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SENSITIVITY OF PLANT α-AMYLASES TO CALCIUM IONS *IN VITRO*

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Key Word Index—Plant α-amylases; Ca^{2+}; inhibition.

Abstract—Plant α-amylases were found to be sensitive to high calcium ion concentration *in vitro*. At 250 mM nearly 70% of the activity of maize, wheat, and barley α-amylases was inhibited, but in contrast, hog pancreatic and bacterial enzymes were not affected. Inhibition of maize α-amylase was competitive, reversed by dialysis and markedly increased by the pre-incubation of the enzyme with Ca^{2+} before the addition of the substrate. The apparent inhibition constant (K_i) and the apparent number of Ca^{2+} (n) bound per enzyme molecule were found to be 85 mM and 1.1, respectively. The latter value indicates the absence of cooperativity phenomenon. The interaction between the maize α-amylase and Ca^{2+} was also studied as a function of temperature. The efficiency of Ca^{2+} interaction decreases with increasing temperature, and as expected, the overall reaction was exothermic with a ΔH value of −13.8 kcal/mol. The values for ΔG and ΔS for the formation of Ca–enzyme complex at 25° were found to be 1.6 kcal/mol and −51.6 cal/mol/deg, respectively. The relatively large negative ΔS value suggests that the enzyme molecule undergoes marked conformational change during its interaction with Ca^{2+}.

INTRODUCTION

During the course of studies on the effect of Ca^{2+} on the inhibition of α-amylases by phytic acid [1] we observed that whereas the wheat and maize α-amylases were quite sensitive to high Ca^{2+} concentration *in vitro*, animal and bacterial enzymes were not inhibited. This unexpected observation prompted us to undertake the present study in which we have investigated the effect of Ca^{2+} on different α-amylases with a view to find out whether or not plant α-amylases interact with Ca^{2+} differently.

RESULTS

Effect of Ca^{2+} *on α-amylases of different origins*

The results summarized in Table 1 show that the effect of Ca^{2+} on the activity of α-amylases derived from a variety of sources is concentration-dependent and varies also with the source of the enzyme. For instance, whereas the activity of bacterial and hog pancreatic α-amylases was not influenced by Ca^{2+} even at 250 mM concentration, the plant enzymes, namely from maize,

Table 1. Effect of Ca^{2+} on the activity of α-amylases derived from different sources*

Enzyme source	Ca^{2+} concentration and inhibition (%)					
	10 mM	25 mM	50 mM	100 mM	200 mM	250 mM
Maize	0	7.5	20	34	54	65
Wheat	0	8	20	40	58	69
Barley	0	9	21	39	58	71
Peanut	0	4	12	20	35	42
Bacillus subtilis	0	0	0	0	0	0
Hog pancreas	0	0	0	0	0	0

* All measurements were made at optimum pH without pre-incubation of enzyme with Ca^{2+}. Equivalent units of enzyme preparations were used from each source. Assay conditions were those described in the Experimental.

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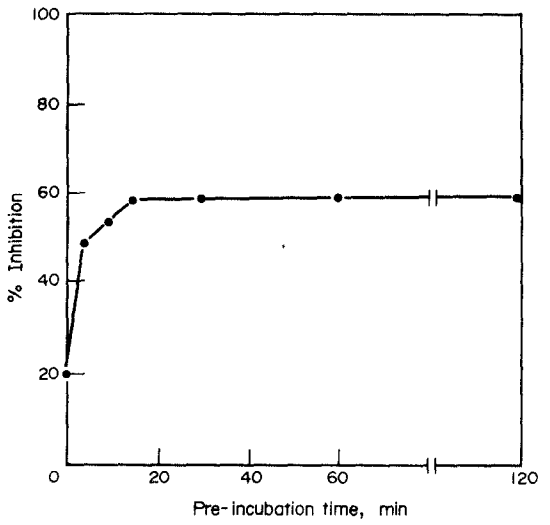

Fig. 1. The inhibitory activity of Ca^{2+} towards maize α-amylase as a function of pre-incubation period of Ca^{2+} with the enzyme. In a typical experiment $CaCl_2$ (100 μmol) was incubated at 30° with 0.3 units of maize α-amylase in 1.5 ml of 0.05M NaOAc buffer (pH 5) containing 25 μmol NaCl. After different durations of pre-incubation the residual α-amylase activity was determined in the usual manner by the addition of 0.5 ml of 0.6% buffered substrate (soluble starch) as described in the text.

wheat, barley and peanuts were potentially inhibited by Ca^{2+} when its concentration in the reaction mixture rose above 50 mM.

