JOURNAL OF BACTERIOLOGY, Oct. 1972, p. 434-438
Vol. 112, No. 1

Printed in U.S.A.

Inositol Phosphate Phosphatases of Microbiological Origin: the Inositol Pentaphosphate Products of *Aspergillus ficuum* Phytases

G. C. J. IRVING AND D. J. COSGROVE
Division of Plant Industry, CSIRO, Canberra City, A.C.T. 2601, Australia

Received for publication 11 May 1972

The fungus *Aspergillus ficuum* NRRL 3135 is known to produce an extracellular nonspecific orthophosphoric monoester phosphohydrolase (EC 3.1.3.2) with a *p*H optimum of 2.0, as well as an extracellular *myo*-inositol hexaphosphate phosphohydrolase (EC 3.1.3.8; phytase) with *p*H optima of 2.0 and 5.5. Both these enzymes are also known to hydrolyze *myo*-inositol hexaphosphate. The pentaphosphates liberated in the first step of this hydrolysis have been isolated and identified by ion-exchange chromatography and optical rotation. The nonspecific orthophosphoric monoester phosphohydrolase produces a single pentaphosphate, D-*myo*-inositol-1,2,4,5,6-pentaphosphate, whereas the phytase, at both *p*H 2.0 and 5.5, produces a mixture of two pentaphosphates. The major component of this mixture is D-*myo*-inositol-1,2,4,5,6-pentaphosphate and the other is D-*myo*-inositol-1,2,3,4,5-pentaphosphate. Thus the pathways of dephosphorylation of *myo*-inositol hexaphosphate by these two enzymes differ from that of wheat-bran phytase which forms L-*myo*-inositol-1,2,3,4,5-pentaphosphate.

Phytases (EC 3.1.3.8) isolated from wheat-bran (12) and a soil bacterium (*Pseudomonas* sp.) (2) have been shown to dephosphorylate *myo*-inositol hexaphosphate in a stepwise manner. The predominant *myo*-inositol pentaphosphates formed by these enzymes are, respectively, L-*myo*-inositol-1,2,3,4,5-pentaphosphate and D-*myo*-inositol-1,2,4,5,6-pentaphosphate. No similar study of phytases of fungal origin has been published although Theodorou (11), by using electrophoresis, has shown that the phytase of a mycorrhizal fungus, *Rhizopogan luteolus*, produces a mixture of at least two pentaphosphates. The major component of this mixture corresponds to the major pentaphosphate liberated by *Neurospora crassa* phytase (7) and by *Pseudomonas* phytase (2), whereas the minor component corresponds to the pentaphosphate liberated by wheat-bran phytase (12).

Shieh, Wodzinski, and Warl (10) recently described the isolation and properties of two fractions with phytase activity from culture filtrates of the fungus *Aspergillus ficuum* NRRL 3135. One of these fractions had a single *p*H optimum at 2.0, whereas the other had *p*H optima at 2.0 and at 5.5.

The work reported in this paper examines the pentaphosphates produced by both the extracellular phytases from *A. ficuum* NRRL 3135 to establish whether the pathways of dephosphorylation of *myo*-inositol hexaphosphate by these enzymes resemble that of wheat-bran phytase (12) or *Pseudomonas* phytase (2).

MATERIALS AND METHODS

All chemicals were reagent grade unless otherwise specified.

Culture of organism. *A. ficuum* 3135 was maintained on agar slopes consisting of Czapek Dox medium (Difco) in which the inorganic orthophosphate had been replaced by dodecasodium *myo*-inositol hexaphosphate (1.75 g/liter; Sigma). Cultures of the organism were prepared by using the low-phosphate medium (2 mg of P per 100 ml) and technique of Shieh and Ware (9).

Enzyme activity. Phytase activity was assayed at 40 C by determining the rate of release of inorganic orthophosphate (5). Glycine-hydrochloric acid was used as buffer at *p*H 2.0, and succinic acid-sodium hydroxide was used as buffer at *p*H 5.5. The assay solutions were 0.02 M with respect to buffer.

Protein determinations. Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as a standard.

Polysaccharide determinations. Polysaccharide was determined by the procedure of Dubois et al. (3) with sucrose as a standard.

