amylase *gt* was determined by incubating the enzyme with different aliquots of NBS at room temperature for 15 min. The number of Trp resiudes (n) oxidized was calculated according to Spande and Witkop [18] using the following equations:

$$n = \Delta\mathrm{OD}\ 280/\mathrm{OD}\ 280 \times 1.31\varepsilon/5,500$$

where n is number of Trps oxidized per mole of α-amylase, ε is molar extinction coefficient of α-amylase at 280 nm, and OD is optical density, and

$$A = \varepsilon Cl$$

where, ε is the extinction coefficient, C is the concentration of the solution, and l is the path length of the light in the sample, usually 1 cm. Absorbance (A) is usually dependent on wavelength.

Stability of α-Amylase *gt* Against Chemical Denaturants and pH

The stability of α-amylase *gt* against different concentrations of Gdn-HCl and urea was studied by monitoring emission of fluorescence intensity at 280 and 305 nm. Protein samples were prepared in different buffer systems (glycine–HCl, pH 4 to 5; phosphate, K_2HPO_4–KH_2PO_4, pH 6.0 to 8.0; glycine–NaOH, 9.0 to 11.0), and changes in their intrinsic fluorescence intensity were recorded at 280 and 305 nm, followed by the enzyme assay.

Analysis of Fluorescence Quenching Data

The fluorescence titration (quenching/ stability) data were analyzed using the Stern–Volmer equation

$$F_o/\Delta F = 1([Q]K_{sv}f_a) + 1/f_a$$

where F_o is the fluorescence intensity in the absence of quencher, ΔF is the difference in fluorescence intensity in the absence and in the presence of quencher, $[Q]$ is the quencher concentration, K_{sv} is the quenching constant, and f_a is the fraction of total fluorescence available to the quencher.

Assay of α-Amylase *gt*

The saccharogenic α-amylase *gt* activity was determined by the modified method of Bernfeld [19] by incubating the reaction mixture containing 0.5 ml of 0.5% of soluble starch prepared in phosphate buffer (pH 8.0) with 0.5 ml appropriately diluted α-amylase for 10 min at 100 °C and determining the liberated reducing sugars using dinitrosalicylic acid reagent. One saccharogenic α-amylase *gt* unit is defined as the amount required for the liberation of 1 μmol of reducing sugars as maltose per milliliter per minute under the assay conditions. The specific enzyme activity (U/mg protein) was calculated by determining the soluble protein in the enzyme sample using bovine serum albumin as the standard according to Bradford [20].

Results

G. thermoleovorans is a fast-growing thermophilic bacterium with a lag phase of ~20 min followed by an exponential phase extended up to 9 h in the SYT medium. α-Amylase *gt* secretion is growth associated, and maximum enzyme production is attained (32 U/ml) in the late exponential and early stationary phases of growth (results not shown).

We used simple GeBAflex tubes (Gene Bio-Application) for electro-eluting α-amylase *gt* band/protein, which accounted for around 75% recovery. The purity of α-amylase *gt* was confirmed by silver-stained native PAGE (data not shown). The far-UV CD spectrum of the protein is shown in Fig. 1. The spectrum of native α-amylase *gt* indicated the presence of

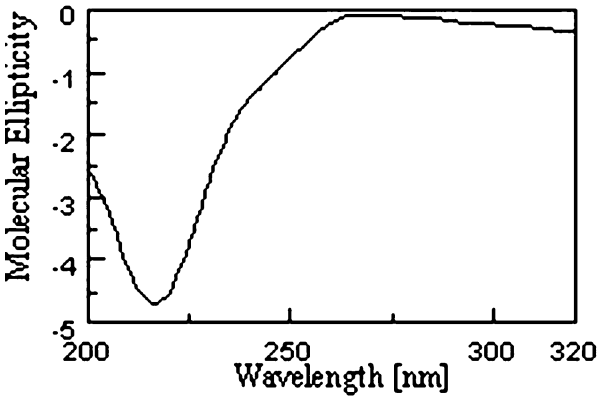

Fig. 1 The purified α-amylase *gt* was thermal-activated at 100 °C for 15 min in 0.1 M phosphate buffer (pH 8.0) and used for secondary structure determination by CD spectroscopy (ellipticity λ at 200 to 320 nm)

25% α-helices, 21% β-sheets, and 54% random coils. In such a fit, the contribution from turns and bends is included in the estimate of the random coil.

