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Alkaline phytase from *Lilium longiflorum*: Purification and structural characterization

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Abstract

Phytases catalyze the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate), the most abundant inositol phosphate in cells. Phytases are of great commercial importance because their use as food and animal feed supplement has been approved by many countries to alleviate environmental and nutritional problems. Although acid phytases have been extensively studied, information regarding alkaline phytases is limited. Alkaline phytases with unique catalytic properties have been identified in plants, however, there is no report on the purification or structural properties. In this paper, we describe the purification of alkaline phytase from plant tissue. The purification was challenging because of contamination from non-specific phosphatases and acid phytases and low endogenous concentration. The purification of alkaline phytase from pollen grains of *Lilium longiflorum* involved selective precipitation by heat and ammonium sulfate followed by anion exchange and chromatofocusing chromatography and, finally, gel electrophoresis. Alkaline phytase was purified ~3000-fold with an overall recovery of 4.2%. The native molecular mass was estimated to be in the range of 118 ± 7 kDa by Ferguson plot analysis and M_r of denatured protein in the range of 52–55 kDa by SDS–PAGE suggesting that the enzyme is a homodimer. Separation by 2-D gel and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis of separated proteins indicates the presence of multiple mass and charge isoforms with p*I* values between 7.3 and 8.3. To our knowledge, this is the first alkaline phytase to be purified from plant sources. The unique properties suggest that the enzyme has the potential to be useful as a feed and food supplement.

Keywords: Alkaline phytase; Enzyme purification; *Lilium longiflorum*; Pollen grains; Phosphate contamination; Animal feed; Ferguson plot

Inositol phosphates play multiple roles in biological processes including signal transduction and calcium regulation [1]. Phytic acid, *myo*-inositol hexakisphosphate, is the most abundant inositol phosphate in cells; in cereal grains and legumes it constitutes 3–5% of the dry weight of seeds (Fig. 1) [2,3]. During seed and pollen development, phytic acid is deposited in membrane-bound bodies as a salt of essential mineral ions such as calcium, magnesium, iron, and zinc [3] and serves as the major storage form of phosphate, inositol, and metal ions for the germinating seed and pollen grains [2,3]. Although high concentrations of phytic acid are present in soybean and corn, the major components of animal feed, the phosphate, inositol, and metal ions in phytate complexes are unavailable to humans and monogastric animals, such as poultry and swine, because they lack the enzymes to hydrolyze phytates [4]. Therefore, for optimal animal growth, supplementation of animal feed with phosphate and essential minerals is necessary [5]. In addition, the excretion of undigested phytate results in elevated levels of organophosphates in soil and water [6]. To address these nutritional and environmental contamination issues, many governments are considering the supplementation of animal feed with phytases, the enzymes that hydrolyze phytic acid [7,8]. The efficacy of supplementing animal

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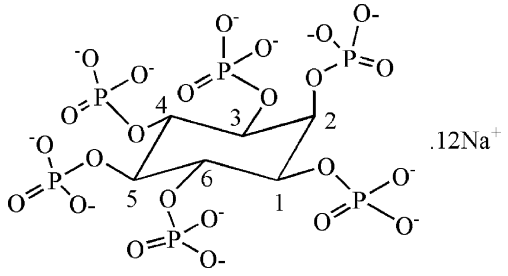

Fig. 1. Sodium salt of phytate.

feed with phytases to increase the bioavailability of inositol and phosphate and decrease phosphate discharge from animal farms has been demonstrated in several experiments [4,5,7,8]. The commercial importance of phytases for animal nutrition has resulted in the need for phytases with a range of catalytic and stability properties for different end applications. This interest in phytases has highlighted the need for a fundamental understanding of structure–activity relationships at the protein level.

