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THE PHYTASES

II. PROPERTIES OF PHYTASE FRACTIONS F_1 AND F_2 FROM WHEAT BRAN AND THE *myo*-INOSITOL PHOSPHATES PRODUCED BY FRACTION F_2

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SUMMARY

Wheat bran phytase (EC 3.1.3.8) was fractionated on DEAE-cellulose to yield two fractions, F_1 and F_2. The pH optima (5.6 and 7.2), K_m values at pH 5 ($2.2 \cdot 10^{-5}$ M and $2 \cdot 10^{-4}$ M), apparent molecular sizes (both 47 000) and electrophoretic mobilities relative to cytochrome *c* in starch gels at pH 3.1 (0.47 and 0.58) were determined for F_1 and F_2, respectively. Fraction F_1 shows competitive inhibition by inorganic phosphate (K_i $3 \cdot 10^{-4}$ M) and F_2 exhibits no detectable inhibition by 10^{-2} M phosphate. *Myo*-inositol phosphates produced in the stepwise dephosphorylation of phytase F_2 have been isolated and characterised as follows: *myo*-inositol 2-dihydrogen phosphate; D- and L-*myo*-inositol 1,2-dikis (dihydrogen phosphate); *myo*-inositol 1,2,3-trikis (dihydrogen phosphate); L-*myo*-inositol 1,2,3,4-tetrakis (dihydrogen phosphate); *myo*-inositol 1,2,3,4,6-pentakis (dihydrogen phosphate); L-*myo*-inositol 1,2,3,4,5-pentakis (dihydrogen phosphate); *myo*-inositol 1,3,4,5,6-pentakis (dihydrogen phosphate).

The results suggest that a variety of phytases are present in biological systems. The initial point of dephosphorylation of phytic acid may occur at the L-1, D-4, 2 or 5 positions of the *myo*-inositol ring.

INTRODUCTION

In Part I of this series it was shown that *myo*-inositol hexakis (dihydrogen phosphate) phosphohydrolase (EC 3.1.3.8) (phytase) from wheat bran may be separated into two fractions (F_1 and F_2) by chromatography on DEAE-cellulose and that F_1 and F_2 differ in their substrate degradation patterns[1]. In the present study, a comparison is made of the properties of the two enzyme fractions. The isolation and

characterization of *myo*-inositol phosphates obtained by the stepwise hydrolysis of phytic acid with the partially purified enzyme fraction F_2 are also described.

MATERIALS AND METHODS

Materials

Corn (*Zea mays*) phytic acid Grade V sodium salt (obtained from Sigma Chemical Co., St. Louis, U.S.A.) was recrystallized according to the method of Johnson and Tate[2]. All other reagents were of analytical grade.

Protein determination

This was determined by the Folin method of Lowry *et al.*[3]. Bovine serum albumin was used as a standard. Empirical determinations were also made by measuring the absorbance at 260 and 280 nm.

Phytase determination

Phytase activity was assayed by measuring the increase in inorganic phosphate by the ascorbic acid method[4]. The reaction mixture was that of Nagai and Funahashi[5]. Incubation was carried out at 37 °C for 30–60 min, and enzyme activity was terminated by adding an aliquot of cold 10% trichloroacetic acid. Enzyme activity is expressed in International Units, *i.e.* 1 unit of enzyme activity is the amount of enzyme which liberates 1 μmole of inorganic phosphate per min.

Partial purification of bran phytases F_1 and F_2

The crude enzyme extract from wheat bran (30 g/100 ml water) was obtained by the osmotic-shock treatment of Nagai and Funahashi[5]. The yellow supernatant fraction left after centrifuging at 7500 $\times$ *g* for 20 min was fractionated with ammonium sulphate.

