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Inositol Phosphate Phosphatases of Microbiological Origin. Some Properties of the Partially Purified Phosphatases of *Aspergillus ficuum* NRRL 3135

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Abstract

Partially purified samples of the acid phosphatase (EC 3.1.3.2) and of the phytase (EC 3.1.3.8) from *A. ficuum* were prepared by gel-filtration and ion-exchange dextran chromatography.

The pH optimum in 0·02M phthalate buffer of the acid phosphatase was found to be 2·2, while in the same buffer the phytase was found to have at least two optima at pH 2·5 and 5·3, with the suggestion of a third at pH 2·8.

The K_m value of the acid phosphatase was $1{\cdot}27 \times 10^{-4}$M with sodium *myo*-inositol hexakisphosphate as substrate at pH 2·2. Similarly the K_m value of the phytase at pH 2·5 was $2{\cdot}44 \times 10^{-5}$M and at pH 5·3 it was $1{\cdot}29 \times 10^{-5}$M.

Of a range of inositol hexakisphosphates tested as substrates the order of ease of hydrolysis was *neo-* > D-*chiro-* > *myo-* > L-*chiro-* > *scyllo-* for the acid phosphatase at pH 2·2, L-*chiro-* > D-*chiro-* > *myo-* > *neo-* > *scyllo-* for the phytase at pH 2·5 and *neo-* > L-*chiro-* > D-*chiro-* > *myo-* > *scyllo-* for the phytase at pH 5·3.

The major pentakisphosphate produced by the acid phosphatase at pH 2·2 from both D-*chiro-* and L-*chiro*-inositol hexakisphosphate was the respective 1,2,3,4,6-pentakisphosphate. In the case of the phytase, at both pH 2·5 and 5·3 the major pentakisphosphate products were the 1,2,3,4,6- and the 1,2,3,5,6-isomers from the D-*chiro-* and L-*chiro-* substrates respectively.

Neither enzyme was inhibited by 1 mM oxalate, 1 mM citrate, 1 mM EDTA or 1 mM (+)-tartrate The activity of the acid phosphatase was reduced to 17% and that of the phytase at pH 2·5 to 69% by 1 mM fluoride. The phytase was not inhibited at pH 5·3 by any of the anions tested.

Introduction

Shieh *et al.* (1969) isolated an acid phosphatase [non-specific orthophosphoric monoester phosphohydrolase (acid optimum); EC 3.1.3.2] with a pH optimum of 2·0 and a phytase (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8) with pH optima at 2·0 and 5·5, from the culture medium of *Aspergillus ficuum* NRRL 3135, a fungus known to occur in soil (Shieh and Ware 1968). They studied the thermal stability, pH optima and substrate specificity and determined the kinetic constants of the two enzymes. The substrate used for the kinetic studies was the calcium salt of *myo*-inositol hexakisphosphate.

In a study of the stereospecificity of these two *A. ficuum* phosphatases, Irving and Cosgrove (1972) examined the optical rotations of the pentakisphosphates produced by the enzymic hydrolysis of sodium *myo*-inositol hexakisphosphate and showed that the acid phosphatase produced only D-*myo*-inositol 1,2,4,5,6-pentakisphosphate.*

* The usage of the prefixes D and L and the numbering of the substituents in inositol pentakisphosphates is in accordance with the recommendations for cyclitol nomenclature of the IUPAC–IUB Commission on Biochemical Nomenclature (IUPAC–IUB 1968).

The phytase produced both D-*myo*-inositol 1,2,4,5,6- and D-*myo*-inositol 1,2,3,4,5-pentakisphosphates.

The aims of the work described in the present paper were to determine, for these two phosphatases from *A. ficuum*, the relative rates of hydrolysis of those isomers of *myo*-inositol hexakisphosphate known to occur in soil, the proportions and types of pentakisphosphates produced by hydrolysis of D-*chiro*- and L-*chiro*-inositol hexakisphosphates and the effects of certain anions on enzyme activity. The kinetic study of Shieh *et al.* (1969) was also repeated, but with sodium *myo*-inositol hexakisphosphate as substrate, and the pH optima were more accurately defined.