Pre-incubation effect

Fig. 1 shows the effect of pre-incubation time on the inhibitory activity of Ca^{2+} toward maize α-amylase. It was observed that pre-incubation of Ca^{2+} with the enzyme before the addition of substrate markedly increases the enzyme inhibition. In fact, 15 min pre-incubation of 50 mM Ca^{2+} with the maize enzyme increased the extent of inhibition from 20% to 60%. Pre-incubation for more than 15 min did not produce any further increase in the inhibition. Thus, it appears that 15 min pre-incubation of the enzyme with appropriate concentration of Ca^{2+} was essential for the maximum effect. It may also be pointed out that pre-incubation of Ca^{2+} with starch (substrate) was ineffective, indicating that calcium ions do not interact with the substrate.

Reversibility of Ca^{2+} inhibition

The reversibility of α-amylase was examined by dialysing the calcium-saturated enzyme samples for 48 hr against 0.05 M NaOAc buffer (pH 5) containing 1 mM EDTA. The results given in Table 2 show that the activity of both maize and wheat α-amylases are restored as soon as excess of Ca^{2+} was removed. In contrast, however, the dialysis of enzyme samples without Ca^{2+} showed an inhibition of *ca* 10%, presumably due to the loss of the less tightly bound calcium necessary for the optimum activity or the stability of the enzyme during dialysis. Surprisingly, this inhibition could not be reversed by the addition of Ca^{2+}. The possibility of partial denaturation of the enzyme during dialysis unrelated to the removal of Ca^{2+} cannot be ruled out at the moment.

Kinetic studies

The Lineweaver–Burk plots (Fig. 2) show that the inhibition of maize α-amylase by Ca^{2+} is of the competitive type. The value of the apparent inhibition constant (K_i) obtained by Dixon plots (Fig. 3) was found to be 85 mM. In addition, the intersection of these plots at a point above the x-axis on the left side of the ordinate further indicates the competitive type of inhibition.

Fig. 4 shows the Hill plots of log $(v_o - v_i)/v_i$ vs log $[Ca^{2+}]$. The velocities of the enzyme catalysed reactions without and with inhibitor are v_o and v_i respectively. The plots are linear and their slope, which equals n, was found to be 1.1, indicating that one calcium

Table 2. Reversal of Ca^{2+} inhibition of maize and wheat α-amylases by dialysis*

Treatment	Residual activity (%)	
	Before dialysis	After dialysis
Maize α-amylase		
No addition (control)	100	91 ± 4
Plus 50 mM $CaCl_2$	40 ± 3	90 ± 4
Wheat α-amylase		
No addition (control)	100	92 ± 4
Plus 50 mM $CaCl_2$	35 ± 4	91 ± 4

*Duplicate samples of three different enzyme preparations of each source (0.3 units) in 0.05M NaOAc buffer, pH 5, without and with $CaCl_2$ (50 mM) were first equilibrated for 30 min followed by a 48 hr dialysis against 4 × 100 vol. of the same buffer containing 1 mM of EDTA at 4°. The activity of the control was assumed as 100% and experiments with control and the Ca^{2+}-treated enzymes were performed simultaneously under identical conditions. Assay conditions were those described in the Experimental.

