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Stability, activity and binding properties study of α-amylase upon interaction with Ca^{2+} and Co^{2+}

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The interaction of α-amylase from *Bacillus amyloliquefaciens* (BAA) with divalent calcium and cobalt cations was studied by equilibrium dialysis, isothermal titration microcalorimetry, UV spectrophotometry and temperature scanning spectrophotometry methods at 27 °C in Tris buffer solution at pH 7.5. There is a set of 17 sites for calcium binding on the enzyme with weak positive cooperativity in binding. The binding of calcium is exothermic ($\Delta H = -16.0$ kJ mol^{-1}) with mean dissociation constant for binding of 0.55 mM. The binding of calcium stabilized the enzyme against surfactant and thermal denaturation. Moreover, the binding of calcium prevents the spontaneous decrease in biological activity of α-amylase. There is a set of 25 non-cooperative sites for cobalt binding on the enzyme. The binding of cobalt is exothermic ($\Delta H = -18.5$ kJ mol^{-1}) with mean dissociation constant for binding of 0.12 mM. The enzyme activity increased significantly with increasing concentration of cobalt; however, the temperature of denaturation of the enzyme decreased. So, divalent calcium and cobalt cations act as stabilizer and activator, respectively, for BAA.

Key words: α-amylase, calcium, cobalt, activator, stabilizer, titration calorimetry.

Introduction

α-Amylase (α-1,4 glucan-4-glucanohydrolase; EC 3.2.1.1) catalyzes the hydrolysis of α-1,4 glycosidic linkages of starch components and glycogen (Gilles et al., 1996; Witt & Sauter, 1996). α-Amylase is widely distributed in plants, animals, and microorganisms and shows varying action patterns depending on the source (Hagenimana et al., 1992). Various aspects of the enzyme have been investigated extensively: its protein structure and function (Brady et al., 1991; Kadziola et al., 1994; Machius et al., 1995), its mechanism of secretion through the cell membrane, and its industrial application (Fogarty, 1983; Fogarty & Kelly, 1990).

Calcium is required to maintain the structural integrity of α-amylase (Vallee & Meunier, 1959). Removal of calcium leads to decreased thermostability and/or decreased enzymatic activity (Violet et al., 1989), or increased susceptibility to proteolytic degradation (Machius et al., 1995). So far, it has not been possible to obtain crystals of α-amylase from *Bacillus amyloliquefaciens* (BAA)

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with bound calcium (MACHIUS et al., 1995). From measurements in the presence of EDTA and Ca^{2+}, it has been reported that BAA is stabilized by the binding of calcium ions (MAASSEN, 1991).

There are some reports on the requirement of Mg^{2+} and Ca^{2+} cations for stability and activation of BAA (OKITA & PREISS, 1980; WITT & SAUTER, 1996). The affinity between divalent metal ions and the α-amylase molecules varies considerably with the source of the enzyme (WINDISH et al., 1965). The inhibition of BAA activity with Hg^{2+} and Cu^{2+} was reported as 27.8 and 19.5%, respectively (MEMON et al., 1987). A lower inhibition (5.6%) at a high concentration of Co^{2+} was also reported for α-amylase from some sources (MEMON et al., 1987; WITT & SAUTER, 1996). The aim of the present investigation was to study the effects of divalent calcium cations, at low concentration, on the stability and thermal denaturation of α-amylase. The interaction of Ca^{2+} and Co^{2+} with the enzyme was investigated by equilibrium dialysis and isothermal titration microcalorimetry techniques to elucidate the stability, activity and metal-binding properties of BAA.

Material and methods

α-Amylase from *Bacillus amyloliquefaciens* (BAA), dodecyl trimethylammonium bromide (DTAB) and Tris-HCl were obtained from Sigma Chemical Co. Dinitrosalicylic acid (DNS), calcium nitrate tetrahydrate, cobalt chloride and soluble starch were purchased from Merck Co. Visking membrane dialysis tubing (MW cut-off 10000-14000) was obtained from Scientific Instrument Center Ltd. (SIC, Eastleigh, Hampshire, UK). All other materials and reagents were of analytical grade, and solutions were made in double-distilled water. Tris-HCl solution at 10 mM concentration, pH = 7.5, was used as a buffer.