Materials and Methods

Reagents

All reagents were of analytical grade unless otherwise specified. The dodecasodium salt of *myo*-inositol hexakisphosphate (98% pure) was obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A.

Source of Enzymes

Batches of culture filtrate were prepared using the procedure of Shieh and Ware (1968).

Enzyme Activity

Phytase activity was determined by following the release of inorganic orthophosphate from 0·7 mM sodium *myo*-inositol hexakisphosphate at 40°C in 20 mM phthalic acid–sodium hydroxide buffer at the appropriate pH. Liberated inorganic orthophosphate was determined using a modification of a previously published method (Irving and Cosgrove 1970). Ethyl acetate was substituted for the heptane–isobutanol mixture and the absorbance measured was that of the unreduced extracted phosphomolybdate complex at 334 nm (De Sesa and Rogers 1954), using a Zeiss PL4 Filter Photometer, instead of that of the reduced complex at 725 nm. The unit of activity is defined as that amount of enzyme which liberated 1 μmol of inorganic orthophosphate per minute under the conditions of assay.

Protein

Protein was estimated by the method of Lowry *et al.* (1951), standardized against bovine serum albumin.

Polysaccharide

Polysaccharide was estimated by the method of Dubois *et al.* (1965), standardized against sucrose.

Enzyme Purification

The acid phosphatase and phytase were isolated and partially purified by a modification of the procedure of Shieh *et al.* (1969). Chromatography and associated manipulations were carried out at 5°C. The modified procedure was as described in the following paragraphs.

1. Sephadex G150 chromatography. Cell-free culture filtrate (48 ml) was applied to a column of Sephadex G150 (37 by 2·5 cm) previously equilibrated with 0·05M sodium citrate buffer (pH 3·0) made 1M with respect to NaCl. The flow rate was approximately 8 ml/h and the fraction volume 5 ml. Activity at both pH 2·0 and 5·5 was detected in fractions 8–30 (116 ml), the fractions being collected from the commencement of sample application.

2. SE-Sephadex C50 chromatography. After buffer exchange into 0·05M sodium citrate buffer (pH 3·0) and concentration to 24 ml using Sephadex G25 (coarse) (Gelotte and Porath 1960) the active eluate from the Sephadex G150 column was applied to a column of SE-Sephadex C50 (40 by 2·5 cm) previously equilibrated with 0·05M sodium citrate buffer (pH 3·0). The enzymes were eluted by applying a linear pH gradient (pH 3·0 → 5·5) in 0·05M sodium citrate buffer. The flow rate was

10 ml/h and the fraction volume 5 ml. Enzymic activity at pH 2·0 was detected in fractions 56–106 and activity at pH 5·5 in fractions 68–106. Fractions 59–64 (30·0 ml) were combined as the acid phosphatase on the basis of their activity at pH 2·0 and lack of activity at pH 5·5 while fractions 90–102 (59·5 ml) were combined as the phytase on the basis of their activity at both pH 2·0 and 5·5. It was found in a subsequent preparation that if the applied sample was allowed to equilibrate with the column overnight at 5°C before beginning elution, then the phytase was more strongly adsorbed and thus not eluted until fractions 124–128, where it was largely resolved from contaminating inactive protein and had approximately twice the specific activity (43·9 units/mg of protein). This preparation was used for all subsequent experiments with the phytase.

3. DEAE-Sephadex A50 chromatography. In an attempt to increase the specific activity of the acid phosphatase eluted from the SE-Sephadex C50 column, 11·0 ml was buffer-exchanged into 0·01 Mtris–HCl buffer (pH 7·0) on a column of Sephadex G25 (24 by 1·5 cm). The eluate (20 ml) containing the activity was applied to a column of DEAE-Sephadex A50 (21 by 1·5 cm) previously equilibrated with 0·01M tris–HCl buffer (pH 7·0). The enzyme was eluted by applying a linear concentration gradient of NaCl (0·0 → 0·3M) in 0·01M tris–HCl buffer (pH 7·0). The flow rate was 10 ml/h and the fraction volume 5 ml. Activity at pH 2·2 was detected in fractions 36–48 (65 ml). This preparation was used for all subsequent experiments with the acid phosphatase.

