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MULTIPLE FORMS OF PHYTASE IN GERMINATING COTYLEDONS OF *CUCURBITA MAXIMA**

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; pumpkin seeds; phytase; myoinositol hexaphosphate; phytic acid.

Abstract—Multiple forms of phytase (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) have been isolated in highly purified forms from germinating *Cucurbita maxima* cotyledons using acetone and ammonium sulphate fractionation, Sephadex gel filtration and ion exchange chromatography on DEAE- and CM-cellulose. Gel filtration produced two peaks of phytase activity; phytase I (high MW) and phytase II (low MW). Phytase I was further resolved into 4 distinct species on CM-cellulose and these were designated phytase IA, IB, IC and ID, according to their elution order. On the other hand, phytase II remained as a single species with a purification of 35-fold. The MWs of each phytase I species were identical (MW 66500 ± 4000) and they were twice the MW of phytase II (MW 32400 ± 4000) indicating that I and II may be structurally related. The properties of various molecular forms were compared. The difference in properties between phytase II and phytase I isoenzymes (IA, IB, IC and ID) was more pronounced than that observed among the isoenzymes of phytase I alone.

INTRODUCTION

Phytase (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) catalyses the hydrolysis of myoinositol hexaphosphate (phytic acid), the primary reserve of organic phosphate in seeds, in the metabolism of the germinating seedlings [1–6]. Recently, Lim and Tate [7, 8] and Ikawa *et al.* [9] reported the presence of multiple forms of phytase in wheat bran and rice bran, respectively. Enzyme preparations of sufficient purity suitable for characterization and structural studies have upto now been unavailable. The present study was undertaken to find a suitable plant source and design a simple scheme for the isolation of multiple forms of phytase. This paper describes the separation and purification of five molecular species of phytase from germinating *Cucurbita maxima* seeds.

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Table 1. Purification of multiple phytases from pumpkin seeds

Purification step	Enzyme (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)*	Yield (%)
Crude extract†	8000	9100	0.88		100
Acetone powder extract‡	7200	6400	1.1	1.25	90
$(NH_4)_2SO_4$ 30–80% fraction	6300	1500	4.2	4.8	78.8
Sephadex G-100 eluate:					
Peak I (phytase I)	4200	70	60	68.7	52.5
Peak II (phytase II)	500	40	12.5	14.2	6.2
DEAE-cellulose of phytase I:					
Peak I (phytase I)	2700	28	96.4	107.9	33.8
Peak II (phytase II)	70	2.2	32	36.4	0.9
DEAE-cellulose of phytase II	330	13	25.4	26	4.1
CM-cellulose of phytase I:					
Peak I (phytase IA)	800	7.3	109	123.8	10
Peak II (phytase IB)	460	4.3	105	119.3	5.8
Peak III (phytase IC)	440	4.6	106	120.2	5.4
Peak IV (phytase ID)	300	2.8	101	121.8	3.8
CM-cellulose of phytase II	200	6.5	30	35	2.5

* Sp. act./sp. act. of crude extract.

† Crude extract was prepared from 20 g cotyledons (fr. wt) and values were multiplied by 10 to obtain the values for 200 g cotyledons.

‡ Acetone powder was prepared from 200 g cotyledons (fr. wt).

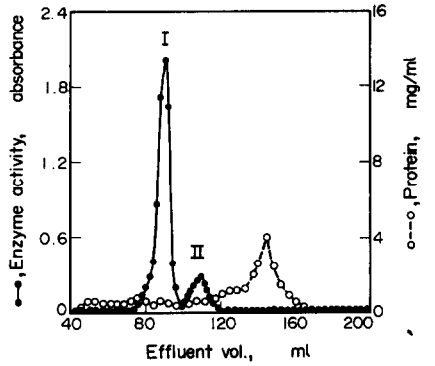

Fig. 1. Elution profile of the $(NH_4)_2SO_4$ fraction from a Sephadex G-100 column (2 × 62 cm). (●——●), Phytase activity expressed as *A* at 660 nm/min/0.1 ml of the eluate; (O-----O), protein by method of Lowry *et al.* [19] expressed as mg per ml. The detailed procedures for gel filtration and enzyme assay are described in the text.