Phytases are a class of phosphatases; they are the primary enzymes responsible for the hydrolysis of phytic acid [9–11]. They catalyze the sequential hydrolysis of phytic acid to less phosphorylated inositol phosphates and, in some cases, to inositol. A number of phytases with varying structural and catalytic properties have been found in plants, yeast, and bacteria [9–11]. Phytases have been classified on the basis of pH optima (acid and alkaline), catalytic mechanisms (histidine acid phosphatase-like phytase, purple acid phosphatase-like phytase, and β-propeller phytase), and specificity of hydrolysis (3-phytase, 6-phytase, and more recently 5-phytases) [9–11]. Although acid phytases have been well studied, information about alkaline phytases is relatively recent [12–19]. Acid and alkaline phytases differ in substrate specificity, metal ion requirement, susceptibility to ethylenediaminetetraacetic acid (EDTA),[1] and final product produced [11–19]. Recent information from bacterial investigations suggests that alkaline phytases differ from acid phytases in structural characteristics including amino acid sequence, catalytic domain, and crystal structure [11,17]. However, information about the structural characteristics of alkaline phytases from plant sources such as amino acid sequence, active site structure, mechanism of action, or crystal structure is unavailable. Towards this end we have been working on the purification of alkaline phytase to homogeneity. The challenges in purifying phytases from plant sources, such as limited stability and contaminating non-specific phosphatases, have been previously noted [10]. In this paper, we describe the purification of alkaline phytase from lily pollen and provide information on the structure and stability of the enzyme.

[1] *Abbreviations used:* EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-[*N*-morpholino]propanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; p*I*, isoelectric point.

Materials and methods

Materials

Pollen grains from *Lilium longiflorum* L. cv Nellie White (1988 and 1991 harvests) were kindly donated by Professor Frank A. Loewus, Washington State University, Pullman, WA. Column chromatography of proteins was conducted on a Fast Protein Liquid Chromatography system (Pharmacia Biotech GradiFrac System, Piscataway, NJ). Dialysis membrane (Spectra/Por 6.4 mm diameter, molecular weight cutoff: 12–14 kDa) was from Spectrum Laboratories, Rancho Dominguez, CA. Ion Exchange columns, HiTrap Q-FF (5 ml), and HiPrep 16/10 Q-FF (20 ml) were purchased from Amersham Biosciences, Piscataway, NJ. Chromatofocusing column (Tricorn column, Mono P 5/200 GL, 5 × 200 mm, 4 ml) was purchased from Amersham Biosciences, Piscataway, NJ. Centriplus and Centricon filtering devices (YM-30, molecular weight cutoff: 30 kDa) were from Amicon, Beverly, MA. Pre-cast mini-gels (4–12%) were purchased from Invitrogen, Carlsbad, CA. Standard proteins for molecular mass estimation were obtained from Sigma Chemical, St. Louis, MO.

Homogenization and protein precipitation

All procedures were conducted at 4 °C unless otherwise noted [12–15]. To 2 g of lily pollen (1988 or 1991 harvests) was added ice-cold Buffer A (16 ml, Tris–HCl, 10 mM, pH 7.0, containing reduced glutathione, 0.5 mM). A comparison of the germination rates of pollen grains from the 1988, 1991, and 2004 harvests showed no significant difference, nearly 90% of the pollen grains germinated in all cases indicating that the 1988 and 1991 pollen grains were viable (data not shown) [20,21]. The suspension was stirred with a glass rod until most of the pollen kit adhered to the glass rod and could be easily removed. To the suspension was added solid cetylpyridinium bromide (80 mg, final concentration 0.5% w/v). The suspension was homogenized with an IKA Euroturrax T20 homogenizer (IKA Works, Wilmington, NC) at 27,000 rpm for 2 × 1 min with a 1 min delay in-between to ensure that the homogenate remains cold. The resulting crude homogenate was centrifuged at 10,000*g* for 30 min. The supernatant which contained alkaline phytase activity was collected and the pellet containing cellular debris was discarded. For large-scale experiments, the supernatants at this stage or ammonium sulfate pellets at a later stage (see below) were combined. Heat labile proteins in the supernatant were precipitated by heating at 55 °C for 80 min in a constant temperature water bath with slow shaking and then allowed to stand on ice for 30 min. The precipitated proteins were removed by centrifugation at 10,000*g* for 20 min and the supernatant was filtered through a syringe filter containing 5 μm cellulose ester filter paper (Advantec MFS, Pleasanton, CA).