Journal of Enzyme Inhibition and Medicinal Chemistry, April 2004 Vol. 19 (2), pp. 157–160

Inhibition of β-Amylase Activity by Calcium, Magnesium and Zinc Ions Determined by Spectrophotometry and Isothermal Titration Calorimetry

M. UMAR DAHOT[a,*], A.A. SABOURY[b] and A.A. MOOSAVI-MOVAHEDI[b]

[a]*Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan;* [b]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*

(Received 1 September 2003; In final form 15 October 2003)

The inhibition effect of metal ions on beta amylase activity was studied. The inhibitor-binding constant (K_i) was determined by spectrophotometric and isothermal titration calorimetric (ITC) methods. The binding of calcium, magnesium and zinc ion as inhibitors at the active site of barley beta amylase was studied at pH = 4.8 (sodium acetate 16 mM) and T = 300 K. The K_i and enthalpy of binding for calcium (13.4, 13.1 mM and −14.3 kJ/mol), magnesium (18.6, 17.8 mM and −17.7 kJ/mol) and zinc (17.5, 17.7 mM and −20.0 kJ/mol) were found by spectrophotometric and ITC methods respectively.

Keywords: β-Amylase; Metal ions; Inhibition; Isothermal titration calorimetry

INTRODUCTION

β-Amylase (α-1, 4-Glucan maltohydrolase E.C. 3.2.1.2) is an exo-enzyme, which hydrolyzes α-D-(1–4) glucosidic linkages in starch from the non-reducing end of the substrate leading to β-maltose.[1,2] Maltose and malto-oligosaccharides produced by amylases find applications in the food, beverage and pharmaceutical industries. The importance of β-amylase is confirmed by the close relation between the β-amylase content of malt and diastatic power of derived wort.[3] β-amylase occurs widely in higher plants, microorganisms and also some animals. The enzymatic properties of bacterial β-amylase are different from those of the plant enzyme in optimum pH and in their ability to digest raw starch granules.

The inhibition of enzyme activity is one of the major regulatory devices of living cells and one of the most importance diagnostic procedures of the enzymologist. It provides valuable information about the specificity of an enzyme, the physical and chemical architecture of the active site, the kinetic mechanism of the reaction and various aspects of enzymatic catalysis and metabolic pathways.[4]

The aim of the present investigation was to study the effect of calcium, magnesium and zinc ions on β-amylase of barley. The type of inhibition mechanism of the binding of metal ions was investigated by spectrophotometric and isothermal titration micro-calorimetric methods.

MATERIAL AND METHODS

Materials

β-Amylase from barley (M_r 152000),[5] dinitrosalicylic acid (DNS), calcium chloride, zinc chloride, magnesium chloride, sodium acetate and soluble starch were purchased from Merck Co. All other materials and reagents were of analytical grade and solutions were made in double distilled water. Sodium acetate-acetic acid (16 mM) was used as a buffer, pH = 4.8.

Methods

β-Amylase Assay Method

β-Amylase activity was determined by the method of Bernfeld.[6] Assay system contained 1.0 ml of enzyme

*Corresponding author. E-mail: udahot@hyd.paknet.com.pk

ISSN 1475-6366 print/ISSN 1475-6374 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/14756360310001650255

solution (0.1%, w/v) and 1.0 ml of 1% soluble starch (pH = 4.8 in acetate buffer) solution. The reaction mixture was incubated in a water bath at 27 ± 1°C for 3 min and the reaction was terminated by the addition of 2.0 ml DNS (3,4-dinitro -salicylic acid) reagent. Colour due to the reducing sugar liberated was developed by heating the reactants in a boiling water bath for 5 min and then rapidly cooling in running tap water. After addition of 20 ml of double distilled water, the absorbance was determined at 540 nm. The blank was prepared in the same manner without enzyme.

One unit of β-amylase activity was defined as the amount of enzyme required to release a reducing group in 3 min from 1% soluble starch corresponding to 1 mg maltose hydrate.

Effect of Metal Ions on β-amylase

The activity of β-amylase was determined after incubation of the enzyme solution with 2–10 mM calcium chloride, magnesium chloride and zinc chloride in sodium acetate buffer, pH = 4.8 for 5 min at 27 ± 1°C. Afterwards, β-amylase activity was determined by the standard assay method described above.