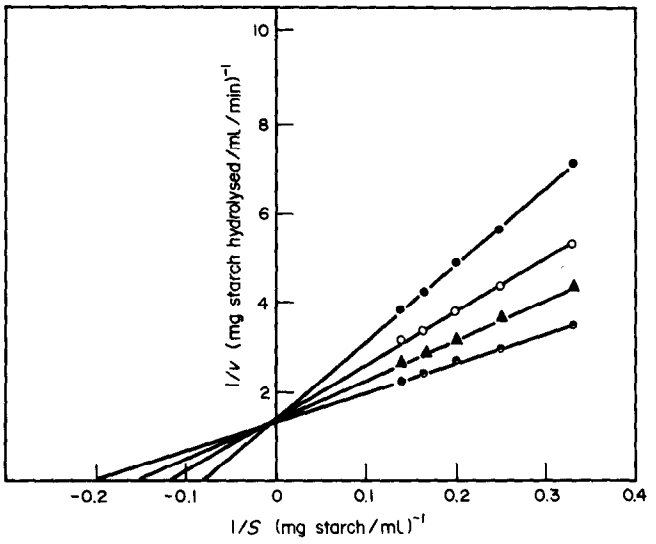

Fig. 2. Lineweaver–Burk plots showing competitive type of inhibition of maize α-amylase by Ca^{2+}. Enzyme (0.3 units) was first pre-incubated with $CaCl_2$ for 30 min as described in Fig. 1. The residual enzyme activity was then assayed with different substrate concentrations as described in Experimental. (◓——◓), (▲——▲), (○——○), and (●——●) are with 0, 50, 100 and 150 mM $CaCl_2$, respectively.

atom binds per enzyme molecule and that the phenomenon of co-operativity is not present [2].

Effect of temperature

The effect of temperature on the interaction between maize α-amylase and Ca^{2+} was studied at four different temperatures, 15°, 20°, 25° and 30°, which lie in the temperature range of 100% stability; thus avoiding the risk of thermal denaturation. The results are given in Table 3. It was found that the extent of inhibition decreases with increasing temperature in the temperature range indicated. The value of K_i also markedly increases with increasing temperature (Table 3). In other words the ability of Ca^{2+} to interact with the enzyme to form an inactive enzyme–calcium complex is a function of temperature; decreasing sharply with increasing temperature.

The effect of temperature on the formation of the enzyme–calcium complex was further analysed in terms of thermodynamic parameters by assuming that K_i values at different temperatures represent association constants for

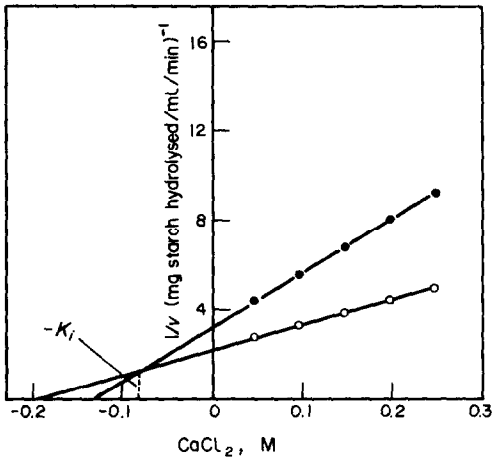

Fig. 3. Plots of $1/v$ vs $CaCl_2$ concentrations. The maize α-amylase (0.3 units) was pre-incubated with varying concentrations of $CaCl_2$ (0–0.4M) without substrate for 30 min as described in Fig. 1. The reaction was started by the addition of buffered starch solution (substrate) and the residual enzyme activity was assayed as described in Experimental. (●——●) and (○——○) represent 0.15 and 0.3% starch concentrations in the assay mixture, respectively. The plots were extended backward to obtain the value of K_i.

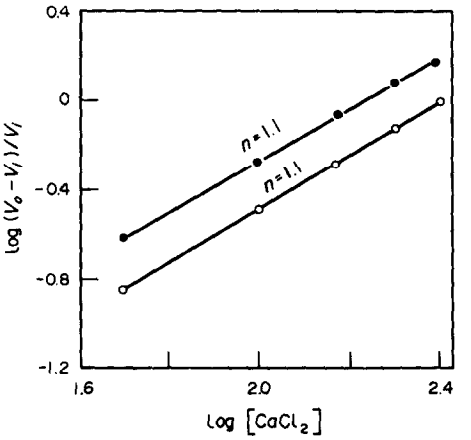

Fig. 4. Plots of $\log (v_o - v_i)/v_i$ vs $\log (CaCl_2)$, inhibitor, where v_o is the control activity without $CaCl_2$ and v_i is the enzyme activity in the presence of $CaCl_2$. Slope of these plots gives the value of n, the apparent number of Ca^{2+} reacting per enzyme molecule to form an inactive enzyme complex, assuming one active site per enzyme molecule. Experimental conditions were those described in Fig. 3. (●——●) and (○——○) represent 0.15 and 0.3% starch concentrations in the assay mixture, respectively.