4. Preparation of chiro-*inositol pentakisphosphates.* The pentakisphosphates from D-*chiro*- and L-*chiro*-inositol hexakisphosphate produced by the action of the acid phosphatase at pH 2·2 and the phytase at pH 2·5 and 5·3 were prepared and isolated as previously described (Irving and Cosgrove 1971*b*).

Results

Partial Purification of A. ficuum *Phosphatases*

The Sephadex G150 chromatography was conducted in buffer made 1M with respect to sodium chloride in order to dissociate any complexes which might have existed between the phosphatases and polysaccharides in the culture medium. Polysaccharide concentrations were accordingly determined at each stage of the purification.

SE-Sephadex C50 was substituted for the CM-cellulose used by Shieh *et al.* (1969) because it is fully ionized above about pH 3·0 whereas CM-cellulose is not fully ionized until the pH is above about 5·0. The use of SE-Sephadex therefore avoided the complication of titrating the exchanger during the pH gradient elution.

Table 1. Purification of *A. ficuum* phosphatases

Values are not quoted for total protein, total polysaccharide and specific activities in the crude filtrate because it contained large quantities of substances of less than macromolecular size which reacted with both the protein and the polysaccharide reagents

Fraction	Volume (ml)	Total activity (units) pH 2·0	Total activity (units) pH 5·5	Total protein (mg)	Total polysaccharide (mg)	Specific activity (units/mg protein) pH 2·0	Specific activity (units/mg protein) pH 5·5	Percentage recovery pH 2·0	Percentage recovery pH 5·5
Crude filtrate	48	139·8	129·6	—	—	—	—	100	100
G150 fractions 8–30	116	136·7	122·2	27·5	60·1	5·0	4·4	97·8	94·3
SE-Sephadex fractions 59–65	30	15·0	1·2	2·8	3·0	5·4	0·4	10·7	0·9
SE-Sephadex fractions 90–102	59·5	46·0	79·5	3·5	1·4	13·1	22·7	32·9	61·3

Yields and specific activities for the various stages of the purification procedure are set out in Table 1. Chromatography of the acid phosphatase on DEAE-Sephadex A50 resulted in a slight increase in specific activity from 5·4 (Table 1) to 6·6 units/mg of protein.

In order to confirm the classification of the enzymes isolated by this modified procedure the activities of the partially purified samples were determined using *p*-nitrophenyl phosphate, fructose 6-phosphate, glucose 1-phosphate, *β*-glycerophosphate and *myo*-inositol hexakisphosphate as substrates. The results are set out in Table 2, and are sufficient, taken together with those of Shieh *et al.* (1969), to characterize the enzymes isolated by the modified procedure as a non-specific orthophosphoric monoester phosphohydrolase and a *myo*-inositol hexakisphosphate phosphohydrolase.

Table 2. Substrate specificity of *A. ficuum* phosphatases

Rates of reaction are expressed as percentages of that for *p*-nitrophenyl phosphate. For assay conditions see Materials and Methods. The substrate concentration was 0·7 mM

Substrate	Acid phosphatase (pH 2·2)	Phytase (pH 5·3)
p-Nitrophenyl phosphate	100	100
Fructose 6-phosphate	41	0
Glucose 1-phosphate	43	0
β-Glycerophosphate	32	0
myo-Inositol hexakisphosphate	16	1051

Properties of Partially Purified A. ficuum *Phosphatases*

1. pH optima. The acid phosphatase preparation has a single pH optimum at pH 2·2 (Fig. 1*a*) while the phytase preparation has two definite optima at pH 2·5 and 5·3 and the suggestion of a third in the region of pH 2·8 (Fig. 1*b*).