Solid ammonium sulphate was slowly stirred into the crude enzyme extract to give 50% saturation. The suspension was allowed to stand for 2 h before centrifuging at 7500 $\times$ *g* for 20 min to remove the residue, which was discarded. Solid ammonium sulphate was then added to the supernatant fraction to complete saturation and allowed to stand for another 2 h. The precipitate obtained after the second centrifugation was dissolved in a minimal volume of cold distilled water and was dialysed overnight against water at 4 °C. The contents of the dialysis bag were centrifuged and the supernatant fraction was used for gel chromatography.

The dialysed fraction was lyophilised and dissolved in 15 ml water, then dialysed against 0.02 M sodium acetate buffer (pH 5.0) for 3 h. It was then loaded on to a column (1.6 cm $\times$ 52 cm) containing Sephadex G-100 which had been previously equilibrated with the same buffer and calibrated with Blue Dextran and tritiated water for distribution coefficient (K_d) measurement. The enzyme eluted with 0.02 M sodium acetate buffer (pH 5.0) was collected in 3–4-ml fractions per tube. Fractions displaying enzyme activity were then bulked together.

The combined fractions were dialysed against cold distilled water before loading on to the DEAE (DE-11)-cellulose which had been treated with bovine serum albumin[1], and washed well with water. Enzyme fraction F_1 was eluted with 0.02 M Tris–HCl, pH 7.3 (200 ml) and enzyme fraction F_2 was then eluted with a linear gradient of

0.02 M (150 ml) to 0.5 M (150 ml) Tris–HCl, pH 7.3. The eluate was collected in 5-ml fractions. Enzyme fractions F_1 and F_2 were obtained by combining tubes from the appropriate peaks of activity, followed by overnight dialysis against cold distilled water.

Large-scale dephosphorylation by enzyme fraction F_2

Recrystallized sodium phytate (10 g) was used as the substrate in the enzymic preparation of inositol phosphate intermediates. The reaction mixture was incubated at 37 °C and in a total volume of 500 ml contained 0.5 M sodium acetate buffer, pH 5.0 (333 ml), recrystallized sodium phytate ($Na_{12}C_6H_6O_{24}P_6.33H_2O$; mol. wt 1533, 10 g) and distilled water. The pH of the reaction mixture was adjusted to pH 5.0 with acetic acid and the F_2 enzyme (67 ml, 13 mg protein, spec. act. 169 μmoles P_i/min per mg protein) was added in portions until 30–50% of the total phosphorus was released as inorganic phosphate. The reaction was terminated by boiling for 5 min and the denatured protein was removed by centrifuging at 2000 $\times$ g for 15 min. The pattern of substrate degradation was examined by the electrophoretic technique of Tate[8]. Inositol phosphates were then separated by anion-exchange chromatography. A small-scale control reaction without enzyme gave no significant release of inorganic phosphate.

Starch gel electrophoresis

The apparatus and procedure are similar to those described by Graham[6]. Samples (0.1 ml) containing 2–5 mg protein were run in 12% starch gels prepared with a buffer of 0.017 M aluminium lactate of ionic strength 0.1, pH 3.1, and 2 M with respect to urea. Electrophoresis was carried out towards the cathode at 3–5 V/cm for 3–6 h at 4 °C. After horizontal slicing the phosphatase bands were detected by the α-naphthyl phosphate procedure of Brewer and Sing[7] or 0.015% (w/v) nigrosine in water–methanol–15 M acetic acid (1400:600:9, v/v/v). Mobility of bands is expressed as

$$M_c = \frac{\text{distance migrated from origin to cathode by band}}{\text{distance migrated from origin to cathode by cytochrome } c}$$

High-voltage electrophoresis

Electrophoretograms were run on Whatman 3 MM paper by either the routine or moving paper electrophoresis techniques[8] in the 0.1 M oxalate buffer (pH 1.5) of Seiffert and Agranoff[9]. Phosphates were detected by the phosphomolybdate dip procedure of Harrap[10].

Paper chromatography

Inositol phosphates were separated on Whatman No. 1 paper which had been chromatographically washed for 17 h with 0.1 M oxalic acid and then with 1.5 M ammonium hydroxide. The descending technique was used at 30 °C for the following solvent systems: (A) isopropanol–ammonia–water (6:1:3, v/v/v) and (B) isopropanol–ammonia–water (8:1:1, v/v/v).

