Phytases are the primary enzymes responsible for the hydrolysis of phytic acid, myo-inositol-1,2,3,4,5,6-hexakisphosphate (I-1,2,3,4,5,6-P6). A number of phytases with varying specificities, properties, and localizations hydrolyze phytic acid present in cells. The specificity of hydrolysis of phytic acid by alkaline phytase from lily (Lilium longiflorum L.) pollen is described. Structures of the intermediate inositol phosphates and the final product were established by a variety of nuclear magnetic resonance techniques ('H-, 31P-, and 3P-'H-detected multiple quantum coherence spectroscopy, and total correlation spectroscopy). On the basis of the structures identified we have proposed a scheme of hydrolysis of phytic acid. Initial hydrolysis of the phosphate ester occurs at the D-5 position of phytic acid to yield the symmetrical I-1,2,3,4,6-P5. The two subsequent dephosphorylations occur adjacent to the D-5 hydroxyl group to yield I-1,2,3-P3 as the final product. Alkaline phytase differs from other phytases in the specificity of hydrolysis of phosphate esters on the inositol ring, its high substrate specificity for phytic acid, and biochemical properties such as susceptibility to activation by calcium and inhibition by fluoride. The physiological significance of alkaline phytase and the biochemical role of I-1,2,3-P3 remain to be identified.

Phytic acid, myo-inositol hexakisphosphate, is a major constituent of seeds and pollen grains (1–5% of dry weight) (Loewus, 1990; Raboy, 1990). In mature lily (Lilium longiflorum L.) pollen and seeds, phytic acid is localized in membrane-bound phytic-rich granules. The presence of phytic acid in plant cells has been known for some time (Loewus, 1990; Raboy, 1990). However, it was only recently recognized that phytic acid is present in virtually all mammalian cells in concentrations higher than most other inositol phosphates (Menniti et al., 1993) and may function as a neurotransmitter in the nervous system (Vallejo et al., 1988). The discovery that I-1,4,5-P3, as the final product by a variety of chromatographic techniques (Lim et al., 1973; Cosgrove, 1980b). In this investigation, structures of the intermediate and final inositol phosphates were established by direct structural analysis (1D- and 2D-NMR techniques). The enzyme exhibited markedly different catalytic characteristics both in the specificity of hydrolysis and in the final product generated.

In this paper we describe our research effort to determine the specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. Previous investigations of phytases have relied heavily on indirect methods such as degradation of inositol phosphates to acyclic sugars by a sequence of reactions (periodate cleavage, reduction, and dephosphorylation) and subsequent identification of the final product by a variety of chromatographic techniques (Lim et al., 1973; Cosgrove, 1980b). In this investigation, structures of the intermediate and final inositol phosphates were established by direct structural analysis (1D- and 2D-NMR techniques). The enzyme exhibited markedly different catalytic characteristics both in the specificity of hydrolysis and in the final product generated.

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; HMQC, 'H-detected heteronuclear multiple quantum coherence; HOHAHA, homonuclear Hartmann-Hann; HVE, high-voltage paper electrophoresis; IP6, IP5, IP4, and IP3, myo-inositol hexakis-, pentakis-, tetrakis-, tris-, bis-, and mono-phosphates, respectively, with numbering of the n configuration as appropriate; Jαα, Jαq, Jαq- spin-spin coupling constants between vicinal diaxial, axial-equatorial, and equatorial protons, respectively; TOCSY, total correlation spectroscopy.
**Materials and Methods**

