Purification and characterization of a phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination

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Phytase (*myo*-inositol-hexakisphosphate phosphohydrolase, EC 3.1.3.8) has been purified from 5–7-day-old maize (*Zea mays*) seedlings, using a four-step purification procedure. The native protein has a molecular mass of about 76 kDa and is built up from two 38 kDa subunits. The pH and temperature optima of the purified enzyme were respectively 4.8 and 55 °C. The apparent K_m for phytate was estimated to be 117 μM. Like other acidic phytases, the maize seedling enzyme exhibited a broad affinity for various phosphorylated substrates and especially for penta- and tri-phosphate esters of *myo*-inositol. The amino acid composition of the h.p.l.c.-purified protein indicated a high hydrophobicity (44 % non-polar amino acids). Rabbit antibodies were produced in response to maize seedling phytase. Western-blot analyses clearly demonstrate that the increase of phytase activity observed during the first 7 days of germination corresponded to an accumulation of the protein in maize seedlings. Phytase accumulated essentially in the shoots (mesocotyl plus coleoptile.

INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) is an abundant plant constituent, comprising 1–5 % (w/w) of legumes, cereals, oilseeds, pollen and nuts (Gibson and Ullah, 1990). It forms a complex salt called phytin with counterions including K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}. In seeds, phytin is usually found in organelles called protein bodies, where it generally constitutes an inclusion, the globoid (Pernollet, 1978). These organelles are localized in the aleurone layer in cereals and in the endosperm and cotyledons in legumes and oilseeds. Maize (*Zea mays*) is an exception to the typical localization pattern in monocotyledons, as 88 % of phytic acid has been reported to be found in the germ rather than in the aleurone layer (Gibson and Ullah, 1990).

The function of these large amounts of phytin is unclear. In seeds the concentration of phytin phosphorus may account for a large part of total phosphorus reserve. Maize seeds, for example, contain 0.89 % (w/w) phytic acid, which represents up to 88 % of total phosphorus (Gibson and Ullah, 1990). Therefore phytin is generally considered as a store of phosphorus and *myo*-inositol, both of which are necessary for seed germination.

Another function of phytin has been reported by Graft et al. (1987); these authors have observed that phytate, by virtue of chelating free iron, is a potent natural antioxidant. They have shown that Fe^{3+}–phytate does not retain a reactive co-ordination site, in contrast with most other known chelates, and consequently it fails to support hydroxyl-radical ($OH^{\cdot}$) generation (Graft et al., 1984). The suppression of these oxidative events, ordinarily catalysed by free iron and many iron chelates, may well be an important function of phytate within plant seeds. Indeed, a large amount of iron in seeds is complexed by phytate (Morris and Ellis, 1976), which may explain the extremely long viability of some seeds despite these dangerous amounts of iron. The liberation of this chelated iron at the beginning of germination by the hydrolysis of phytate may be necessary for the seedling development.

Germination of seeds or pollen leads to a rapid disappearance of phytin inclusions accompanied by a large increase in activity of the enzyme responsible for phytin degradation, phytase (*myo*-inositol-hexakisphosphate phosphohydrolase, EC 3.1.3.8) (Gibson and Ullah, 1990; Loewus et al., 1990). In most cases it is not clear whether this rise in phytase activity is the result of the activation of pre-existing enzyme or of *de novo* synthesis of the protein.

Phytase activities have been partially purified and characterized from many germinating seeds and pollens. However, only two phytases have been purified to homogeneity from cotyledons of *Phaseolus aureus* (mung bean) (Maiti and Biswas, 1979) and of *Glycine max* (soybean) (Gibson and Ullah, 1988).

As this enzyme may play an important role during germination, our objective was to isolate and purify maize seed phytase in order to study the regulation of its synthesis and to try to understand its function, as well as that of phytin, during germination.

MATERIALS AND METHODS

Plant cultures

Maize seeds (*Zea mays* c.v. MO17; Rhone Poulenc) were soaked for 24 h in distilled aerated water and then grown in wet vermiculite at 28 °C with a 16 h photoperiod (80 μE/s per m^2).