Inositols and polyols were separated on Whatman No. 1 using Solvent C, acetone–water (4:1, v/v) (8 h), and D, ethyl acetate–pyridine–water (8:2:1, v/v/v)

(29 h). Phosphates were detected by the method of Harrap[10] and inositols and polyols by the method of Anet and Reynolds[11].

Gas chromatography

Acetylated polyols were separated on 1.8 m columns of chromosorb W coated with 10% ECNSS-M as described by Oades[12].

Ion-exchange chromatography

The *myo*-inositol phosphate esters from the large-scale dephosphorylation were separated on Dowex-1 X-2 (Cl^-) resin column (6.2 cm × 37 cm). Phosphates were eluted with LiCl, first as a gradient of 0.1 M (2 l) to 0.7 M (2 l) and then from 0.7 M (2 l) to 1 M (2 l). Fractions (18 ml per tube) were collected at room temperature. An aliquot from each fraction was examined by high-voltage electrophoresis. Only fractions which showed single identical components on electrophoresis were combined and mixed fractions were discarded. Pooled fractions were concentrated to 10 ml on the rotary evaporator at 20 °C and were desalted by passage through a column of Sephadex G-10 (3.5 cm × 39 cm), which was eluted with water. The anion-exclusion effect[13] results in elution of a phosphate as a skewed peak near the void volume.

Characterization of inositol phosphates

(a) Phosphorus:inositol ratio. Phosphorus to inositol ratios were only performed on samples which were shown to be electrophoretically homogeneous. Total phosphorus determinations were carried out by the method of Bartlett[14]. A known quantity of L-*chiro*-inositol was added to the *myo*-inositol phosphate before hydrolysis (6 M HCl, 110 °C for 4 days) to act as an internal standard. The hydrolysate was then evaporated to dryness and acetylated (pyridine–acetic anhydride, 1:1, v/v, 110 °C for 2 h). The acetylated polyols were evaporated to dryness, dissolved in chloroform and analysed by gas chromatography. The *myo*-inositol content was calculated from the measured area ratio of the *myo*-inositol and L-*chiro*-inositol hexa-acetate peaks and the original L-*chiro*-inositol content.

(b) cis-Migration[8]. The inositol phosphate was heated in 1 M HCl, 100 °C for 15–20 min and examined electrophoretically and chromatographically (Solvents A and B) for isomer formation.

(c) trans-Migration[8]. The phosphate was heated in 1 M HCl, 110 °C for 90 min and examined electrophoretically.

(d) Periodate oxidation–reduction and dephosphorylation. Periodate oxidations were performed at room temperature in the dark on the lithium salt (100–200 mg) of the electrophoretically homogeneous phosphate in the presence of excess 0.04 M sodium metaperiodate (25 ml) at pH 6.5. The course of the oxidation may be conveniently followed in a 1-cm cuvette at 330 nm or at lower wavelengths nearer to the absorption maximum at 222.5 nm by dilution of suitable aliquots. The progress of the oxidation was also examined by electrophoresis at pH 1.5. When the rapid consumption of periodate had ceased (14–40 h), the reaction mixture was cooled to 0 °C and a solution of sodium borohydride (500 mg) in ice water (12.5 ml), was added in small portions with magnetic stirring. The reaction mixture was allowed to stand at room temperature overnight and the pH adjusted to 4.8 with glacial acetic acid (1.25 ml). The reaction mixture was heated at 100 °C for 3 h and then heated in a sealed tube

for 4 h at 120 °C to complete the dephosphorylation. The cooled reaction mixture was passed through a Dowex 50 (H^+) resin (4.0 cm × 2 cm) column and the eluate evaporated to dryness. The residue was then evaporated to dryness with methanol (5 × 200 ml) to remove boric acid and finally passed through a Dowex 1 X-8 Ac resin (10 cm × 1 cm) column to remove acidic components. The neutral polyols were examined by paper chromatography in Solvents C and D. An aliquot was also acetylated and examined by gas chromatography. The above conditions were suitable for the oxidation of di- and triphosphates but attempts to oxidise tetraphosphates suspected of bearing a *trans*-vicinal glycol grouping were unsatisfactory, although a similar procedure was successfully used to oxidise a tetraphosphate bearing a *cis*-vicinal glycol[2].