**Preparation of Alkaline Phytase**

Preparation of alkaline phytase followed the method of Baldi et al. (1988). *Lilium longiflorum* L. cv Nellie White (1991 harvest) was kindly supplied by F.A. Loewus of the Institute of Biological Chemistry at Washington State University. All enzyme preparation procedures were carried out on an ice bath or in a cold room at 4°C. Ten grams of pollen was suspended in 50 mL of 0.01 M Tris-HCl, pH 7.0, containing 0.5 mM GSH. The suspension was stirred with a glass rod until most of the pollen had adhered to the rod and could be easily removed. An additional 50 mL of the same buffer containing 1% cetylpyridinium bromide was added to the pollen suspension to provide a final concentration of 0.5% detergent. The suspension was homogenized in a motorized glass-glass homogenizer with the aid of a small amount of clean sand. Homogenization proceeded until microscopic examination revealed that most of the pollen grains had been broken. This was generally accomplished after 20 up-and-down strokes with the motorized homogenizer. The homogenate was centrifuged at 20,000g for 20 min and the supernatant was filtered through Whatman No. 4 filter paper. The filtered solution was dialyzed against 0.01 M Tris-HCl buffer (pH 7.0) containing 0.5 mM GSH at 4°C. In some experiments the filtered extract was used without further purification. In other cases the alkaline phytase was further purified by CaCl₂ precipitation and ion-exchange chromatography as described below.

The filtrate was brought to 0.01 M CaCl₂ by addition of an appropriate amount of 1 M CaCl₂ and then stirred for 15 min. The resulting precipitate was removed by centrifugation as described above and the supernatant, containing most of the alkaline phytase, was applied to a column (26-mL volume) of DEAE Sepharose CL-6B that had been equilibrated with 0.01 M Tris-HCl, pH 7.0, containing 0.05 mM GSH. The column was washed with 100 mL of buffer followed by a linear gradient of 0 to 0.6 M KCl in the buffer. Most of the alkaline phytase eluted between 0.02 and 0.17 M KCl.

Both crude, filtered extracts and DEAE-purified alkaline phytase preparations were dialyzed, concentrated by ultrafiltration, and stored at 4°C. Specific activity of alkaline phytase preparations varied from 0.1 nkat/mg protein in crude, filtered extracts to 0.32 nkat/mg protein in DEAE-purified preparations. In the presence of sodium fluoride (20 mM), an inhibitory effect of phytase activity was observed. The reaction mixture for the phytase assay contained 0.1 M Tris-HCl (pH 8.0), 1 mM sodium phytate, 1 mM CaCl₂, and an aliquot (usually 0.1 mL containing protein) of phytase sample in a total volume of 1.2 mL. After incubation at 37°C for 60 min, the reaction was stopped by addition of 0.8 mL of 10% TCA. The precipitated protein was removed by centrifugation and the supernatant was analyzed for Pi (Ames, 1966). Protein concentration was determined by the Coomassie blue dye-binding method (Bradford, 1976).

**HVE Analysis of Inositol Phosphates**

To analyze the inositol phosphates released by alkaline phytase treatment of phytic acid, a solution of 0.2 M Tris-HCl, pH 8.0, 2 mM CaCl₂, and 20 mM sodium fluoride was mixed with an equal volume of phytase preparation. Sodium fluoride was included in the reaction mixture to inhibit acid phytase activity, which was present in the alkaline phytase preparations. The reaction mixture was allowed to incubate on a water bath at 37°C. Aliquots were removed at timed intervals and spotted onto Whatman No. 1 chromatography paper, and the inositol phosphates were separated by HVE in a Gilson model D electrophorator (Agranoff et al., 1983). Electrophoresis was carried out at 4 kV for 15 min in 0.06 M sodium oxalate buffer (pH 1.54). After electrophoresis, the dried paper was sprayed with Clark/Dawson spray reagent (Clark et al., 1981) to locate the inositol phosphates. Rᵢ values of inositol phosphates formed in the reaction and those of other inositol phosphates are as follows: IP₄, 1.0; 1-1,2,3,5,6-P₅, 0.92; 1-1,2,3,4,6-P₅, 0.88; 1-1,2,5,6-P₄, 0.86; 1-1,2,3,6-P₄, 0.81; 1-1,2,3-P₃, 0.72; 1-1,2,6-P₃, 0.68. Preparative HVE of inositol phosphates was performed by streaking 0.4-mL samples of reaction mixture along a 16-cm line on Whatman No. 1 chromatography paper followed by electrophoresis as described above. Inositol phosphate-containing areas of the paper were cut out and washed with absolute ethanol to remove oxalic acid and Varsol oil remaining from electrophoresis. The paper was air dried and then extracted twice with distilled water to elute the inositol phosphates. The washes were combined, passed through a 0.45-µm filter, and lyophilized prior to NMR analysis.