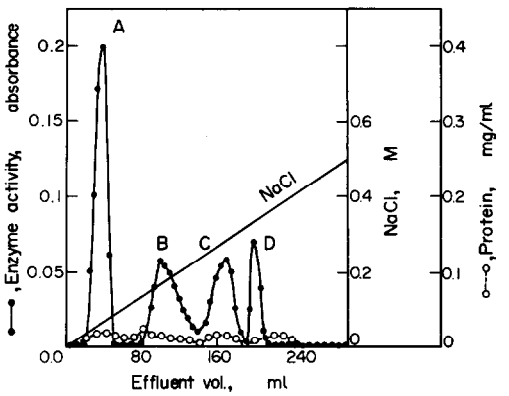

Fig. 3. CM-cellulose chromatography of the phytase I fraction obtained from the DEAE-cellulose column (peak I, Fig. 2). (●——●), Phytase activity as *A* at 660 nm/min/0.1 ml of the eluate; (O-----O), protein as mg per ml. Peaks A, B, C and D represent various forms of phytase. The detailed procedures for chromatography and enzyme assay are described in the text.

RESULTS AND DISCUSSION

Table 1 summarizes the separation and purification procedure of the multiple forms of phytase from the germinating pumpkin cotyledons. Treatment with chilled acetone was a useful step in removing the lipids which would otherwise interfere with the isolation procedure. This step also resulted in 1.25-fold purification with a yield of 90%. The increase of activity after acetone treatment could have been due to the removal of some inhibitory lipid substance present in the cotyledons.

The $(NH_4)_2SO_4$ fractionation enriched the enzyme further and the protein fraction precipitating between 0.3 and 0.8 saturation contained the bulk of the enzyme activity. Gel filtration of this fraction on Sephadex G-100 produced two phytase-containing peaks representing the high MW (phytase I) and the low MW (phytase II) enzymes (Fig. 1). Phytase I appeared to be dominant, accounting for nearly 90% of the combined activity in I and II. At this stage, the purification of I and II was 68.7- and 14.2-fold with 52.5 and 6.2% yields, respectively.

The results of DEAE-cellulose chromatography of phytase I are shown in Fig. 2. The enzyme was resolved into two peaks eluting at 0.23 and 0.3 M salt gradient. The peak at 0.23 M NaCl was longest and contained *ca* 97% of the total activity recovered from the column. The small peak at 0.3 M NaCl accounted for less than 3% of the total activity and appeared to be a contamination of phytase II (peak II in Fig. 1) as the latter also eluted at 0.3 M NaCl gradient from the DEAE-cellulose column using the same experimental conditions. This small fraction in Fig. 2 was not analysed further. In this step the phytase I was substantially purified.

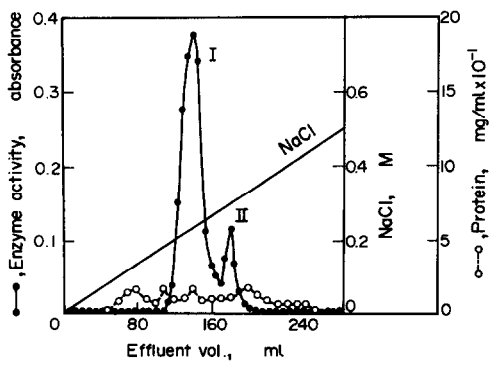

Fig. 2. DEAE-cellulose chromatography of the phytase I fraction obtained from the Sephadex column. (●——●), Phytase activity expressed as *A* at 660 nm/min/0.1 ml of the eluate; (O-----O), protein expressed as mg per ml. The detailed procedures are given in the text.

The phytase II fraction chromatographed as a single peak on the DEAE-cellulose column. It was also substantially purified (26-fold) with a yield of 4.1%.

Fig. 3 shows the elution profile of phytase I fraction from the CM-cellulose column. It was observed that phytase I was resolved into 4 distinct phytase-containing peaks (A, B, C and D) eluting at 0.06, 0.17, 0.28 and 0.33 M NaCl gradients (Fig. 3). These results indicated the presence of 4 distinct species of phytase in fraction I obtained from the DEAE-cellulose column. These were designated as phytase IA, IB, IC and ID according to the elution order and accounted for *ca* 40, 23, 22 and 15% of the total activity eluted, respectively. Phytase II, on the other hand, eluted from the CM-cellulose column in a single sharp peak at 0.15 M NaCl gradient. All phytase preparations appeared homogeneous by polyacrylamide gel-electrophoresis.