Enzyme extraction and purification

The following procedure was adapted from that described by Gibson and Ullah (1988) for soybean phytase. All the steps were performed at 4 °C. Seedlings (5 or 6 days old), resulting from the germination of about 200 g of dry seeds, were extensively washed with distilled water and ground in a cold Waring blender in 800 ml of 100 mM sodium acetate (pH 4.8)/10 mM $CaCl_2$/1 mM dithiothreitol/1 mM phenylmethanesulphonyl fluoride. After filtration through two layers of 300 μm-mesh nylon filter and centrifugation at 8000 *g* for 20 min, the supernatant was adjusted to 30 % $(NH_4)_2SO_4$ saturation. After centrifugation at 8000 *g* for 20 min, the supernatant was adjusted to 60 % $(NH_4)_2SO_4$ saturation. The 30–60 %-satd.-$(NH_4)_2SO_4$ pellet was solubilized in about 50 ml of extraction buffer and extensively dialysed against 20 mM Tris/acetate, pH 7.5. The precipitate formed during

dialysis was removed by centrifugation (8000 *g*, 30 min); it did not contain appreciable phytase activity.

The dialysed $(NH_4)_2SO_4$ fraction was loaded on to a DEAE-Trisacryl M (IBF) anionic-exchanger column (3 cm × 7 cm), equilibrated with 20 mM Tris/acetate, pH 7.5, at a flow rate 30 ml/h. The column was washed with 100 ml of loading buffer, and eluted with a linear gradient from 20 to 300 mM Tris/acetate, pH 7.5 (160 ml). The fractions (3 ml) containing phytase activity were pooled and concentrated by addition of $(NH_4)_2SO_4$ to 65% saturation. After centrifugation at 8000 *g* for 30 min, the pellet was solubilized in 2.5–3 ml of 100 mM sodium acetate (pH 4.8)/0.25 M KCl.

The solubilized pellet was then loaded on to an Ultrogel AcA 34 (IBF) column (1.4 cm × 120 cm) and eluted (3 ml fractions) with the same buffer at a flow rate of 13 ml/h. The fractions containing phytase activity were pooled, added to 10% glycerol and dialysed against 50 mM sodium acetate, pH 4.8, containing 10% glycerol. All the subsequent operations were done in the presence of 10% glycerol.

Further purification was obtained by chromatography of the dialysed fraction on a SP-Trisacryl M (IBF) cationic-exchanger column (0.8 cm × 4 cm) equilibrated with the loading buffer [50 mM sodium acetate (pH 4.8)/10% glycerol]. After washing with 30 ml of the same buffer, the column was eluted with a linear gradient from 0.05 to 0.4 M NaCl in the same buffer (50 ml) at a flow rate of 30 ml/h.

The active fractions were pooled, dialysed against the same buffer without NaCl, and concentrated on a small volume of SP-Trisacryl (500 μl), washed with loading buffer containing 0.1 M NaCl, and eluted with 500 μl of 0.3 M NaCl in the loading buffer.

In some experiments, especially at the end of purification (after the Ultrogel AcA 34 filtration), the dialysis steps were substituted by filtration on a Bio-Gel P6DG (Bio-Rad) column.

Protein estimation

Protein concentrations were determined as described by Bradford (1976), with BSA as standard.

Enzyme-activity assays

Phytase activity was measured at 55 °C by the method of Gibson and Ullah (1988), except that sodium phytate (Sigma) was 2 mM in the assay instead of 0.5 mM, and that 2 mM $CaCl_2$ was added. The activity of the purified enzyme was found to be stabilized by addition of BSA to the assay at a concentration 2–3-fold that of the enzyme. For the determination of the enzymic characteristics of the purified phytase, BSA was systematically added. Enzyme activity was expressed as nmol of P_i liberated/min per mg of protein.

Acid phosphatase activity was measured at 400 nm by monitoring the release of *p*-nitrophenol as described by Gibson and Ullah (1988). Enzyme activity was expressed as nmol of *p*-nitrophenol liberated/min per mg of protein.

Gel electrophoresis

Standard native 7.5%-PAGE (Davis, 1964) was carried out at 4 °C. Phosphatase activities were detected on the gel with 1-naphthyl phosphate coupled with Fast Blue RR as described by Gabard and Jones (1986).

Estimation of the molecular mass of native phytase was done using a 4–25% polyacrylamide gradient gel run at 100 V for 24 h in 89 mM Tris/borate buffer, pH 8.4.

Electrophoresis under denaturing conditions was performed with a 12.5%-polyacrylamide/SDS gel as described by Laemmli (1970). Proteins were stained with Coomassie Blue R-250.

Antibody production

Purified maize seed phytase (150 μg) was injected into a rabbit. The two first injections were carried out at 14-day intervals and the two others at 21-day intervals. The titre of the antibodies was determined after the third injection, prior to final bleeding 10 days after the fourth injection; 10 ng of the purified protein were detected with the serum diluted at 1:5000.