Ion-exchange dextran chromatography of culture filtrate. Cell-free culture filtrate (50 ml) was buffer exchanged, at room temperature, into citrate buffer (0.05 M; *p*H 3.5) by using a column of Sephadex (2.5 by 40 cm; G25 Pharmacia). The buffer-exchanged, active material (70 ml) was chromatographed at room temperature by using a column of SE-Sephadex (2.5 by 34 cm; C50 Pharmacia). A linear *p*H gradient in citrate buffer (0.05 M) was used for elution. The mixing chamber contained 200 ml of *p*H 3.5 buffer, whereas the reservoir contained 200 ml of *p*H 5.5 buffer. The fraction volume was approximately 7.0 ml.

Enzyme hydrolysis of myo-inositol hexaphosphate. Dodecasodium *myo*-inositol hexaphosphate (50 mg; Sigma) was dissolved in 3.0 ml of the appropriate 0.05 M buffer, glycine-hydrochloric acid at *p*H 2.0 or succinic acid-sodium hydroxide at *p*H 5.5. The *p*H was adjusted to that of the buffer, and sufficient further buffer was added to bring the final incubation mixture volume to 10.0 ml. An appropriate volume of enzyme solution was added to initiate hydrolysis, and the mixture was incubated at 40 C until between 5.8 and 9.1% of the total ester-phosphate had been released as inorganic orthophosphate. The reaction was terminated by the addition of sufficient 1 M NaOH to raise the *p*H to about 10. The resulting solution was suitable for immediate ion-exchange chromatography. In preparations of pentaphosphates for optical rotation determinations, the quantity of dodecasodium *myo*-inositol hexaphosphate was increased to 5.0 g, and the incubation mixture volume was increased to 100 ml. The products of the large-scale preparations were isolated as their barium salts which were dissolved in 0.48 N HCl for the ion-exchange isolation of the pentaphosphates or in 0.28 N HCl for the isolation of the tetraphosphates.

Ion-exchange chromatography of myo-inositol penta- and tetraphosphates. The enzymic hydrolysates from the small-scale preparations were chromatographed by using method B described by Cosgrove (1). The fraction volume was 10.0 ml. The barium salts of the products of the large-scale preparations were chromatographed under similar conditions, but the column dimensions were increased to 2.5 by 80 cm and the fraction volume was increased to 25.0 ml. The resolved penta- and tetraphosphates were isolated as their barium salts.

Optical properties. Optical rotatory dispersion curves and optical rotations at 300 nm were determined with a Perkin-Elmer P22 spectropolarimeter. Rotations were determined by using the free acids of the tetraphosphate and of the pentaphosphates. The free acids were formed by treating the barium salts with a fivefold excess of Dowex AG-50W (x8; H^+ form; 100–200 mesh) for 4 hr at room temperature. Previous experience has shown that this technique yields a sample free from Ba^{2+} ions, by the sulfate test.

RESULTS

Ion-exchange dextran chromatography. The phytase activity of the culture filtrate was resolved into two major and possibly three minor peaks (Fig. 1). Peak A had a single activity maximum at *p*H 2.0, whereas peak E had activity maxima at both *p*H 2.0 and 5.5. Peak E was largely free of polysaccharide and inactive protein. The contents of tubes 1 through 10 were combined, as were the contents of tubes 44 through 57. These two solutions were used as enzyme stock solutions for the subsequent experiments.

Ion-exchange chromatography. The phytase from peak A produced a single pentaphosphate peak (Fig. 2) and a single tetraphosphate peak (Fig. 3). The pentaphosphate corresponds to a 1,2,4,5,6-pentaphosphate (1), whereas the tetraphosphate corresponds to a 1,2,5,6-tetraphosphate (1). The phytase from peak E produced a mixture of two pentaphosphates at both *p*H 2.0 and 5.5 (Fig. 4 and 5). The major pentaphosphate component corresponds to a 1,2,4,5,6-pentaphosphate (1), and the minor pentaphosphate corresponds to a 1,2,3,4,5-pentaphosphate (1). The major tetraphosphate (Fig. 6) component corresponds to *myo*-tetra II (1), a 1,2,5,6-tetraphosphate, and the minor component corresponds to *myo*-tetra I (1).