Table 3. Effect of temperature on the maize α-amylase inhibition by Ca^{2+}*

Temperature	Ca^{2+} concentrations and inhibition (%)					
	50 mM	100 mM	150 mM	200 mM	250 mM	K_i† (mM)
15°	40	56	67	70	74	27 ± 2
20°	31	46	56	64	68	40 ± 2
25°	25	39	50	56	60	67 ± 4
30°	20	31	41	48	51	85 ± 4

* Experiments were carried out without pre-incubation of the enzyme with Ca^{2+}.

† K_i, apparent inhibition constant. Values were obtained from Dixon plots [20]. The values are the mean of triplicate estimations.

Table 4. Thermodynamic parameters for the interaction between Ca^{2+} and the α-amylase from maize (ΔH^* = −13.8 kcal/mol)

Temperature	ΔG† (kcal/mol)	ΔS‡ (cal/deg/mol)
15°	2.06	−55.0
20°	1.87	−53.4
25°	1.60	−51.6
30°	1.49	−50.4

* ΔH was calculated from the van't Hoff plots shown in Fig. 5.

† ΔG was calculated as $-4.57 \times T \times pK_i$.

‡ ΔS was calculated from $\Delta G = \Delta H - T\Delta S$.

the enzyme–calcium complex formation. This assumption allows the calculation of the enthalpy change (ΔH), involved in the formation of enzyme–calcium complex, from the plot of pK_i as a function of the reciprocal of absolute temperature (Fig. 5) as described by Taketa and Pogell [3]. The ΔH was found to be −13.8 kcal/mol indicating the exothermic nature of the reaction between Ca^{2+} and maize α-amylase.

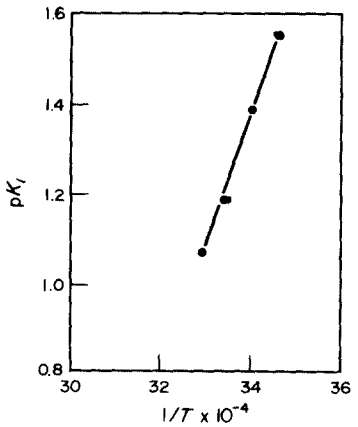

Fig. 5. Effect of temperature on apparent overall association constant, K_i. Values of K_i at different temperatures were obtained by Dixon plots as described in Fig. 3. The molar enthalpy change, ΔH, was calculated from the slope of this line according to the equation $\Delta H = -4.57 \times$ slope.

From the K_i values, the free energy change (ΔG) values and the entropy change (ΔS) values of the enzyme–calcium complex formation were calculated at different temperatures by the following equations: $\Delta G = -RT \ln K_i$ and $\Delta G = \Delta H - T\Delta S$. The results are summarized in Table 4. It was observed that ΔG is a positive value significantly decreasing with increasing temperature. On the other hand ΔS is negative and remains fairly constant throughout the temperature change.

DISCUSSION

The present study has shown that whereas plant α-amylases, namely, maize, wheat, barley, and peanut enzymes are sensitive to high Ca^{2+} concentration *in vitro*, the activity of hog pancreatic and bacterial (*Bacillus subtilis*) α-amylases was not affected. This appears to be a novel feature which distinguishes the plant α-amylases from those of animals and bacterial sources, and to the best of our knowledge, studies involving high Ca^{2+} concentrations with α-amylases of different origins, especially of plants have not been reported before.

The calcium requirement and the calcium-binding ability of α-amylases vary with the source of the enzyme [4, 5]. For instance, mammalian α-amylases bind calcium more tightly than those of higher plants [6]. It is, however, not clear at the moment if the preferential inhibition of plant α-amylases at high Ca^{2+} concentration is related to the calcium-binding ability of different α-amylases.