The excitation spectrum of native α-amylase *gt* showed peaks at 305 and 280 nm, when emission was fixed at 340 and 325 nm, respectively (results not shown). The fluorescence emission spectrum was high toward the blue-shifted signal. In the presence of KI, a significant increase in α-helix content and a marked decrease in random coil were recorded. In contrast, only a minor variation in β-sheet or turn was observed (results not shown). The KI treatment led to a loss of both fluorescence intensity (Fig. 2a) and α-amylase *gt* activity (Fig. 2b). The experimental K_{sv} and f_a values at 280 and 305 nm were found to be 11.1 and 9.75 and 1.0 and 0.4, respectively. The quenching fluorescence data of α-amylase *gt* in the native state and in the presence of different concentrations of acrylamide are shown in Fig. 3a.

The quenching data for all the quenchers were analyzed by the Stern–Volmer equation. A decrease in the fluorescence intensity was observed on adding acrylamide, and further addition resulted in only minimal changes. It is surprising to note that there

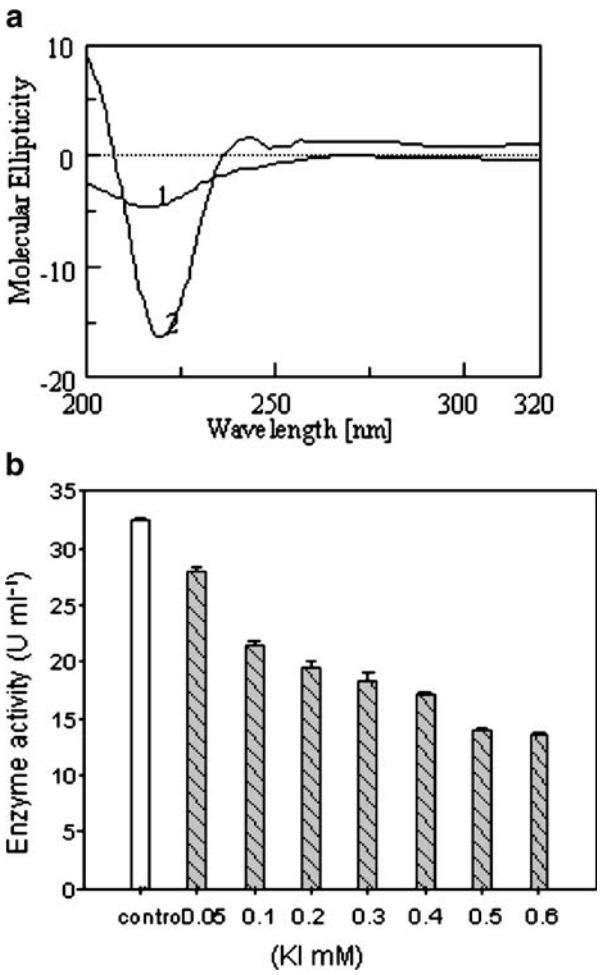

Fig. 2 Effect of anion quencher KI: **a** on secondary structure of α-amylase *gt* (spectra *1*, *2* represent native α-amylase *gt*, KI with native α-amylase *gt*; experimental conditions as in Fig. 1) and **b** on activity of native α-amylase *gt* in 0.1 M phosphate buffer (pH 8.0) at 100 °C for 10 min