(e) Optical rotations. Suitable aliquots of an electrophoretically homogeneous phosphate of known phosphorus to inositol ratio n were adjusted to the appropriate pH with Dowex 50 (H^+) or concentrated ammonium hydroxide, the phosphorus content c in g/l was measured[14] for each solution and finally the optical rotation α was measured in a tube of length l in decimetres with a Bellingham and Stanley (London, Great Britain) visual polarimeter at ambient temperature t with a sodium D lamp as light source. The molecular rotation was then calculated with the relationship $[M]^t_D = 310 \cdot n \cdot \alpha / c \cdot l$, which is equivalent to the relationship $[M]^t_D = 0.01 \cdot \text{mol. wt} \cdot [\alpha]^t_D$.

(f) Electrophoretic pattern after further enzymic degradation. This "fingerprinting" technique is useful for distinguishing between *myo*-inositol 1,3,4,5,6 pentakis (dihydrogen phosphate) and D- or L-*myo*-inositol 1,2,4,5,6 pentakis (dihydrogen phosphate). It is also of value for establishing product precursor relationships such as the formation of a common tetraphosphate from two separable pentaphosphates.

RESULTS

Properties of enzyme fractions F_1 and F_2

Table I shows the purification scheme used for the isolation of phytase fractions F_1 and F_2. The purifications (F_1 285-fold and F_2 200-fold) are low and starch gel electrophoresis at pH 3.1 showed that the protein of the phosphatase bands for fractions $F_1(M_c, 0.47)$ and $F_2(M_c, 0.58)$ was below the level of detection. The mobilities of the inactive protein bands were as follows: $F_1(M_c, 0.61$ strong, 0.74 weak), and $F_2(M_c, 0.46$ weak, 0.54 weak, 0.76 strong).

Table II compares some of the properties of the enzymes. Although a difference in pH optima is evident, enzyme F_1 has a relatively sharp optimum at pH 5.6, whereas for F_2, with an optimum near pH 7, the profile is broad. Hence the F_2 enzyme still contributes significantly to the enzymic degradation at pH 5. The K_m value for F_1 ($2.2 \cdot 10^{-5}$ M) is noticeably lower than for F_2 ($2 \cdot 10^{-4}$ M). F_1 was competitively inhibited by inorganic phosphate, as shown in Fig. 1, with an inhibitor constant K_i of $3 \cdot 10^{-4}$ M; by contrast, enzyme F_2 showed no detectable inhibition by levels of phosphate up to 10^{-2} M. Enzyme fractions F_1 and F_2 have similar molecular sizes (47 000) as shown by their distribution coefficients (K_d values) on Sephadex G-100, but differ significantly in their charge characteristics at pH 3.1.

TABLE I

PURIFICATION OF WHEAT BRAN PHYTASES F_1 AND F_2

Fraction	*Volume (ml)*	*Protein (mg)*	*Activity (units)*	*Spec. act.*	*Purification*
1. Crude aqueous extract supernatant fraction left after centrifuging at 7500 × *g* for 20 min	76	1140	785	0.7	1
2. 50–100% $(NH_4)_2SO_4$ saturation of Fraction 1. Fraction dialysed overnight at 4 °C against double-distilled water	15	240	373	1.6	2
3. Fraction 2 passed through a 1.6 cm × 5.2 cm Sephadex G-100 column. Active enzyme fractions collected	28	2.8	350	125	178
4. Fraction 3 passed through a DE-11-cellulose column (1.8 cm × 18 cm)					
(a) Fraction F_1 eluted by 0.02 M Tris–HCl (pH 7.3)	30	1	200	200	285
(b) Fraction F_2 eluted by a gradient of 0.02–0.5 M Tris–HCl (pH 7.3)	50	0.5	70	140	200

myo-Inositol phosphate intermediates in the hydrolysis of phytic acid by wheat bran phytase fraction F_2

