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MYOINOSITOL HEXAPHOSPHATE AS A POTENTIAL INHIBITOR OF α-AMYLASES

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Key Word Index—*Triticum vulgare*; Gramineae; wheat; α-amylase; myoinositol hexaphosphate; phytic acid; inhibitor.

Abstract—Myoinositol hexaphosphate (MHP) strongly inhibited α-amylases of different origins. The inhibition of wheat α-amylase is noncompetitive with an apparent K_i value of 1 mM, pH dependent and markedly increased by the preincubation of enzyme with MHP before the addition of substrate. Addition of Ca^{2+} did not reverse the inhibition of α-amylase indicating that its inhibition was not due to the binding of Ca^{2+} by MHP.

INTRODUCTION

Myoinositol hexaphosphate (MHP), commonly known as phytic acid, is widely distributed in nature and is generally believed to be the primary reserve phosphate in seeds [1–4]. Cawley and Mitchell [5] reported that MHP suppressed the α-amylase activity in sprouted wheatmeal by chelating the Ca^{2+} necessary for the enzyme activity [6–8]. However, the interaction between MHP and the purified enzyme preparations has not been examined in detail, and the mechanism of their interaction is still unknown.

In this communication we report a more detailed investigation on the interaction between MHP and the wheat α-amylase *in vitro*. It has been shown that the inhibitory action of MHP is noncompetitive, pH dependent and markedly influenced by the preincubation of the enzyme with inhibitor, but not by Ca^{2+} as suggested by Cawley and Mitchell [5].

RESULTS

Inhibition of α-amylases

Table 1 summarizes the results of the percentages of inhibition of α-amylases derived from a variety of sources by MHP. The data clearly show that MHP is a potent inhibitor of α-amylases. Thus, its inhibitory activity toward α-amylase appears to be a general phenomenon, although the extent of the inhibition varies from source to source.

Table 1. The effect of myoinositol hexaphosphate on α-amylases derived from a variety of sources*

Enzyme source	Inhibitor conc and inhibition (%)				
	2 mM	4 mM	6 mM	8 mM	10 mM
Wheat	25.0	52.0	68.2	78.0	84.5
Maize	24.3	53.6	66.8	83.5	91.4
Chick peas	21.1	55.5	73.4	77.8	
Barley	22.2	55.5	60.0	67.0	
Peanut	17.4	34.8	39.2	45.6	54.0
Bacillus subtilis	58.0	76.0	84.3	90.0	93.2

*All measurements were made at pH 4 without peincubation of enzyme with MHP. Equivalent units of enzyme preparations were used from each source. The reaction mixture and other conditions were those as described in the Experimental at inhibitor concentrations indicated.

Effect of pH

Figure 1 shows the activity of the wheat α-amylase and the percentage of inhibition by MHP as a function of pH. From these results it is apparent that the inhibitory

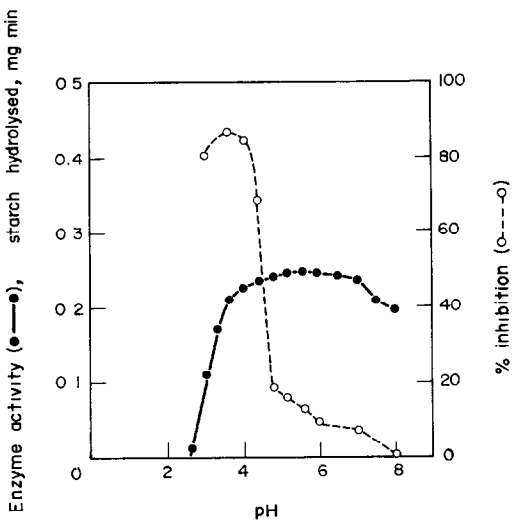

Fig. 1. The effect of pH on wheat α-amylase inhibition by MHP. Enzyme activity without MHP (●——●); and inhibition percent (○——○). Experiments with and without MHP were carried out simultaneously. The enzyme activity at each pH in the absence of MHP was used as 100%. The reaction mixture and assay conditions were same as described in the Experimental. Buffers were 0.05 M acetate (pH 3.6–5.6) and 0.05 M phosphate (pH 6–8) containing 0.05 M NaCl. The soln of MHP was adjusted to the desired pH with the help of 2 M HOAc before adding it to the reaction mixture.