The crude antiserum was then affinity-purified as described by Lin et al. (1989); briefly, purified phytase (30 μg) was subjected to SDS/PAGE and the gel was subsequently electroblotted on to a nitrocellulose membrane. The region on the nitrocellulose corresponding to the phytase subunit was excised and incubated with the crude rabbit anti-phytase immune serum, diluted 10-fold. The bound antibodies were eluted with 0.5 ml acidic washes and, after neutralization, affinity-purified antibodies were stored at −80 °C.

Western blots

Total proteins were extracted from 0.2 g of frozen seedlings as described by Nechustai and Nelson (1985). Proteins were subjected to SDS/12.5%-PAGE and electroblotted on to nitrocellulose membrane. Probing the blots with purified maize phytase antibodies was done as described by Hahn and Stiegler (1986), using anti-rabbit IgG sheep immunoglobulins coupled with peroxidase (Biosys).

Determination of amino acid composition

Purified phytase (100 μg) was precipitated with 4 vol. of acetone at −20 °C. The pellet was resuspended into 200 μl of Tris/HCl (pH 7.6)/150 mM LiCl/6 M urea/0.4 M mercaptoethanol, before injection for h.p.l.c. on to a 30 nm (300 Å)-pore-size C3 silica column (Ultrapore RPSC; Beckman). The column was eluted with a 30–70%-acetonitrile gradient in 0.1% trifluoroacetic acid. After freeze-drying, maize phytase was hydrolysed for 24 h under reduced pressure at 110 °C in constant-boiling HCl. Analysis were performed on a Beckman amino acid analyser, model 6300, with the standard sodium citrate eluting buffer.

RESULTS

Increase of phytase activity during maize seed germination

Proteins, extracted from 30 seedlings at various times of germination, were precipitated with $(NH_4)_2SO_4$ to 60% saturation. After solubilization of the pellet and dialysis against 100 mM sodium acetate pH 4.8, protein concentrations and phytase activities were determined. Figure 1 shows that phytase activity per seedling increased from day 1 to day 5. No significant activity could be detected at zero time (after 24 h hydratation in distilled water as described in the Materials and methods section). Phytase activity reaches a plateau between days 5 and 7. During this time the amounts of total proteins extracted from 30 seedlings did not change significantly. For the purification of phytase, seedlings were taken at 5 or 6 days.

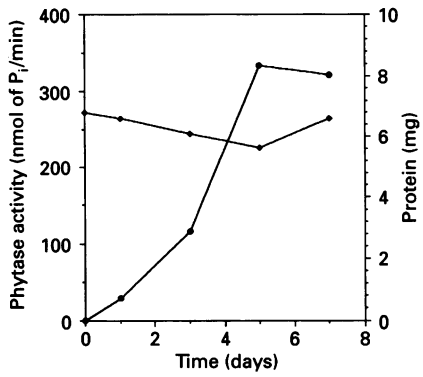

Figure 1 Time course of phytase activity in maize seedlings during germination

The 0–60%-satd.-$(NH_4)_2SO_4$ fractions of proteins extracted from 30 seedlings were analysed for phytase activity (●) and protein levels (◆) after various times of germination. The results correspond to 30 seedlings.

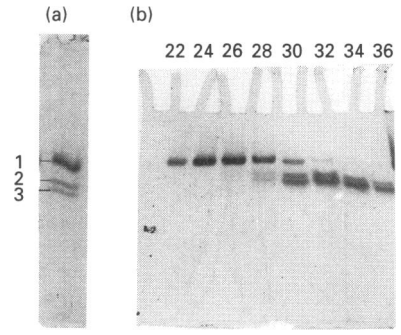

Figure 2 Detection of phosphatase activities on a native polyacrylamide gel

(**a**) A 20 μg portion of protein of the 30–60%-satd.-$(NH_4)_2SO_4$ fraction were electrophoresed. Phosphatase activities were detected using 1-naphthyl phosphate and Fast Blue RR. (**b**) Electrophoretic analysis of phosphatase activities of different fractions of the DEAE-Trisacryl column (see Figure 3b). A 10 μl portion of each fraction was loaded on a 7.5% non-denaturing polyacrylamide gel.

Purification of maize seed phytase

One difficulty encountered in phytase purification is to separate phytase activity from other acid phosphatases. In the protocol described by Gibson and Ullah (1988), the authors succeeded in separating the soybean phytase activity from the other acid phosphatase activities by a one-step chromatography on a strong cationic-exchange column (SP Trisacryl M) at pH 4.5. Soybean phytase activity was not retained on the column, whereas the major phosphatase activity was eluted by a saline gradient. This step did not allow us to separate phytase from other acid phosphatase activities in the case of maize seedling extracts. Both activities were retained on the column and eluted by the same concentration of NaCl.

