Substrate Selectivity in *Aspergillus ficuum* Phytase and Acid Phosphatases Using *myo*-Inositol Phosphates

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The three extracellular acid phosphatases produced by *Aspergillus ficuum* have varying affinities for *myo*-inositol hexakis-, pentakis-, tetrakis-, and trisphosphate. Phytase (EC 3.1.8) has previously been shown to degrade phytate at pH 5.5 and 2.5, but similar activity has not been demonstrated in the concurrently produced extracellular acid phosphatases. Data obtained in this study demonstrate that the acid phosphatase with an optimum at pH 2.5 is a potent phytase at this pH. However, the pH 6.0 optimum acid phosphatase hydrolyzes phosphate from the *myo*-inositol backbone very poorly. The kinetic parameters obtained for these enzymes indicate the potential value of both phytase and pH 2.5 acid phosphatase as feed additives for monogastric animals.

INTRODUCTION

If *Aspergillus ficuum* NRRL 3135 is cultured in hylon starch medium with limiting phosphate, it produces three extracellular acid phosphatases (Irving and Cosgrove, 1972; Ullah and Gibson, 1987; Ullah and Cummins, 1987, 1988). One of these is the previously identified phytase (*myo*-inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8), with pH optima of 5.5 and 2.5. The other two are acid phosphomonoesterases (EC 3.1.3.2) with pH optima of 2.5 and 6.0. These enzymes were purified to near homogeneity and their physicochemical and catalytic properties and N-terminal sequences determined (Ullah and Cummins, 1987, 1988; Ullah, 1988b). Substrate selectivity studies showed that while phytase was able to degrade phytate, both pH 2.5 optimum and pH 6.0 optimum acid phosphatases were unable to hydrolyze phytate at pH 5.0 (Ullah and Cummins, 1988). The ability of fungal phytase to hydrolyze IP_6 sequentially to *myo*-inositol monophosphate has been demonstrated in a bioreactor constructed with immobilized phytase (Ullah and Phillippy, 1988). The kinetic parameters for this sequential degradation of phytate by phytase, however, were not ascertained due to scarcity of IP_5, IP_4, and IP_3. A preliminary report of the cloning of *A. ficuum* phytase has been published (Mullaney et al., 1991). Fungal phytase and the other two extracellular acid phosphatases have recently been cloned and characterized in our laboratory (unpublished data). The active site of fungal phytase was chemically probed and identified (Ullah et al., 1991; Ullah and Dischinger, 1992, 1993a); a homologous site has been located in pH 2.5 acid phosphatase (Ullah and Dischinger, 1993b). It is likely that improvement of the kinetic parameters of these enzymes can be achieved by site-directed mutagenesis. As a prelude to protein engineering, we report here the kinetic parameters of these enzymes for phytate and lower forms of inositol phosphates. Furthermore, we report that pH 2.5 optimum acid phosphatase hydrolyzes phytate at pH 2.5 and qualifies as a *myo*-inositiol hexakisphosphate phosphohydrolase (EC 3.1.3.8).

EXPERIMENTAL PROCEDURES

Enzyme Production and Purification. *A. ficuum* NRRL 3135 was grown in a medium with hylon starch as the principal carbon source and limiting phosphate (Ullah and Gibson, 1987). The secreted phytase and acid phosphatases were purified as previously described (Ullah and Gibson, 1987; Ullah and Cummins, 1987, 1988). The specific activities of purified phytase, pH 2.5 acid phosphatase, and pH 6.0 acid phosphatase were 2200, 3000, 20 000 nkat/mg, respectively.

Enzyme Assays. Each assay contained 5.5 pmol of phytase plus 37.5 μmol of sodium acetate, pH 5.0; 6.3 pmol of pH 2.5 optimum acid phosphatase plus 37.5 μmol of glycine–HCl; or 5.7 pmol of pH 6.0 optimum acid phosphatase plus 37.5 μmol of imidazole. The enzyme reaction was initiated by adding 10–750 nmol of IP_6, IP_5, IP_4, or IP_3. Final volumes after addition of substrate were 1 mL/assay. Assays were incubated at 58 °C for 3 min for phytase and pH 2.5 optimum acid phosphatase and for 10 min for pH 6.0 optimum acid phosphatase. Liberated inorganic orthophosphate was measured by the procedure of Heinonen and Lahti (1981). One unit of enzyme activity was defined as nanomoles of inorganic orthophosphate liberated per second at 58 °C (nkat). The Michaelis constant (K_m), maximal velocity (V_{max}), and maximal catalytic rate when substrate is saturating (K_{cat}) were determined from assay data.