Isothermal Titration Microcalorimetric Method

The isothermal titration microcalorimetric experiment was 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 semi conducting thermopile (multi-injection thermocouple plates) positioned between the vessel holder and the surrounding heat sink. The insertion vessel was made from stainless steel. Metal solutions (5 mM) were injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 ml enzyme (1.0 mg/ml), including acetate buffer. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of calcium, zinc and magnesium solution into the perfusion vessel was repeated 20 times and each injection was for 35 μl reagent. The calorimetric signal was measured by a digital voltmeter that was part of a computerized system. The heat of each injection was calculated by the "Thermometric Deigitam 3" software program. The heat of dilution of the calcium, zinc and magnesium solutions were 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 the enzyme was negligible. The microcalorimeter was frequently calibrated during the course of the study.

RESULTS AND DISCUSSION

The data were obtained in experiments to determine the effect of calcium, magnesium and zinc on beta-amylase and to establish the type of inhibition mechanism. A series of straight lines of reciprocal velocity (1/V) *vs* inhibitor concentration was observed, which converged on the abscissa at a point away from the origin (Figure 1a, b and c). This reflects competitive inhibition since the lines intersect each other above the horizontal axis in the left hand upper quadrant.[7,8]

The data obtained from isothermal micro-calorimetry of the β-amylase interaction with calcium, magnesium and zinc ions is shown in Figure 2. Figure 2a shows the heat of each injection

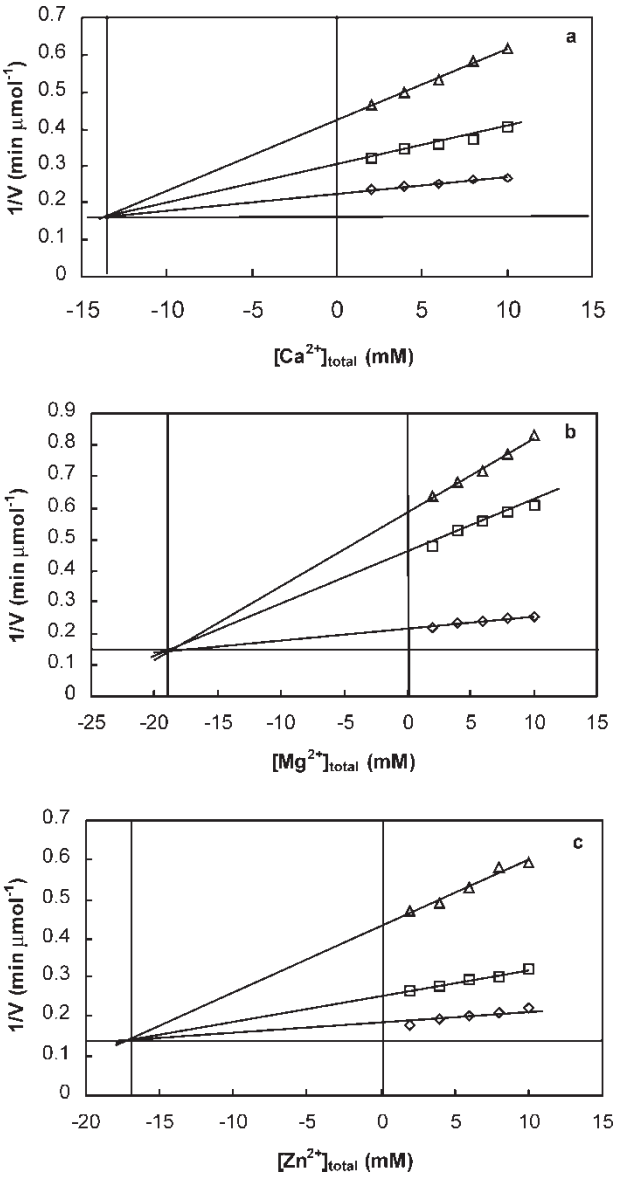

FIGURE 1 Dixon plots for kinetics of β-amylase at pH = 4.8 and T = 300 K in the presence of different fixed concentrations of substrate 2% (Δ), 5% (□) and 10% (◇) in the presence of Ca^{2+}(a), Mg^{2+}(b) and Zn^{2+}(c).