The experimental scheme described in the Materials and methods section permitted the purification of maize seed phytase. This scheme is given in Table 1. When the 30–60%-satd.-$(NH_4)_2SO_4$ fraction was analysed by native gel electrophoresis and stained for phosphatase activities, three bands of activity were detected (Figure 2a): a major one (1) and two minor bands (2) and (3). The major acid phosphatase activity was partially separated from phytase activity on the first anionic-exchange DEAE-Trisacryl column (Figure 3a). The large peak of phosphatase activity corresponded to the major activity stained on the polyacrylamide gel (Figure 2b, fractions 22–28), whereas the peak of phytase activity contained the two low phosphatase activities (Figure 2b, fractions 30–36).

A gel-filtration step on AcA34 gel was then employed; after this step the remaining major phosphatase activity was completely separated from the phytase activity (Figure 3b).

Further purification was achieved by chromatography on a cationic-exchange SP-Trisacryl column: phytase was eluted for a 0.25 M NaCl concentration (Figure 3c). After this step, the enzyme was diluted in about 20 ml of buffer. Different procedures of concentration were tried, leading to a high loss of activity. The best procedure to concentrate the enzyme was the use of a micro column of SP-Trisacryl, which allowed a 20–40-fold concentration and eliminated traces of contaminant proteins.

It is noteworthy that, after the AcA34 step, the phytase activity was generally very unstable. Addition of 10% glycerol and 2 mM $CaCl_2$ was found to stabilize this activity somewhat; however, the apparent specific activity of purified phytase varied considerably from one experiment to another.

Electrophoresis analysis of purified phytase

Non-denaturant PAGE of the purified fraction after concentration on SP-Trisacryl, showed two bands stained by Coomassie Blue (a and b; Figure 4a). These proteins corresponded to the phosphatase activities 2 and 3 shown in Figure 2. Under longer conditions of electrophoresis on a gradient of polyacrylamide, only one large band was observed, with an apparent molecular mass of 76 kDa. When this fraction was analysed on a 12.5%-polyacrylamide/SDS reducing gel, only one subunit, of 38 kDa, was observed (Figure 4b). Therefore the two bands, a and b, observed on Figure 4(a) appeared to differ only by their charge. The native maize seed phytase would seem to be a dimer of a 38 kDa subunit.

When gels were stained with periodic acid–Schiff reagent

Table 1 Purification of maize seed phytase

Step	Volume (ml)	Enzyme activity (nmol of P_i/min)	Protein (mg)	Specific activity (nmol of P_i/min per mg)	Purification (fold)	Yield (%)
30–60%-satd.-$(NH_4)_2SO_4$ pellet	150.0	4111	130	31	1.0	100
DEAE-Trisacryl column	27.0	2745	17	161	5.0	66
AcA 34 filtration	27.0	1250	3.2	416	13.0	30
SP-Trisacryl						
Column	22.0	492	0.5	1041	32.0	12
Concentration	1.0	389	0.17	2288	73.0	10

