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Communication

A Calcium-Activated Phytase from Pollen of *Lilium longiflorum*[1]

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ABSTRACT

A phytase was isolated and partially purified from the pollen of *Lilium longiflorum* Thunb. Optimum activity was at pH 8.0. The phytase was activated by Ca^{2+} and Sr^{2+} but not by the other divalent cations tested. Activity was inhibited by ethylenediaminetetraacetate. The phytase had a temperature optimum of 55 to 60°C and an activation energy of about 12,700 calories/mole. Extraction of *L. longiflorum* pollen with 0.1% Triton X-100 increased recovery of the phytase by nearly 4-fold. The phytase had a molecular weight of about 88,000 as determined by gel filtration chromatography and a K_m value of 7.2 micromolar for phytic acid in the presence of Ca^{2+}.

Mature pollen grains from many plants store phytic acid (9), which is degraded during pollen germination by one or more phytases (*myo*-inositol hexakisphosphate phosphohydrolase) (10, 11). The *myo*-inositol moiety released during phytate degradation is utilized for phosphatidylinositol and pectin biosynthesis, supplying the needs of the elongating pollen tube (8).

Phytases have been found in pollen (6, 11) and phytase activities which appear only after pollen germination have also been reported (10, 11). Previously, two phytases had been discovered in *Lilium longiflorum* pollen, one, a pH 5 enzyme, which is constitutive in the mature pollen grain, and the other an induced pH 6.5 form which appears only after germination (11). Another constitutive phytase found in *Typha latifolia* pollen, has its pH maximum at 8.0 and is activated by Ca^{2+} (6). This paper deals with the isolation, partial purification and characterization of a similar Ca^{2+}-activated phytase from pollen of *L. longiflorum*.

MATERIALS AND METHODS

Chemicals. Sodium phytate was obtained from Sigma Chemical Co. and purified by recrystallization of the sodium salt from aqueous methanol. β-Glycerophosphate was obtained from Sigma Chemical Co.

Isolation and Purification of Phytase. Freezer-stored mature pollen (10 g) from *Lilium longiflorum* Thunb. cv. Nellie White was suspended in 10 mM Tris-HCl (100 ml) containing 0.5 mM GSH, 0.1 mM $CaCl_2$, and 0.1% Triton X-100 (pH 7.6). Pollenkitt was removed by stirring with a glass rod until all pollenkitt adhered to the rod. The suspension was ground in a Kontes Duall glass-glass homogenizer and then centrifuged at 20,000*g* for 20 min. The supernatant was dialyzed overnight at 4°C against the Tris buffer (pH 7.6) (without Triton X-100). Solid $(NH_4)_2SO_4$ was added to the dialysate to 25% saturation. After equilibration for 20 min at 4°C, the precipitate was removed centrifugation at 12,000*g* for 10 min and additional $(NH_4)_2SO_4$ was added to give 55% saturation. After equilibration, the precipitate was collected and dissolved in 10 ml of the above buffer and dialyzed against two changes of the same buffer (4 h, 4°C).

The dialyzed material was applied to a column of Sephadex G-200 (1.2 × 95 cm) that had been equilibrated in 10 mM Tris-HCl, containing 0.5 mM GSH and 0.1 mM $CaCl_2$. Protein was eluted with the equilibration buffer. Two-ml fractions were collected and assayed for phytase, β-glycerophosphatase, and protein. Mol wt of the phytase was estimated by comparison of elution volume with protein standards of known mol wt.

Fractions from the G-200 column with phytase activity were pooled and applied to a column of DEAE cellulose (1.0 × 20 cm) which had been equilibrated in 10 mM Tris-HCl, containing 0.5 mM GSH and 0.1 mM $CaCl_2$. After loading, the column was washed with 30 ml of buffer, followed by a linear gradient of 0 to 0.6 M NaCl in the Tris buffer. The phytase, which eluted between 0.17 and 0.26 M NaCl, was concentrated by lyophilization and finally dialyzed overnight at 4°C against the Tris buffer.