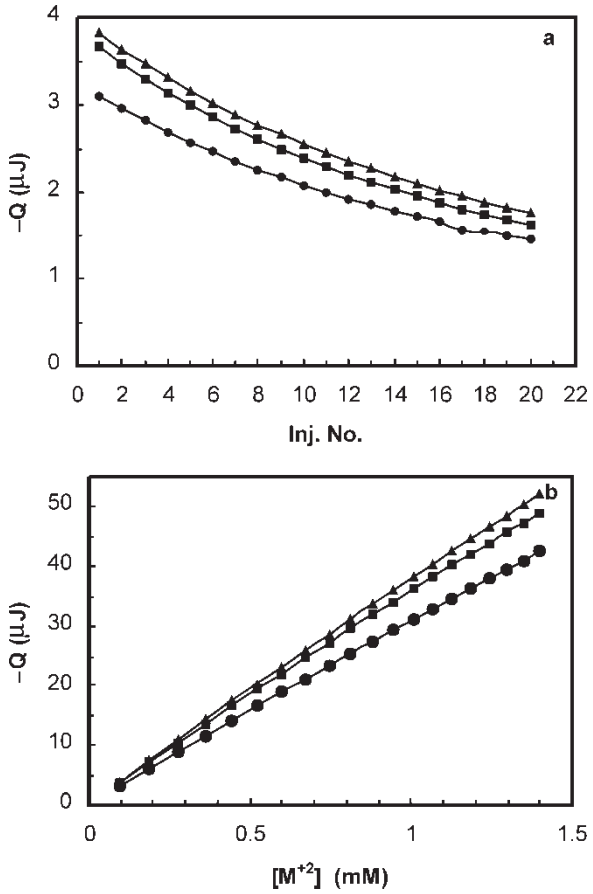

FIGURE 2 a) The heat of metal binding to β-amylase for 20 automatic cumulative injections, each of 35 μl, of metal ion solution, 5 mM, into the sample cell containing 1.8 ml β-amylase solution at a concentration of 1.0 mg/ml. b) The heat of binding versus total concentration of metal ion, calculated from Figure 2a: (■) for Ca^{2+}, (●) for $Mg^{2+}$and (▲) for Zn^{2+}.

and Figure 2b shows the heat related to the total concentration of calcium, magnesium and zinc ions. The following equation was used for the calorimetric data analysis.[9–12]

$$1/Q = (K_i/\Delta H)(1/[M]) + (1/\Delta H) \quad (1)$$

Where Q is the heat value, ΔH is the enthalpy of binding and [M] represents the concentration of metal ions.

A plot of 1/Q versus 1/[I] is shown in Figure 3a, b and c for calcium, magnesium and zinc, respectively. Each titration curve in Figure 2b should form a great portion of a single hyperbolic curve, where its double reciprocal graph gives a very good linear plot ($R^2 = 0.999$). The linear plots show that the assumption of a 1:1 ratio for the metal binding to the enzyme is true and that weaker additional binding sites were not involved. These linear plots have been analyzed and values of K_i and ΔH obtained by spectrophotometric and ITC methods are shown in Table I. A good correlation between the radius of the metal ion and the K_i value was observed in the present study. From the data obtained, it can be seen that

