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THE HYDROLYSIS OF INOSITOL PHOSPHATES BY *AEROBACTER AEROGENES*

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SUMMARY

(1) Examination of the phytase activity of *Aerobacter aerogenes* showed that most of the enzyme is bound to the insoluble debris obtained after ultrasonic disintegration of washed cells.

(2) The activity of the debris was shown to be optimal between pH 4 and 5 and to be inhibited by high substrate concentration and by inorganic orthophosphate. With *myo*-inositol hexaphosphate as substrate the Michaelis constant was found to be approx. 0.135 mM.

(3) The rate of hydrolysis of various inositol phosphates is affected by the number of substituent phosphate radicals and their spatial arrangement.

(4) The dephosphorylation of *myo*-inositol polyphosphates occurs in a step-wise manner resulting in the appearance of several intermediate inositol polyphosphates. These were separated by ion-exchange chromatography and compared with the esters produced during hydrolysis by wheat-bran phytase and by 5 M HCl.

INTRODUCTION

The enzyme "phytase", which hydrolyses phytic acid, has a wide distribution amongst plant and animal tissues, certain fungi, yeasts and bacteria (SLOANE-STANLEY[1]). Few data are available on the hydrolysis of inositol phosphates by microbial enzymes. Plant enzymes have been used exclusively in studies of the effect of phytase in producing mixtures of inositol phosphoesters, by partial dephosphorylation of phytic acid (POSTERNAK AND POSTERNAK[2], COSGROVE[3]), but no results have been reported on the action of microbial enzymes in this respect.

In this investigation the hydrolysis of inositol phosphates by *Aerobacter aerogenes* has been studied and shown to be similar to that effected by phytase from plant tissues.

MATERIALS AND METHODS

Organism

The culture of *Aerobacter aerogenes* (Strain 55) was obtained from the late Dr. S. E. JACOBS, Imperial College, London.

Growth

The organism was grown in batch cultures in 2 l of nutrient broth (Lab Lemco, 3 g; Marmite, 1 g; NaCl, 5 g; peptone, 10 g; deionised water, 1000 ml) which was inoculated with 100 ml of a 16-h culture grown in the same medium. The pH of the culture was maintained at 6.8 by the automatic addition of 0.5 M KOH or 0.25 M H_2SO_4 as required. The temperature was maintained at $27\pm0.5°$ and aeration regulated at approx. 50 cm^3/min, the culture being continuously agitated by a rotating paddle at a constant speed of 400 rev./min. Approx. 0.5 ml of antifoam (Silicone MS Antifoam A, Hopkin and Williams Ltd., Chadwell Heath, Essex, England) was added at the start of incubation. The cells were harvested after 16 h growth by centrifuging at 10 000 $\times$ *g* in a refrigerated centrifuge, washed twice with ice-cold distilled water and finally resuspended in distilled water at a concentration of about 20 mg dry wt. per ml.

Preparation of enzymically active material

Suspensions of washed cells of *A. aerogenes* were treated in an ultrasonic disintegrator with a power output of 60 W (Measuring and Scientific Equipment Ltd., London S.W.1) until examination by phase-contrast microscopy (magnification $\times$ 2000) showed that the large majority of the cells were disrupted. The extent of disruption was confirmed by examination with an electron microscope. During treatment the vessel containing the cell suspension was kept cold by immersion in an ice bath. The suspension of cell debris was centrifuged at 27 000 $\times$ *g* for 30 min in the refrigerated centrifuge, the sediment washed twice in double distilled water and finally resuspended in distilled water at a rate of 10 mg dry wt. per ml.

Samples (20 ml) of "cell-free" supernatant were freed of inorganic orthophosphate by elution from a 15 cm $\times$ 2.5 cm column of Sephadex G-25 (fine grade; Pharmacia, Uppsala, Sweden) using 0.2 M acetate buffer (pH 5.0) or 5 mM Tris buffer (pH 7.5). The flow rate was adjusted to 0.5 ml/min, 2-ml fractions collected and analysed for inorganic orthophosphate and phytase activity. Additional phytase determinations were made using untreated "cell-free" supernatant, a suspension of whole washed cells and a similar suspension which had been frozen at $-10°$ for 24 h and thawed. All the determinations of phytase activity were made using aliquots of enzyme source equivalent to 10 mg dry wt. of cell debris.