**NMR Spectroscopy**

Spectra were recorded on a 500-MHz Varian VXR-500 spectrometer. Dried samples (0.5–2.0 mg) were dissolved in D₂O (0.7 mL) and pH adjustments were made with perdeuterated acetic acid. All spectra were recorded at 25°C. 1-D ¹H NMR spectra were obtained at 499.84 MHz. ¹H chemical shifts were referenced to the residual proton absorption of the solvent, D₂O (δ 4.67). The acquisition conditions were as follows: spectral windows, 6738 Hz; pulse width, 50 to 90° tipping angle. Typically, 64 scans with a recycle delay of 6 s between acquisitions was collected. The residual H₂O resonance was suppressed by a 2-s selective presaturation pulse. Proton-coupled or decoupled ³¹P-NMR spectra were obtained at 203.33 MHz. ³¹P chemical shifts were referenced to external phosphoric acid (85%). The acquisition conditions were as follows: spectral window, 10,000 Hz; pulse width, 15 μs; delay between pulses, 3 s. ³¹P-HMQC was obtained using an inverse detection probe. The pulse sequence was that of Summers et al. (1986). Experimental details and processing parameters are given in the figure legends.

TOCSY, also called HOHAHA, data sets were obtained with a ¹H probe using the pulse sequence of Griesinger et al.
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(1988). Experimental details and processing parameters are given in the figure legends.

RESULTS

Products of alkaline phytase-catalyzed phytic acid hydrolysis were separated by HVE. Comparison of migratory behavior of the inositol phosphates formed with reference compounds showed the formation of IP₅, IP₄, and IP₃. The successive appearance of IP₅, IP₄, and IP₃ with time (Fig. 1) suggests sequential hydrolysis of phosphate esters of phytic acid, which is consistent with observations of acid phytase such as wheat bran phytase-catalyzed reactions (Lim et al., 1973; Cosgrove, 1980). However, unlike the products formed by wheat bran phytase, only one isomer of IP₅, IP₄, and IP₃ was observed by HVE under conditions that clearly separated all isomers of IP₅, IP₄, and IP₃ that were in our possession (Rₐ values are presented in “Materials and Methods”). Also, as seen in Figure 1, the final product of the reaction is IP₃. Consistent with previous observations, further hydrolysis of IP₃ was not detected for an additional 48 h (Baldi, 1988).

To isolate IP₅, IP₄, and IP₃ produced by alkaline phytase, an aliquot of the reaction mixture was frozen at different times, the products were separated by HVE, and the inositol phosphates were extracted from the paper. To establish the structure of the inositol phosphates as well as to establish the purity of the compounds, the inositol phosphates were analyzed by 1D(¹H and ³¹P)- and 2D-NMR spectroscopy. NMR investigation was based on the premise that dephosphorylation of phytic acid would affect the NMR spectroscopic characteristics of phytic acid in a diagnostic manner. (a) Dephosphorylation would lead to upfield shift of the α proton by approximately 0.5 to 1.0 ppm (Cerdan et al., 1986).

Figure 1. HVE separation of inositol phosphates produced by alkaline phytase-catalyzed hydrolysis of phytic acid. Aliquots of the reaction mixture were removed at the times indicated and immediately frozen. The reaction mixtures were separated by HVE and sprayed with molybdate spray reagent to visualize the phosphates.

Figure 2. ¹H-NMR spectra of IP₅ and inositol phosphates derived from the action of alkaline phytase on IP₅. Reaction was stopped at different times by freezing. The inositol phosphates were separated by HVE, extracted from the paper, and lyophilized. A, IP₅ at pH 12.0. B, IP₅ at pH 5.0. C, Mixture of IP₄ and IP₃ at pH 5.2. D, IP₄ at pH 5.0. E, IP₃ at pH 5.0. Exponential line broadening of 0.5 Hz was applied. Digital resolution is 0.2 Hz/point. The singlet, labeled S, at 5.358 Hz in C and E is due to contamination from a new batch of filter paper used during HVE. See also legend to Figure 4.
Figure 2. The pH was 5.0. The sweep width was 410 Hz in the F1 dimension and 2099 Hz in the F2 dimension. A total of 128 t1 increments, each consisting of 24 transients with relaxation delay of 2.5 s between successive transients, were obtained. A mixed Lorentzian and Giaan window was applied in the F2 dimension and a shifted Giaan window was applied in the F1 dimension. The data matrix was expanded to a 512 × 128 real matrix, resulting in digital resolution of 3.2 Hz/point in the F1 and 2.1 Hz/point in the F2 dimensions.