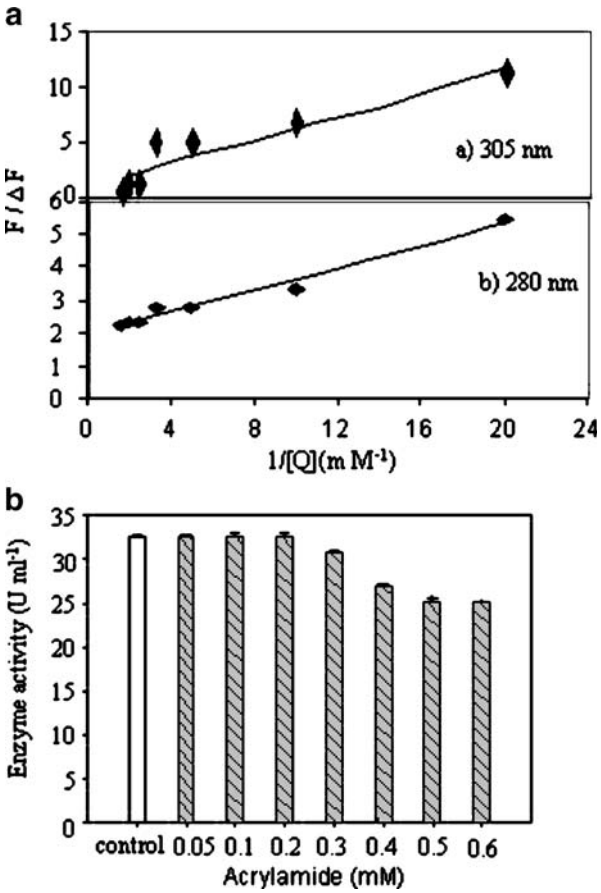

Fig. 3 Effect of non ionic quencher acrylamide: **a** Fluorescence quenching spectra, *A* different concentrations of acrylamide with thermal-activated α-amylase *gt* excited λ at 305 nm, *B* different concentrations of acrylamide with thermal-activated α-amylase *gt* excited λ at 280 nm, and **b** activity on native α-amylase *gt* (experimental conditions as in Fig. 2b)

was no quantitative change in the activity of the α-amylase *gt* even at high concentration of acrylamide (0.5 mM; Fig. 3b). The experimental K_{sv} and f_a values at 280 and 305 nm were found to be 3.4 and 2.56 and 0.49 and 0.28, respectively. The addition of CsCl decreased the fluorescence quenching of α-amylase *gt* (Fig. 4a) as compared to the anionic quencher (KI), which caused markedly higher quenching. The experimental K_{sv} and f_a values at 280 and 305 nm were found to be 1.5 and 2.0 and 0.25 and 0.23, respectively. These local environmental changes, however, had no effect on α-amylase *gt* activity (Fig. 4b).

The decrease in fluorescence intensity with different concentrations of Gdn-HCl at 305 and 280 nm excitation of native α-amylase *gt* (Fig. 5a). The Stern–Volmer binding constant (K_{sv}) was 4.13 M^{-1}, and the fraction of the total fluorescence available to the quencher (f_a) was 0.39, which indicated the total number of Trps exposed, in the presence of Gdn-HCl, to be ~2.0. On the other hand, as the Gdn-HCl concentration was increased, the loss of the whole conformational structure occurred, and also the amylolytic activity decreased drastically at 0.4 M Gdn-HCl (Fig. 5b).

Stabilization of the secondary structure was carried out by incorporating starch (0.1%) to a fixed concentration (0.1 mg/ml) of α-amylase *gt* and recording the conformational changes and α-amylase *gt* activity; this confirmed the protective nature of starch against

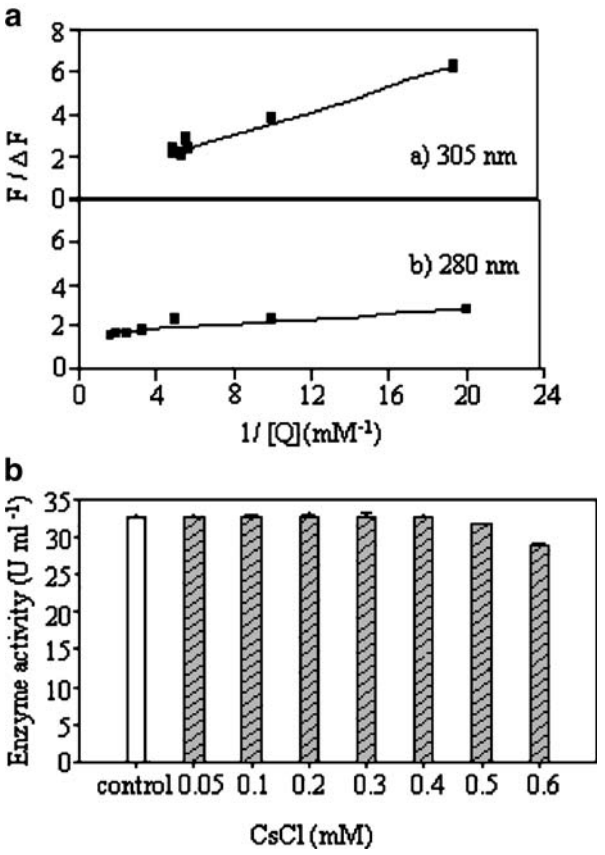

Fig. 4 Effect of cationic quencher CsCl: **a** Fluorescence quenching spectra, *A* different concentrations of CsCl with thermal-activated α-amylase *gt* excited λ at 305 nm, *B* different concentrations of CsCl with thermal-activated α-amylase *gt* excited λ at 280 nm, and **b** on activity of native α-amylase *gt* (experimental conditions as in Fig. 2b)

denaturation (Gdn-HCl [0.1 M]). Starch with α-amylase *gt* provided protection against denaturation with Gdn-HCl (Fig. 5c).