Multiple forms of phytase were also reported in wheat [7, 8] and rice [9], but these were not purified and their number was also not certain. The present study is probably the first which provides the purified preparations of 5 distinct molecular species of phytase suitable for characterization and structural studies.

A comparison of the properties of various phytases is shown in Table 2. Phytase IA, IB, IC and ID had identical MWs (66 500), but phytase II had a MW of 32 400. Thus, on the basis of MW, it appears that the multiple forms of phytase in pumpkin seeds are of two types: the high MW type (phytase IA, IB, IC and ID) and the low MW type (phytase II). The two types exhibit entirely different properties. On the other hand, various phytase I forms show identical properties except the electrophoretic

Table 2. Some properties of various molecular forms of *Cucurbita maxima* phytase

Properties	Forms of phytase IA	IB	IC	ID	II
MW*	66500	66500	66500	66500	32400
Optimum pH	4.8	4.8	5.2	5.2	5.5
Optimum temp.	48°	50°	50°	52°	55°
Thermal stability % residual activity after 60 min at:					
60°	60 ± 3	70 ± 2	75 ± 2	70 ± 2	90 ± 4
70°	35 ± 2	45 ± 2	40 ± 2	42 ± 2	60 ± 3
80°	10 ± 2	20 ± 3	8 ± 2	12 ± 2	20 ± 2
Apparent K_m (mM)	6.7	2.8	2.2	4.5	1.5
V_{max} (μmol/hr/mg)	66.5	39.0	40.0	66.0	14.2
Energy of activation (kcal/mol)	13.8	8.3	11.0	10.0	13.8
Electrophoretic mobility†	0.35	0.38	0.24	0.25	0.42
Inhibition by F^- K_i (mM)	2.5	2.0	3.5	2.7	1.0

* MW was determined by Sephadex G-100 gel filtration method [20].

† Electrophoretic mobility was determined at pH 8.2 in phosphate buffer using bromophenol blue as reference.

mobilities and elution patterns from the CM-cellulose column. At the present time it is not certain whether phytase II is derived from any of phytase I forms *in vivo* or whether both are produced independently.

The enzymic dephosphorylation of phytic acid is known to occur in a stepwise manner involving multiple substrate pathways [7, 8, 10, 11]. There are 6 possible sites on the phytate molecule for enzyme attack and different isomeric myoinositol pentaphosphates are known to be produced during the hydrolysis of phytate by enzymes derived from different sources [8, 12–16]. Based on such results, Lim and Tate [8], Johnson and Tate [13] and Greaves *et al.* [16] suggested that in biological systems multiple phytases are involved in the dephosphorylation of phytate.

In general, various phytase forms showed significantly different phytate hydrolysis rates. Since different spatial arrangements of the substituent phosphate radicals of phytate molecule will greatly affect the rate of hydrolysis [16], it seems likely that different enzyme forms attack at different sites on the phytate molecule. This view would also be consistent with the 'two-point attachment' mechanism between the substrate and the enzyme proposed by Gibbins and Norris [17]. Although the mode of dephosphorylation of phytate by pumpkin seed phytases has not been investigated, their occurrence in the germinating pumpkin seeds is significant and lends further support to the above hypothesis.

EXPERIMENTAL

Materials. Sodium phytate, $C_6H_6(OPO_3, Na_2)_6$was obtained from B.D.H. Sephadex G-100, BSA and CM-cellulose were from Sigma and Cellex-D (DEAE-cellulose) was from Bio-Rad. Pumpkin seeds were obtained locally.

Enzyme assay. Phytase activity was assayed with phytic acid as the substrate and by measuring the amount of Pi liberated by the method of ref. [18]. The reaction mixture contained the following in a total vol. of 2 ml, unless otherwise stated: 0.64 mM of phytic acid; 0.17 mM of NaOAc buffer (pH 4.8) and 0.1–1 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 45° 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 one μmol of Pi per min under assay conditions. Protein was measured by the method of ref. [19], using BSA as a standard.

MW was measured by gel filtration using a Sephadex G-100 column (2 × 65 cm) as described in ref. [20]. Cytochrome *c*, BSA and alkaline phosphatase were used as protein standards.

Seed germination. Seeds were sterilized and germinated in dark at 30° under aseptic conditions as described in ref. [21].