Enzyme Assays. The reaction mixture for phytase assay contained 0.1 M Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 2 mM sodium phytate, and an appropriate dilution of enzyme sample in a total reaction volume of 1.2 ml. Phytase activity at pH 5.0 was determined in 0.1 M sodium acetate buffer. After incubation at 37°C for 30 min, the reaction was stopped by addition of 0.8 ml of 10% TCA, centrifuged to remove precipitate, and analyzed for Pi (1). One unit of phytase was that amount of enzyme which

Table I. *Extraction of Pollen Phytase with Triton X-100*

One g pollen was homogenized in 10 ml of 10 mM Tris-HCl, containing 0.5 mM GSH, 0.1 mM $CaCl_2$, and Triton X-100 at the indicated concentration.

Concentration of Triton X-100	Phytase Activity[a]	
	pH 5	pH 8
%	*relative*	
0	100	100
0.1	174	392
1.0	134	380

[a] Activity is relative to that extracted without Triton X-100 and recovered in the crude supernatant. Total recovery from 1 g pollen in control samples was 10.4×10^{-2} units pH 5 phytase and 5.7×10^{-2} units pH 8 phytase.

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Table II. *Summary of Phytase Purification*

Purification Stage	Volume	Protein	Phytase			β-Glycerophosphatase	
			Activity	Specific activity	Yield	Specific activity	Yield
	ml	*mg*	*units*	*units/mg*	%	*units/mg*	%
Crude supernatant	88	317	2.10	0.007	100.0	0.052	100.0
$(NH_4)_2 SO_4$ precipitation	22	263	1.82	0.007	86.5	0.039	61.7
Gel filtration	18	25.8	1.42	0.055	67.6	0.060	9.3
DEAE cellulose	24	8.2	0.54	0.066	25.7	0.032	1.6

Table III. *Effect of Divalent Cations on Pollen Phytase Activity*

Cation Added	Phytase Activity[a]	
	pH 5	pH 8
2 mM	*relative*	
None	100	100
$CaCl_2$	119	313
$MgCl_2$	99	66
$MnCl_2$	110	63
$CoCl_2$	109	83
$CuSO_4$	57	99
$Sr(NO_3)_2$	111	236

[a] Mean of two or more determinations (SD ± 10%).

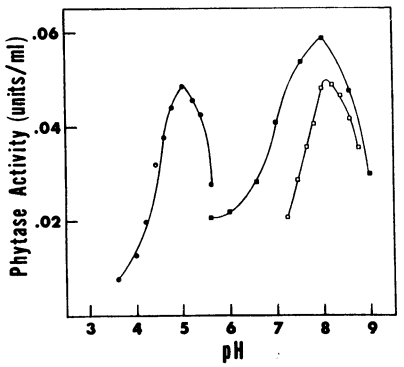

FIG. 1. pH profile for phytases isolated from pollen of *L. longiflorum*. Activities were determined in 0.1 M sodium acetate (●), 0.1 M Tris-maleate (■), and 0.1 M Bicine (□).

liberated 1 μmol of Pi from sodium phytate per min under these conditions. The reaction mixture for β-glycerophosphatase assay contained 0.1 M Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 2 mM β-glycerophosphate, and enzyme in a total volume of 1.2 ml. The reaction was carried out under the same conditions as for phytase determination. Protein contents were determined by the Coomassie blue method (3).

