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INHIBITION OF PLANT PHYTASES BY PHLOROGLUCINOL

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; pumpkin seeds; phytase; phytic acid; polyphenol; phloroglucinol; inhibitor.

Abstract—Plant phytases (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) were inhibited by phloroglucinol (1,3,5-benzenetriol) *in vitro*. The inhibition of the *Cucurbita maxima* phytase was found to be non-competitive and pH dependent with an apparent inhibition constant (K_i) value of 2.3×10^{-2} M at optimum pH (4.8) and temperature (50°). The apparent number of inhibitor molecules (n) bound per enzyme molecule was found to be 2.2 suggesting that the positive cooperativity phenomenon may be present.

INTRODUCTION

Polyphenols occur widely in plants and are known to inhibit seed germination [1, 2] and many enzyme systems [3–7]. Phytase (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) occurs widely in germinating seeds and plays a vital role in mobilizing the phytate phosphorus during seed germination [8–10]. Its interaction with phenolic compounds was, therefore, considered to be of physiological significance, especially from the germination point of view. These considerations prompted us to ascertain the significance of polyphenol phytase interaction in seed metabolism. This paper deals with the interaction of phloroglucinol (1,3,5-benzenetriol) with pumpkin seed phytase.

RESULTS

The results given in Table 1 showed that phloroglucinol was a potential inhibitor of plant phytases though the extent of inhibition varied from source to source. The inhibition of pumpkin seed phytase was maximal followed by barley, maize and peanut enzymes. Surprisingly, however, the wheat and chick pea enzymes were only poorly inhibited.

Table 2 shows the relative effects of some structurally related phenols (phloroglucinol, orcinol and resorcinol) on the activity of pumpkin phytase *in vitro*. The effect of phloroglucinol was significantly greater compared to orcinol and resorcinol. In fact, the effect of both orcinol and resorcinol appeared to be insignificant. In view of the strong inhibitory activity of phloroglucinol towards pumpkin seed phytase, the interaction between the two has been investigated in detail.

Table 1. Inhibition of phytases by phloroglucinol

Inhibitor concn (mM)	Enzyme source and inhibition (%)					
	Pumpkin seed	Maize	Barley	Peanut	Wheat	Chick pea
20	25.0	13.0	16.6	10.0	3.5	1.5
30	45.0	25.0	26.6	18.0	7.5	4.0
40	55.0	38.0	40.0	27.2	13.0	8.5
50	60.0	45.0	50.8	38.6	18.5	11.0
60	68.5	50.0	57.0	54.5	22.5	12.0
70	—	53.0	60.0	59.0	25.0	12.5

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Table 2. Effect of some structurally related phenols on the dephosphorylation of phytic acid by phytase *in vitro*

Reaction system*	Relative activity (% of control)
Control	100
+ Phloroglucinol	40
+ Resorcinol	100
+ Orcinol	89.5

* The final concentration of the added phenols was 50 mM. Control was without added phenols. The reaction mixture and conditions for enzyme assay were those as described in the text.

Effect of pH

Fig. 1 shows the effect of pH on the inhibition of phytase by phloroglucinol. The maximum inhibition occurred around optimum pH (4.8). On either side of the pH optimum, the inhibition decreased rather sharply. For instance, the inhibition at pH 4.8 was 80%, but it decreased to nearly 11% both at pH 4 and 6.5. From the inhibition constant (K_i) data at various pH values (Fig. 1) it is apparent that the affinity ($1/K_i$) of phloroglucinol for the enzyme and enzyme–substrate complex to form inactive enzyme–inhibitor (EI), and enzyme–substrate–inhibitor (ESI) complexes was the highest at pH optimum, 4.8, while on either side of this pH it decreased sharply. Thus, the ionic state of the enzyme appears to be critical for the formation of inactive EI and ESI complexes. The decreased inhibition at higher pHs indicates the involvement of a positively charged imidazole group of the protein in the binding of phloroglucinol [11].