(b) Dephosphorylation would significantly affect the splitting pattern of the inositol ring proton because of loss of the proton-phosphorous coupling. The splitting pattern of protons on the inositol ring is due to coupling with two vicinal protons, one on either side (JH-P about 2–3 Hz, Jv-a, about 8–10 Hz, and Jv-eq about 2–3 Hz) and with the phosphorus (JH-P about 8–10 Hz); therefore, the presence or absence of H-P coupling would be obvious. (c) The presence of a plane of symmetry in the inositol phosphates formed would be indicated by the number of chemically distinct sets of resonances. Four distinct sets of resonances would result if there were a plane of symmetry and six would result if there were none. (d) 31P-HMQC would provide information on H-P connectivities and thereby provide additional proof of structural assignment. (e) Further proof of structures could be obtained by specific and broad-band homonuclear and heteronuclear decoupling experiments.

The 1H-NMR spectra of IP6, IP4, and IP3 isolated from alkaline phytate-catalyzed reaction mixture are compared in Figure 2. The spectrum of IP6 (Fig. 2A) consists of three sets of protons in the ratio 1:2:3. Consistent with observations that equatorial proton resonances are generally downfield of axial protons, the one equatorial proton H(2), at δ 4.8, is split into a triplet of doublets, which appears as broad doublets because of one large Jv-a, coupling and two small Jv-eq vicinal coupling to H(1) and H(3). The enantiotropic H(4) and H(6) protons at δ 4.35 are split into quartets due to two Jv-a, vicinal couplings with protons on either side and one JH-P coupling, all of similar magnitude. The enantiotropic H(1) and H(3) protons overlap with the H(5) proton at approximately δ 4.08. The H(5) proton is split into a quartet because of two vicinal Jv-a, coupling and one JH-P coupling, all of similar magnitude. The H(1) and H(3) protons are split into broad triplets because of one Jv-a, coupling and a JH-P coupling of similar magnitude and a small Jv-eq coupling with the H(2) proton.

IP3 (Fig. 2B) shows four sets of resonances in the intensity ratio 1:2:2:1, indicating a plane of symmetry in the molecule. The presence of a plane of symmetry suggests that the free hydroxy group must be at the C(2) or C(5) position. The most noticeable change compared to IP6 is the upfield shift of the H(5) proton by about 0.5 ppm, the change in splitting pattern from a quartet to a sharp triplet, and the change in the reduction in intensity of the multiplet at about δ 4.1. The presence of the broadened doublet due to H(2) at δ 4.8 suggests that IP3 is 1-1,2,3,4,6-P5. Consistent with the assignment, 31P-HMQC (Fig. 3) shows H-P connectivities at the H(2), H(1), H(3), H(4), and H(6) protons and none for the H(5) proton. The 31P resonances in the F1 dimension in the ratio 2:2:1 again indicate a plane of symmetry in the molecule.