On the addition of urea, the fluorescence intensity of α-amylase *gt* decreased (Fig. 6a) and blue shifted, indicating a decrease in the polarity of the Trp environment. The experimental K_{sv} and f_a values at 280 and 305 nm were found to be 6.12 and 5.0 and 0.46 and 0.37, respectively. Around 50% decrease in the fluorescence intensity was observed with urea (0.6 M). The CD spectra in the presence of urea clearly revealed the unfolding of α-amylase *gt*, which led to changes in both α-helix as well as β-sheet content (Fig. 6b). These conformational changes occurring in the protein could be responsible for a decline in the α-amylase activity (Fig. 6c).

Trp residues exhibited the unique spectroscopic features, and a heterogeneous Trp environment was seen at λ_{280} and λ_{305} (Fig. 7a). NBS treatment led to the loss of both activity and fluorescence intensity, which is believed to be due to the oxidation of Trp to oxi-indole. The experimental K_{sv} and f_a values at 280 and 305 nm were found to be 2.21 and 1.92 and 0.27 and 0.21, respectively. In the presence of NBS (14 mM), α-helix content and α-amylase *gt* activity decreased with no observable change in β-sheets (Fig. 7b and c).

The fluorescence intensity increased with a shift in pH from neutral to the acidic range, while it decreased with increasing pH (results are not shown). As the pH was changed from

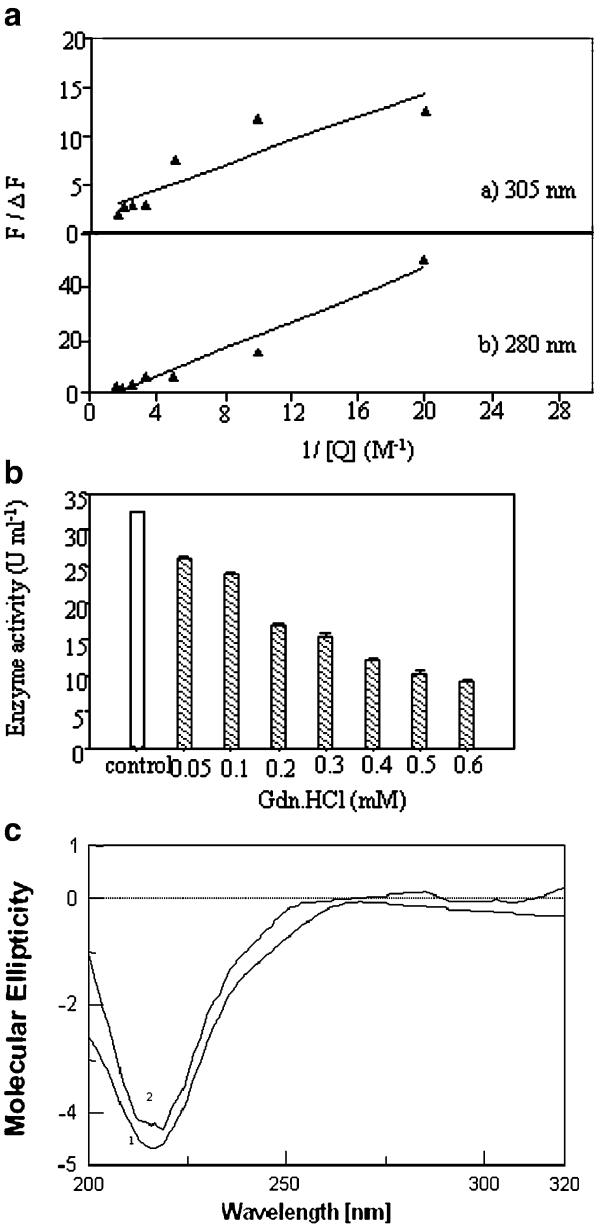

Fig. 5 Effect of inhibitor Gdn-HCl: **a** fluorescence-quenching spectra, *A* different concentrations of Gdn-HCl with thermal-activated α-amylase *gt* excited λ at 305 nm, *B* different concentrations of Gdn-HCl with thermal-activated α-amylase *gt* excited λ at 280 nm, and **b** on activity of native α-amylase *gt* (experimental conditions as in Fig. 2b), **c** on secondary structure of α-amylase *gt* (spectra *1*, *2* represent native α-amylase *gt*, native α-amylase *gt* with Gdn-HCl [0.1 mM], and starch [0.1%])