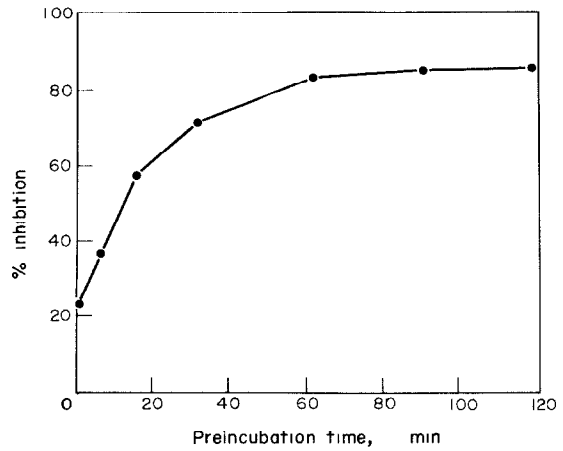

Fig. 2. Dependence of inhibition on preincubation time of wheat α-amylase with MHP. In a typical experiment 0.5 ml of the enzyme (*ca* 5 units) were incubated with 100 μmol of MHP or without MHP at 30° in a total vol. of 2 ml containing 100 μmol of acetate buffer (pH 4). At timed intervals, aliquots (0.2 ml) were assayed for α-amylase. The enzyme activity without MHP was assumed as 100%.

activity of MHP toward α-amylase decreases sharply (from 87 to 18%) with increasing pH between pH 3.5 and 5 followed by a gradual decrease (from 18 to 6%) in the increasing pH range between pH 5 and 7. In contrast, there is little pH effect on the enzyme activity without MHP between pH 3.5 and 6. Identical inhibition patterns were observed for enzymes derived from other sources, namely, maize, chick peas, barley and peanuts

Effect of preincubation

Figure 2 shows the dependence of inhibition on preincubation time of α-amylase with MHP. The results show that preincubation of the enzyme with MHP before the addition of substrate significantly increases the extent of the enzyme inhibition. The maximum effect was achieved in *ca* 60 min. Incubation longer than 60 min did not result in any further increase in the inhibition of α-amylase. It may be pointed out that the preincubation of MHP with starch did not affect the inhibitory power of MHP indicating that there is no interaction between inhibitor and the substrate.

Effect of Ca ions

The effects of Ca^{2+} on the activity of wheat, maize and bacterial α-amylases are summarised in Table 2. It was found that the addition of Ca^{2+} up to 8 mM had no effect on α-amylase activity with or without MHP. It was noticed, however, that in the case of wheat and maize enzymes, Ca^{2+} above 10 mM concentration become fairly inhibitory both with or without MHP. In fact, in the presence of 50 mM Ca^{2+} the inhibition of wheat and maize enzymes by 4 mM of MHP was *ca* 98 and 90% compared to only 66 and 68% without Ca^{2+} respectively. Although the combined effect of Ca^{2+} and MHP was additive, the two inhibitors seem to function independently, as the presence of one is not essential for the inhibitory activity of the other. That the inhibitory action of MHP is independent of Ca^{2+} was further supported by the results that even the relatively high concentration of Ca^{2+} (75 mM), sufficient to bind *ca* 12 mM of MHP, did not reverse the inhibition of the bacterial α-amylase (Table 2).