In Part I of this series[1] it was shown that the phytic acid degradation pattern of phytase fraction F_2 differed markedly from fraction F_1. In particular, enzyme fraction F_2 showed the presence of three pentaphosphates and only one tetraphosphate. Table III lists the electrophoretic mobilities, structural assignments and molecular rotations at pH 1 and 11 of the seven electrophoretically homogeneous inositol phosphates obtained by ion-exchange chromatography of a phytic acid hydrolysate produced by wheat bran phytase fraction F_2.

TABLE II

COMPARISON OF SOME PROPERTIES OF PHYTASE FRACTIONS F_1 AND F_2

The buffers used for pH determination were 0.5 M sodium acetate for pH 4–5.6 and 0.5 M Tris–HCl for pH 6–8. The enzyme solution was equilibrated with the appropriate buffer before measurement of phytase activity. K_m and K_i values were determined from the double-reciprocal plots of reaction rate against substrate (phytate) concentrations as described by Lineweaver and Burk[31]. Apparent molecular weights were obtained by measuring the distribution coefficients (K_d) values on Sephadex G-100 and interpolation of these points on a standard curve of log molecular weight *versus* distribution coefficient, as described by Andrews[32]. Electrophoretic mobilities (M_c) are expressed relative to cytochrome *c*.

	F_1	F_2
pH optimum	5.6	7.2
K_m value (pH 5)	$2.2 \cdot 10^{-5}$ M	$2 \cdot 10^{-4}$ M
KH_2PO_4 as inhibitor K_i value (pH 5)	$3 \cdot 10^{-4}$ M	no inhibition at 10^{-2} M
Distribution coefficient (K_d) on Sephadex G-100	0.25	0.25
Apparent molecular weight	47 000 ± 2000	47 000 ± 2000
Electrophoretic mobility (M_c) at pH 3.1	0.47	0.58
Lipid cofactor	lysolecithin	not detectable

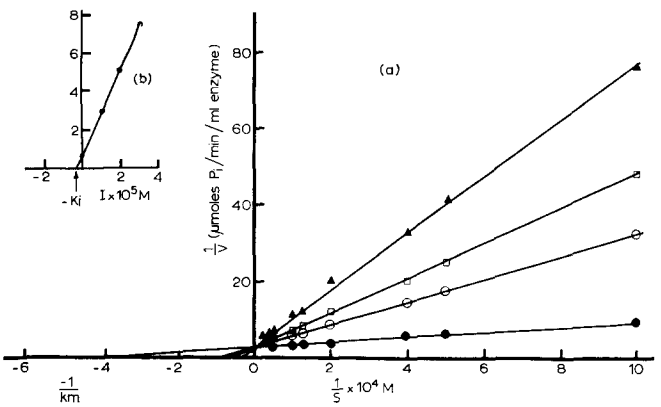


Fig. 1. (a) Lineweaver–Burk[31] reciprocal plots of velocity (v) against substrate concentration [S] for enzyme fraction F_1. Velocity is expressed as μmoles of P_i liberated per min per ml of enzyme. The substrate concentration represents various amounts of phytate with or without KH_2PO_4 as the inhibitor. ●—●, without inhibitor; ○—○, with 1 mM KH_2PO_4; □—□, with 2 mM KH_2PO_4; ▲—▲, with 3 mM KH_2PO_4. The K_m value is $2.2 \cdot 10^{-5}$ M. (b) Replots of the slope (K_m/V) of (a) against various inhibitor (I) concentrations. The K_i value is $3 \cdot 10^{-4}$ M.