Protein Estimation. Total protein concentration was estimated by Coomassie Brilliant Blue G-250 dye binding using ovalbumin as standard (Sedmak and Grossberg, 1977).

***myo*-Inositol Phosphates.** Sodium salt of *myo*-inositol hexakisphosphate (IP_6) was obtained from Sigma Chemical Co. (St. Louis, MO). *myo*-Inositol pentakis-, tetrakis-, and trisphosphates were prepared from sodium phytate by hydrolysis at 15 psi and 121 °C (Phillippy et al., 1987). The resulting inositol phosphates were separated by gradient ion chromatography with postcolumn derivatization (Phillippy and Bland, 1988). The isomers of *myo*-inositol pentakisphosphate (IP_5) and *myo*-inositol tetrakisphosphate were identified as $I(1,2,4,5,6)P_5$ and $I(1,2,5,6)$-P_4 by NMR (Phillippy and Bland, 1988). The isomers of *myo*-inositol triphosphate (IP_3) were present as a mixture.

RESULTS

Substrate Selectivity of Fungal Phytase and Acid Phosphatases for IP_6, IP_5, IP_4, and IP_3. The only kinetic parameters previously available were for the use of IP_6 by phytase (EC 3.1.3.8; Ullah and Gibson, 1987). The kinetic parameters of all three enzymes for IP_6, IP_5, IP_4, and IP_3 are in Table 1. With phytase, IP_6 had the lowest K_m (27 μM) followed by IP_5 (161 μM), IP_3 (200 μM), and IP_4 (1 mM). With the pH 2.5 optimum acid phosphatase, IP_6 had the lowest K_m (103 μM) followed by IP_5 (330 μM), IP_4 (690 μM), and IP_3 (2 mM). With the pH 6.0 optimum acid phosphatase, IP_6 had the lowest K_m (315 μM) followed by IP_4 and IP_3 (400 μM) and IP_5 (1.2 mM). The V_{max} of these three proteins for various species of *myo*-inositol phos-

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Table 1. Kinetic Parameters of *A. ficuum* Phytase and Acid Phosphatases (APase) for IP_6, IP_5, IP_4, and IP_3

enzyme	substrate	K_m (μM)	V_{max} (nkat/mL)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
phytase	IP6	27	386	348	1.29×10^7
phytase	IP5	161	529	477	2.96×10^6
phytase	IP4	1000	1724	1554	1.55×10^6
phytase	IP3	200	182	164	8.20×10^5
pH 2.5 APase	IP6	103	425	628	6.10×10^6
pH 2.5 APase	IP5	330	1000	1479	4.48×10^6
pH 2.5 APase	IP4	690	1330	1967	2.85×10^6
pH 2.5 APase	IP3	2000	5000	7395	3.70×10^6
pH 6.0 APase	IP6	315	13.92	2.55	8.10×10^3
pH 6.0 APase	IP5	1265	210	38.53	3.05×10^4
pH 6.0 APase	IP4	400	56.24	10.31	2.58×10^4
pH 6.0 APase	IP3	400	13.91	2.55	6.38×10^4

phates had a wider range than the K_m (Table 1). As expected, phytase hydrolyzed IP_6 quite efficiently (386 nkat/mL). The phytase's V_{max} for IP_5 and IP_4 increased remarkably to 529 and 1724 nkat/mL, respectively. The lower affinity of IP_5 and IP_6 for phytase caused this increase. IP_3 was hydrolyzed inefficiently by phytase as reflected by both K_m (200 μM) and V_{max} (182 nkat/mL). The overall throughput of the phytase's catalytic activity is reflected by the K_{cat}/K_m (Table 1, last column). As expected, IP_6 had the highest value (1.29×10^7 M^{-1} s^{-1}) followed by IP_5, IP_4, and IP_3. The low K_{cat} value of phytase for IP_3 indicates that the rate of dissociation of the ES complex to enzyme and product is diminished severely, despite the higher affinity of the substrate for the enzyme (K_m = 200 μM). The pH 2.5 optimum acid phosphatase bound to all of the tested inositol phosphates as substrates with various affinities at the optimum pH of 2.5. IP_6 had the highest affinity (K_m = 103 μM) followed by IP_5, IP_4, and IP_3. None of these substrates, however, were hydrolyzed by this enzyme at pH 5.0. The K_{cat} values of pH 2.5 optimum acid phosphatase for tested inositol phosphates were much higher than obtained for phytase; the range was 628–7395 s^{-1} (Table 1). The K_{cat}/K_m values were all in a narrow range (2.85–6.1 × 10^6 M^{-1} s^{-1}). The pH 6.0 optimum acid phosphatase hydrolyzed the inositol phosphates very poorly as reflected by the extremely low K_{cat} and K_{cat}/K_m values for all of the species of IPs tested. The K_m values for IP_6, IP_4, and IP_3 were, however, in the acceptable range, *i.e.*, 315–400 μM. Only the IP_5 had low affinity for this enzyme.