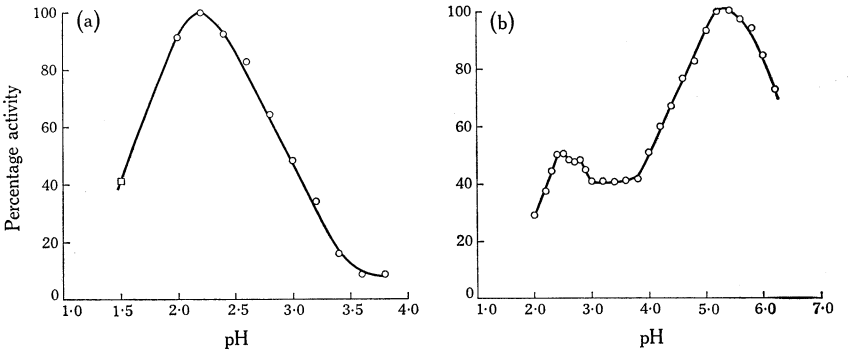

Fig. 1. Effect of pH on the activity of *A. ficuum* acid phosphatase (*a*) and phytase (*b*). For assay conditions see Materials and Methods. The relative rates are expressed as percentages of the initial reaction rate at pH 2·2 for the acid phosphatase and pH 5·3 for the phytase. Each point is the mean of two determinations. □ Point determined in NaCl–HCl buffer (0·02M, pH 1·5).

2. Kinetics. The plots of $[S]/v_0$ against [S] (Fig. 2) suggest that both enzymes follow classical Michaelis–Menten kinetics. This form of plot was chosen because it gives greatest weight to the most accurate points, those at highest substrate concentration. At pH 2·5 the phytase is inhibited by substrate concentrations in excess of about 0·6 mM (Fig. 2). The inhibition constant (K_s') is $2{\cdot}03 \times 10^{-2}$M. At pH 5·3 this inhibition occurs at substrate concentrations in excess of about 2 mM (Fig. 2).

3. Relative rates of hydrolysis of inositol hexakisphosphates. The relative rates of hydrolysis of those isomers of inositol hexakisphosphate known to occur in soil (Cosgrove 1962; Cosgrove and Tate 1963) are recorded in Table 3, together with the relative rates for binary equimolar (0·7 mM) mixtures of each of the isomers with *myo*-inositol hexakisphosphate. The use of the binary mixtures was to determine if the two isomers were hydrolysed by the same or by different active centres on the enzyme.

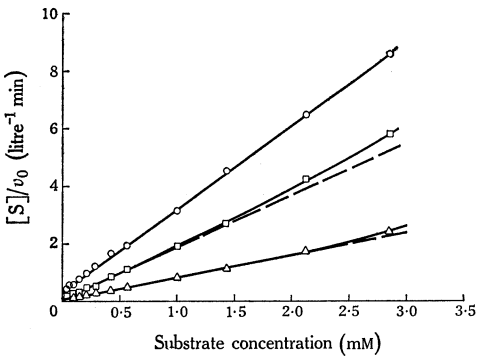

Fig. 2. Linear plots of activity–substrate saturation data for *A. ficuum* acid phosphatase at pH 2·2 and phytase at pH 2·5 and 5·3.
○ Acid phosphatase (pH 2·2). Each point is the mean of three determinations; computed K_m is $1{\cdot}27 \times 10^{-4}$M.
□ Phytase (pH 2·5). Each point is the mean of five determinations; computed K_m is $2{\cdot}44 \times 10^{-5}$M.
△ Phytase (pH 5·3). Each point is the mean of five determinations; computed K_m is $1{\cdot}29 \times 10^{-5}$M.