Optical properties. The optical rotatory dispersion curves of the pentaphosphates were plain and negative in the range 300 to 600 nm. The absolute magnitude of the rotation increased towards the shorter wavelength. The molecular rotations at 300 nm are recorded in Table 1.

DISCUSSION

By the use of ion-exchange chromatography (1), it is now possible to decide whether a *myo*-inositol pentaphosphate is a 1,3,4,5,6-, a 1,-2,4,5,6-, a 1,2,3,4,6-, or a 1,2,3,4,5-pentaphosphate. The *myo*-inositol pentaphosphates isolated in this study were identified by using a two-stage ion-exchange chromatographic procedure standardized against *myo*-inositol pentaphosphates isolated and identified in a previous investigation (1). The assignment of structures to these standard *myo*-inositol pentaphosphates is supported by a separate investigation (6) which involved phosphorus:*myo*-inositol ratios, paper chromatography, and electrophoresis.

The assignment of sign of optical rotation to

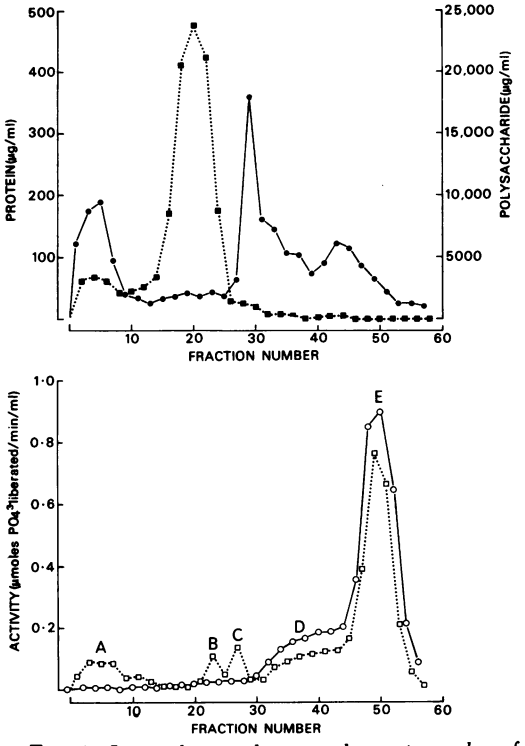


FIG. 1. *Ion-exchange dextran chromatography of A. ficuum NRRL 3135 culture filtrate. Symbols:* □, *phytase activity at pH 2.0;* ○, *phytase activity at pH 5.5;* ●, *protein;* ■, *polysaccharide.*

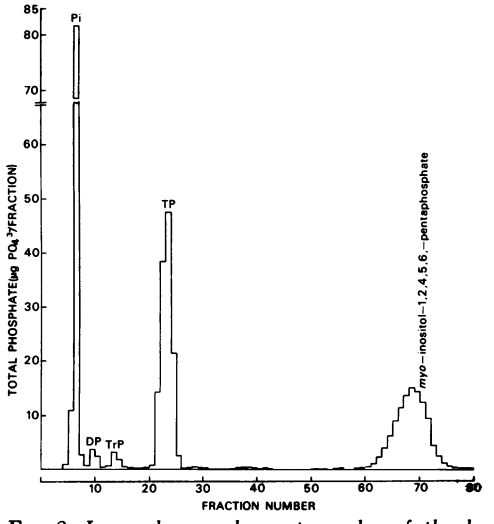


FIG. 2. *Ion-exchange chromatography of the hydrolysate (pH 2.0) of myo-inositol hexaphosphate by the phytase of peak A. Elution was by 0.48 N HCl, and fraction volume was 10 ml. Abbreviations: Pi, inorganic phosphate; DP, myo-inositol diphosphate; TrP, myo-inositol triphosphate; TP, myo-inositol tetraphosphate.*

