International Journal of Biological Macromolecules 51 (2012) 146–152

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

A differential behavior of α-amylase, in terms of catalytic activity and thermal stability, in response to higher concentration $CaCl_2$

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ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 8 April 2012
Accepted 12 April 2012
Available online 20 April 2012

Keywords:
α-Amylase
Thermal stability
Thermal denaturation
$CaCl_2$
Activation energy

ABSTRACT

A differential relationship was observed between thermal stability and catalytic activity of α-amylase in the presence of different concentrations of $CaCl_2$. The enzyme displays optimum catalytic activity in the presence of 1.0–2.0 mM $CaCl_2$. Further addition of $CaCl_2$ leads to inhibition of the enzyme, however, at the same time the enzyme gains an additional resistance against thermal denaturation. It was evident that the enzyme is thermodynamically more stable (compared to the active enzyme) in the presence of inhibitory concentration of $CaCl_2$. For example, the thermal transition temperature (T_m) of optimally active α-amylase was found to be 64 ± 1 °C, whereas, for the less active enzyme (in the presence 10 mM $CaCl_2$) the value was determined to be 71 ± 1 °C. Similarly, the activation energy of thermal inactivation (*Ea*) was found to be 228 ± 12 kJ/mol and 291 ± 15 kJ/mol for the optimally active enzyme and the enzyme in the presence of 10 mM $CaCl_2$, respectively. Biophysical analysis of different states of the enzymes in response to variable calcium level indicates no significant change in the secondary structure in response to different concentration of $CaCl_2$, however the less active but thermodynamically stable enzyme (in the presence of higher concentration of $CaCl_2$) was shown to have relatively more compact structure. The results suggest that the enzyme has separate catalytic and structure stabilizing domains and they significantly vary in their functional attributes in response to calcium level.

1. Introduction

The structural stability of an enzyme is one of the most important parameters that decide its utility and commercial applicability. From the industrial application point of view, α-amylases (α-1,4-D-glucan glucanohydrolase, EC:3.2.1.1) constitute major group of enzymes catalyzing random hydrolysis of α-1,4-glycosidic bonds in starch and results in the formation of maltose and malto-oligosaccharides with retention of α-anomeric configuration [1,2]. They belong to the family 13 of glycosyl transferases which is characterized by the presence of $(\beta/\alpha)_8$ barrel. The structure of enzyme is constituted by three domains called as domain A, B and C. Domain A consists of a central $(\beta/\alpha)_8$ barrel which forms the core of the enzyme molecule. Domain B is formed by a protrusion between third α-helix and third β-sheet of $(\beta/\alpha)_8$ barrel [3]. The size and structure of the domain B varies among various molecules of the α-amylase family [4]. Domain C is located at the C-terminal part of the amino acid sequence of enzyme. α-Amylases have wide range of application in clinical and analytical chemistry [5–9]. They have major role in starch saccharification and liquefaction in food, textile, paper, brewing and distillery industries.

The structural stability of α-amylases depends on various intrinsic (e.g., amino acid sequence) and extrinsic (e.g., solution conditions, such as pH, presence of cofactors, metal ions, etc.) factors [10–16]. Calcium is an essential metal required for catalytic activity and structural stability of most of the α-amylases [14]. Metal ions are essential for both activity and structural integrity of several metalloproteins [15,16]. The right stoichiometry of metal ions and proteins/enzymes is necessary for displaying their optimum activity and stability. The sub-optimal level of calcium may have great influence on structure, activity and thermal stability of the enzyme [17–20]. Almost all the α-amylases contain at least one calcium ion per enzyme molecule for their catalytic activity [21]. It has been shown that the removal of calcium from barley α-amylase irreversibly inactivates the enzyme, whereas the bacterial α-amylases regain the activity after addition of calcium [22]. Several reports indicate that the role of calcium ion in α-amylases is mainly structural [4,23,24] and their catalytic site is located far away from the calcium-binding site. There are certain α-amylases which catalyze the reaction independent of calcium ion concentration [25]. However, in majority of cases it is evident that the

Abbreviations: CD, circular dichroism; *Ea*, activation energy of thermal inactivation; EGTA, ethylene glycol-bis (β-amino-ethylether)-N,N,N′,N′-tetra-acetic acid tetrasodium; kr, thermal inactivation rate constants; K_{sv}, Stern–Volmer constant; T_m, thermal transition temperature; λ_{max}, fluorescence emission maximum.

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http://dx.doi.org/10.1016/j.ijbiomac.2012.04.013

activity and structural stability of the enzymes is largely influenced by variation of calcium level [17,26,27]. Enhancement in the structural stability of enzymes by various means lead to rearrangement of interacting forces that may or may not have an effect on activity. Considering the above perspectives, the objective of the present study was to investigate the concentration dependent effect of calcium on activity and stability of α-amylase and to establish a correlation between activity and stability.

2. Materials and methods

2.1. Materials

α-Amylase type II (A6380, from *Bacillus amyloliquefaciens*), dinitrosalisylic acid, corn starch, $CaCl_2$, Potassium-Sodium-tartarate and ethylene glycol-bis (β-amino-ethylether)-N,N,N′,N′-tetra-acetic acid tetrasodium (EGTA) were procured from Sigma Chemical Company, St. Louis, MO, USA. Citric acid, *tri*-sodium citrate, NaOH and acrylamide were procured from E-Merck, Mumbai, India. Regenerated cellulose dialysis membrane was procured from Spectrum Laboratories Inc., Rancho Dominguez, CA. Enzyme solutions were prepared in 20 mM citrate buffer (pH 5.9) and dialyzed extensively against the same buffer to remove the added excipients, freeze dried and stored at 0 °C for the future use. In order to remove the intrinsic calcium the enzyme was dialyzed against 4 mM EGTA overnight followed by dialysis against the buffer to remove the EGTA. Quartz triple distilled water was used throughout the experiments.