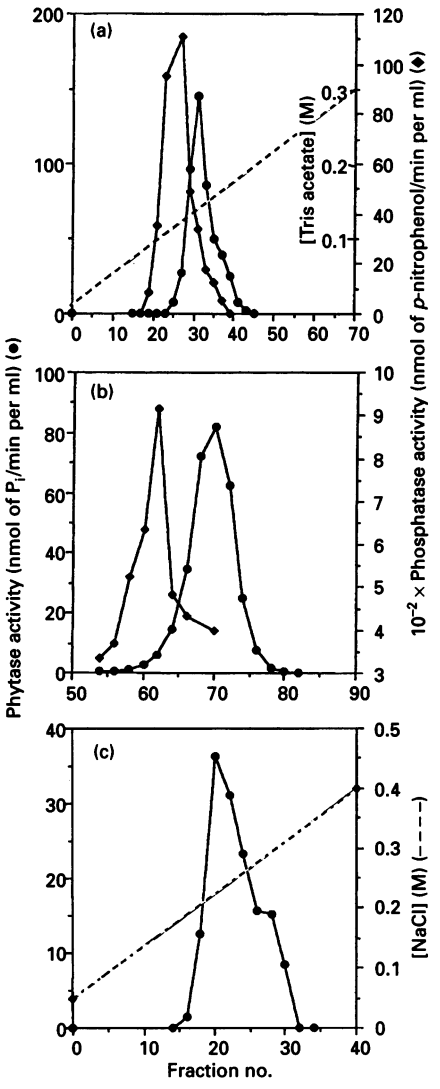

Figure 3 Purification of maize seedling phytase

(**a**) DEAE-Trisacryl M column. The dialysed 30–60%-satd.-$(NH_4)_2SO_4$ fraction from 6-day germinating seeds was chromatographed on a 3 cm × 7 cm column in 20 mM Tris/acetate, pH 7.5. Elution was developed with a linear gradient of 0.02–0.3 M NaCl in the same buffer, 2.5 ml fractions being collected. Fractions were analysed for phytase (●) and phosphatase (◆) activity. (**b**) AcA34 gel column. The fractions containing phytase activity (28–40 in **a**) were loaded on an AcA34 gel-filtration column (1.4 cm × 120 cm) using 100 mM sodium acetate buffer (pH 4.8)/0.25 M KCl, and 2.5 ml fractions being collected. The fractions were analysed for phytase (●) and phosphatase (◆) activity. (**c**) SP-Trisacryl column. The fractions containing phytase activities (66–75 in **b**) were further purified by chromatography on a SP-Trisacryl M column (0.8 cm × 4 cm) equilibrated with 50 mM sodium acetate, pH 4.8, containing 10% glycerol. Elution was developed with a 0.05–0.4 M NaCl gradient. Fractions were analysed for phytase activity (●) and for protein (Figure 4**a**, lanes 2 and 3).

(Kapitany and Zebrowski, 1973) for detection of carbohydrate residues, no staining of phytase was observed, whereas ovalbumin and Cl inhibitor at an equivalent protein concentration were stained (results not shown).

Enzyme-activity analysis

Temperature optimum

The temperature profile of purified phytase was conducted from

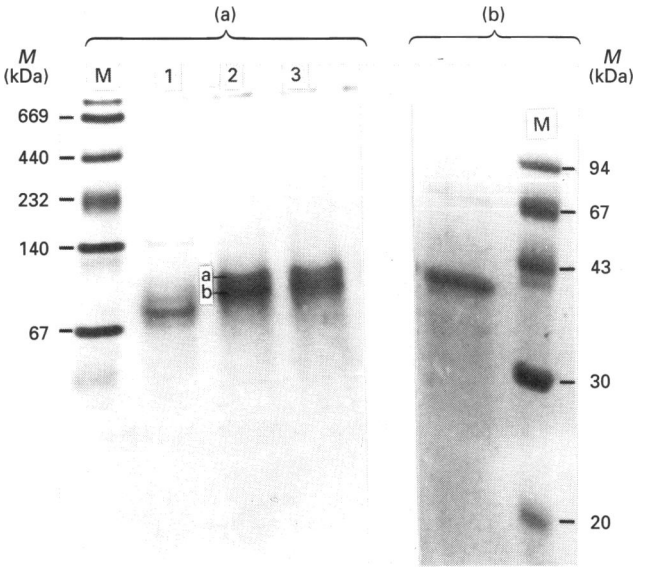

Figure 4 Electrophoretic analysis of purified phytase

(**a**) Coomassie Blue staining of purified phytase on a 4–25% non-denaturing polyacrylamide gradient gel. Lane 1, fraction not retained on the microcolumn of SP-Trisacryl; lanes 2 and 3, 5 μg (lane 2) and 4 μg (lane 3) of SP-Trisacryl concentrated phytase; (**b**) Coomassie Blue staining of a SDS/12.5%-polyacrylamide gel loaded with 1 μg of purified phytase.

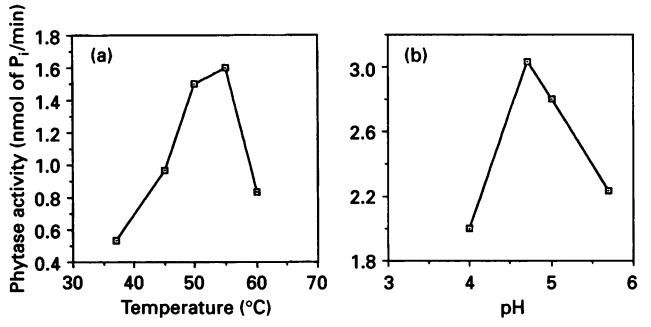

Figure 5 Temperature and pH optima

Assays were performed under standard conditions in the presence of 2 μg of phytase and 5 μg of BSA per ml. Acetate buffer (50 mM) was used for pH optimum determination.