Isothermal titration microcalorimetric method
The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel is made from stainless steel. Metal ion solution (6 mM for calcium and 24 mM for cobalt) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 2 mL enzyme solution, 1.23 mg/mL for calcium interaction and 1.00 mg/mL for cobalt interaction. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of calcium solution into the perfusion vessel was repeated 20 times, using 35 μL reagent for each injection. Injection of cobalt solution into the perfusion vessel was repeated 15 times, each injection involving 50 μL reagent. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the metal ion solution was measured as described above except enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of enzyme-metal interaction. The enthalpy of dilution of enzyme is negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

Equilibrium dialysis
Experiments were carried out at 300 K using a BAA solution with a concentration of 1.23 mg/mL for calcium interaction and 0.4 mg/mL for cobalt interaction, of which 2 mL aliquots were placed in dialysis bags and equilibrated with 2 mL of the metal ion solution, covering the required concentrations range, for over 96 h. Corrections for inequalities arising from Donnan effects were negligible at the ionic strength used. The free metal ion concentrations in equilibrium with complexes of enzyme-metal were assayed by the atomic absorption (Perkin Elmer, model 603) method. The molecular weight of BAA was taken to be 54,800.

Temperature-scanning spectroscopy
Absorbance profiles describing the thermal denaturation of BAA were obtained from a Gilford model 2400-S spectrophotometer fitted with a temperature programmer, which controls the speed of temperature change in melting experiments. The cuvette holder can accommodate four samples: one acts as a reference buffer solution and the others are for each experimental determination. All reference and samples cells had identical concentrations of calcium. The concentration of enzyme in the sample cells was 0.50 mg/mL for calcium interaction and 0.25 mg/mL for cobalt interaction. The recording chart reads the temperature, reference line (from the reference cuvette) and the absorbance change at 280 nm for each of the three samples in the cuvette.

Spectrophotometric study of denaturation
DTAB denaturation curves were obtained by measuring the maximum absorbance (280 nm) of the solutions containing 1.2 mg/mL BAA using a Shimadzu model UV-3100 spectrophotometer and 1 cm cuvettes thermostated to maintain the temperature at 27.0±0.1 °C. All measurements were made after BAA and DTAB had been incubated for over 5 min, after which time the absorbance did not change.

Assay of α-amylase activity
BAA was determined by the method of Bernfeld (BERNFELD, 1955). The assay system contained 1.0 ml of enzyme solution (with concentration of 0.1%, w/v) and 1.0 ml of 1% soluble starch (pH 7.5 in Tris buffer) solution. This reaction mixture was incubated at 27 °C for 3 minutes. The reaction was terminated

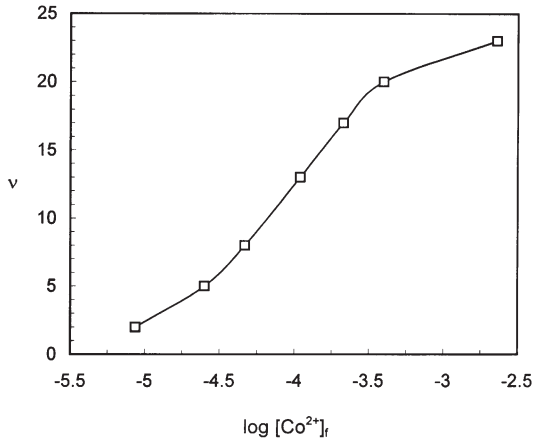


Fig. 1. Binding isotherm for divalent cobalt ions interacting with α-amylase at pH 7.5 and 27 °C.

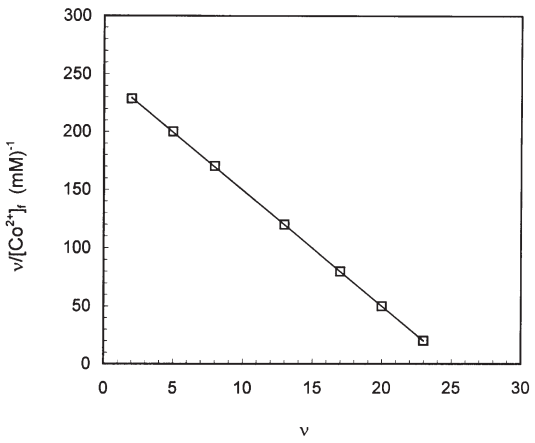


Fig. 2. The Scatchard plot for binding Co^{2+} to α-amylase at pH 7.5 and 27 °C, according to the Scatchard equation; $\nu/[Co^{2+}]_f = (g - \nu)/K$.