2.2. α-Amylase activity

The α-amylase activity was measured according to the method of Bernfeld [28] for the estimation of reducing sugars using starch as substrate. The solution of α-amylase was prepared in 20 mM citrate buffer (pH 5.9) for the activity measurement. To 1 ml of enzyme solution, 1 ml of 1% (w/v) starch solution was added and the reaction mixture was incubated at 37 °C in a temperature controlled shaking water bath for 5 min. The reaction was terminated by addition of 2 ml of 1% alkaline dinitrosalisylic acid and then the reaction mixture (4 ml) was subjected in boiling water bath for 10 min, cooled down to room temperature (25 °C) and diluted five times using triple distilled water. After thorough mixing the absorbance was recorded at 540 nm in Shimadzu UV-1601 UV-Visible spectrophotometer, and reducing sugar equivalent was determined using maltose standard. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of maltose equivalent from 1 ml of 1% starch solution at 37 °C in 5 min at pH 5.9.

2.3. Protein estimation

Protein concentration of α-amylase solution was determined by using its extinction coefficient ($E^{1\,cm}_{1\%}$) in aqueous medium. The absorbance of enzyme solution was recorded at 280 nm in Shimadzu UV-1601 UV-Visible spectrophotometer and concentration was calculated using $E^{1\,cm}_{1\%}$ value of α-amylase as 14.46 [29]. Alternatively protein concentration of the enzyme was estimated by Lowry method [30] using BSA as standard.

2.4. Kinetics of thermal inactivation

The enzyme solutions were prepared in 20 mM citrate buffer (pH 5.9) and incubated at different temperatures in presence of different concentrations of $CaCl_2$. The samples were withdrawn after regular interval, cooled down on ice bath and residual activity was measured at 37 °C. The percentage residual enzyme activity was calculated based on the original activity of enzyme (as 100%, without inactivation). Thermal inactivation of calcium-depleted α-amylase was carried out in the temperature range of 40–55 °C, where as in presence of 2, 5 and 10 mM $CaCl_2$ it was performed in the range of 50–70 °C. The thermal inactivation rate constants (kr) were determined by plotting the percentage residual activity vs incubation time at each temperature. The activation energy (*Ea*) and other thermodynamic parameters of thermal inactivation of enzyme were obtained from Arrhenius plot [31,32].

2.5. Thermal denaturation of α-amylase

The thermal denaturation of enzyme in presence of different concentrations of $CaCl_2$ was monitored by recording the absorbance at 287 nm in the range of 30–90 °C, using temperature scan rate of 1 °C/min in a Cary Varian 100-bio UV-Visible spectrophotometer. The protein concentration in all the samples was maintained as 0.15 mg/ml. The T_m was obtained after normalizing the absorbance of native and denatured enzyme between 0 and 1. The fraction unfolded (F_u) was plotted against temperature and T_m was determined by interpolating the curve on temperature axis at which the F_u was found to be 0.5. By assuming the simple two state mechanism of protein denaturation the standard enthalpy change (ΔH°_{Tm}) and standard entropy change (ΔS°_{Tm}) at T_m were determined using well established equations [31,32].

$$F_u = \frac{A_T - A_N}{A_D - A_N} \quad (1)$$

$$K_D = \frac{F_u}{1 - F_u} \quad (2)$$

where A_N and A_D are the spectral properties of native and denatured enzyme respectively. A_T is the spectral property of enzyme at temperature T (°C) and K_D is the thermal denaturation equilibrium constant. The ΔG°_{25} for thermal denaturation of protein was calculated by using standard equations [32].

2.6. Thermal aggregation of α-amylase

Thermal aggregation of α-amylase was measured in presence of different concentration of $CaCl_2$ using centrifugation [33]. The enzyme solutions (0.15 mg/ml) were incubated in presence of different concentrations of $CaCl_2$ for 30 min at 60 °C, cooled on ice bath and centrifuged at 10,000 rpm for 1 h and protein concentration in the supernatant was determined by Lowry method [30]. The enzyme sample without added $CaCl_2$ was taken as control and the percentage aggregation was calculated based on values obtained before and after the heat treatment.

2.7. Intrinsic fluorescence and acrylamide quenching

Intrinsic fluorescence spectra of α-amylase (50 μg/ml) were recorded at 25 °C in presence of different concentration of $CaCl_2$ in a Shimadzu spectrofluorophotometer model RF-5000, equipped with temperature-controlled water bath. The fluorescence excitation wavelength was set at 280 nm and emission spectra were recorded in the range of 300–400 nm using slit width of 10 and 5 nm for excitation and emission, respectively. Acrylamide quenching of α-amylase in the presence of different concentration of $CaCl_2$ was carried out by sequential addition of acrylamide aliquots to enzyme solution and subsequent monitoring of reduction in fluorescence intensity. Aliquots of 10 μl of 2 M acrylamide stock solution were added to 2 ml of α-amylase (50 μg/ml) solution each time and mixed thoroughly by inverting. The fluorescence excitation wavelength was set at 280 nm and emission spectra were recorded in the range of 300–400 nm using slit width of 10 and 5 nm for excitation and emission, respectively. The fluorescence quenching