On the basis of the data available in the literature it is generally thought that tightly bound calcium holds the enzyme molecule in the correct conformation for maximum activity without participating directly in the formation of the enzyme–substrate complex [5, 7, 8], and the weakly bound calcium stabilizes the enzyme molecule against protease digestion [2, 9, 11] and thermal inactivation [4, 5, 11]. In fact, Fischer and Stein [5] and Stein *et al.* [10] reported that removal of weakly bound calcium from α-amylase results only in minor conformational changes. In agreement with these observations we found that 48 hr dialysis of maize and wheat α-amylases against 1 mM EDTA solution at pH 5 produced only about 10% inactivation which could not be restored by the addition of Ca^{2+} to the enzyme solution (Table 2). On the contrary, addition of a large excess of Ca^{2+} caused significant inhibition of these enzymes. We believe that the partial loss of enzymic

activity was probably due to the removal of some of the tightly bound Ca^{2+} leading to an irreversible inactivation.

The nature of inhibition of maize, and perhaps of other plant α-amylases also, is of the competitive type indicating that calcium ions when present in large excess compete with the substrate for the active site of the enzyme. Since, carboxylate and imidazolium groups are assumed to participate in the breakdown of enzyme–substrate complex [12–14], the carboxylate group will be the most likely site for the Ca^{2+} attack. Another possibility is the formation of higher aggregates of enzyme molecule at high Ca^{2+} concentration as suggested in the case of Zn^{2+} involving the imidazole group of histidine [5, 15]. We also found that zinc ions are competitive inhibitors of maize α-amylase (unpublished data).

Up to now all attempts to classify α-amylases on the basis of calcium requirement have been unsuccessful. However, on the basis of the responses of various α-amylases toward high Ca^{2+} concentration *in vitro*, these can be divided into two main types; those inhibited or not inhibited by Ca^{2+}. Plant α-amylases which also bind calcium less tightly than mammalian enzymes [6] appear to belong to the former type. It may, however, be necessary to investigate a large variety of α-amylases before a definite generalization could be made in regard to the interaction between calcium and plant α-amylases. Nevertheless, the present study indicates that plant α-amylases are structurally different from those of animal and bacterial enzymes. In addition, the inhibition of plant α-amylases by the high Ca^{2+} concentration may be of physiological significance since Ca^{2+} ions are reported to play a regulatory role in the biosynthesis of α-amylases at the molecular level [16].

EXPERIMENTAL

Materials. The bacterial α-amylase was purchased from E. Merck. Hog pancreatic α-amylase was from Sigma. Wheat, maize, barley and peanut enzymes used in this study were prepared as described in ref. [1]. The prepns were not homogeneous, but were free from β-amylase. Soluble starch was from B.D.H. Various seeds used as enzyme source were purchased locally. Unless otherwise stated all other chemicals were of reagent grade.

Enzyme assay. α-Amylase activity was assayed by using soluble starch as the substrate and by measuring the decrease of substrate concn with I_2/KI reagent as described in ref. [17]. The reaction mixture, except where noted, contained the following in a final vol. of 2 ml: 6 mg of soluble starch (0.5 ml of buffered substrate soln); 200 μmol of NaOAc buffer (pH 5); 25 μmol of NaCl and 0.1–1.0 units of α-amylase. Varying concns of Ca^{2+} as $CaCl_2$ or $Ca(OAc)_2$ (0–250 mM) were added to the starch soln before the addition of the enzyme. For kinetic and thermodynamic studies the enzyme was pre-incubated with Ca^{2+} for *ca* 30 min at the reaction temp. and pH. The enzyme action was then started by adding the buffered substrate soln to the reaction mixture. The incubation was carried out at 30° for 5 min and the decrease was measured as described above at 620 nm. One unit of α-amylase is the amount of enzyme that digests 1 mg starch per min under assay conditions. Protein was estimated by the method of ref. [18] using BSA as a standard.

Kinetic studies. The nature of inhibition was determined by Lineweaver–Burk plots [19] by using at least 5 substrate concns. Apparent inhibition constant (K_i) was obtained from Dixon plots of $1/v$ vs Ca^{2+} concns [20]. For these plots enzyme was assayed by using 3 and 6 mg of substrate concns and 5 different concns of the inhibitor, Ca^{2+}. The K_i values were also obtained by replotting the slopes of double reciprocal plots (Fig. 2) vs Ca^{2+} concns [21]. The values so obtained were in close agreement with those obtained from Dixon plots. The interaction constant (n) was determined by the method of ref. [22].

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