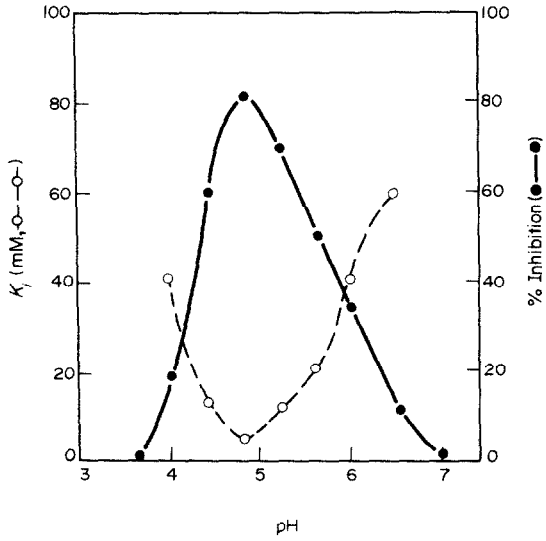

Fig. 1. Inhibitory activity of phloroglucinol towards pumpkin seed phytase and apparent inhibition constant (K_i) as a function of pH. % Inhibition (—●—●—) and K_i (—○—○—). Experiments with and without inhibitor (80 mM) at each pH were carried out simultaneously and the enzyme activity without inhibitor was used as 100%. Buffer was 0.05 M acetate for pH range 3.5–5.6, and the pH between 6 and 7 was adjusted by 2M NaOH. Assay conditions were as described in the text.

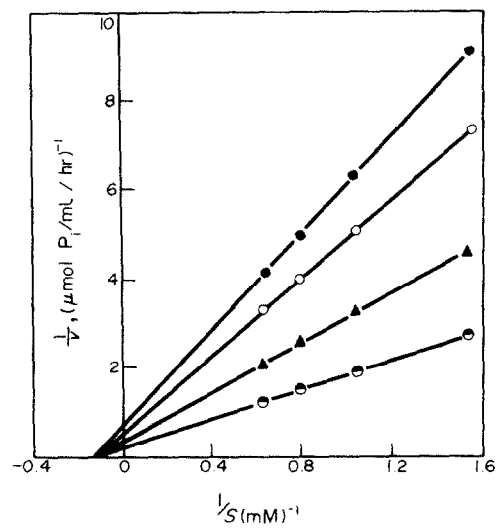

Fig. 2. Lineweaver–Burk plots showing the non-competitive type of inhibition of pumpkin seed phytase by phloroglucinol. (—●—●—), (—▲—▲—), (—○—○—) and (—●—●—) are with and without 20, 40 and 60 mM phloroglucinol, respectively.

Effect of PVP on the phloroglucinol inhibition of phytase

The results of the reactivation of the inactive EI and ESI complexes are given in Table 3. It was found that over 90% of enzyme activity was restored by the addition of PVP (3 mg/ml) to the reaction mixture.

Kinetic studies

The Lineweaver–Burk plots with and without the inhibitor (Fig. 2) indicated that the inhibition was of the non-competitive type. The value of K_i was 23 mM. The plots of relative enzyme activity against the inhibitor concentration (Fig. 3A) gave a sigmoid-shaped curve rather than a rectangular hyperbola suggesting that more than one inhibitor molecule per enzyme molecule participated in the formation of inactive enzyme–inhibitor complex [11, 12]. As expected, the plots $1/V$ vs inhibitor concentration (Fig. 4) were non-linear. These were concave upward at low inhibitor concentrations (0–25 mM), but above this range (between 25 and 30 mM) the plots were quite linear. Thus, the sigmoidal saturation function seems to limit the inhibitor control to a narrow and selected range of inhibitor concentrations.

From the inhibition data, a linear Hill plot of log $(V_o - V_i)/V_i$ vs log phloroglucinol concentration (Fig. 3B), where V_o and V_i are the reaction velocities without and with phloroglucinol at a fixed substrate concentration, respectively, was a straight line with a slope of

Table 3. Effect of PVP on phloroglucinol inhibition of phytase*

Additions (final concn)	Relative activity (% of control)
None (control)	100
Phloroglucinol (40 mM)	45 ± 3
Phloroglucinol (40 mM) + PVP (1 mg/ml)	66 ± 4
Phloroglucinol (40 mM) + PVP (2 mg/ml)	85 ± 4
Phloroglucinol (40 mM) + PVP (3 mg/ml)	94 ± 4

* Reaction mixture and conditions of enzyme assay were as described in the text except that enzyme–inhibitor complex was pre-incubated for 30 min with PVP before the addition of the substrate.