by the addition of 2.0 mL of DNS (3,4 dinitro salicylic acid) reagent solution (including calcium nitrate tetrahydrate and NaOH). Colour due to the reducing sugar liberated was developed by heating the reactants in a boiling water bath for 5 min and then rapidly cool in running tap water. After addition of 20 mL of double distilled water, the extinction value was determined at 540 nm. The blank was prepared in the same manner without enzyme.

One unit of α-amylase activity was defined as the amount of enzyme required to release, in 3 minutes from 1% soluble starch, reducing groups corresponding to 1 mg maltose hydrate.

Results and discussion

The binding isotherm has been plotted as the average number of cobalt ions bound to one macromolecule of α-amylase, ν, versus $\log[Co^{2+}]_f$, where $[Co^{2+}]_f$ is the free concentration of cobalt ion, as shown in Figure 1. In this case, the Scatchard plot is linear, as shown in Figure 2. Therefore, the binding of cobalt ions is non-cooperative (Scatchard, 1949; Saboury & Moosavi-Movahedi, 1994; Saboury et al., 1996). The number of binding sites for cobalt ion is 25, obtained from the intercept on the ν-axis. This value is slightly less than half the value of the number of amino acid residues with negative charges on the surface of protein. Also, the dissociation equilibrium constant can be obtained from the slope of the Scatchard linear plot; $K = 0.1$ mM.

The binding isotherm for the interaction of calcium ions with BAA has been plotted in Figure 3. In this case, the Scatchard plot is not linear, as shown in Figure 4. Therefore, the binding of calcium ion is cooperative (Saboury & Moosavi-Movahedi, 1994). The number of binding sites g, the dissociation equilibrium constant K and the Hill coefficient n can be obtained by fitting the experimental data to the Hill equation (Hill, 1910):

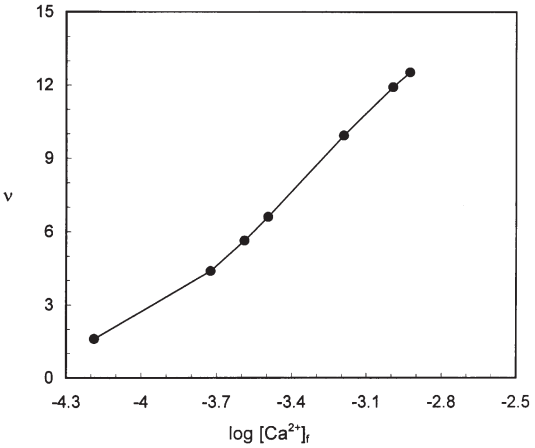


Fig. 3. Binding isotherm for divalent calcium ions interacting with BAA at pH 7.5 and 27 °C.

$$\nu = \frac{g(K_a[Ca^{2+}])^n}{1 + (K_a[Ca^{2+}])^n}$$

where K_a is the association equilibrium constant ($K_a = 1/K$). The number of binding sites for calcium ion is 17. Also, the dissociation equilibrium constant and the Hill coefficient are 0.50 ± 0.05

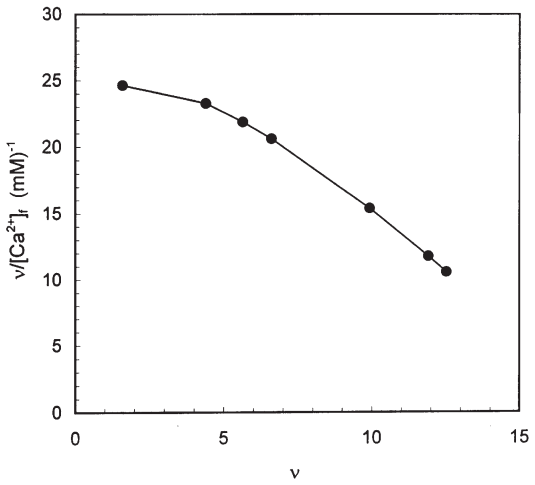


Fig. 4. The Scatchard plot for divalent calcium ions interacting with BAA at pH 7.5 and 27 °C.