Nomenclature

It is important to note that cyclitol nomenclature employed in this paper and another from this laboratory[15] and also from Cosgrove[16] is the nomenclature described in the IUPAC–IUB *Tentative Rules for Cyclitols*[17], whereas earlier papers by Tomlinson and Ballou[18] and Cosgrove[19] used the numbering procedure of Fletcher *et al.*[20].

To aid in the correct interpretation of chiral structures, the formulae in this paper will have the *meso* "2" position and the appropriate D-1 and L-1 positions designated. This means that those formulae which are named according to IUPAC-IUB nomenclature (Rule *I*-5)[17] as belonging to the D series (*e.g.* IVa) are numbered in an anti-clockwise direction and, conversely, those which are to be named as the L series (*e.g.* Vb) are numbered in a clockwise direction. For symmetrical *meso* structures

TABLE III

PRODUCTS OF THE HYDROLYSIS OF PHYTIC ACID BY PHYTASE (FRACTION F_2)

The phosphorus-to-inositol ratio for each compound is denoted by the numerical subscript following the letter P. Structures refer to Fig. 2, and electrophoretic mobilities (M_{PP_i}) in 0.1 M oxalate buffer, pH 1.5, are calculated with reference to inorganic pyrophosphate. Molar rotations were obtained with a visual polarimeter using the sodium D line as light source.

Compound	*Phosphorus to inositol ratio*	*Structures of main components*	*Electrophoretic mobility* (M_{PP_i})	$[M]_D^{25}$ *pH 1*	$[M]_D^{25}$ *pH 11*
F_2P_1	1.1	Ia	0.48	0	0
F_2P_2	2.0	IIa, IIb	0.71	−15 ± 1	− 5 ± 1
F_2P_3	3.0	III	0.86	14 ± 14	0 ± 15
F_2P_4	4.0	IVa	0.99	+30 ± 15	−70 ± 17
F_2P_5C	5.0	Vc	1.06	0 ± 13	0 ± 19
F_2P_5B	5.1	Vb	1.14	+ 9 ± 4	+27 ± 5
F_2P_5A	5.2	Va	1.23	+18 ± 36	+15 ± 30

(*e.g.* IVc) only the *meso* 2 position is designated and the prefix D or L is omitted as set out in IUPAC–IUB Rule *I*-5. In the formulae an unsubstituted vertical stroke denotes a free hydroxyl and those strokes terminating in "R" represent dihydrogen phosphate ester groups (R = OPO_3H_2).

Symbolic names for electrophoretically homogeneous inositol phosphates

Because of the large number of inositol phosphates which have been isolated from different enzymic hydrolysates, it has become necessary to use a systematic means for characterizing them and establishing relationships among them, prior to structural assignments. The following scheme has been employed for this series of papers: Electrophoretically homogeneous compounds were given a symbolic name, in which the first two symbols (F_n) describe the enzyme fraction employed in the dephosphorylation, the second two symbols (P_n) denote the degree of phosphorylation of the inositol ring as measured by the phosphorus: inositol assay and the final letter is used to differentiate isomeric phosphates in decreasing order of mobility. Where two isomeric phosphates are known to be present, but are not electrophoretically separable, two letters are appended, and where only a single phosphate is present, the final letter is omitted. For example, F_2P_5C signifies the pentaphosphate (P_5) of lowest mobility (C) isolated from the dephosphorylation of phytic acid by wheat bran phytase fraction F_2.