Determination of phytase activity

Aliquots of the enzyme source were incubated, in the presence of 1 drop of toluene, with *myo*-inositol hexaphosphate (final concentration 0.11 mM) in 0.2 M acetate buffer (pH 5.0) for 16 h at 25°. The total volume of the reaction mixture was 5.0 ml. After incubation suitable aliquots were taken, mixed with 1 ml 1 M HCl and centrifuged. Inorganic orthophosphate was determined in the supernatant, by a modification of the method of DICKMAN AND BRAY (MEHTA *et al.*[4]). Similar determina-

tions of inorganic orthophosphate in the supernatants of cell debris and substrate controls were made and the phosphorus released from the substrate by enzyme action calculated by difference.

Pure *myo*-inositol hexaphosphate was obtained from a commercial product (British Drug Houses, Poole, Dorset, England); by anion-exchange resin chromatography. *Scyllo*-inositol hexaphosphate was separated in a similar way from a mixture of inositol phosphates extracted from soil (ANDERSON[5]). Two *myo*-inositol pentaphosphate fractions (I and II) were obtained after partial dephosphorylation of *myo*-inositol hexaphosphate with 5 M HCl at 100° for 2 h. Partial dephosphorylation of *myo*-inositol hexaphosphate using wheat-bran phytase (ANDERSON[6]), gave a mixture of *myo*-inositol pentaphosphate I, tetra-, tri- and diphosphates. The mixtures of inositol polyphosphates obtained by these methods were separated by anion-exchange resin chromatography. Samples of *myo*-inositol monophosphate produced by acid and enzymatic hydrolysis of *myo*-inositol hexaphosphate were kindly supplied by Professor A. DESJOBERT. Sodium β-glycerophosphate was obtained commercially.

Anion-exchange resin chromatography

The method of COSGROVE[3], modified by collecting 25-ml fractions of eluant, was used to separate mixtures of *scyllo*- and *myo*-inositol hexaphosphates and *myo*-inositol pentaphosphates but was found to give poor resolution of the lower esters when large amounts were added to the column. The lower esters, *myo*-inositol tetra-, tri- and diphosphates, were therefore separated by gradient elution with ammonium formate solution from a Dowex 1 (formate form) resin column (ANDERSON[5]). Analysis of total phosphorus in the fractions obtained during the elutions, using the method of MEHTA *et al.*[4] after oxidation of the phosphorus with magnesium nitrate, indicated the positions at which the various inositol phosphates were eluted. The contents of the tubes corresponding to these individual phosphates were bulked and made alkaline with 1 M aq. ammonia using 2 drops of 0.25% *p*-nitrophenol as indicator. Excess 10% barium acetate and 2 vol. of 95% ethanol were added and the solutions stood overnight in the refrigerator. The precipitated phosphates were collected by centrifuging, washed three times and dissolved in distilled water using excess Amberlite IR-120 (H^+) ion-exchange resin. The phosphates were identified by their characteristic position on the elution pattern and by comparison with known standards on paper chromatograms. It is not possible to separate *myo*-inositol hexa- and pentaphosphates on paper chromatograms but they are resolved by anion-exchange resin chromatography.

Paper chromatography

The chromatography paper (Whatman No. 1) was washed with 0.5% (w/v) solution of disodium EDTA made alkaline with a little conc. ammonia solution. Before the paper was completely dry the EDTA was removed by washing three times with distilled water, partially drying the paper between washes. After applying suitable amounts (about 5–10 μg P) of the individual phosphates, papers were run for about 48 h at room temperature, in a draught-free room, using the *n*-propanol–ammonia–water (5:4:1, v/v) solvent of DESJOBERT AND PETEK[7]. The dried papers were sprayed with the sulphosalicylic acid–$FeCl_3$ reagent of WADE AND MORGAN[8] to locate the phosphates.

TABLE I

THE PHYTASE ACTIVITY OF *A. aerogenes*

The figures show the percentage of substrate phosphorus released, as inorganic orthophosphate, by comparable aliquots of the enzyme sources. Phytase activity was determined as described in the text.

Untreated cells	*Frozen–thawed cells*	*Cell debris*	*Untreated "cell-free" supernatant*	*Desalted "cell-free" supernatant*
20	30	65	Nil	5

RESULTS

Phytase activity of untreated cells, frozen–thawed cells, cell debris, etc.

The results obtained (Table I) show an increase in activity with increasing severity of treatment of the cells, the most active preparation being the washed cell debris suspension. This material was used as the phytase source in all subsequent experiments. No activity was detected in the untreated "cell-free" supernatant but after gel filtration of this material traces of activity were detected in the phosphate-free protein fractions. This activity was less than 10% of that found on the debris. Attempts to correlate the phytase activity with a particular fraction of the cell debris have not been successful.