Table 3. Relative rates of hydrolysis of inositol hexakisphosphates

Rates of reaction are expressed as percentages of that for *myo*-inositol hexakisphosphate. For assay conditions see Materials and Methods. The substrate concentration was 0·7 mM for the individual isomers and 0·7 mM isomer plus 0·7 mM *myo*-inositol hexakisphosphate for the binary mixtures

Inositol hexakisphosphates	Acid phosphatase (pH 2·2)	Phytase (pH 2·5)	Phytase (pH 5·3)
myo-	100	100	100
D-*chiro*-	119	104	144
L-*chiro*-	80	131	155
neo-	210	77	167
scyllo-	21	38	87
D-*chiro*- + *myo*-	168	92	124
L-*chiro*- + *myo*-	154	111	131
neo- + *myo*-	205	73	127
scyllo- + *myo*-	107	81	92

4. Pentakisphosphates from D-chiro- *and* L-chiro-*inositol hexakisphosphates.* In an attempt to define the steric requirements of the active centres of the two enzymes their action on the stereoisomers D-*chiro*- and L-*chiro*-inositol hexakisphosphate was examined. Table 4 lists the relative amounts of the pentakisphosphates isolated from the acid phosphatase hydrolysates of D-*chiro*- and L-*chiro*-inositol hexakisphosphate at pH 2·2 and from the corresponding phytase hydrolysates at pH 2·5 and 5·3. Also included are data for *myo*-inositol pentakisphosphates computed from data in a previous publication (Irving and Cosgrove 1972).

5. Effects of anions on enzyme activity. The effects of selected anions, at 1 mM concentration, on the activity of both the acid phosphatase and the phytase are

summarized in Table 5. The anions were selected on their ability to complex cations and their known effects on other acid phosphatases.

Table 4. Percentages of D-*chiro*- and L-*chiro*-inositol pentakisphosphates detected in enzymatic hydrolysates of the respective hexakisphosphates

Pentakisphosphate	Acid phosphatase (pH 2·2)	Phytase (pH 2·5)	Phytase (pH 5·3)
D-*chiro*-1,2,3,5,6-	35·8	4·3	26·4
D-*chiro*-1,2,3,4,6-	36·5	94·0	60·9
D-*chiro*-1,2,3,4,5-	27·7	1·7	12·7
L-*chiro*-1,2,3,5,6-	14·4	95·4	69·9
L-*chiro*-1,2,3,4,6-	54·1	1·4	17·5
L-*chiro*-1,2,3,4,5-	31·5	3·2	12·6
D-*myo*-1,2,4,5,6-[A]	100	94·3	81·3
D-*myo*-1,2,3,4,5-[A]	0	5·7	18·7

[A] From Irving and Cosgrove (1972).

Table 5. Effects of anions on the activity of the acid phosphatase and the phytase

Rates of reaction are expressed as percentages of that for *myo*-inositol hexakisphosphate. For assay conditions see Materials and Methods. The substrate concentration was 0·7 mM

Anion	Acid phosphatase (pH 2·2)	Phytase (pH 2·5)	Phytase (pH 5·3)
None	100	100	100
1 mM citrate	116	97	104
1 mM EDTA	111	99	103
1 mM fluoride	17	69	102
1 mM oxalate	105	102	102
1 mM (+)-tartrate	99	100	104

Discussion

Partial Purification of A. ficuum *Phosphatases*

The increases in the specific activity of the acid phosphatase and the phytase (Table 1) are small but not unreasonable since the culture medium was synthetic and would contain only those proteins excreted by *A. ficuum*. Similar small increases in specific activity in the purification of extracellular fungal enzymes have been reported for a crystalline preparation of a proteinase from *A. sydowi* (purification = ·45) (Danno and Yoshimura 1967) and for leucine aminopeptidase I from *A. oryzae* (purification = 4·4) (Nakadai *et al.* 1973).

Properties of Partially Purified A. ficuum *Phosphatases*

(i) *pH optima*

The pH optima (Fig. 1) differ only slightly from those reported by Shieh *et al.* (1969). These deviations are possibly due to the optima being more closely defined

by the smaller increments of pH used in this present work. A peak in enzymic activity in the region of pH 3·4 is discernible in the results of Shieh *et al.* (1969), but they did not comment on it. It is not possible at this stage to say if these multiple pH–activity peaks of the phytase preparation (Fig. 1*b*) are due to more than one enzyme or not; however, the pH 2·5 and the pH 5·3 activities could not be resolved using the gel-electrophoresis method of Margolis and Kenrick (1968) and the visualization method of Brewer and Sing (1970).