Structural assignments (see Table III and Fig. 2)

(a) Inositol pentaphosphates F_2P_5A, F_2P_5B and F_2P_5C and tetraphosphate F_2P_4

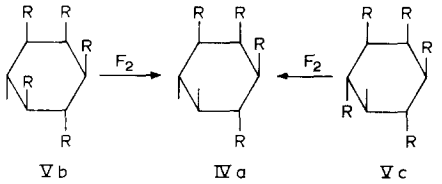
Compound F_2P_5A. Compound F_2P_5A was shown to be the somewhat unexpected *myo*-inositol 1,3,4,5,6 pentakis (dihydrogen phosphate) Structure Va, which was earlier[2] isolated from chicken blood. Structure Va was established by the following criteria: electrophoretic mobility by moving paper electrophoresis[8], acid-catalysed *cis* migration to give the D- and L-1,2,4,5,6 pentakis (dihydrogen phosphates)[8], absence of significant optical activity, an indistinguishable enzymic F_1 degradation pattern to the degradation pattern of authentic *myo*-inositol 1,3,4,5,6 pentakis (dihydrogen phosphate).

Compound F_2P_5B. Compound F_2P_5B was characterized as indistinguishable from the L-*myo*-inositol 1,2,3,4,5 pentakis (dihydrogen phosphate) Structure Vb from crude bran phytase degradations by its electrophoretic mobility[8], stability to *cis* migration conditions[8], isomerisation pattern produced under *trans* migration conditions and optical activity[15].

Compound F_2P_5C. Compound F_2P_5C was characterised as *myo*-inositol 1,2,3,4,6 pentakis (dihydrogen phosphate) Structure Vc by its electrophoretic mobility, stability to *cis* migration conditions, the electrophoretic pattern produced under *trans* migration conditions and lack of significant optical activity. This product has only hitherto been detected in chemical degradations of phytic acid[8,19].

Compound F_2P_4. Further enzymic degradation of either F_2P_5B or F_2P_5C by enzyme fraction F_2 gives an electrophoretically homogeneous tetraphosphate F_2P_4 which is indistinguishable from the minor tetraphosphate F_1P_4B produced by the F_1 enzyme, and as noted previously[1] there is no tetraphosphate corresponding to the F_1P_4A. F_2P_4 is stable to *cis* migration conditions, it is optically active and its formation from both F_2P_5B and F_2P_5C define the presence of L-*myo*-inositol 1,2,3,4 tetrakis

(dihydrogen phosphate) IVa. The *trans* vicinal glycol is resistant to the conditions employed for periodate oxidation.



(b) Triphosphate F_2P_3

It was shown previously[1] that the triphosphate F_2P_3 listed in Table III has a distinctly lower paper chromatographic mobility than the triphosphate F_1P_3 produced by enzyme F_1. Its lack of optical rotation, stability to *cis* migration conditions and conversion to ribitol by the periodate oxidation–borohydride reduction–hydrolysis sequence clearly establish the structure as *myo*-inositol 1,2,3 trikis (dihydrogen phosphate) III.

(c) Diphosphate F_2P_2

The periodate oxidation–borohydride reduction–hydrolysis sequence gave erythritol. Mild alkaline hydrolysis[18] gave *myo*-inositol 1- and 2-monophosphates. The magnitude and sign[15] of the molecular rotations of F_2P_2 in acid and alkali suggest it is a mixture of the enantiomorphs IIa and IIb in which D-*myo*-inositol 1,2 dikis (dihydrogen phosphate) IIa predominates. What is believed to be relatively pure IIa has been isolated from the action of *Neurospora crassa* phytase[15] and will be discussed in more detail in a later paper.

(d) Monophosphate F_2P_1

myo-Inositol-2-dihydrogen phosphate (Ia) was the only detectable monophos-

TABLE IV

% RATIOS OF PRODUCTS FROM THE HYDROLYSIS OF PHYTIC ACID BY PHYTASE (FRACTION F_2)

Ratios are expressed as a percentage of the total phosphorus P_t and also as moles per 100 moles of original substrate (phytate). For each series an aliquot of the reaction mixture containing 40–50 μg P_t was separated by electrophoresis in 0.1 M oxalate buffer, pH 1.5, on a 1-cm-wide strip of Whatman 3MM. After detection with the molybdate reagent the strip was divided into 35 segments and analysed for total phosphorus by the method of Bartlett[14]. Inositol mole % values were obtained by the difference between the sum of the mole percentages of inositol phosphates and 100.

