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EFFECT OF Zn^{2+} ON PLANT α-AMYLASES *IN VITRO*

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Key Word Index—*Zea mays*; *Triticum vulgare*; *Hordeum vulgare*; maize; wheat; barley; Gramineae; *Bacillus subtilis*; α-amylase; EDTA; zinc acetate; inhibitor.

Abstract—A detailed study of the *in vitro* interaction between Zn^{2+} and α-amylases from different origins has shown that under physiological conditions, plant α-amylases are strongly inhibited by zinc ions. The inhibition is pH-dependent and can be reversed by extensive dialysis against buffer. In addition, it can also be removed by the treatment with EDTA, particularly at low pH values. However, EDTA itself does not have any effect on enzymatic activity. The inhibition for maize α-amylase at its optimum pH (5.5), was competitive and the K_i was 10 mM. The Hill coefficient value (n) was 1, suggesting that Zn^{2+} interacts with α-amylase in a simple manner with no co-operativity.

INTRODUCTION

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are metalloenzymes requiring calcium both for activity and maximum stability [1–3]. There are sufficient data to show that calcium forms an integral part of the enzyme molecule. In the case of *B. subtilis* α-amylase, zinc was also reported to be integrated with the enzyme molecule in addition to calcium [4, 5]. However, unlike calcium, zinc was involved in inducing the dimerization of the enzyme molecule. Isemura and Kakiuchi [6, 7] reported the formation of higher aggregates than the dimer in the presence of higher concentrations of zinc ions. But the interesting finding was that the phenomenon of dimerization appeared to be peculiar to *B. subtilis* α-amylase only and no other α-amylase showed this behaviour. Secondly, dimerization was induced only by zinc and not by calcium [4]. Ono and Hiromi [8], on the other hand, reported zinc ions to be inhibitive towards bacterial α-amylase, though the mechanism of this inhibition has not been described.

Whereas the role of zinc in bacterial α-amylase is well established, its effect on plant α-amylases has not been studied in detail. Therefore, with a view to investigating the effect of zinc ions on plant α-amylases, the present study was undertaken.

RESULTS

Inhibition of α-amylases

The effect of Zn^{2+} on α-amylases from different sources is summarized in Table 1. Zn^{2+} is a potent inhibitor of α-amylases both from bacterial as well as plant sources. The inhibitory action of Zn^{2+} towards bacterial α-amylase has already been reported by Ono and Hiromi [8]. The extent of inhibition depends on the source of enzyme (Table 1); thus, under identical conditions bacterial α-amylase is inhibited comparatively less than each of the plant α-amylases tested.

Nature of binding between Zn^{2+} and α-amylase

Experiments involving extensive dialysis and treatment with EDTA were carried out. Table 2 shows the results of dialysis against buffer. These results indicated that on prolonged dialysis of the enzyme–Zn^{2+} complex against buffer, nearly 92% enzymatic activity was restored, suggesting that the inhibition of α-amylases by Zn^{2+} is reversible.

Effect of pH *and EDTA*

Table 3 summarizes the effect of pH and EDTA on Zn^{2+} interaction with α-amylases. Interaction between Zn^{2+} and α-amylase is highly pH-dependent. At pH 4.5, α-amylase activity was not affected by Zn^{2+} even at 40 mM. However, on either side of this pH value, Zn^{2+} appreciably affected the enzyme activity. Below pH 4.5, Zn^{2+} showed an activating effect, i.e. the activity of α-amylase was increased by the addition of Zn^{2+}. This activation was further increased with decreasing pH. On

Table 1. Inhibition of α-amylases by Zn^{2+}

Zinc acetate (mM)	Enzyme source and inhibition (%)			
	Maize	Wheat	Barley	*Bacillus subtilis*
5	28	22	25	15
10	46	41	48	22
20	62	58	61	38
30	72	67	70	56
40	80	73	74	68
50	84	78	78	72
60	86	80	82	78

All measurements were made at pH 5.5 using equivalent units of enzyme from each source. Substrate and inhibitor (Zn^{2+}) were mixed simultaneously and the reaction was started by the addition of enzyme solution. The reaction mixture and other conditions were the same as described in Experimental.