General properties of the cell debris phytase activity

Using a range of substrate concentrations of 0.02 mM to 0.40 mM at pH 5.0 it was found that above a concentration of 0.30 mM the activity of the debris was reduced. The Michaelis constant was calculated to be approx. 0.135 mM. Optimal activity was found between 45° and 50° and pH 4.0–5.0, using acetate or citrate buffers. Tris–maleate and cacodylate buffers were inhibitory. No activity was detected below pH 3.0 or above pH 6.5 using KCl–HCl and carbonate–bicarbonate buffers, respectively. The glycerophosphatase activity of the debris was optimal at about pH 6.5–7.0. The concentration of all the buffers used was 0.2 M.

Inorganic orthophosphate, added as KH_2PO_4, was inhibitory at concentrations above 0.5 mM, particularly if added to the debris suspension before the substrate.

Hydrolysis of various phosphate esters

Initial velocities of the reactions were calculated for the period when the first 15% of the substrates, phosphorus was released as inorganic orthophosphate. The results (Fig. 1) show a decreasing rate of hydrolysis with decreasing number of substituent phosphate radicals. However expression of the orthophosphate released as a proportion of the total phosphate originally present in the substrate showed that most of the esters are very similar in stability. The cell debris suspension was found to be inactive on *scyllo*-inositol hexaphosphate and to have only a weak activity with *myo*-inositol pentaphosphate (II). The initial velocity of hydrolysis of both *myo*-inositol monophosphates, was greater than that observed for any of the other substrates. The presence of phosphomonoesterase on the debris made it impossible to determine the specificity of the phytase.

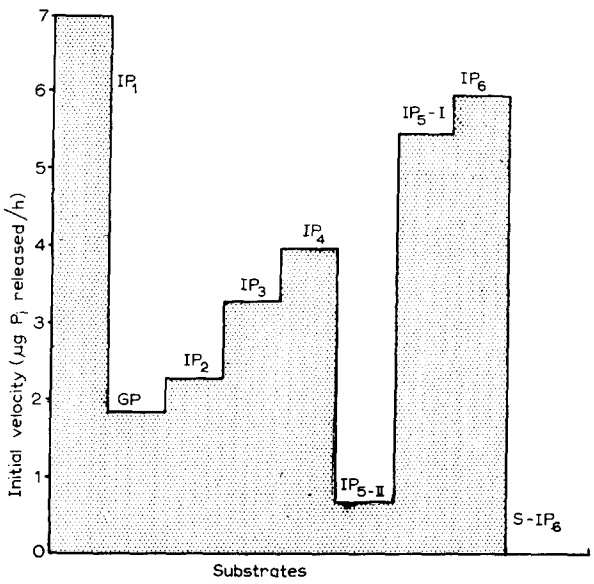

Fig. 1. Comparison of the initial velocities of hydrolysis of various inositol phosphates. Initial velocities, expressed as μg inorganic orthophosphate phosphorus released from the substrates per h by 10 mg dry wt. of cell debris, were calculated for the period during which the first 15% of the substrate phosphorus was released. The abbreviations for the substrates are as follows: GP, sodium β-glycerophosphate; IP_1, IP_2, IP_3, IP_4, IP_5 and IP_6, *myo*-inositol mono-, di-, tri-, tetra-, penta- and hexaphosphate, respectively; S-IP_6, *scyllo*-inositol hexaphosphate; IP_5-I and IP_5-II, *myo*-inositol pentaphosphate fractions obtained after partial dephosphorylation of *myo*-inositol hexaphosphate.

Separation of intermediates

In order to produce sufficient quantities of inositol polyphosphate intermediates for clear separation by anion-exchange chromatography and identification on paper chromatograms the normal phytase assay was modified somewhat. *Myo*-inositol hexaphosphate (final concn. 0.55 mM) was incubated with 2.0 ml cell debris suspension. When phosphate analysis showed that about 30% hydrolysis had occurred the pH of the reaction mixture was adjusted to about 10 using conc. aq. ammonia solution and the suspension centrifuged at 27 000 $\times$ g for 30 min. Adjustment of the pH to 10 prevents loss of phosphoric esters, which are strongly adsorbed by protein at acid pH values, during centrifugation. The supernatants of four such incubation mixtures were bulked, applied to the resin columns and eluted. Cell debris and substrate control were treated similarly. *Myo*-inositol tetraphosphate was also hydrolysed in this way and the products examined. The results are shown in Table II.