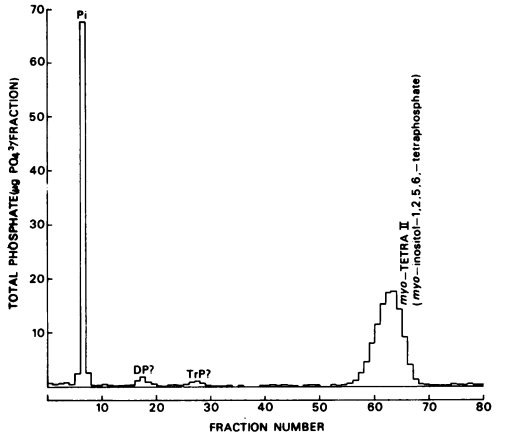


FIG. 3. *Ion-exchange chromatography of the hydrolysate (pH 2.0) of myo-inositol hexaphosphate by the phytase of peak A. Elution was by 0.28 N HCl, and fraction volume was 10 ml.*

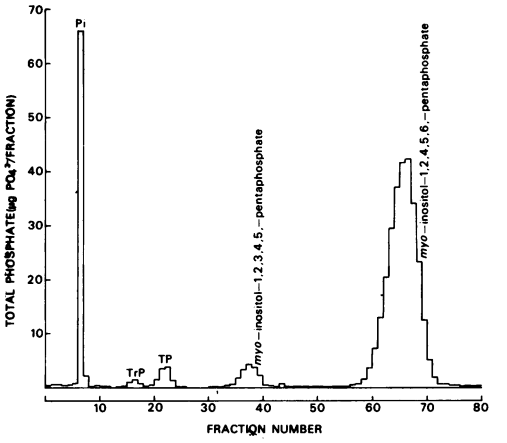


FIG. 4. *Ion-exchange chromatography of the hydrolysate (pH 2.0) of myo-inositol hexaphosphate by the phytase of peak E. For conditions see Fig. 2.*

the enantiomers of the *myo*-inositol-1,2,3,4,5-pentaphosphates rests on the work of Tomlinson and Ballou on wheat-bran phytase (12). They deduced that the major *myo*-inositol pentaphosphate produced by wheat-bran phytase is L-*myo*-inositol-1,2,3,4,5-pentaphosphate because its further enzymic dephosphorylation yielded D-*myo*-inositol-1,2,6-triphosphate and also *myo*-inositol-1,2,3-triphosphate. Johnson and Tate (7) confirmed this conclusion and found that L-*myo*-inositol-1,2,3,4,5-pentaphosphate has a positive optical rotation at *p*H 1 and 589 nm. The assignment of sign of optical rotation to the enantiomers of the *myo*-inositol-1,2,4,5,6-pentaphosphates rests on the work of Johnson and Tate on the phytase from a strain

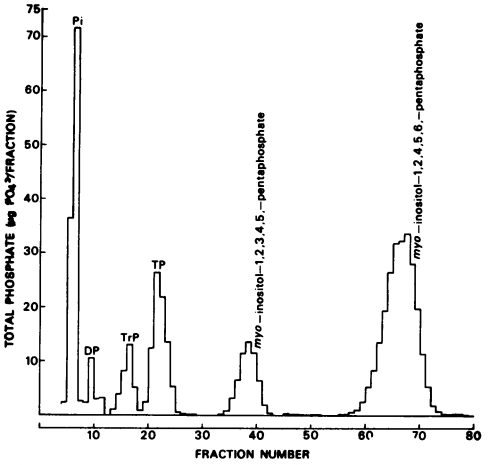


FIG. 5. *Ion-exchange chromatography of the hydrolysate (pH 5.5) of myo-inositol hexaphosphate by the phytase of peak E. For conditions see Fig. 2.*

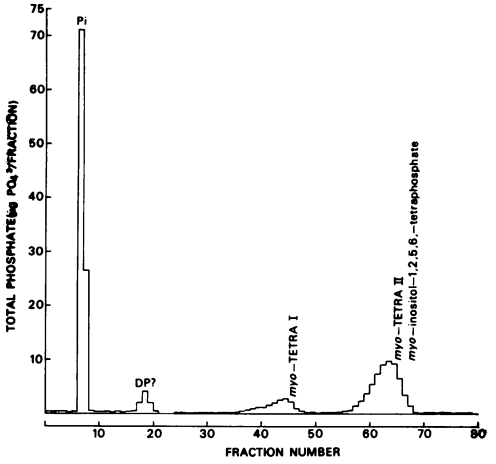


FIG. 6. *Ion-exchange chromatography of the hydrolysate (pH 5.5) of myo-inositol hexaphosphate by the phytase of peak E. For conditions see Fig. 3.*