When a mixture of IP4 and IP3 was analyzed, a more complicated proton spectrum (Fig. 2C) was obtained. To separate resonances due to IP4 from those due to IP3, a TOCSY experiment was performed (Griesinger, 1988; Nakashiki, 1990). The TOCSY technique, very similar to the HOHAHA technique, is useful for determining a network of mutually coupled protons, because in a TOCSY experiment multiple-bond coherence transfer can take place (Nakanishi, 1990). Since all protons on an inositol ring are part of a connected spin system, all protons on IP4 should show connectivity either due to direct coupling or due to long-range magnetization transfer. The 2D spectrum (Fig. 4) shows two networks of spin systems, one giving rise to four peaks as indicated by arrow A, and another giving rise to six peaks as indicated by arrow B. The presence of six sets of resonances in spin system B indicates a lack of symmetry in the molecule. The most noticeable change in spin system B compared to IP3 is the upfield shift of only one resonance, the H(4) or enantiotropic H(6) resonance, by about 0.5 ppm and the change in splitting pattern from a quartet to a triplet, indicating loss of a coupling partner. Resonances close to δ 4.8, δ 4.3, and δ 4.1 have not changed appreciably in chemical shift.
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I

F1 'K *

5 1,3 4,6

F2 (ppm)

Figure 4. Two-dimensional TOCSY spectra of a mixture of IP4 and IP3 with attached 'H spectra. The pH was 5.2. The sweep width employed in both the F1 and F2 dimensions was 2200 Hz. A total of 512 t; increments, each consisting of 24 transients with relaxation delay of 2 s between successive transients, were obtained. A sinebell window was applied in both dimensions and the data matrix was expanded to a 512 x 512 real matrix. Digital resolution was 4.3 Hz/point in both dimensions. The singlet at δ 3.65, labeled S, is due to contamination from a new batch of filter paper used during HVE.

or splitting pattern, indicating phosphorus couplings. Therefore, spin system B must be the tetraphosphate. H(5) of IP4 shows direct coupling to H(4) and H(6) and long-range connectivities to H(1), H(3), and H(2). Additional confirmation of these structures was obtained by purifying IP4 and analyzing the NMR spectra (Fig. 2D). The spectrum in Figure 2D is very similar to spin system B (Fig. 4). Enantiotopic protons cannot be distinguished by NMR, so IP4 could be the structure I-1,2,3,6-P4, shown in Figure 2D or its enantiomer I-1,2,3,4-P4. Further confirmation of this assignment of phosphorylated positions is provided by 31P-HMQC (Fig. 5), which shows H-P connectivities at H(1), H(2), H(3), and H(6) or its enantiotopic H(4).

The presence of four sets of resonances in spin system A (Fig. 4) suggests a plane of symmetry in the molecule. The upfield shift of H(6) [or enantiotopic H(4)] compared to IP3 and the change of quartet to triplet are consistent with removal of phosphate from H(6) [or H(4)]. The chemical shifts of H(2), H(1), H(3), and H(4) show relatively small changes compared to IP4, and the splitting patterns of H(1), H(2), and H(3) show the presence of H-P coupling. H(5) of IP3 shows direct coupling to H(4) and H(6) and also clearly shows connectivities to H(1), H(3), and H(2). Additional confirmation of this structure was obtained by purifying IP3 and analyzing the NMR spectrum (Fig. 2E), which is very similar to that suggested by spin system A in Figure 4. The NMR spectra in Figure 4 and Figure 2D are consistent with the structure I-1,2,3-P3.

DISCUSSION

A complete understanding of the biological role of phytic acid and inositol phosphates requires detailed knowledge about the reaction specificity and biochemical characteristics of the enzymes involved in its metabolism. Phytases are the primary enzymes that catalyze the hydrolysis of phytic acid. In this study the specificity of hydrolysis of phytic acid by alkaline phytase was established. Establishing the specificity
of hydrolysis of phytases has always been a challenge because of the need to identify the structures of individual inositol phosphates in a mixture containing regio- and stereoisomers. In this investigation, the structures of the intermediates and final products were determined by analytical methods that would provide direct structural information. The use of a variety of 1D- and 2D- (TOCSY, $^{31}$P-HMQC) NMR techniques allowed us to establish unambiguously the structures of IP$_3$ and IP$_4$. NMR evidence suggests that the IP$_4$ formed is due to hydrolysis of phosphate adjacent to the D-5 position. However, since NMR cannot distinguish between enantiomeric protons, at this time we cannot suggest which enantiomer of IP$_4$, I-1,2,3,4-P$_4$, or I-1,2,3,6-P$_4$, is produced. TOCSY is commonly used to determine networks of mutually coupled protons in macromolecules (Griesinger et al., 1988; Nakanishi, 1990). In this investigation, the TOCSY experiment was successfully used for a different purpose, to provide information that allows us to establish structures of individual compounds in mixtures, without resorting to separation. Our results point out the tremendous potential of TOCSY experiments for this purpose.