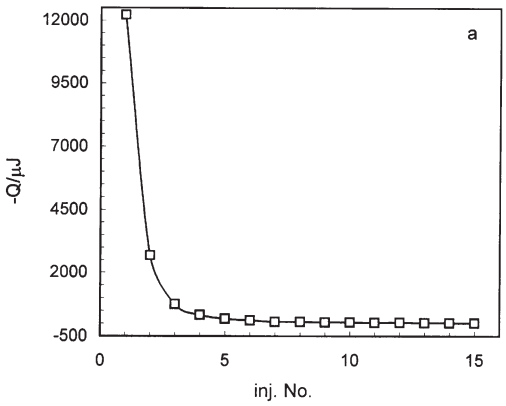
mM and 1.1 ± 0.05, respectively. So, calcium binding sites have weak positive cooperativity (it may be considered as a noncooperative system).

If the multiple binding sites on a macromolecule are identical and independent, by measuring the total heat of reaction Q at any fixed concentration of metal ligand L, the dissociation equilibrium constant K and the molar enthalpy of binding (ΔH) for the ligand L can be obtained from the linear plot of $Q/[\mathrm{L}]$ versus Q, according to the equation (SABOURY, 2000)

$$\frac{Q}{[\mathrm{L}]} = \frac{1}{K}(\Delta H - Q) \qquad (2)$$

The data obtained from isothermal titration microcalorimetry of BAA with cobalt ligand L is shown in Figure 5. Figure 5a shows the heat of each injection, and Figure 5b shows the cumulative heat related to each total concentration of L. The total concentration of cobalt ions after the 10 injections is much more than the total concentration of binding sites on a macromolecule with one binding site. Therefore, it can be assumed that the total and free concentrations of ligand are approximately equal. The linear plot of $Q/[\mathrm{L}]$ versus $-Q$ is shown in Figure 6. It was obtained by considering $g = 25$ (from Fig. 2). The values of K and ΔH obtained from axis intercepts are:

$$K = 0.12 \text{ mM} \qquad \Delta H = -18.5 \text{ kJ mol}^{-1}$$



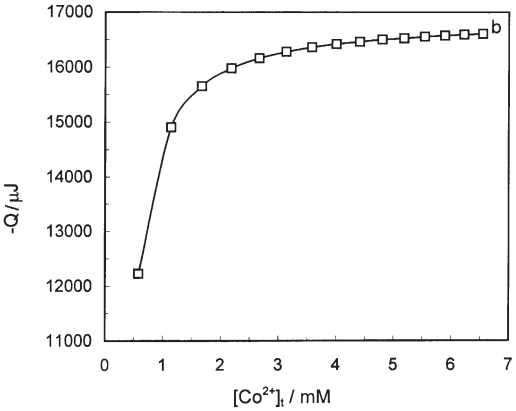


Fig. 5. (a) The heat of cobalt binding to α-amylase for 15 automatic cumulative injections, each of 50 μL of 24 mM cobalt solution, into the sample cell containing 2 mL α-amylase solution at a concentration of 1 mg/mL at pH 7.5 and 27 °C. (b) The cumulative heat related to the total concentration of cobalt, calculated per mole of binding site.

It is observed that the dissociation equilibrium constant value obtained from the Scatchard plot is approximately equal to the calorimetric value.

Equation (2) cannot be used for isothermal titration calorimetric data of BAA-calcium ion interaction, because the binding process is cooperative. In this case, equation (3) is useful (GHADERMARZI et al., 1998).

$$\Delta H = 1/A_\mathrm{i}\{(B_\mathrm{i} + K) - [(B_\mathrm{i} + K)^2 - C_\mathrm{i}]^{1/2}\} \qquad (3)$$

where $B_\mathrm{i} = [\mathrm{BAA}]_\mathrm{total} + [\mathrm{L}]_\mathrm{total}$, $C_\mathrm{i} = 4[\mathrm{BAA}]_\mathrm{total} \cdot [\mathrm{L}]_\mathrm{total}$, $A_\mathrm{i} = V_\mathrm{i}/2Q_\mathrm{i}$, V_i is the volume of the