neutral to basic, there was no significant change in the α-helix content (Table 1). This was in accordance with the activity of the enzyme that changed marginally. Under acidic conditions, the enzyme showed a complete loss of both α-helix and β-sheet or turns (Table 1). The α-amylase *gt* activity decreased with the lowering of pH, and the highest activity was recorded at pH 8.0. Only a slight decline in α-amylase *gt* activity was seen with further increase in pH (Fig. 8a).

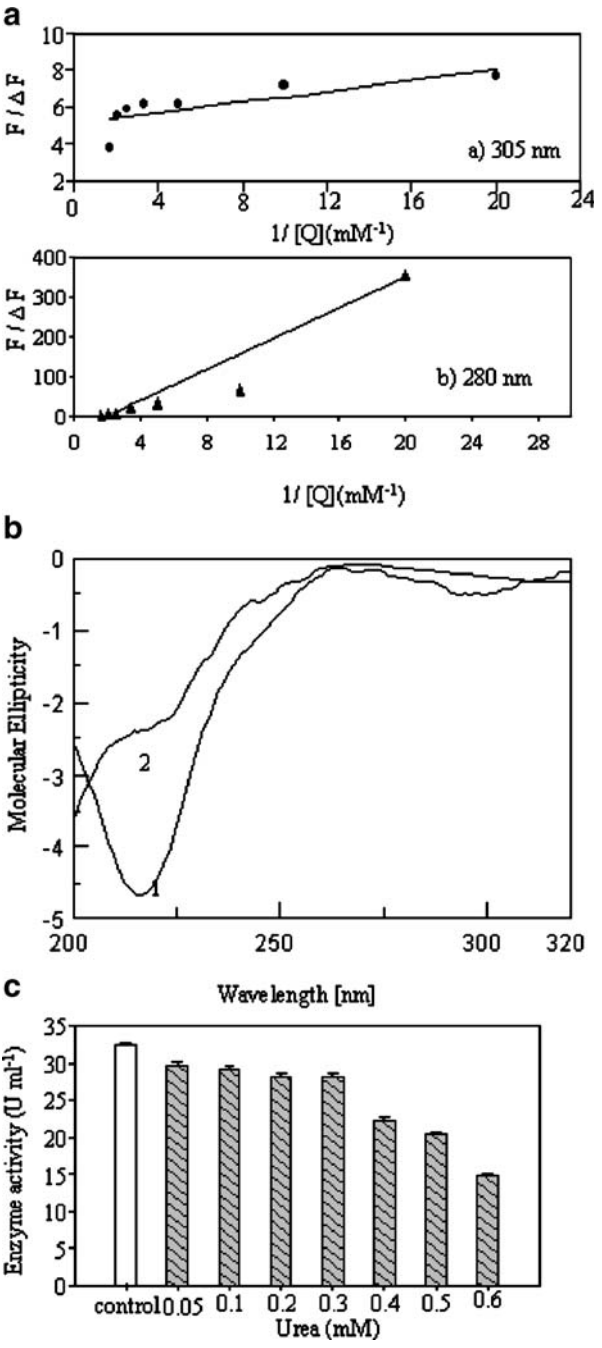

Fig. 6 Effect of urea: **a** Fluorescence-quenching spectra, *A* different concentrations of urea with thermal-activated α-amylase *gt* excited λ at 305 nm, *B* different concentrations of urea with thermal-activated α-amylase *gt* excited λ at 280 nm, **b** on secondary structure of α-amylase *gt* (spectra *1* and *2* represent native α-amylase *gt* and urea [0.4 mM] with native α-amylase *gt*), and **c** on activity of native α-amylase *gt* (experimental conditions as in Fig. 2b)

As the temperature was increased, no change in the α-helix content was observed, which remained around 22% at all temperatures (Table 2). The enzyme activity also enhanced constantly with increase in temperature from 50 to 100 °C, and thereafter, it declined (Fig. 8b).