On the basis of the structures identified, we propose that the sequence of dephosphorylation of phytic acid by alkaline phytase is as shown in Figure 6. Alkaline phytase from lily pollen hydrolyzed the phosphate at the D-5 position of phytic acid to yield a symmetrical IP$_3$. Further hydrolysis occurs adjacent to the free hydroxyl group. The two subsequent dephosphorylations occur on either side of the 5-hydroxyl group to yield I-1,2,3-P$_3$, a symmetrical molecule, as the final product. Unpublished information had suggested that alkaline phytase from Typha latifolia also produces I-1,2,3-P$_3$ (Loewus et al., 1990). Consistent with characteristics of other phytases (Cosgrove, 1980b), hydrolysis occurs adjacent to the free hydroxyl group and, therefore, the initial position of hydrolysis is a major determinant of subsequent points of attack. Attack adjacent to a free hydroxyl group could be due to greater nucleophilicity of the hydroxyl oxygen compared to the ester bond oxygen (Cosgrove, 1980b). The rates of

\[
\begin{align*}
(I-1,2,3,4,5,6-P_6) & \rightarrow (I-1,2,3,4,6-P_5) \\
(I-1,2,3,4,6-P_5) & \rightarrow (I-1,2,3,4-P_4) \\
(I-1,2,3,4-P_4) & \rightarrow (I-1,2,3-P_3) \\
& \text{OR} \\
(I-1,2,3,4-P_4) & \rightarrow (I-1,2,3,6-P_4)
\end{align*}
\]

Figure 6. Alkaline phytase-catalyzed dephosphorylation of phytic acid.
removal of the second and third phosphates are significantly lower, possibly due to reduced specificity for IP₃ and IP₄ (Baldi, 1988) and inhibition by the phosphate released. Alternative routes of hydrolysis of the intermediates leading to formation of multiple isomers of inositol phosphates were not observed. Formation of 1,2,3-P₃ as one of three inositol trisphosphate intermediates, which is subsequently hydrolyzed to 1,2- or 2,3-IP₂ and finally to IP₁, has been observed with wheat bran phytase (Lim et al., 1973; Cosgrove, 1980b).

Phytases that differ in substrate specificity, position of hydrolysis on the inositol ring, final product produced, and biochemical characteristics such as pH optima and susceptibility to inhibitors and activators have been found, sometimes in the same tissue (Cosgrove, 1980a, 1980b; Baldi et al., 1988). For example, unlike alkaline phytase, most acid phytases studied hydrolyze phytic acid at the 0-3 or 1-6 (or 0-4) positions. Also, acid phytases are able to catalyze complete dephosphorylation of phytic acid to inositol, whereas alkaline phytase is unable to catalyze dephosphorylation of IP₂. In addition, unlike alkaline phytase, alternative pathways of hydrolysis of IP₁ to yield multiple isomers of IP₂, IP₃, and IP₄ are observed with acid phytases such as wheat bran phytase (Lim et al., 1973).

The narrow substrate specificity of alkaline phytase along with other characteristics such as higher specificity for phytic acid, more stringent specificity of hydrolysis leading to uncommon isomers of inositol phosphates, such as 1-2,3-P₉, and dependence on calcium ions for activation all raise questions regarding the potential physiological role, such as a regulatory role, for this enzyme. In vivo, phytic acid can be hydrolyzed by a number of phytases. The biological significance of the numerous phytases and the intracellular roles of the products produced are unknown. In addition to 1-4,5-P₅, biological functions for a number of inositol phosphates such as 1-3,4,5-P₄ (Berridge et al., 1989) and IP₈ (Vallejo et al., 1988) have been suggested. It is likely that other inositol phosphates may have messenger functions. 1-2,3,5-P₃ joins the growing list of inositol phosphates whose physiological roles remain to be established.

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