of *Neurospora crassa* (7). The *myo*-inositol-1,2,4,5,6-pentaphosphate produced by this enzyme was identified as D-*myo*-inositol-1,-2,4,5,6-pentaphosphate because its further enzymic dephosphorylation yielded D-*myo*-inositol-1,2-diphosphate. D-*myo*-inositol-1,-2,4,5,6-pentaphosphate was found to have a negative optical rotation at *p*H 1 and 589 nm. The sign of optical rotation was confirmed by Cosgrove (2) with D-*myo*-inositol-1,2,4,5,6-pentaphosphate produced by a phytase from a bacterium of the genus *Pseudomonas*.

Without a knowledge of the molecular rotations of the pure enantiomers it is not possible to know whether the molecular rotations recorded in Table 1 are due to mixtures of enantiomers or not. It is therefore only possible to identify the major component of a possible mixture of enantiomers. The sign of rotation (Table 1) and the chromatographic behavior (Fig. 2) of the pentaphosphate produced by peak A phytase identify it as D-*myo*-inositol-1,2,4,5,6-pentaphosphate (1), and, similarly, the tetraphosphate as D-*myo*-inositol-1,2,5,6-tetraphosphate (1). The signs of rotation (Table 1) and the chromatographic behavior (Fig. 4 and 5) of the pentaphosphates produced by peak E phytase at both *p*H 2.0 and 5.5 identify the major pentaphosphate component as D-*myo*-inositol-1,2,4,5,6-pentaphosphate (1) and the minor pentaphosphate component as D-*myo*-inositol-1,2,3,4,5-pentaphosphate (1). The structures of D-*myo*-inositol-1,2,4,5,6-pentaphosphate and D-*myo*-inositol-1,2,3,4,5-pentaphosphate are illustrated in Fig. 7.

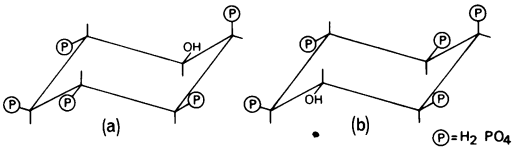
TABLE 1. *Molecular rotations of the free acids of myo-inositol tetra- and pentaphosphates*[a]

Compound	Enzyme source	Concn (g/100 ml)	$[M]^{25}_{300}$
myo-Inositol-1,2,5,6-tetraphosphate	Peak A, *p*H 2.0	0.383	−233°
myo-Inositol-1,2,4,5,6-pentaphosphate	Peak A, *p*H 2.0	0.449	−115°
myo-Inositol-1,2,4,5,6-pentaphosphate	Peak E, *p*H 2.0	0.390	−147°
myo-Inositol-1,2,4,5,6-pentaphosphate	Peak E, *p*H 5.5	0.400	−160°
myo-Inositol-1,2,3,4,5-pentaphosphate	Peak E, *p*H 5.5	0.341	− 37°

[a] Molecular rotations were determined at 300 nm and 25 C by using aqueous solutions of the free acids. The pathlength was 1 dm. The concentrations of the samples were calculated from determinations of total phosphate. Determinations were made at 300 nm because at the conventional 589 nm the measured rotations were negative but of the order of 10^{-3} degrees. The use of 300 nm increased the measured rotation by a factor of up to 22 over that at 589 nm and left no doubt as to the sign of rotation.

The extracellular phytase of *A. ficuum* NRRL 3135 produces the same pentaphosphate as the major pentaphosphate component produced by *Pseudomonas* phytase (2). The pathway of hydrolysis of *myo*-inositol hexaphosphate by these enzymes from *A. ficuum* NRRL 3135 therefore resembles that of *Pseudomonas* phytase (2) and not that of wheat-bran phytase (12).