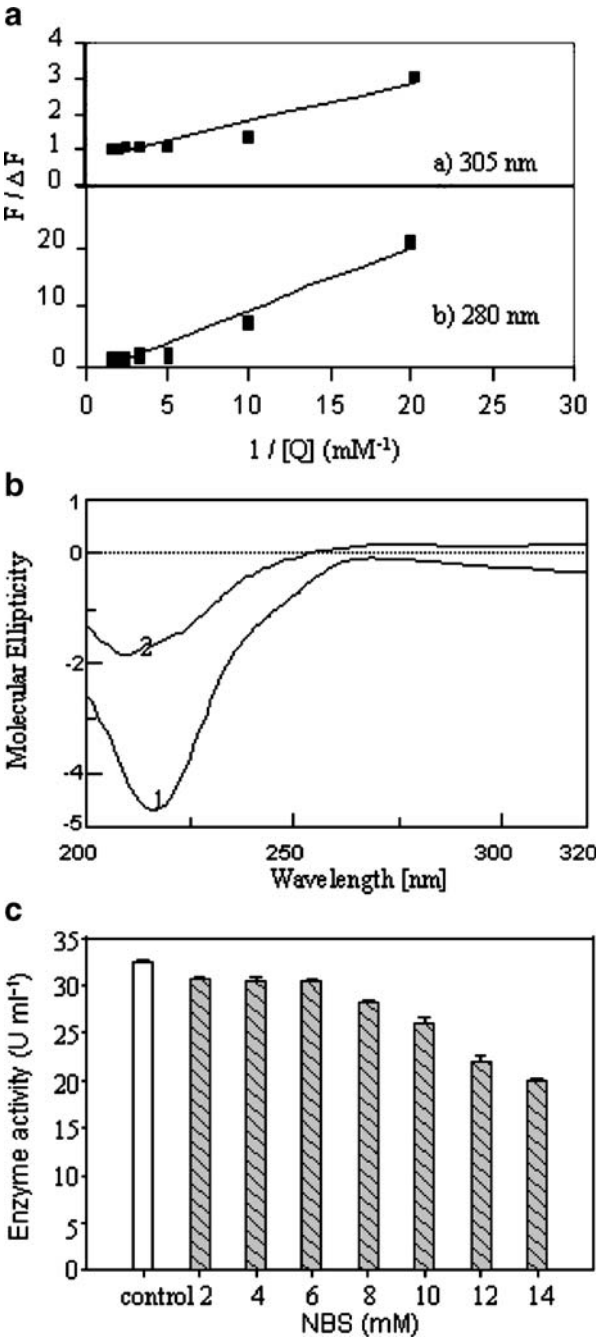

Fig. 7 Effect of NBS: **a** Fluorescence quenching spectra, *A* different concentrations of NBS with thermal-activated α-amylase *gt* excited λ at 305 nm, *B* different concentrations of NBS with thermal-activated α-amylase *gt* excited λ at 280 nm, **b** on secondary structure of α-amylase *gt* [spectra 1 and 2 represents native α-amylase *gt* and native α-amylase *gt* with NBS), and **c** on activity of native α-amylase *gt* (experimental conditions as in Fig. 2b)

Table 1 Effect of pH on secondary structure of α-amylase *gt* of an extreme thermophile *G. thermoleovorans*.

	pH		
	4.0 (%)	8.0 (%)	10 (%)
α-Helix	0.0	25	22
β-Sheet	100	21	36
Random coil	0.0	54	42

Discussion

Thermophilic organisms are important sources for the production of efficient thermostable enzymes [13]. Thermostable α-amylases are important not only for the study of biocatalysis and protein stability at extreme conditions but also for many biotechnological applications in food, chemical, and pharmaceutical industries [5].