RESULTS

Extraction and Purification of the Phytase. As shown in Table I, extraction of mature *L. longiflorum* pollen with 0.1% Triton X-100 increased the recovery of two phytase activities. The effect of Triton X-100 was most dramatic for the pH 8 phytase, resulting in a nearly 4-fold increase in the yield of this enzyme. Increasing the concentration of Triton X-100 did not improve the recovery of either phytase. Table II summarizes the purification of the pH 8 phytase. Pollen extracts contained large amounts of β-glycerophosphatase activity in addition to the two phytases. The phytase was purified approximately 10-fold, and was not homogeneous. Even though the β-glycerophosphatase activity was less than 2% of the initial yield, the final phytase preparation still contained considerable β-glycerophosphatase activity. The differences in yields and specific activities at each purification step, however, suggests that they are separate enzymes. Part of the β-glycerophosphatase activity was likely due to *myo*-inositol-1-phosphatase which has been isolated from *L. longiflorum* pollen (12). The DEAE cellulse-purified enzyme had no measureable phytase activity at pH 5. The pH 8 phytase had a mol wt of approximately 88,000 based on its elution volume from the Sephadex G-200 column.

Effect of Divalent Cations on Phytase Activities. As shown in Table III, Ca^{2+} at concentrations equimolar to the substrate enhanced phytase activity at pH 8 by more than 300%. At higher concentrations, Ca^{2+} became inhibitory (data not shown), possibly due to precipitation of the substrate as calcium phytate. Sr^{2+} could substitute for Ca^{2+} to a great extent, but the other cations that were tested failed to enhance the pH 8 phytase activity. Mg^{2+} and Mn^{2+} were somewhat inhibitory. None of the divalent cations tested had much effect on the pH 5 phytase activity with the exception of Cu^{2+}, which was inhibitory. EDTA at 0.5 mM inhibited the pH 8 phytase by 35% and at 5 mM by 94%. Inhibition by 0.5 mM EDTA was overcome by the addition of 2 mM $CaCl_2$ to the reaction mixture. EDTA had no effect on the pH 5 phytase.

Properties of the Ca^{2+}-Activated Phytase. Figure 1 shows the pH profile for the two constitutive phytases from *L. longiflorum* pollen. The Ca^{2+}-activated phytase had a pH optimum of about 8.0 and was most active in Tris-maleate, rather than in Bicine buffer.

The optimum temperature for the pH 8 phytase activity was between 55 and 60°C and an activation energy of 12,700 calories/mol was calculated between 30 and 50°C. The pH 5 phytase had a slightly lower temperature optimum (45–50° C) and an activation energy of about 11,500 calories/mol.

Phytase activity at pH 8 was determined over a range of concentrations of sodium phytate in the presence of equimolar concentrations of Ca^{2+}. A Lineweaver-Burk plot of the data gave a K_m value of 7.2 μM.

DISCUSSION

These results demonstrate the presence of an alkaline phytase in *L. longiflorum* pollen which is activated by Ca^{2+}. Enhanced extraction of the phytase with Triton X-100 suggests that it has significant hydrophobic character and may be associated with a membranous structure in the intact pollen grain. Most plant phytases have pH optima between 4.0 and 5.6, although a few are reported to have pH optima of 7.0 and above (5, 6). The *L. longiflorum* pollen phytase reported here resembles the *T. latifolia* phytase, although the effect of Ca^{2+} was much greater in the former.

The activation of the phytase by Ca^{2+} raises the intriguing possibility that its activity may be regulated by the intracellular Ca^{2+} concentration. Since phytic acid degradation occurs as pollen germination begins (10, 11), it is of interest to consider the role of Ca^{2+} in activating the enzymes required for phytic acid hydrolysis. Evidence has recently appeared to suggest that the phosphotidylinositol cycle occurs in plants (2, 4, 14) and may regulate intracellular Ca^{2+} concentrations in a manner similar to that postulated for animal cells (13). The discovery of phospho-

tidylinositol-hydrolyzing enzyme activities in *L. longiflorum* pollen (7) suggests that phosphotidylinositol turnover may regulate events during pollen germination, including phytic acid breakdown. Further purification and characterization of the Ca^{2+}-activated phytase from *L. longiflorum* pollen and its regulation during pollen development are in progress.

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