Table 2. The effect of Ca^{2+} on the inhibitory activity of MHP toward wheat, maize and bacterial α-amylases*

Experiment	% Inhibition	
	pH 4	pH 5
Wheat α-amylase		
(i) enzyme + 8 mM $CaCl_2$	0 0	0.0
(ii) enzyme + 8 mM $CaCl_2$ + 4 mM MHP	66.0 ± 4	13.8 ± 2
(iii) enzyme + 4 mM MHP	64.0 ± 4	13.5 ± 2
(iv) enzyme + 50 mM $CaCl_2$	40 0 ± 4	41 3 ± 4
(v) enzyme + 50 mM $CaCl_2$ + 4 mM MHP	98.4 ± 5	50 0 ± 4
Maize α-amylase		
(i) enzyme + 8 mM $CaCl_2$	0 0 +	0 0
(ii) enzyme + 8 mM $CaCl_2$ + 4 mM MHP	68.5 ± 4	37.2 ± 3
(iii) enzyme + 4 mM MHP	67 6 ± 4	38.2 ± 3
(iv) enzyme + 50 mM $CaCl_2$	44.0 ± 4	45 6 ± 4
(v) enzyme + 50 mM $CaCl_2$ + 4 mM MHP	90 8 ± 6	61.4 ± 4
Bacillus subtilis α-amylase		
(i) enzyme + 75 mM $CaCl_2$	0.0	0.0
(ii) enzyme + 75 mM $CaCl_2$ + 4 mM MHP	60.0 ± 5	34.6 ± 3
(iii) enzyme + 4 mM MHP	60 0 ± 5	35.0 ± 3

*In all experiments $CaCl_2$ was added immediately after mixing the enzyme with MHP. Enzyme action was started by adding buffered starch soln Results are average of triplicate experiments. MHP is myoinositol hexaphosphate.

Nature of inhibition

The nature of α-amylase inhibition by MHP was evaluated according to the method of Lineweaver–Burk [9]. It was found to be perfectly noncompetitive, i.e. the inhibitor had no effect on K_m [10]. The value of apparent K_i for the wheat α-amylase as computed from the replots of the slopes of Lineweaver–Burk plots, obtained in the presence of at least three different levels of MHP, against the MHP concentration [11] was found to be 1.0 mM.

DISCUSSION

The data reported show that MHP inhibits α-amylases of different origins. In this respect the inhibitory activity of MHP is identical to that of wheat kernel albumins [12–16]. In dormant seeds MHP is mainly localised in aleurone particles [3, 4] which are the major components of the α-amylase producing aleurone cells in seeds [17–18]. Its activity, like that of abscisic acid [19] as inhibitor of α-amylase, may therefore be of physiological significance during the process of seed germination and seed maturation.

The results of pH effect on the interaction between MHP and α-amylase suggest that the ionic state of MHP which is a function of pH [20] is critical for the formation of the inactive enzyme–inhibitor or enzyme–inhibitor–substrate complex. Low pH (between 3.5 and 4) appears to be ideal for this interaction. Although, the mechanism of the MHP interaction with α-amylase is still unknown, the decreased inhibition at higher pH values would be in

agreement with the involvement of a positively charged imidazole group of the protein in the binding of inhibitor [21].

It is well known that Ca^{2+} are required for the activity and maximum stability of α-amylases [6, 7]. Removal of metal ions by the chelating agents or by dialysis results in reversible inactivation of enzyme as the activity may be completely restored on the addition of Ca^{2+} [22]. Because MHP forms insoluble calcium phytate [23], Cawley and Mitchell [5] concluded that inhibition of α-amylase in sprouted wheatmeal was due to the binding of Ca by MHP. Our results of the inhibition of maize, wheat and *Bacillus subtilis* α-amylases are not in agreement with the above conclusion since addition of Ca^{2+}, sufficient to bind the entire amount of MHP, did not reverse the inhibition caused by MHP. This is further supported by the result indicating that effectiveness of MHP as inhibitor of α-amylase decreases with increasing pH; while on the contrary its strength to bind Ca increases with increasing pH [20]. Thus, the inhibitory activity of MHP appears to be related to its direct interaction with the enzyme (protein), probably at an allosteric site, rather than its Ca-binding property. This is also in agreement with the findings that MHP forms complexes with plant and animal proteins [24].