pH Profile of pH 2.5 Optimum Acid Phosphatase for IP_6. When the pH 2.5 optimum acid phosphatase was assayed with IP_6 at pH 2.5, the enzyme hydrolyzed inorganic orthophosphates from the *myo*-inositol backbone with a high turnover number (K_{cat}). To determine the pH optimum of this enzyme for IP_6, enzyme assays were performed from pH 1.5 through pH 5.5. The results are in Figure 1. A single optimum was observed at pH 2.5. The pH profile thus paralleled the earlier determination of the pH profile for *p*-nitrophenyl phosphate (Ullah and Cummins, 1987).

Rate Linearity of pH 2.5 Optimum Acid Phosphatase with IP_6. Assays were performed with various amounts of enzyme, using IP_6 as substrate. The results are in Figure 2. Release of orthophosphate by increasing amount of enzyme increased linearly from 0.5 to 5.0 pmol of enzyme. On average, 196 pmol of inorganic phosphates was released s^{-1} (pmol of enzyme)$^{-1}$. The enzymatic hydrolysis of IP_6 by the pH 2.5 optimum acid phosphatase clearly establishes the enzyme to be a phytase.

***myo*-Inositol Hexakisphosphate Concentration vs Phytase Activity of pH 2.5 Optimum Acid Phosphatase.** Fungal phytase activity is inhibited by phytate concentration higher than 750 μM (Ullah, 1988a). The concentration-dependent product formation does not follow the typical sigmoidal curve. To determine whether similar inhibition of activity by phytate occurs at high concentration in pH 2.5 optimum acid phosphatase, assays were performed from 50 to 2000 μM sodium phytate. The results are in Figure 3. Maximum activity was at 500 μM phytate concentration. Higher concentration had pronounced inhibition on activity. Phytase activity was inhibited 11, 37, 71, and 79% with 0.75, 1.0, 1.5, and 2.0 mM sodium phytate, respectively.

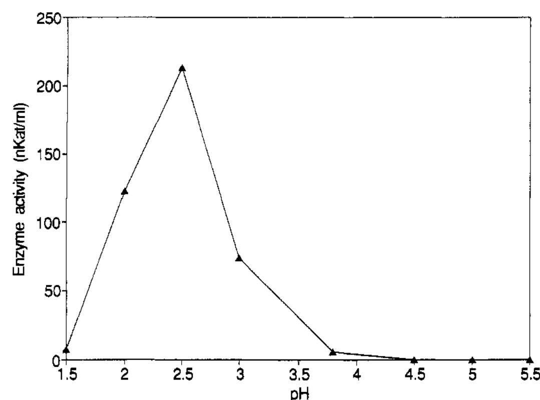

Figure 1. pH optimum profile of phytase activity of pH 2.5 optimum acid phosphatase.

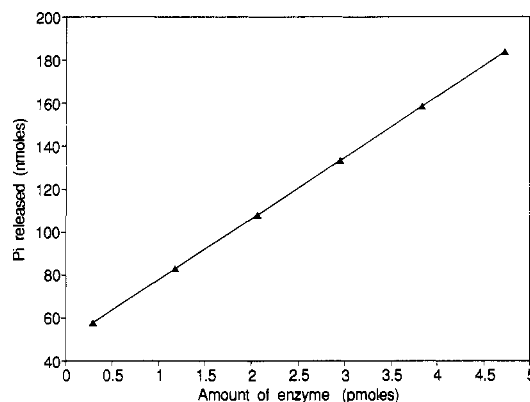

Figure 2. Rate linearity of phytase activity of pH 2.5 optimum acid phosphatase, activity vs enzyme concentration.