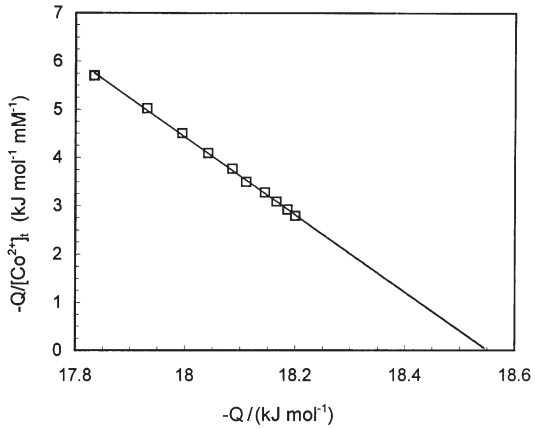


Fig. 6. $Q/[\mathrm{L}]$ versus Q, similar to the Scatchard linear plot, according to the equation (2), using data shown in Figure 5b.

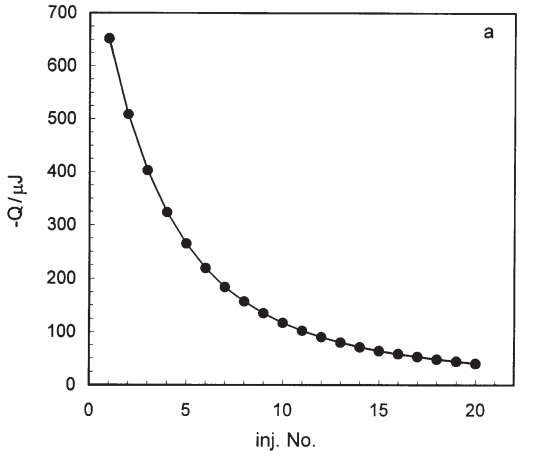
Table 1. The relative activity and denaturing temperature of α-amylase in the presence of different concentrations of Co^{2+}.

$[Co^{2+}]$/mM	% Activity	T_m/K
0	100	336
6	129	328
30	131	323
60	135	–
65	129	320

reaction solution. Equation (3) contains two unknowns, K and ΔH. A_i, B_i and C_i can be calculated for each injection, and then by fitting these known parameters to equation (3) using a computer program for nonlinear least-square fitting (JAMES et al., 1985), ΔH and K values may be obtained. The data obtained from isothermal titration microcalorimetry of α-amylase interaction with calcium ions is shown in Figure 7. Figure 7a shows the heat of each injection and Figure 7b shows the cumulative heat related to the total concentration of calcium ion. The results are:

$$K = 0.55 \pm 0.05 \text{ mM} \quad \Delta H = -16 \pm 0.5 \text{ kJ/mol}$$

The relative activities of the BAA were determined in the presence of different concentrations of divalent cobalt cation. These results are summarized in Table 1. The BAA activity increased significantly with Co^{2+} concentration. Even though



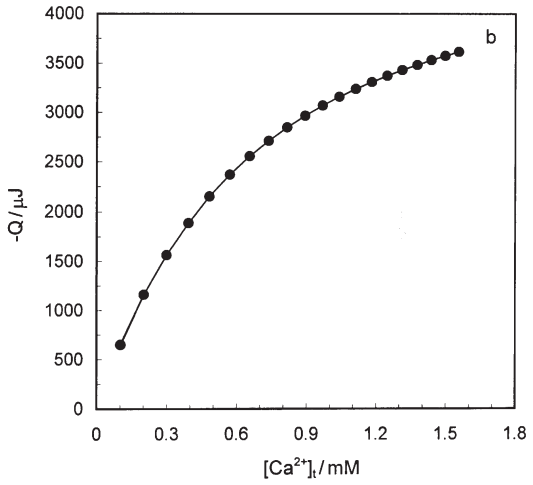


Fig. 7. (a) The heat of calcium binding to BAA for 20 automatic cumulative injections, each of 35 μL of 6 mM Ca^{2+} solution, into the sample cell containing 2 mL BAA solution at a concentration of 1.233 mg/mL at pH 7.5 and 27 °C. (b) The cumulative heat related to the total concentration of calcium, calculated per mole of binding site.