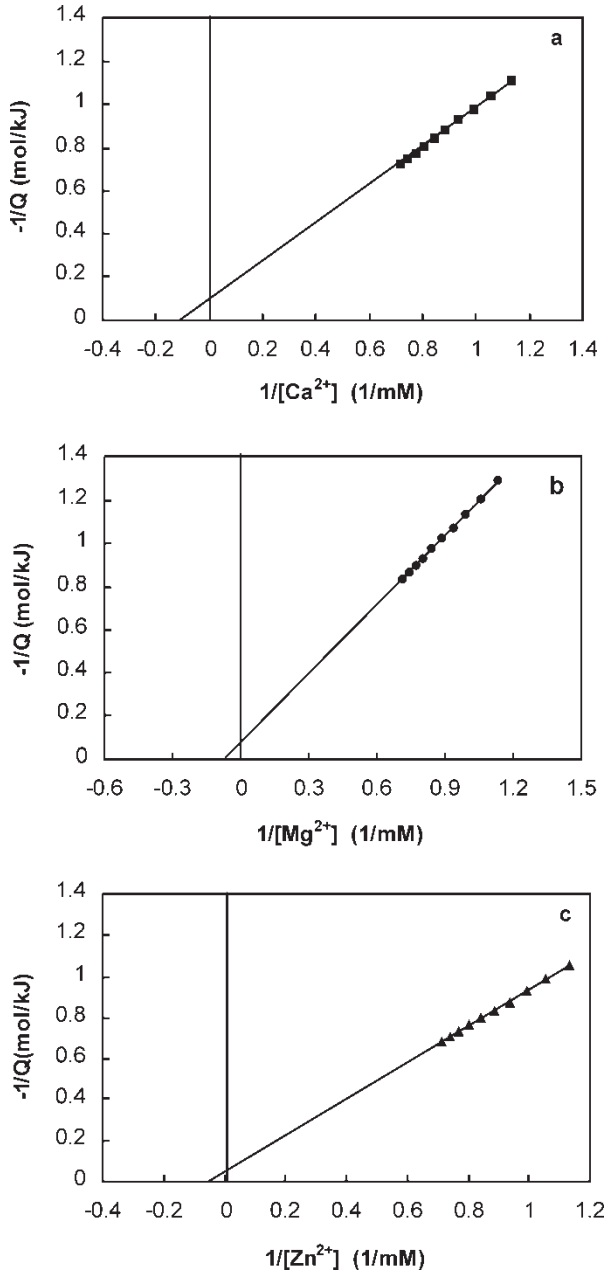

FIGURE 3 The linear plot of 1/Q versus 1/[Metal Ion] for 20 automatic cumulative injections of Ca^{2+}(a), Mg^{2+}(b), and Zn^{2+}(c).

the value of K_i increases with the decrease in radius of the metal ions.

Many enzymatic reactions rely on the cooperative action of divalent metal cofactors that are incorporated into protein as positively charged ions.[13] Although quite well established their functional role has not yet received a satisfactory explanation and is still a matter of debate and speculation. It is also established that what is decisive for the dramatically different behavior of magnesium (a co-catalyst) and calcium (an inhibitor) in certain cases are kinetic factors and not the properties of the pre-reactive state of the enzymes.[14] In this study, simple techniques of spectrophotometric and

TABLE I The values of K_i and ΔH of metal ions binding to beta-amylase

Metal ions		Spectrophotometric method	Isothermal calorimetric method	Radius
Ca^{2+}	K_i values ΔH	13.4 mM	13.1 mM $-14.3\,kJ\,mol^{-1}$	99 Å
Mg^{2+}	K_i values ΔH	18.6 mM	18.6 mM $-17.7\,kJ\,mol^{-1}$	65 Å
Zn^{2+}	K_i values ΔH	17.5 mM	17.7 mM $-20.0\,kJ\,mol^{-1}$	74 Å

isothermal titration calorimetry were successfully applied to monitor the type of inhibition of beta-amylase by calcium, magnesium and zinc.

CONCLUSION

Two independent spectrophotometric and calorimetric methods were used for the determination of the inhibition type and kinetic parameters of an enzyme reaction. Competitive inhibition gives a series of lines with increasing slope with increasing substrate concentration. The dissociation binding constant (K_i) obtained from thermodynamic and kinetic studies supports our assumption that the values of K_i increases with the decrease in radius of metal ions.

Acknowledgements

The financial support of the Research Council of the University of Tehran is gratefully acknowledged.

References

[1] Robyt, J.F. and Whelan, W.J. (1968) "β-amylases", In: Radley, J., ed, *Starch and its Derivatives* (Chapman and Hall, London, U.K.), pp 430–476.
[2] Sopanen, T. and Lauviere, C. (1989) *Plant Physiol.* **89**, 244–249.
[3] Santos, M.M. and Riis, P. (1996) *J. Inst. Brew.* **102**, 271–275.
[4] Whileley, C.G. (2000) *Cell Biochem. Biophys.* **33**, 217–225.
[5] The Merck Index, pp. 95, 11th ed. (1989) Merck & Co. Inc. USA.
[6] Bernfeld, P. (1955) In: Colowick, S.P. and Kaplan, N.O., eds, *Meth. Enzymol.* (Academic Press Inc. Publishers, New York) Vol. **1**, pp 149–158.
[7] Dixon, M. (1953) *Biochem. J.* **55**, 170–171.
[8] Saboury, A.A., Divsalar, A., Ataie, G., Moosavi-Movahedi, A.A., Housaindokht, M.R. and Hakimelahi, G.H. (2002) *J. Biochem. Mol. Biol.* **35**, 302–305.
[9] Saboury, A.A., Dahot, M., Umar, Ghobadi, S., Chamani, J. and Moosavi-Movahedi, A.A. (1998) *J. Chin. Chem. Soc.* **45**, 667–671.
[10] Saboury, A.A. and Moosavi-Movahedi, A.A. (1997) *J. Enz. Inhib.* **12**, 273–279.
[11] Sarraf, N.S., Saboury, A.A. and Moosavi-Movahedi, A.A. (2002) *J. Enz. Inhib. Med. Chem.* **17**, 203–206.
[12] Saboury, A.A. (2003) *J. Therm. Anal. Cal.* **72**, 93–103.
[13] Cowan, J.A. (1998) *Chem. Rev.* **98**, 1067–1087.
[14] Mordasini, T., Curioni, A. and Andreoni, W. (2003) *J. Biol. Chem.* **278**, 4381–4384.