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Table 2. Reversal of inhibition by dialysis

Experiment*	Inhibition (%)	Reversal of inhibition (%)
Enzyme + zinc acetate (incubated for 30 min)		
(i) Undialysed mixture	90	—
(ii) After 24 hr dialysis	36	60
(iii) After 48 hr dialysis	20	78
(iv) After 72 hr dialysis	7	92

*A mixture of maize α-amylase (1 unit) and zinc acetate (250 mM) in 0.05 M NaOAc buffer, pH 5.5 was incubated for 30 min and then dialysed for 72 hr against four changes of the same buffer containing EDTA (1 mM) at 4°. In another experiment, an equivalent amount of enzyme without Zn^{2+} was simultaneously incubated and dialysed under all the identical conditions. The activity of this untreated enzyme was taken as 100%. At different time intervals, aliquots were withdrawn and the degree of inhibition determined. These results are the average of triplicate experiments.

Table 3. Effect of pH and EDTA on the maize α-amylase inhibition by Zn^{2+}

	Per cent inhibition		
pH*	Zinc acetate (10 mM)	10 mM Zinc acetate + EDTA† (10 mM)	Reversal of inhibition by EDTA‡ (%)
3.6	−44	55	—
4.0	−20	13	—
4.5	0	0	—
5.0	24	8	67
5.5	45	17	62
6.0	57	30	47
6.5	79	54	31
7.0	80	72	10

*The buffers used were 0.05 M NaOAc (pH 3.6–5.6); 0.05 M NaOAc adjusted with 1 N NaOH (pH 6–6.5) and 0.05 M Tris–HCl (pH 7).

†The final pH of the reaction mixture was adjusted with 1 N NaOH after the addition of Zn^{2+} and EDTA. The activity in control without containing Zn^{2+} and EDTA was used as 100%. EDTA at 10 mM gave no inhibition.

‡The values of per cent inhibition caused by Zn^{2+} in presence of EDTA were used to calculate the per cent reversal of inhibition. Per cent inhibition by Zn^{2+} at each pH value was assumed as 100%.

the other hand, above pH 4.5, Zn^{2+} exhibited a significant inhibition of enzyme activity. The degree of inhibition increased with increasing pH and, thus, at pH 7, the inhibition by 10-mM zinc acetate was nearly 80%. EDTA alone did not have any effect on enzyme activity. However, when it was added in the presence of Zn^{2+}, it reversed the effect caused by the latter. Furthermore, the reversal by EDTA was also pH-dependent and the extent of reversal decreased with increasing pH. Thus, whereas at pH 5, the reversal of inhibition was 67%, at pH 7 it was only 10% with the same concentration of EDTA (10 mM). Below pH 4.5, EDTA not only reversed the activating effect of Zn^{2+} but also inhibited the enzyme activity; while in the absence of Zn^{2+} it had no effect on the activity. The mechanism of the inhibitory effect of EDTA in the presence of Zn^{2+} at low pH values is not known. These results are contrary to our finding on the inhibitory effect of Ca^{2+} towards α-amylases, where the reversal by EDTA increases with increasing pH [9].

Kinetic studies

The Lineweaver–Burk plots with and without inhibitor (Fig. 1) were linear and intersected at a single point on the *y*-axis. This indicated that the α-amylase inhibition by Zn^{2+} is of the perfectly competitive type, i.e. the inhibitor had no effect on V_{max} whereas K_m value was significantly increased with increasing inhibitor concentration [10]. The value of K_i for maize α-amylase, as computed from Dixon plot (Fig. 2) was 10 mM. K_i evaluated by replotting the slopes of the double reciprocal plots in Fig. 1 vs inhibitor concentration according to ref. [11] was 10.4 mM. This value was in good agreement with the K_i value obtained from Dixon plots.