α-Amylase *gt* was also purified by conventional chromatography [10], and, based on its location on the polyacrylamide gel, it was ensured that the electro-eluted protein band and the chromatographically purified protein were the same. The extraction of proteins from the polyacrylamide gel has been performed for many years by electro-elution [21], although it often yields low recovery and involves a high risk of contamination. To avoid these problems, we used simple GeBAflex tubes (Gene Bio-Application) for electro-eluting the α-amylase *gt* band/protein, which accounted for good recovery. The purity of α-amylase *gt* was confirmed by silver-staining native PAGE (data not shown). The α-amylase *gt* from *G. thermoleovorans* is a monomeric enzyme with a molecular mass of 26 kDa and p*I* of 5.4 [10]. The high-maltose-forming α-amylase *gt* is optimally active at 100 °C and pH 8.0, and

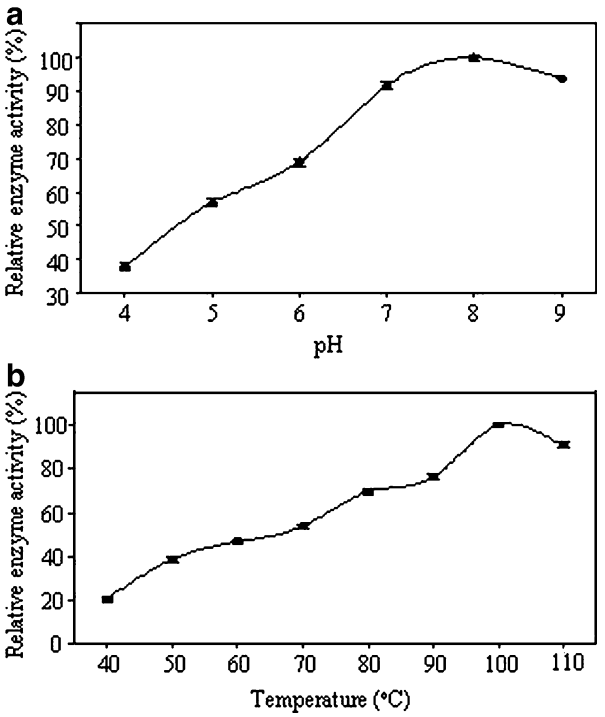

Fig. 8 **a** Effect of pH on α-amylase *gt* activity (experimental conditions as in Fig. 2b) and **b** effect of temperature on α-amylase *gt* activity (experimental conditions as in Fig. 2b)

Table 2 Effect of temperature on secondary structure of α-amylase *gt* of *G. thermoleovorans*.

	20 °C thermal activated at 100 °C (%)	60 °C (%)	70 °C (%)	80 °C (%)	90 °C (%)
α-Helix	25.0	25.3	22.3	22.1	22.4
β-Sheet	21.0	14.0	12.5	7.2	9.2
Random coil	54.0	60.7	65.2	70.7	68.4

its activity/stability is not affected by the addition of Ca^{2+} [10, 22] as observed in α-amylases from *Pyrococcus furiosus* [23] and *P. woesei* [24].

Modifications of crucial amino acids in the active site of an enzyme are believed to bring about important changes in their biological character. These changes in the secondary structure and the positions of important aromatic residues can be monitored by CD and fluorescence studies, and subsequently, their effect can be analyzed by estimating the enzyme activity. The secondary structure of the purified α-amylase *gt* was determined by CD spectroscopy. The CD spectrum of α-amylase *gt* showed similarity with that reported for *Bacillus* spp. [25].

The structural investigation included different experimental conditions such as using different quenchers, such as neutral (acrylamide), anionic (I^-), and cationic (Cs^+) quenchers, and denaturants. In the presence of KI, a significant increase in α-helix content and a remarkable decrease in random coil were recorded. In contrast, only a minor variation in β-sheet or turn was observed. The KI treatment led to a loss of both fluorescence intensity and α-amylase *gt* activity. The α-amylase was sensitive to local change effects; quenching of α-amylase *gt* with KI revealed the charged state of the local environment around Trp fluorophores. According to Gao et al. [26], the Trp residues of α-amylase *gt* are located in a relatively positively charged environment. The related K_{sv} values suggested that the optimal Van der Waals distances and electrostatic interactions between the enzyme and ions increased with addition of low concentrations of KI (0.6 mM). Charged quenchers are known to probe only the surface-exposed Trps, while the polar and uncharged acrylamide can diffuse into the protein matrix and quench the fluorescence of surface as well as buried Trp residues [27]. The quenching data for all the quenchers was analyzed by the Stern–Volmer equation. A decrease in the fluorescence intensity was observed on adding acrylamide, and further addition resulted in only minimal changes. It is surprising to note that there was no quantitative change in the activity of the α-amylase *gt* even at a high concentration of acrylamide. This suggests an interaction of acrylamide with the enzyme by means of a simple collision process and does not apparently interfere with the substrate-binding site [26].