Preincubation of MHP with α-amylase for a certain minimum time was found essential for maximum inhibition. Exactly similar results have been reported by Petrucci *et al.* in the case of human saliva α-amylase by 0.19 albumin fraction from wheat kernel [25]. In agreement with these authors. the preincubation results were interpreted to mean that the formation of enzyme–inhibitor–substrate complex was much slower than the formation of enzyme–substrate complex. As pointed out earlier in this paper, preincubation of MHP with starch before the addition of enzyme did not affect the inhibitory power of MHP. In this respect its effect is different from abscisic acid which when mixed with starch before adding the enzyme did not produce any inhibition of α-amylase [17].

It may be pointed out here that whereas α-amylases can be completely inhibited by MHP, the β-amylases are not inhibited by it (unpublished data). It then follows that in case β-amylase is present as contamination in α-amylase, the total inhibition of amylolytic activity would not be possible. Since more than 99% of the total amylolytic activity present in the enzyme preparations used in this study can be inhibited by using an appropriate amount of MHP, the enzyme preparations were considered to be relatively free from the β-amylase contamination. As such, trace contamination of β-amylase is unlikely to affect the results of α-amylase inhibition by MHP significantly.

EXPERIMENTAL

Material. All chemicals used in this study were reagent grade unless stated otherwise. Sodium phytate, $C_6H_6(OPO_3.Na_2)_6$, and soluble starch were obtained from B.D.H. Chemicals, Poole (U.K.). BSA and Sephadex B-100 were obtained from Sigma Chemical Co. (U.S.A.). Sweet potato β-amylase was from the V.P. Chest Institute, Delhi (India). Various seeds used as enzyme source were purchased from the local seed stores.

Enzyme preparation. All operations were carried out at 0–4° unless otherwise stated. Seeds were germinated in dark at 25°. Cotyledons (50 g) were separated from the 6–8 days old germinating seedlings and homogenised in ice-cold 0.1 M acetate buffer (pH 5) containing 0.1 M NaCl for 1 min. The clear homogenate was obtained by centrifugation at 12000 g for 30 min. The homogenate was then subjected to $(NH_4)_2SO_4$ pptn, and the protein fraction precipitating between 0.2 and 0.7 satn was collected by centrifugation at 20000 g for 1 hr. From this fraction α-amylase was separated by gel filtration on Sephadex G-100 as described in ref. [12]. The enzyme prepn so obtained was used without any further purification, since the β-amylase contamination was negligible (less than 1%). Bacterial α-amylase was from E. Merck AG, Darmstadt.

Enzyme assay. The α-amylase activity was assayed by the method of ref. [26] using soluble starch as the substrate and following the decrease of substrate concn with I_2/KI reagent. Unless otherwise stated the reaction mixture contained the following in a final vol. of 2 ml: 6 mg of soluble strach (0.5 ml buffered substrate soln); 200 μmol of acetate buffer (pH 4); 12 μmol of NaCl; and 0.1–1.0 units of α-amylase. Unless stated otherwise, inhibitor (1–8 mM) was added to the starch soln before the addition of the enzyme. Incubation was at 30° for 10 min and the decrease in starch concn was measured as described above at 620 nm. One unit of α-amylase is the amount of enzyme that digests 10 mg starch in 30 min under assay conditions. This method was used in all expts except kinetic studies. The measurements of α-amylase activity for Lineweaver–Burk plots (1/V versus 1/S) were made in the following manner: one unit of the enzyme was first preincubated without and with 0.65 mM, 1.3 mM, and 1.95 mM of MHP for 1–2 hr (time required for complete equilibrium between enzyme and MHP) at 30° and pH 4. The enzyme action was then started by adding the buffered starch soln to the reaction mixture. After 5 min incubation at 30°, the activity was measured with 3,5-dinitrosalicylate reagent [13]. One unit of α-amylase is that amount of enzyme which liberates 1 μmol of maltose in 1 min at 30° from 1% starch soln in 0.01 M acetate buffer, pH 4. The assay of β-amylase was performed by the same procedure and experimental conditions were also identical to those used for α-amylase. Protein was measured by the method of ref. [27] using BSA as standard.