(ii) *Kinetics*

The values of the Michaelis constants (Fig. 2) differ by up to two orders of magnitude from the value of $1 \cdot 25 \times 10^{-3}$M determined by Shieh *et al.* (1969) for both enzymes. These differences are probably due to the fact that the assay solution used by the latter workers would have been 15 mM with respect to calcium ion. Such a concentration of divalent cation could be expected to increase the K_m value of a phytase, since it has been shown that 5 mM magnesium ion competitively inhibits the phytase of *Pseudomonas* sp., increasing the K_m value at pH 5·5 from $1 \cdot 63 \times 10^{-5}$ to $3 \cdot 9 \times 10^{-3}$M (Irving and Cosgrove 1971*a*), a difference of two orders of magnitude. The K_m value for *A. ficuum* phytase at pH 5·3 (Fig. 2) is similar to that of *Pseudomonas* phytase at pH 5·5 (Irving and Cosgrove 1971*a*). In both cases the substrate was sodium *myo*-inositol hexakisphosphate.

(iii) *Relative rates of hydrolysis of inositol hexakisphosphates*

The order of ease of hydrolysis of the isomers of *myo*-inositol hexakisphosphate by the phytase at pH 5·3 (Table 3) is the inverse of the order of their abundance in soil (Cosgrove 1963; Cosgrove and Tate 1963). This suggests that extracellular phytases produced in soil by fungi similar to *A. ficuum* NRRL 3135 could play a part in determining the relative abundance of these isomers in soil.

The relative rate of hydrolysis for a binary equimolar mixture is very much less than the sum of that for the corresponding isomer with that for *myo*-inositol hexakisphosphate (Table 3). It is probable therefore that all the isomers are hydrolysed at the same active centres on the enzyme.

(iv) *Pentakisphosphates from* D-chiro- *and* L-chiro-*inositol hexakisphosphates*

The pattern of pentakisphosphates produced by *A. ficuum* phytase at both pH 2·5 and 5·3 (Table 4) is the same as that produced by *Pseudomonas* phytase (Irving and Cosgrove 1971*b*). In addition, these two enzymes produce the same pentakisphosphates from *myo*-inositol hexakisphosphate (Irving and Cosgrove 1971*b*, 1972). It is highly probable therefore that both *A. ficuum* phytase and *Pseudomonas* phytase have active centres that require the same stereochemistry in the substrate for active binding. This requirement appears to be the possession of a vicinal *trans*-equatorial pair of phosphate groups with the same stereochemistry as that of the phosphate groups in (+)-*trans*-cyclohexane-1,2-diol diphosphate (Irving and Cosgrove 1972).

The acid phosphatase is less specific in its action on D-*chiro*- and L-*chiro*-inositol hexakisphosphates (Table 4). This is in accord with the wide substrate specificity of *A. ficuum* acid phosphatase (Shieh *et al.* 1969) but is in contrast to the fact that it produces a single pentakisphosphate from *myo*-inositol hexakisphosphate (Irving and Cosgrove 1972).

(v) *Effects of anions on enzyme activity*

Of those anions tested only fluoride inhibited the enzymes. The inhibition increased with decreasing pH in the case of the phytase. This increase in inhibition with decreasing pH can be understood if the inhibiting ion is the HF_2^- ion. The concentration of this ion would increase with increased hydrogen ion concentration and Reiner *et al.* (1955) have published evidence that it is the ionic species responsible for the fluoride inhibition of prostatic acid phosphatase. The lack of inhibition by the chelating agents (Table 5) suggests that if cation activation is important with these enzymes then the activating cation must either be tightly bound to the enzyme or present in the assay solution in excess. The assay solution contained sodium and potassium ions. These cations were not tested for activating properties.

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