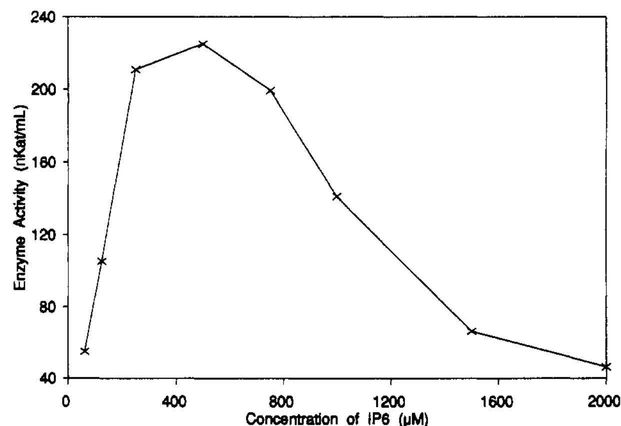

Figure 3. *myo*-Inositol hexakisphosphate concentration vs phytase activity of pH 2.5 optimum acid phosphatase.

DISCUSSION

Both phytase (EC 3.1.3.8) and certain acid phosphatases (EC 3.1.3.2) can accommodate *myo*-inositol phosphates

as substrate. These enzymes have value in feeds containing high phytate as in the case of soybeans and cotton seed. *A. ficuum* phytase, described by Shieh and Ware (1968), has been targeted by both academic and industrial researchers for genetic manipulation (Mullaney et al., 1991) and overexpression. Our research team is concentrating on protein engineering-mediated improvement of kinetic parameters and stability of this fungal phytase. Before the study reported here was initiated, a complete examination of kinetic parameters and the substrate binding domain of the enzyme, vital for any successful enzyme engineering, was lacking. Although the kinetic parameters of phytase for its natural substrate (phytate) and for a synthetic substrate (*p*-nitrophenyl phosphate) were known (Ullah, 1988b), values had not been determined for IP_5, IP_4, and IP_3. The results reported in Table 1 fill this void. Both phytase and pH 2.5 optimum acid phosphatase can efficiently hydrolyze the tested forms of *myo*-inositol phosphates. Poor hydrolysis of tested forms of *myo*-inositol by pH 6.0 optimum acid phosphatase is demonstrated by the low V_{max} and K_{cat} values. A comparison of the primary structures of the active site and substrate binding site domains of these enzymes may provide clues to the relatively low catalytic activity in pH 6.0 optimum acid phosphatase. The kinetic efficiency of these phosphomonoesterases is reflected in the K_{cat}/K_m values for tested substrates. The highest values for phytase and pH 2.5 optimum acid phosphatase were obtained for phytate, followed by IP_5 and IP_4 (Table 1). Structural modification of the active site with a target K_{cat}/K_m value of 1.0×10^8 M^{-1} s^{-1} will result in more efficient enzymes for industrial applications.

Results obtained from substrate selectivity studies with the pH 2.5 optimum acid phosphatase confirmed that enzyme to be a phytase. A similar conclusion was drawn previously (Irving and Cosgrove, 1972), but the results were not based on homogeneous enzyme preparations. The pH profile of this enzyme for IP_6 demonstrates that the enzyme has a single pH optimum at 2.5 (Figure 1). Phytase, however, has two pH optima for phytate, at pH 5.0 and pH 2.5 (Ullah and Gibson, 1987; Ullah, 1988b). The difference in pH profiles for these enzymes indicates that the catalytic domains are not identical. We have recently elucidated a 23-residue peptide containing the active site of pH 2.5 optimum acid phosphatase (Ullah and Dischinger, 1993b) and compared the sequence with the active site region of phytase (Ullah et al., 1991). The pH 2.5 acid phosphatase was found to have the three residues, Arg-His-Gly, that are widely conserved in acid phosphatases (Ullah and Dischinger, 1993b). Of the other 20 residues in this peptide, 8 were conserved and 5 were conservatively replaced, when compared to phytase. In an earlier study, it was shown that the phytate-driven phosphohydrolase activity of phytase does not follow the typical sigmoidal curve in response to increasing amounts of substrate (Ullah, 1988a). Similar behavior was exhibited by the pH 2.5 optimum acid phosphatase (Figure 3). In addition, both phytase and acid phosphatase were inhibited by higher concentrations of phytate. In high concentrations, the charge due to the phosphate groups may affect the local environment of the catalytic domain of the protein. This might inhibit conversion of the enzyme–substrate complex to enzyme and product. Availability of a complete three-dimensional structure of these proteins may help explain the role of excess substrate inhibition. Site-directed mutagenesis of the key residues in the active center may result in lowered inhibition and permit more products to be formed. Achievement of this goal would result in the production of a highly desirable enzyme for the conversion of phytate in foodstuffs and animal feed.

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