Co^{2+} is an activator for α-amylase, the denaturing temperature (T_m) of the enzyme is decreased with increasing concentration of Co^{2+} (see Table 1). The values of T_m in different concentrations of Co^{2+}were obtained from the midpoint of the change of optical density for the enzyme at $\lambda_{max} = 280$ nm with increasing temperature (see Fig. 8).

The thermal denaturation curves for BAA are

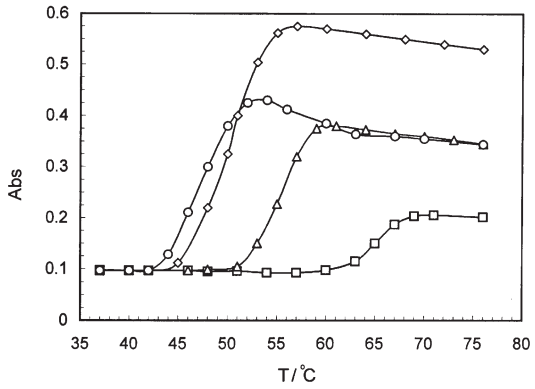
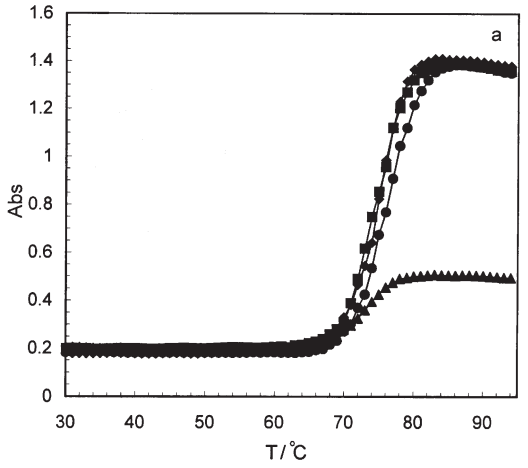


Fig. 8. The change of optical density of α-amylase (0.25 mg/mL) at $\lambda_{max} = 280$ nm with increasing temperature at different fixed concentrations of cobalt ion: 0 mM (□), 6 mM (△), 30 mM (◇) and 65 mM (○).



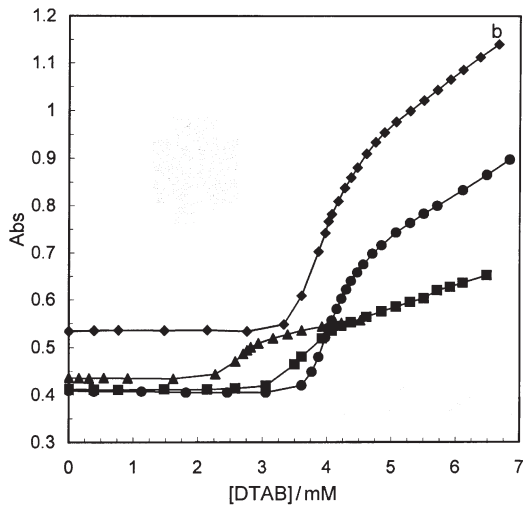


Fig. 9. The change of absorbance of BAA (0.5 mg/mL) at $\lambda_{max} = 280$ nm with increasing (a) temperature and (b) concentration of DTAB at different fixed concentrations of calcium ion: 0 mM (▲), 2 mM (■), 4 mM (♦) and 6 mM (●). The concentration of BAA was 0.5 mg/mL for thermal denaturation and 1.2 mg/mL for denaturation by DTAB at 27 ℃.

shown in Figure 9a. In all cases, denaturation was followed by measuring the absorbance at 280 nm in different concentration of calcium. The denaturing temperature (T_m) of the enzyme increases with increasing concentration of calcium. The values of T_m in different concentrations of calcium were obtained from the midpoint of the change of absorbance with increasing temperature. The values of T_m are 72.5, 74.4, 74.6 and 76.0 ± 0.5 ℃ in the absence and in the presence of 2, 4 and 6 mM calcium, respectively. Therefore, the presence of calcium led to more thermal stability. The essential feature of this study is the observed similarity between the effect of DTAB, a denaturant cationic surfactant, and temperature on the process of denaturation. The profiles of denaturation of BAA by DTAB are shown in Fig. 9b. The concentration of DTAB in the midpoint of the transition, $[DTAB]_{1/2}$, increases with increasing calcium concentration. So, calcium binding to BAA also stabilized the enzyme against denaturation by surfactant.