Ammonium formate gradient elution of the mixture of polyphosphate intermediates produced during hydrolysis of hexaphosphate separated it into fractions which were identified as *myo*-inositol tetra-, tri- and diphosphates *plus* an additional fraction containing a phosphate which behaved identically to *myo*-inositol hexaphosphate on a paper chromatogram. Separation of a similar mixture of intermediates by HCl gradient elution showed a similar pattern of lower esters and resolved the final fraction mentioned above into two components, a *myo*-inositol pentaphosphate (I) and residual *myo*-inositol hexaphosphate. The intermediates present after about 30% hy-

TABLE II

THE LOWER ESTERS PRODUCED DURING ENZYMATIC HYDROLYSIS OF *myo*-INOSITOL TETRA- AND HEXAPHOSPHATES

The esters were separated by anion-exchange resin chromatography as described in the text. The products are expressed as percentages of the original substrate.

Hydrolysis products	*Ammonium formate elution*		*HCl elution*	
	Myo-inositol tetraphosphate + cell debris	*Myo-inositol hexaphosphate + cell debris*	*Myo-inositol hexaphosphate + cell debris*	*Myo-inositol hexaphosphate + wheat bran*
Inorganic orthophosphate	21.0	32.0	30.0	33.0
Myo-inositol monophosphate	—	—	—	7.0
Myo-inositol diphosphate	13.0	3.0	—	2.5
Myo-inositol triphosphate	36.0	3.5	4.0	5.0
Myo-inositol tetraphosphate	30.0	7.0	8.0	9.0
Myo-inositol pentaphosphate (I)	—	52.5	36.0	30.0
Myo-inositol pentaphosphate (II)	—		—	—
Myo-inositol hexaphosphate	—		22.0	22.0

drolysis of *myo*-inositol hexaphosphate by wheat-bran phytase (ANDERSON[6]) and by 5 M HCl at 100° were also separated by HCl gradient elution. Both the bacterial and wheat-bran enzymes produced only one pentaphosphate fraction (I). Acid hydrolysis however was shown to produce two *myo*-inositol pentaphosphates (I and II). It was observed that the *myo*-inositol pentaphosphate fraction I tended to accumulate after hydrolysis of the hexaphosphate by both bacterial and wheat-bran phytases. A similar accumulation of *myo*-inositol triphosphate was found after partial dephosphorylation of *myo*-inositol tetraphosphate.

DISCUSSION

The existence of an enzyme which is specific for inositol polyphosphates is uncertain since the preparations so far described are very active with glycerophosphate. The term "phytophosphatase" has been used to describe such preparations having activity towards both glycerophosphate and inositol hexaphosphate (COURTOIS[9]). The activity of the cell debris preparation on β-glycerophosphate was optimal at about pH 6.5 and comparatively low at the level (pH 4.0–5.0) where activity towards *myo*-inositol hexaphosphate was maximal. This evidence leads us to suspect that these substrates are being hydrolysed by two distinct enzymes.

GIBBINS AND NORRIS[10] suggested that the inhibition of phytase by high substrate concentrations may indicate that a "two-point attachment" exists between substrate and enzyme. The observations that phytase gave different rates of hydrolysis with different isomeric forms of inositol polyphosphates indicates that the enzyme is greatly affected by the spatial arrangement of the substituent phosphate radicals. The most striking examples of this were the total lack of activity of the enzyme toward *scyllo*-inositol hexaphosphate, with its all-equatorial substituent groups, and the large difference in the hydrolysis rates of the two *myo*-inositol pentaphosphate fractions. These observations may be considered as evidence supporting the suggestion

of a "two-point attachment" between substrate and enzyme, which would be greatly affected by different spatial arrangements of the substituent phosphate radicals of the substrate.

Examination of the products of hydrolysis of *myo*-inositol hexa- and tetraphosphates from *A. aerogenes* phytase action showed a very similar pattern to those produced by wheat-bran phytase and in all cases the first product of hydrolysis appears to accumulate. The absence of one of the *myo*-inositol pentaphosphate fractions (II) from the mixtures of polyphosphates produced during enzymatic hydrolysis of the hexaphosphate is interesting (*cf.* COSGROVE[3]). The possibility that it is formed and immediately hydrolysed can be ignored in view of the evidence presented about its high resistance to hydrolysis by bacterial phytase. The observed patterns of intermediate production confirm the observation (DESJOBERT AND PETEK[7]) that the dephosphorylation of inositol phosphates occurs in a step-wise fashion.

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