The phytase activity of peak A is more stereospecific in its action than either *Pseudomonas* phytase or the phytase activity of peak E, since it produces a single pentaphosphate, whereas the other enzymes produce two. This conclusion is supported by the fact that peak A phytase also produces a single tetraphos-



D-*myo*-inositol-1,2,4,5,6,-pentaphosphate D-*myo*-inositol-1,2,3,4,5,-pentaphosphate

FIG. 7. *a, The structure of* D-*myo-inositol-1,-2,4,5,6-pentaphosphate; b, the structure of* D-*myo-inositol-1,2,3,4,5-pentaphosphate.*

phate. Such stereospecificity was unexpected since Shieh, Wodzinski, and Ware (10) had reported that the nonspecific orthophosphoric monoester phosphohydrolase had a much wider substrate specificity than the *p*H 5.5 phytase.

The phytase activity of peak E closely parallels that of *Pseudomonas* phytase, since both produce the same two pentaphosphates. Previous work on *Pseudomonas* phytase (4) had shown that the minor pentaphosphate component produced by that enzyme was either L-*myo*-inositol-1,2,3,4,5-pentaphosphate, its enantiomer or a mixture. Not enough of this minor component was available for determination of an optical rotation, but it is probable on the basis of the work reported in this paper that the minor pentaphosphate component of *Pseudomonas* phytase is also D-*myo*-inositol-1,2,3,4,5-pentaphosphate.

The action of the phytase of peak A at *p*H 2.0 and of the phytase of peak E at both *p*H 2.0 and 5.5 is consistent with the model of the active center of a bacterial (*Pseudomonas* sp.) phytase proposed by Irving and Cosgrove (4). In all cases, the phosphate group which is hydrolyzed to yield the pentaphosphate is a member of a vicinal *trans*-equatorial pair with the same stereochemistry as (+)-*trans*-cyclohexane-1,2-diol-diphosphate.

The major pentaphosphate component produced by wheat-bran phytase is L-*myo*-inositol-1,2,3,4,5-pentaphosphate (7). It is therefore now possible, using both the *A. ficuum* NRRL 3135 enzymes and the phytase from wheat-bran, to prepare three of the four possible optically active forms of *myo*-inositol pentaphosphate.

ACKNOWLEDGMENTS

The authors would like to thank A. Sargeson, Australian National University, for the use of the Perkin-Elmer spectropolarimeter.

LITERATURE CITED

1. Cosgrove, D. J. 1969. Ion-exchange chromatography of inositol polyphosphates. Ann. N.Y. Acad. Sci. **165**:677-686.
2. Cosgrove, D. J. 1970. Inositol phosphate phosphatases of microbiological origin. Inositol phosphate intermediates in the dephosphorylation of the hexaphosphates of *myo*-inositol, *scyllo*-inositol, and D-*chiro*-inositol by a bacterial (*Pseudomonas* sp.) phytase. Aust. J. Biol. Sci. **23**:1207-1220.
3. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1965. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
4. Irving, G. C. J., and D. J. Cosgrove. 1971. Inositol phosphate phosphatases of microbiological origin. Observations on the nature of the active centre of a bacterial (*Pseudomonas* sp.) phytase. Aust. J. Biol. Sci. **24**:559-564.
5. Irving, G. C. J., and D. J. Cosgrove. 1971. Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. Aust. J. Biol. Sci. **24**:547-557.
6. Johnson, L. F., and M. E. Tate. 1969. Structure of "phytic acids." Can. J. Chem. **47**:63-73.
7. Johnson, L. F., and M. E. Tate. 1969. The structure of *myo*-inositol pentaphosphates. Ann. N.Y. Acad. Sci. **165**:526-551.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
9. Shieh, T. R., and J. H. Ware. 1968. Survey of microorganisms for the production of extracellular phytase. Appl. Microbiol. **16**:1348-1351.
10. Shieh, T. R., R. J. Wodzinski, and J. H. Ware. 1969. Regulation of acid phosphatases by inorganic phosphate in *Aspergillus ficuum*. J. Bacteriol. **100**:1161-1165.
11. Theodorou, C. 1971. The phytase activity of the mycorrhizal fungus *Rhizopogon luteolus*. Soil Biol. Biochem. **3**:89-90.
12. Tomlinson, R. V., and C. E. Ballou. 1962. *myo*-Inositol polyphosphate intermediates in the dephosphorylation of phytic acid by phytase. Biochemistry. **1**:166-171.