Separation and purification of multiple forms of phytase. All operations were carried out at 0–4° unless otherwise stated. Phytase activity was determined at each stage of purification. The following steps were used:

Me_2CO *powder extract.* Cotyledons (200 g) of 6-day-old seedlings were defatted 3× by homogenizing in a Waring blender with chilled (−20°) Me_2CO. The homogenate was filtered under suction and dried *in vacuo*. Defatted meal (Me_2CO powder) (*ca* 100 g) was extracted in 1 l. of 0.1 M NaOAc buffer (pH 5) for 12 hr. The homogenate was centrifuged for 1 hr at 10000 *g* and the supernatant fraction collected by decantation. It was referred to as Me_2CO powder extract.

$(NH_4)_2SO_4$ *fractionation.* The protein fraction precipitating between 0.3 and 0.8 satn contained all the phytase activity. It was collected by centrifugation for 1 hr at 20000 *g*, dissolved in a min vol. of 0.05 M NaOAc buffer, pH 5 and dialysed 18 hr against the same buffer. The contents of the dialysis bag were centrifuged as before to remove the inactive protein ppt. The supernatant fraction was concd by ultrafiltration and subjected to the following purification steps.

Gel-filtration on Sephadex G-100. A 1.5 ml sample (*ca* 150 mg protein) was loaded on a 2 × 62 cm column of Sephadex G-100 made up in 0.05 M NaOAc buffer, pH 4.8. Elution was with the same buffer at a flow rate of 8 ml/hr (2 ml fractions). Aliquots from each fraction (0.1 ml) were assayed both for the phytase and protein content. As shown in Fig. 1, two phytase-containing peaks were obtained. The tubes representing each enzyme peak were pooled separately, precipitated by $(NH_4)_2SO_4$ at 100% satn and centrifuged for 1 hr at 20000 *g*. The ppt. was dissolved in 25 mM Tris–HCl buffer (pH 7.2) and dialysed 18 hr against the same buffer. The fast eluting (large MW) and the slow eluting (small MW) enzyme fractions were referred to as phytase I and phytase II, respectively.

DEAE-cellulose column chromatography. The dialysed enzyme fractions (phytase I and II) (15 ml: 4 and 7 mg protein, respectively) were separately applied to the DEAE-cellulose columns

(2.5 × 31 cm) which were pre-equilibrated with 25 mM Tris–HCl buffer (pH 7.2). The columns were washed with 200 ml of buffer to remove unabsorbed protein. The absorbed proteins were eluted by a linear NaCl gradient (0–0.5 M NaCl) using a single mixing container with 150 ml 25 mM Tris–HCl buffer (pH 7.2) and a reservoir with 150 ml of the same buffer containing 0.5 M NaCl. Fractions (5 ml) were collected at a flow rate of 1 ml/min and aliquots (0.1 ml) from each fraction were analysed for the protein content and phytase activity. Fractions with enzyme activity were pooled and precipitated with $(NH_4)_2SO_4$ at 100% satn.

CM-cellulose column chromatography. Phytase I and II fractions obtained from the DEAE-cellulose columns were dissolved in 50 mM NaOAc buffer, pH 4.8 and dialysed for 18 hr against the same buffer. *Ca* 2.7 mg protein in 15 ml were applied to a CM-cellulose column (1.25 × 30 cm), previously equilibrated with 50 mM NaOAc buffer (pH 4.8), and the column was washed with 200 ml of buffer to remove unbound protein. The absorbed protein was eluted by a linear NaCl gradient (0–0.5 M) using a single mixing vessel with 150 ml 50 mM NaOAc buffer and a reservoir with 150 ml of the same buffer containing 0.5 M NaCl. Fractions (5 ml) were collected at a flow rate of 1 ml/min. Aliquots (0.1 ml) from each fraction were assayed for phytase activity. Tubes in each peak showing phytase activity were pooled separately and concd by ultrafiltration.

Disc electrophoresis was carried out on 5% acrylamide gel in 50 mM NaPi buffer (pH 8.2) as described in ref. [22]. Protein samples (40–60 μg) were layered through the electrode buffer in 40% sucrose soln. Bromophenol blue was used as the tracker dye. A constant current of 5 mA per gel was applied for 2 hr. After the run, the protein bands were located by staining with Coomassie blue [23] and destaining with 3% HOAc.

Kinetic studies. Energy of activation was determined by Arrhenius plots [24]. K_m and V_{max} values were obtained from Lineweaver–Burk plots [25] using at least 5 substrate concns. Values of apparent inhibition constant (K_i) were obtained from Dixon plots [26]. In all expts, conditions of incubation were as described under enzyme assay.

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