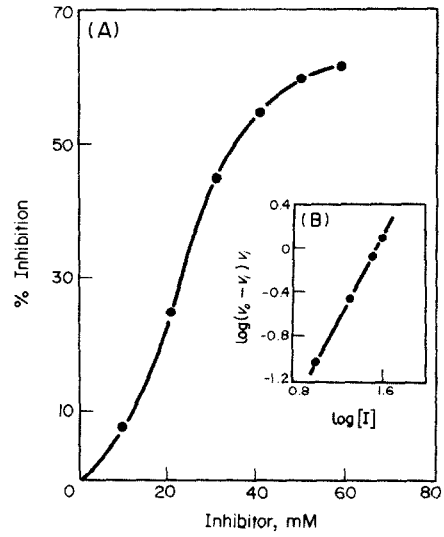

Fig. 3. (A) Plot of % inhibition vs phloroglucinol concentration showing sigmoid-shaped curve. (B) Hill plot of log $(V - V_i)/V_i$ vs log phloroglucinol concn, where V_o and V_i are the velocities of the enzyme catalysed reaction without and with inhibitor, respectively. The slope of this plot is Hill coefficient (n) and is a measure of the 'sigmoidicity' of the overall reaction between phloroglucinol and the enzyme. Conditions of enzyme assay were the same as described in the text.

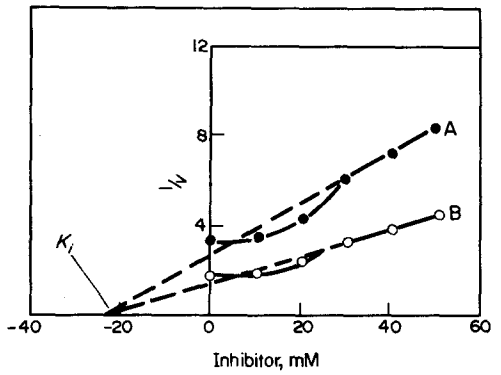

Fig. 4. Plots of $1/V$ vs phloroglucinol concentration at fixed substrate concentrations. Assays were carried out in the presence of varying inhibitor concentrations (0–50 mM) at 0.64 and 1.28 mM (A and B) substrate concentrations. The linear portion of the plots was extended backwards to obtain the value of K_i. Other experimental conditions were as described in the text.

2.2, which equals the apparent number of inhibitor molecules (n) bound per enzyme molecule. The data also indicated the presence of positive cooperativity, a phenomenon associated with most systems involving allosteric interactions [11, 13].

Thermodyanamic studies

From the inhibition data obtained at various temperatures, the entropy change (ΔS) for the overall reaction between phloroglucinol and phytase was calculated as described in ref. [11]. The values of ΔS at 20, 30, 40 and 50° were found to be − 173.6, −168.4, − 161.6 and − 156.1 J/deg/mol, respectively. The relatively large negative values of ΔS indicated that the formation of the inactive phytase–phloroglucinol complex involved marked conformational change in the enzyme protein.

DISCUSSION

Our results show that phloroglucinol is a powerful inhibitor of plant phytases. In general, phenolic compounds reduce the enzyme activity firstly by reducing the solubility of the enzyme proteins by forming insoluble protein–phenolics complexes [2], and secondly by direct inhibition of the enzymes by forming a soluble, but inactive enzyme–inhibitor complex [14–17]. The inhibitory activity of phloroglucinol appears to be of the second type since enzyme protein was not precipitated by the addition of phloroglucinol, and also according to Williams [2], a minimum MW of 500 or more is essential for phenols to produce effective inhibition by means of enzyme protein precipitation. Thus, phloroglucinol with MW 126 would not be suitable for the protein precipitation. The fact that orcinol and resorcinol, which are structurally related to phloroglucinol showed little or no inhibitory activity towards phytase is at present difficult to explain.