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REFERENCES

1. Posternak, S. (1905) *Compt. Rend.* **140**, 322.
2. Asada, K. and Kasai, Z. (1962) *Plant Cell Physiol.* **3**, 397.
3. Dieckert, J. W., Snowden, J. E., Moore, A. T., Heinzehman, D. C. and Altschul, A. M. (1962) *J. Food. Sci.* **27**, 321.
4. Sharma, C. B. and Dieckert, J. W. (1975) *Physiol. Plantarum* **33**, 1.
5. Cawley, R. W. and Mitchell, T. A. (1968) *J. Sci. Fd Agric.* **19**, 106.
6. Buonocore, V., Poerio, E., Silano, V. and Tomashi, M. (1967) *Biochem. J.* **153**, 621.
7. Caldwell, M. L. and Kung, J. T. (1953) *J. Am. Chem. Soc.* **75**, 3132.
8. Sandstedt, R. M., Kneen, E. and Blish, M. J. (1939) *Cereal Chem.* **16**, 712.
9. Lineweaver, H. and Burk, D. J. (1934) *J. Am. Chem. Soc.* **56**, 658.
10. Friedenwald, J. S. and Maengwyn-Davies, G. D. (1954) *A Symposium on the Mechansim of Enzyme Action* (McElroy, W. D. and Glass, B. eds) p. 159. The Johns Hopkins Press, Baltimore.
11. Cleland, W. W. (1970) *The Enzymes* (Boyer, P. D., Lardy, H. and Myrback, K. eds) Vol. II, p. 19. Academic Press, New York.
12. McGeeney, K. F. and O'Donnell, M. D. (1976) *Biochim. Biophys. Acta* **422**, 159.
13. Silano, V., Pocchiari, F. and Kasarda, D. D. (1973) *Biochim. Biophys. Acta* **317**, 139.

14 Saunders, R. M. and Lang, J. A. (1973) *Phytochemistry* **12**, 1237.
15. Silano, V., Furia, M., Gianfreda, L., Macri, A., Palescandolo, R., Rab, A., Scardi, V., Stella, E. and Vafré, F. (1975) *Biochim. Biophys Acta* **391**, 170.
16. Shainkin, R. and Birk, Y. (1970) *Biochim. Biophys. Acta* **221**, 502.
17. Ogawa, M., Tanaka, K. Kasai, Z (1975) *Agr. Biol. Chem.* **39**, 695.
18. Varner, J. E. (1965) *Plant Biochemistry* (Bonner, J. and Varner, J. E. eds) p. 763. Academic Press, New York.
19. Hemberg, T. (1975) *Physiol. Plantarum* **35**, 11.
20. Brown, E. C., Heit, M. L. and Ryan, D. E. (1961) *Can. J. Chem.* **39**, 1290.
21. Taketa, K. and Pogell, B. M. (1965) *J. Biol. Chem.* **240**, 651.
22. Greenwood, C. T. (1970) *The Carbohydrates* (Pigman, W. and Horton, D. eds) Vol. II B, p. 471. Academic Press, New York.
23. Gosselin, R. E. and Coghlan, E. R (1953) *Arch. Biochem.* **45**, 301.
24. Bourdillon, J. (1951) *J. Biol. Chem.* **189**, 65.
25. Petrucci, T., Rab, A., Tomashi, M. and Silano, V. (1976) *Biochim. Biophys. Acta* **420**, 288.
26. Smith, B. W. and Roe, J. H. (1949) *J. Biol Chem.* **179**, 53.
27 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265