A simple hyperbolic curve of per cent inhibition vs inhibitor concentration (Fig. 3A) suggests that only one Zn^{2+} per enzyme molecule participates in the formation of inactive enzyme inhibitor complex. In addition, these results also indicate the absence of any co-operativity during the interaction of Zn^{2+} with α-amylases. The same was also supported by the value of the interaction constant (n), i.e. the number of Zn^{2+} combining with one molecule of enzyme. The value of n was determined from the slope of the Hill plot obtained by plotting log $(V_0 - V_i)/V_i$ against log (Zn^{2+} conc.), where V_0 and V_i represent the reaction velocities without and with inhibitor using soluble starch as the substrate. Linear plot showing a slope of 1.0 was obtained (Fig. 3B). The value of the Hill coefficient ($n = 1$) indicated that Zn^{2+} does not show any co-operativity while interacting with α-amylase.

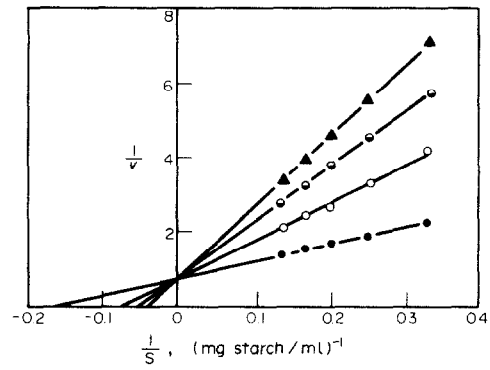

Fig. 1. Lineweaver–Burk plots showing competitive type of inhibition of maize α-amylase by zinc acetate. The enzyme was incubated with varying substrate concentration (3–7 mg starch/ml) in the absence (●) and presence of 12 mM (○), 24 mM (◒) and 36 mM (▲) zinc acetate at pH 5.5. The velocity was expressed as mg starch hydrolysed/min per ml of the reaction mixture. Other conditions of enzyme assay were as given in Experimental.

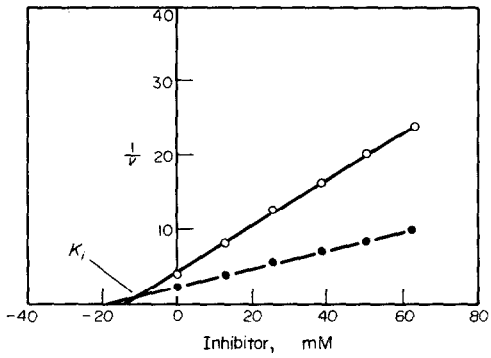

Fig. 2. Dixon plots of $1/v$ vs Zn^{2+} concentrations. Maize α-amylase was assayed at pH 5.5 with varying zinc acetate concentration (0–60 mM) at 3 mg starch/ml (○) and 6 mg starch/ml (●) of the reaction mixture. The point of intersection, obtained by back extrapolation of the two straight lines, gave the value of K_i.

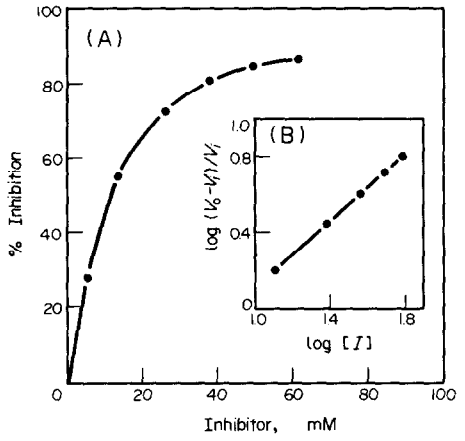

Fig. 3. (A) Plot of per cent inhibition vs zinc acetate concentrations showing a simple hyperbolic curve. (B) Hill plot of log $(V_o - V_i)/V_i$ vs log zinc acetate concentration, where V_o and V_i represent the velocities of the enzyme-catalysed reaction without and with inhibitor, respectively. The slope of this plot is the Hill coefficient (n) and is equal to 1. Other conditions of enzymes assay were the same as those described earlier.