The results of kinetic studies indicated that the phytase inhibition by phloroglucinol, like that of the digestive enzymes by other phenolic compounds [4, 5], is of the non-competitive type. The kinetic and thermodynamic data for the interaction between phloroglucinol and the enzyme are very similar to those of the allosteric inhibition of fructose 1,6-diphosphatase by AMP [11]. However, whether the former is actually a case of allosteric inhibition is uncertain and further studies are required to clarify this point.

EXPERIMENTAL

Material. All chemicals used in this study were Analytical Reagent grade unless otherwise stated. Sodium phytate, $C_6H_6(OPO_3 \cdot Na_2)_6$, orcinol and resorcinol were obtained from B.D.H. Phloroglucinol was from Riedel, Hannover, (Germany). Sephadex G-100 and BSA were obtained from Sigma. 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 of *Cucurbita maxima* were sterilized by dipping in 1% NaOCl soln for 1–2 min, rinsed thoroughly with glass distilled H_2O and germinated in the dark at 30° under aseptic conditions as described by ref. [18]. Six-day-old germinating cotyledons (50 g) were homogenized with chilled Me_2CO (−10°) in a Waring blender for 1 min and then filtered. The residue was washed ×3 with chilled Me_2CO and dried *in vacuo*. About 25 g of the Me_2CO powder so obtained was homogenized in 250 ml 0.1 M acetate buffer (pH 5.0) for 1 hr. The homogenate was centrifuged at 10000 *g* for 1 hr, the clear supernatant was decanted and subjected to $(NH_4)_2SO_4$ precipitation. The protein fraction precipitating between 0.2 and 0.7 saturation contained all the phytase activity. It was collected by centrifugation at 20000 *g* for 1 hr, dissolved in a minimal vol. of 0.05 M acetate buffer and dialysed overnight against the same buffer. The contents of the dialysis bag were centrifuged as before to remove any inactive protein ppt. The clear enzyme soln thus obtained was concd by ultrafiltration and further purified by gel filtration. A sample (1.5 ml, *ca* 100 mg protein) was loaded on a 2 × 65 cm column of Sephadex G-100 which had previously been equilibrated with 0.05 M acetate buffer, pH 5.0. Elution was done with the same buffer at a flow rate of 8 ml/hr and 2 ml fractions were collected. Aliquots from each fraction were assayed for phytase activity. The enzyme-containing fractions were pooled and concd by dialysis against polyethylene glycol. At this stage the purification was *ca* 70-fold. This partially purified enzyme prepn was used in the present study.

Enzyme assay. Phytase activity was assayed by using phytic acid as the substrate and by measuring the amount of inorganic P liberated by the method of ref. [19]. The reaction mixture contained the following in a total vol. of 2.0 ml unless otherwise stated: 0.64 mM of phytic acid; 0.17 mM of acetate buffer (pH 4.8); and 0.1–1.0 unit of phytase. For inhibition studies the inhibitor (0–70 mM) was first pre-incubated with enzyme for *ca* 15 min at the reaction temp. and pH. The enzyme action was then started by adding the buffered substrate soln to the reaction mixture. Incubation was carried out at 50° for 30–60 min, and the enzyme activity was terminated by adding cold 10% TCA. One unit of enzyme activity is the amount of enzyme in mg which liberated 1 μmol of inorganic P per min under assay conditions. Protein was measured by the method of ref. [20], using BSA as a standard.

Kinetic studies. The nature of phytase inhibition by phloroglucinol was evaluated according to the method of ref. [21]. Values of apparent inhibition constant (K_i) were determined from Dixon plots of $1/v$ vs inhibitor concn [22]. For these plots enzyme was assayed by using 0.64 and 1.28 mM substrate concns and 5 different inhibitor concns in the range 0–50 mM. Other experimental conditions were the same as those described under enzyme assay. In the low inhibitor concn range (0–25 mM),

the plots were non-linear, but above this concn (between 25 and 50 mM) the plots were quite linear (Fig. 4). It was extended backwards to obtain the values of K_i. The K_i values so obtained were in agreement with those obtained by replotting the slopes of double reciprocal plots (Fig. 2) vs inhibitor concns [23]. Interaction constant (n) was determined by the method of ref. [24].

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