37 to 60 °C using a 30 min incubation period under standard assay conditions. As for other plant phytases (Gibson and Ullah, 1990), the optimum temperature was found to be about 55 °C (Figure 5a). To check for temperature stability, 20 μg of the enzyme was preincubated for 10 min in 50 mM acetate, pH 4.8, at 55 °C and then assayed for activity. No loss in activity was observed, whereas a preincubation at 65 °C resulted in a 70% loss of activity.

pH optimum

Enzyme assays were performed from pH 4.0 to pH 6.0; the pH optimum was determined to be about 4.8. Activity fell rapidly for pH higher than 5.0 (Figure 5b). No significant activity was measured at higher pH (6.0–8.0).

Table 2 Effect of bivalent cations

The values result from the mean of at least two measurements (S.D. ± 10%). Measurements were made after 30 min under standard assay conditions in the presence of 2 mM phytate, 2 μg of enzyme and 6 μg of BSA. Mg^{2+} and Mn^{2+}, when tested from 0.25 to 2 mM, had no significant effect.

Cation	Concn (mM)	Activity (% of control)
None	–	100
Ca^{2+}	0.25	100
	0.50	105
	1.00	110
	2.00	135
Zn^{2+}	0.25	76
	0.50	62
	1.00	55
	2.00	41
Fe^{2+}	0.25	76
	0.50	64
	1.00	62
	2.00	44

Table 3 Affinity of phytase for various substrates

Measurements were made for 30 min under standard conditions with 2 μg/ml enzyme and 5 μg/ml BSA.

Substrate	K_m (μM)	$V_{max.}$ (nmol of P_i/h)
Phytate	117	400
myo-InsP_5*	133	714
myo-InsP_3†	250	333
ATP	227	500
Sodium pyrophosphate	181	500
p-Nitrophenyl phosphate	555	400

* 1,3,4,5,6-pentakisphosphate (Boehringer).
† 86% 1,4,5 isomer/14% 2,4,5 isomer (Sigma).

Effect of bivalent cations

Different cations were tested for their effect on phytase activity from 0.25 to 2 mM (Table 2). Only Ca^{2+} was found to exert a light stimulatory effect, approx. 35% over the control at 2 mM. Mg^{2+} and Mn^{2+} had no effect, whereas Zn^{2+} and Fe^{2+} were inhibitory.

Affinity for various substrates

A number of substrates were tested with the concentrated purified fraction. The Lineweaver–Burk plots of the data gave the K_m and the $V_{max.}$ reported on Table 3. From the different compounds tested, phytate gave the lowest K_m value (117 μM). The maize seedling phytase was able to catalyse the hydrolysis of the two intermediate forms of *myo*-inositol phosphates tested (*myo*-inositol 5- and 3-phosphates), but with a higher K_m than for phytate. The $V_{max.}$ for the various substrates tested were not very different, except for *myo*-inositol 5-phosphate, which gave a significantly higher $V_{max.}$. It is noteworthy that the K_m and $V_{max.}$ values given for phytate are only apparent values, since the phosphate liberated in the presence of this substrate results not only from the hydrolysis of the *myo*-inositol 6-phosphate, but also of intermediate phosphoric esters of *myo*-inositol.

Table 4 Amino acid composition of maize phytase

	Composition		
	Maize phytase		Soybean phytase*
Amino acid	(Residues/mol)	(Residues/ 100 residues)	(Residues/ 100 residues)
Asp/Asn	37	10.6	11.9
Thr	20	5.8	3.6
Ser	21	6.2	5.9
Glu/Gln	31	8.9	9.1
Pro	21	6.2	5.9
Gly	36	10.5	9.5
Ala	36	10.3	5.9
Cys	0	0	1.4
Val	27	7.9	7.1
Met	6	1.6	2.2
Ile	17	4.9	5.0
Leu	31	8.9	10.1
Tyr	7	2.1	5.6
Phe	15	4.2	4.6
His	5	1.55	2.2
Lys	23	6.6	4.8
Arg	13	3.6	4.6
Trp	n.d.†	n.d.	n.d.

* Calculated from the amino acid composition given by Gibson and Ullah (1988).
† n.d., not determined.

Amino acid composition

The amino acid composition of maize phytase (Table 4) was determined with the protein purified by reverse-phase h.p.l.c. The maize phytase was eluted at 53% acetonitrile, which is consistent with its high percentage (44%) of non-polar amino acids. Basic amino acids represent 10% and acidic amino acids 19% of maize phytase. These percentages are very similar to those reported by Gibson and Ullah (1988) for soybean phytase: 41% for non-polar amino acids, 9% for basic and 21% for acidic amino acids.