The free energy of protein unfolding ΔG° was calculated as a function of DTAB concentration by assuming a two-state mechanism and using equations (Pace, 1990)

$$F_D = \frac{A_N - A_{obs}}{A_N - A_D} \tag{4}$$

$$\Delta G^\circ = -RT \ln \frac{F_D}{1 - F_D} \tag{5}$$

where A_{obs} is the observed absorbance used to follow unfolding in the transition region, and A_N and A_D are the values of absorbance of the native and denatured conformations of the protein, respectively. Figure 10 shows the free energy of unfolding, calculated from equation (5) using the data in the Figure 9b; the free energy varies linearly with DTAB concentration over a limited region. The simplest method of estimating the conformational stability in the absence of denaturant, ΔG°

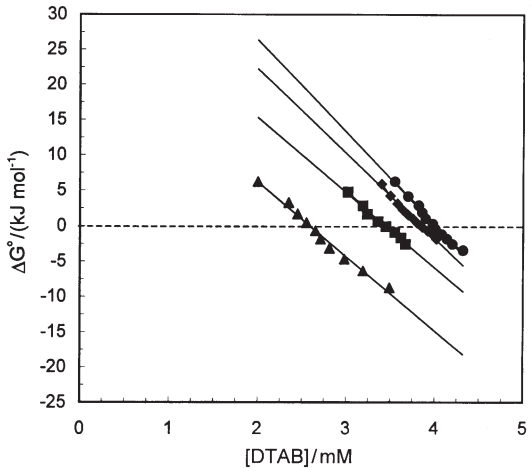


Fig. 10. The free energy of unfolding (calculated from eqn. (5) by assuming a two-state mechanism) vs. DTAB concentration, at different concentrations of calcium ion: 0 mM (▲), 2 mM (■), 4 mM (♦) and 6 mM (●), based on the data shown in Figure 9b.

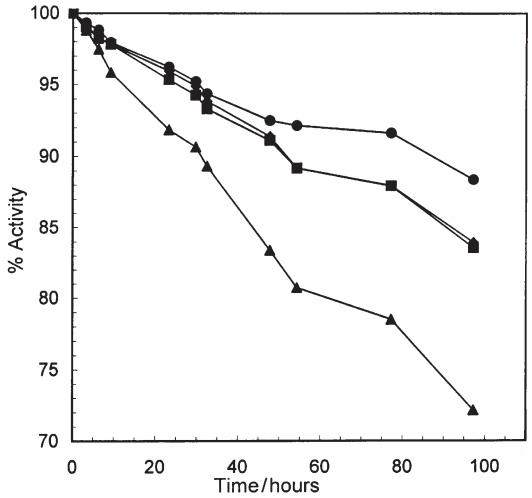


Fig. 11. Spontaneous inactivation of BAA at different concentrations of calcium ion: 0 mM (▲), 2 mM (■), 4 mM (♦) and 6 mM (●).

(H_2O), in different concentration of calcium, is to assume that linear dependence continues to zero concentration and to use a least-square analysis to fit the data to the equation (6) (Greene & Pace, 1974).

$$\Delta G^\circ = \Delta G^\circ(H_2O) - m[\mathrm{DTAB}] \qquad (6)$$

where m is a measure of the dependence of ΔG° on DTAB concentration. The values of $\Delta G^\circ(H_2O)$ are 27.2, 36.5, 46.1 and 52.7±0.2 kJ/mol in the absence and in the presence of 2, 4 and 6 mM calcium, respectively. Therefore, the existence of calcium led to greater stability of the enzyme.

The relative activities of the BAA were determined in the presence of different concentrations of divalent calcium cation. These results are shown in Figure 11. As can be seen, excess binding of calcium ion to BAA led to a decrease of spontaneous inactivation of the enzyme.

It is concluded that the binding of divalent cobalt cations on the surface of the α-amylase globular macromolecule causes an increase in enzyme activity by increasing flexibility (decreasing T_m). Also, the binding of divalent calcium cations on the surface of the BAA globular macromolecule causes an increase in enzyme thermal stability by decreasing flexibility (increasing T_m) and also increases enzyme stability against DTAB, a surfactant denaturant.

Acknowledgements

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