DISCUSSION

Most of the enzymes including α-amylases are mainly produced in aleurone grains [12, 13]. These aleurone grains also store phytate and several metallic ions [14]. Thus, in view of their coexistence, it appears quite likely that both phytate and metallic ions might be playing an important role in the regulation of α-amylase activity during germination and maturation of plant seeds. Working in this direction, we have already reported the effect of phytate and high Ca^{2+} concentration on α-amylases in our previous papers [9, 15]. In the present communication we have described the effect of another important metallic ion, i.e. Zn^{2+}. These data show that at physiological pH, Zn^{2+} inhibits each of the plant α-amylases tested. Thus, in their inhibitory effect they are identical to phytate [15] but different to Ca^{2+} [9]. The inhibition by Zn^{2+} is reversible and it may be completely removed either by extensive dialysis or by treatment with EDTA. This indicates that the interaction of Zn^{2+} with α-amylase results in the formation of a weak dissociable complex [16]. Furthermore, this inhibition is a function of pH and increases with increasing pH. Since carboxylate and imidazolium groups are assumed to participate in the breakdown of enzyme substrate complex [17–19], the COO^- group will be the most probable site for the binding of Zn^{2+} and Ca^{2+} in the enzyme–inhibitor complex. The activating effect of Zn^{2+} at low pH values (below pH 4.5) was attributed to their possible role in facilitating the enzyme–substrate complex formation by a mechanism different from the one involved in their inhibitory action. It is worth noting that the inhibition of plant α-amylases by Zn^{2+} is not due to the formation of higher aggregates of enzyme molecules as occurs in bacterial α-amylase [4, 5], because in that stage the α-amylase–Zn^{2+} interaction would follow complex kinetics whereas in the present case it has been found to follow a simple Michaelis–Menten kinetics. This demonstrates that Zn^{2+} directly binds to enzyme molecule in the ratio of 1:1 to form an inactive enzyme inhibitor complex.

EXPERIMENTAL

Material. Soluble starch was obtained from B.D.H. (U.K.). Bacterial α-amylase (sp. act. 200 units/mg protein), zinc acetate and EDTA were purchased from Merck. Sephadex G-100 and BSA were from Sigma. Various seeds used as enzyme source were purchased from the local seed store.

Enzyme preparation. The extraction and purification of α-amylase from each plant source was carried out as described in our previous paper [15].

Enzyme assay. The α-amylase activity was assayed by the method of ref. [20] using soluble starch as the substrate and following the decrease of substrate concn with I_2/KI reagent. The reaction mixture contained the following in a total vol of 2 ml unless otherwise stated: 6 mg of soluble starch (0.5 ml buffered substrate soln); 200 μmol of NaOAc buffer (pH 5.5); 12 μmol of NaCl and 0.1–1.0 units of α-amylase. In the inhibition studies, the inhibitor (0–62 mM) and the substrate were added simultaneously and the reaction was started by the addition of enzyme soln. Incubation was carried out at 30° for 5 min and the reaction was terminated by adding 1 M HCl. The decrease in starch concn was measured as described above at 620 nm. One unit of α-amylase was defined as the amount of enzyme in mg that digests 1 mg starch/min under the above assay conditions. Protein concn was measured by the method of ref. [21].

Kinetic studies. The nature of maize α-amylase inhibition by Zn^{2+} was determined by the method of ref. [22]. The α-amylase activity was assayed at 5 different substrate concns (varying from 3 to 7 mg starch/ml) both in the absence as well as in the presence of 12, 24 and 36 mM concns of Zn acetate. Finally $1/v$ was plotted vs $1/S$ to obtain the Lineweaver–Burk plots. The value of apparent inhibition constant (K_i) was determined by a Dixon plot [23]. In this expt enzyme was assayed at two different substrate levels (3 and 6 mg starch/ml) using various inhibitor concns in the range of 0–60 mM Zn acetate. The value of K_i was given by the point of intersection of two straight lines obtained by plotting $1/v$ vs inhibitor concn (I). The K_i value was also evaluated by replotting the slopes of double reciprocal plots (Fig. 1) vs inhibitor concns [11]. The interaction constant (n) was determined by the method of ref. [24].

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