Western-blot analysis of proteins extracted from seedlings at different steps of germination

In order to investigate whether maize phytase was synthesized during germination or whether the phytase pre-existed in the seeds in a pro-enzyme form, proteins extracted from seedlings at different steps of germination were examined by immunoblot analysis (Figures 6a and 6b): a band migrating at the level of the phytase subunit (38 kDa) cross-reacted with the antiserum. The results clearly show that the amount of phytase for the same amount of total proteins increased during germination from day 1 to day 7. We have shown that, during this period, the total amounts of proteins extracted from the same number of seedlings did not change significantly. Therefore the increase of phytase activity reported on Figure 1 *does* correspond to an accumulation of the protein during germination.

To estimate the relative abundance of phytase in different parts of the seedlings, Western-blot analysis was performed with proteins extracted from shoots, roots or kernels of 5-day seed-

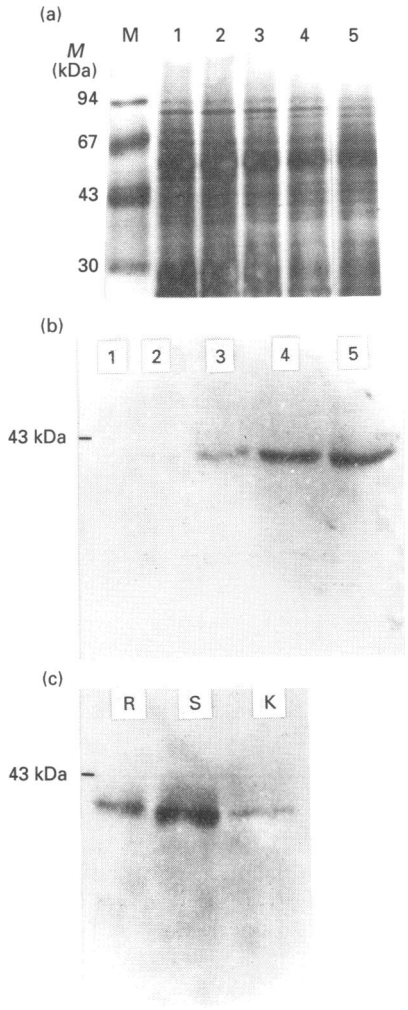

Figure 6 Immunoblot analysis of proteins extracted from maize seedlings at different times of germination

(**a**) Coomassie Blue staining of an SDS/12.5%-polyacrylamide gel loaded with 10 μg of total protein extracted from seedlings after 1 (lane 1), 2 (lane 2), 3 (lane 3), 5 (lane 4) and 7 (lane 5) days of germination. (**b**) Immunoblots of a duplicate of (**a**) probed with the purified antibodies. Purified maize seed phytase antibodies were diluted at 1:100 and anti-rabbit IgG sheep immunoglobulins coupled with peroxidase were diluted at 1:800. (**c**) Immunoblots of the total protein extracted from different parts of the 5-day seedlings (R, roots; S, shoots; K, kernels; 10 μg of proteins was loaded in each lane).

lings. Figure 6(c) shows that phytase accumulated essentially in the shoots (mesocotyl plus coleoptile). Only a very low amount of phytase was detected in the kernels.

DISCUSSION

Phytase activities have been reported in a wide range of seeds of higher plants containing phytin such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize, rye (*Secale cereale*), soybean, mung bean and other legumes or oilseeds (Gibson and Ullah, 1990). However only phytases from wheat bran (Lim and Tate, 1971, 1973), from mung-bean cotyledons (Maiti and Biswas, 1979) and, more recently, from soybean cotyledons (Gibson and Ullah, 1988), have been extensively characterized, and only mung-bean and soybean phytases have been purified to homogeneity.

In maize seeds, phytin constitutes a large reserve of phosphorus, and a 200% increase in phytase activity has been reported following germination (Chang, 1967). The present paper describes, for the first time, the purification and characterization of maize phytase.

First attempts to separate maize phytase activity from the major acid phosphatase activity by the one-step procedure described by Gibson and Ullah (1988) for soybean phytase purification were unsuccessful. This procedure was based on the difference of isolectric points of the two soybean enzymes (pI 5.5 for phytase and 6.7 for phosphatase). When chromatographed on a strong cationic-exchanger (SP-Trisacryl) column at pH 4.5, soybean phosphatase was retained on the column, whereas phytase was excluded. Under the same conditions, both maize activities were retained and eluted at the same saline concentration.

A four-step purification procedure is described, consisting of $(NH_4)_2SO_4$ fractionation, anion-exchange chromatography, gel filtration and cation-exchange chromatography. Phytase was completely separated from the major acid phosphatase activity after the third step. The last step, followed by concentration of the enzyme on a micro column of SP-Trisacryl, allowed us to obtain a homogeneous preparation of maize phytase.

