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Alkaline phytase from lily pollen: Investigation of biochemical properties

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Abstract

Phytases catalyze the hydrolysis of phytic acid ($InsP_6$, *myo*-inositol hexakisphosphate), the most abundant inositol phosphate in cells. In cereal grains and legumes, it constitutes 3–5% of the dry weight of seeds. The inability of humans and monogastric animals such as swine and poultry to absorb complexed $InsP_6$ has led to nutritional and environmental problems. The efficacy of supplemental phytases to address these issues is well established; thus, there is a need for phytases with a range of biochemical and biophysical properties for numerous applications. An alkaline phytase that shows unique catalytic properties was isolated from plant tissues. In this paper, we report on the biochemical properties of an alkaline phytase from pollen grains of *Lilium longiflorum*. The enzyme exhibits narrow substrate specificity, it hydrolyzed $InsP_6$ and *para*-nitrophenyl phosphate (pNPP). Alkaline phytase followed Michaelis–Menten kinetics with a K_m of 81 μM and V_{max} of 217 nmol Pi/min/mg with $InsP_6$ and a K_m of 372 μM and V_{max} of 1272 nmol Pi/min/mg with pNPP. The pH optimum was 8.0 with $InsP_6$ as the substrate and 7.0 with pNPP. Alkaline phytase was activated by calcium and inactivated by ethylenediaminetetraacetic acid; however, the enzyme retained a low level of activity even in Ca^{2+}-free medium. Fluoride as well as *myo*-inositol hexasulfate did not have any inhibitory affect, whereas vanadate inhibited the enzyme. The enzyme was activated by sodium chloride and potassium chloride and inactivated by magnesium chloride; the activation by salts followed the Hofmeister series. The temperature optimum for hydrolysis is 55 °C; the enzyme was stable at 55 °C for about 30 min. The enzyme has unique properties that suggest the potential to be useful as a feed supplement.

Keywords: Phytate; Alkaline phytase; Pollen grains; *Lilium longiflorum*; Enzyme activation; Hofmeister series; Phosphate contamination; Animal feed

Phytases, a class of phosphatases, are the primary enzymes responsible for the hydrolysis of phytic acid [1–6]. They catalyze the sequential hydrolysis of phytic acid to less phosphorylated inositol phosphates and, in some cases, to inositol [7]. A number of phytases with varying structural and catalytic properties have been found in plants, yeast, and bacteria [2–4].

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, EC.3.1.3.8, 6-phytase, EC.3.1.3.26, and more recently 5-phytases, EC.3.1.3.72) [1–4]. Although acid phytases have been extensively studied, investigations of alkaline phytases have been relatively few [3,4]. Alkaline phytases were first reported in pollen grains of cattail, *Typha latifolia* [8], and *Lilium longiflorum* [9]. Subsequently, the presence of alkaline phytase in a number of legume seeds was reported [10]. The membrane-associated alkaline phytase from lily pollen has unique catalytic properties; it has a pH optimum of 8.0, is activated by calcium ions, not inhibited by fluoride, and yields Ins-1,2,3-P_3 as the final product (Fig. 1) [11–13]. In addition to pH optima, acid and alkaline

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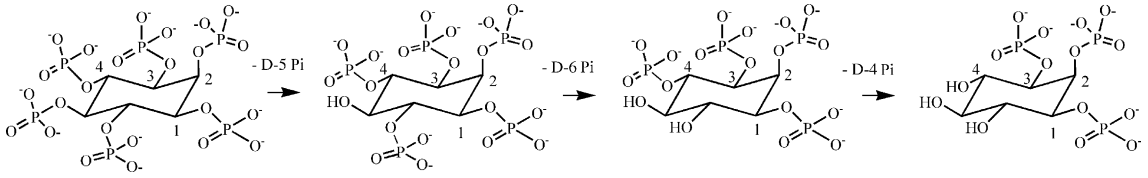

Fig. 1. Hydrolysis of phytic acid by alkaline phytase from lily pollen.

phytases differ in substrate specificity, specificity of hydrolysis, final products produced and metal ion requirement [3,4]. Alkaline phytase from bacterial sources (*Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*) exhibit catalytic properties similar to plant alkaline phytases; bacterial enzymes also require calcium for activity, are inhibited by EDTA, and yield $InsP_3$ ($Ins(1,3,5)P_3$ and $Ins(2,4,6)P_3$) as the final product [14–21]. In addition to the differences in catalytic properties, the crystal structure of alkaline phytase from *B. amyloliquefaciens* is structurally distinct, it lacks the active site motif RHGxRxP found in many acid phytases, and has a six-bladed propeller structure rather than the α/β domain structure of acid phytases [2,4,21].

Phytate is the major storage form of phosphate and inositol in seeds [22]. It exists complexed with essential mineral ions such as Na^+, K^+, Zn^{2+}, and Fe^{2+} as well as proteins [22]. Monogastric animals such as poultry and swine do not have phytase in their digestive tract and are not able to utilize the phosphates, inositol, or metal ions in phytate [1–4,19]. The unabsorbed phytate is excreted and contributes to phosphate pollution in water bodies downstream of agriculturally intensive areas [23]. In addition, animal feed has to be supplemented by phosphate and mineral ions. The detrimental affect of high concentrations of phytic acid in corn and soybeans on animal nutrition, as well as soil and water contamination and eutrophication is well established [25]. To alleviate the detrimental affects of high concentrations of phytate, pig and poultry feed is supplemented by phytases [24–26]. Depending on the target application, there is a need for phytases with a range of differing biochemical and biophysical properties. The commercial significance of phytases has triggered great interest in phytases exhibiting a range of characteristics as well as the need to understand the underlying basis for structure–activity relationships. In this paper, we investigate the biochemical properties of an alkaline phytase from lily pollen.

Materials and methods

Materials

Pollen grains from *L. 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). Dialysis membrane (Spectra/Por 6.4 mm diameter, molecular weight cutoff: 12–14 kDa) was from Spectrum Laboratories, CA. Ion-exchange columns, HiTrap Q-FF (5 and 20 ml), were purchased from Amersham Biosciences. Chromatofocusing column (Tricorn column, Mono P 5/200 GL, 5 × 200 mm, 4 ml) was purchased from Amersham Biosciences. Centriplus and Centricon filtering devices (YM-30, molecular weight cutoff: 30 kDa) were from Amicon. Sodium phytate and *myo*-inositol hexasulfate (MIHS)[1] were purchased from Sigma Chemicals, St. Louis, MO. Calculations to determine the concentration of free calcium in assay media were conducted using the program, Maxchelator 2004 (WEBMAXC STANDARD at http://www.stanford.edu/~cpatton/maxc.html).

Homogenization and protein purification

All procedures were conducted at 4 °C unless otherwise noted [12]. To 2 g of lily pollen (1988 or 1991 harvests) was added ice-cold Buffer A (16 ml of 10 mM Tris–HCl, 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 [27,28]. 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 cool the homogenate. The resulting crude homogenate was centrifuged at 10,000*g* for 30 min. The supernatant containing alkaline phytase activity was collected and the pellet containing cellular debris was discarded. 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 cen-

[1] *Abbreviations used:* MIHS, *myo*-inositol hexasulfate; EDTA, ethylenediaminetetraacetic acid; pNPP, *para*-nitrophenylphosphate.