The addition of CsCl increased the fluorescence intensity of α-amylase *gt* as compared to the anionic quencher (KI), which caused markedly higher quenching. The increase in fluorescence could be attributed to the interactions of monovalent cesium ions with tryptophan residues present on the surface of the α-amylase *gt*.

Gdn-HCl is known to react reversibly with the carboxyl groups of amino acids [28]. At low concentrations, Gdn-HCl is believed to shield the carboxyl ends of Trp residues in the active site. Stabilization of secondary structure was carried out by incorporating starch (0.1%) to a fixed concentration (0.1 mg/ml) of α-amylase *gt* and recorded the conformational changes and α-amylase *gt* activity; this confirmed the protective nature of starch against denaturation (Gdn-HCl [0.1 M]). Starch appears to interact noncovalently

with α-amylase *gt* and provide protection against denaturation with Gdn-HCl. Similar observations have been reported in *B. licheniformis* [14].

On adding urea, the fluorescence intensity of α-amylase *gt* decreased and blue shifted, indicating decrease in the polarity of the Trp environment. Around 50% decrease in the fluorescence intensity was observed with urea (0.6 M), which may either be due to the quenching of Trp residues or unfolding of α-amylase *gt* that exposed the buried residues to water as observed in *Bacillus firmus* [29]. The CD spectra in the presence of urea clearly revealed the unfolding of α-amylase *gt* that led to changes in both the α-helix as well as β-sheet; these conformational changes occurring in the protein could be responsible for a decline in the α-amylase activity [26]. These experimental observations suggested that Trp residues are located in the carbohydrate-binding site and are involved directly in maintaining the secondary structure as observed in *Bacillus* sp. [16].

The role of Trp residues in α-amylase *gt* was investigated by analyzing the changes in fluorescence of the NBS-treated α-amylase *gt* at λ_{280} and λ_{305}. NBS treatment led to the loss of both activity and fluorescence intensity, which is believed to be due to the oxidation of Trp to oxi-indole. Similar results have been reported in case of α-amylase of *Bacillus* sp. IMD 434 [16, 30]. A decrease in α-amylase *gt* activity was observed at higher concentration of NBS; this observation suggested that the Trp residues are not involved in the active site of α-amylase *gt* but are important in maintaining the secondary structure and coordinating the α-amylase *gt* activity [31]. High concentration of NBS led to a loss of α-amylase *gt* activity. There was, however, no change in α-amylase *gt* activity at low concentration, which was probably not sufficient to reach the hydrophobic core of Trp for oxidation.

When the fluorescence spectra of α-amylase *gt* were also recorded as a function of pH, the fluorescence intensity increased with a shift in pH from neutral to an acidic range, while it decreased with increasing pH. Increase in fluorescence intensity at low pH is attributed to the lifting of Trp residues in the hydrophobic core of the protein. Similar results have been observed in protein concanavalin A [15, 32]. As the pH was changed from neutral to basic, there was no significant change in the α-helix content. This was in accordance with the activity of the enzyme that changed marginally. Under acidic conditions, the enzyme showed a complete loss of both the α-helix and β-sheet or turns. These changes suggested a loss of the ordered secondary structure that led to a decrease in the α-amylase *gt* activity. The α-amylase *gt* activity decreased with the lowering of pH, and the highest activity was recorded at pH 8.0. Only a slight decline in α-amylase *gt* activity was seen with a further increase in pH.

When the temperature was increased, there was no change in the α-helix content, and this remained around 22% at all temperatures. The enzyme activity also enhanced constantly with an increase in temperature from 50 to 100 °C, and thereafter, it declined. This is in agreement with the fact that the activity of α-amylase *gt* was retained even at elevated temperatures. Thus, α-helix appeared to play a major role in the catalytic activity of α-amylase *gt*.

In conclusion, the observations presented in this communication provide some mechanistic features related to the role of aromatic Trp moieties and the positive nature of the microenvironment around the Trp moieties in stabilizing the hyperthermostable, Ca^{2+}-independendent, and high-maltose-forming α-amylase of *G. thermoleovorans*. There was no change in the α-helix content or the enzyme activity with an increase in temperature (60–100 °C), suggesting a critical role of the α-helix content in maintaining the catalytic activity of α-amylase.

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