The molecular mass of the purified protein was estimated to be 76 kDa, which is higher than that reported for soybean phytase (60 kDa) or for wheat bran phytase (47 kDa), but lower than that reported for mung-bean phytase (158 kDa). The maize phytase, unlike the other reported plant seed phytases, appears to be a dimer of a 38 kDa subunit. Furthermore, analysis on non-denaturing 7.5%-PAGE shows two closely migrating bands which seem to differ only by their charge, since longer migration on gradient PAGE results in only one band. This suggests that the protein might undergo post-translational modifications such as phosphorylation. As reported for soybean phytase, no carbohydrate residue was detected.

Up until now, two main types of plant phytases have been identified: acidic phytases with a pH optimum around 5.0, and alkaline phytases with an optimum around 8.0. The first group includes the soybean seed phytase (Gibson and Ullah, 1988), the F1 phytase of wheat bran (Lim and Tate, 1971, 1973), the pH 5.0 phytases of *Lilium longiflorum* pollen (Baldi et al., 1988) and of *Petunia hybrida* pollen (Jackson and Linskens, 1982). Acidic phytases exhibit a broad affinity for various phosphorylated substrates (Gibson and Ullah, 1990), and the wheat bran pH 5.0 phytase catalyses the hydrolysis of virtually all intermediate forms of *myo*-inositol phosphate from phytic acid to *myo*-inositol 2-phosphate (Lim and Tate, 1971). Mung-bean cotyledon phytase, in spite of a pH optimum of 7.5, exhibits the enzymic properties of the acidic group.

On the other hand, alkaline pH 8.0 phytases have been identified in different types of pollen: *Lilium longiflorum* (Lin et al., 1987; Baldi et al., 1988) and *Typha latifolia* (Hara et al., 1985). The extraction of these phytases is strongly enhanced by the presence of Triton X-100, suggesting an association with membranous structures. Unlike the pH 5.0 phytases, the pH 8.0 forms are highly specific for phytate, and none of the *myo*-inositol phosphates containing three or fewer ester groups can act as substrate (Baldi et al., 1988).

The maize seedling phytase characterized here is more similar to the first group: its optimum pH is 4.8. The apparent K_m of the maize enzyme for phytate is 117 μM. This K_m is twice that reported for soybean phytase (48 μM), but more than 10-fold higher than that of the *Lilium* pollen pH 8.0 phytase (7.2 μM). Like other acidic phytases, maize phytase exhibits a significant affinity for various phosphorylated substrates, and especially for 5- and 3-phosphate esters of *myo*-inositol. As with other acidic

phytases, its activity is only slightly stimulated by Ca^{2+} (130 % of the control at 2 mM $CaCl_2$), whereas, under the same conditions, the activity of the pH 8.0 phytase from *Lilium* pollen was strongly stimulated (300 % of the control).

Although large increases in phytase activities have been extensively reported in germinating seedlings as well as in germinating pollens, only a few reports have dealt with the regulation of these activities. Two reports on pollen phytases (Jackson and Linskens, 1982; Lin et al., 1987) suggest that phytases induced during germination may be synthesized from long-lived, pre-existing, mRNA. These results were based on differential sensitivities to antibiotics; appearance of phytase activities was prevented by cycloheximide, but not by cordicepin or actinomycin D. No information concerning the regulation of the increase of phytase activity in germinating seeds is available. In their work on soybean seed phytase, Gibson and Ullah (1988) conclude that "further studies are necessary to investigate whether the phytase is synthesized during germination or whether it exists in a proenzyme form". The Western-blot analysis obtained with purified maize phytase antibodies clearly demonstrates that the protein accumulates in maize seedlings during the first 7 days of germination, corresponding to the period of increase in phytase activity. Surprisingly, the enzyme appears to accumulate essentially in shoots (mesocotyl plus coleoptile). This may be related to the fact that, in maize seeds, phytin has been detected essentially in the germ (Gibson and Ullah, 1990).

Gibberellic acid is known to trigger the synthesis of several hydrolytic enzymes during seed germination (Panabières et al., 1989) and has been suggested to increase phytase activity in the aleurone layer of barley (*Hordeum vulgare*) (Gabard and Jones, 1986). Western-blot analysis (results not shown) of proteins extracted from maize seedlings germinating in the presence of different concentrations of gibberellic acid or of an inhibitor of gibberellic acid synthesis (paclobutrazol) did not show any effect on phytase accumulation, even at concentrations which significantly affected shoot elongation. Therefore gibberellic acid did not appear to trigger the synthesis of maize phytase during seed germination.

Further experiments are needed to isolate the cDNA of maize seed phytase and to study the control of its expression during seed germination.

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