Compound	*Structure of main components*	*Electrophoretic mobility (M_{PP_i})*	*% P_t*	*Mole %*	*% P_t*	*Mole %*	*% P_t*	*Mole %*	*% P_t*	*Mole %*
P_i		0.30	15.5	15.5	44.3	44.3	55.7	55.7	86.2	86.2
F_2P_6	VI	1.27	51.0	51.0	2.5	2.5	2.0	2.0	0	0
F_2P_5A	Va	1.23	3.0	3.6	0.5	0.6	0	0	0	0
F_2P_5B	Vb	1.14	24.7	29.6	7.7	9.3	0	0	0	0
F_2P_5C	Vc	1.06	3.3	4.0	19.2	23.0	10.3	12.4	0	0
F_2P_4	IVa	0.99	2.0	2.9	11.4	17.2	7.6	11.4	0	0
F_2P_3	III	0.86	0.7	1.4	10.5	20.9	15.0	30.0	0	0
F_2P_2	IIa, IIb	0.71	0	0	2.5	7.5	7.4	22.2	0	0
F_2P_1	Ia	0.48	0	0	1.5	9.0	2.0	12.1	13.8	82.3
Inositol		0	—	7.5		10.0	—	9.9	—	17.7

phate by prolonged paper chromatography using solvent system B (80:10:10, v/v/v) and electrophoresis in 0.1 M citrate, pH 5.0. Further treatment with enzyme fraction F_2 hydrolysed this phosphate to the free *myo*-inositol. If D-*myo*-inositol-1-phosphate (Ib) or its enantiomorph was present, it must have been below the level of detection ($\approx 5\%$ of Ia).

Product ratios (Table IV)

The relative ratios of the various components of the F_2 phytase reactions at 15.5, 44.3, 55.7 and 86.2% release of inorganic phosphate are listed in Table IV.

DISCUSSION

In Part I of this series[1], it was shown that wheat bran phytase fraction F_1 was specifically activated by lysolecithin and no such activation was observed for enzyme fraction F_2. Fig. 1 shows that enzyme fraction F_1 also exhibits competitive end-product inhibition by inorganic phosphate. In marked contrast, enzyme fraction F_2 was not inhibited by phosphate at 10^{-2} M. Inhibition of cereal grain phytases by inorganic phosphate has been previously noted by Peers[21] (wheat bran), Luers and Silbereisen[22,23] (malt barley), Hoff-Jorgensen[24] (rye bran) and Yoshida[25] (rice bran). However the purified wheat bran phytase preparation of Nagai and Funahashi[5] was not inhibited by inorganic phosphate and the reason for this exception is uncertain, but may lie in varietal differences.

Preliminary experiments with dissected germinating wheat grains, have shown that the characteristic electrophoretic substrate degradation pattern for the F_2 enzyme fraction is associated with the embryo. This may be a consequence of morphological separation of the F_1 and F_2 enzyme fractions, or alternatively it may be due to end-product inhibition of the susceptible F_1 enzyme, by the high levels of inorganic phosphate which are known[26] to be present in the embryo during germination.

Mayer[27] detected a phytase with an optimum at pH 7 in lettuce seed, and the pH profile for enzyme fraction F_2 also shows a maximum near pH 7. However, the pH profile is broad and for comparison with F_1 enzyme and with the experiments of Tomlinson and Ballou[18], the large-scale dephosphorylation was carried out at pH 5. No detectable differences in electrophoretic substrate degradation patterns were observed between incubations at pH 5 and 7. In the present study emphasis has been placed on identifying the inositol phosphates produced by the F_2 enzyme fraction because of the marked dissimilarity of its electrophoretic substrate degradation pattern[1] to that of the crude enzyme, which has already been studied by Tomlinson and Ballou[18].

The stepwise hydrolysis of phytic acid by enzyme fraction F_2 is summarised in Fig. 2, and shows clear distinctions from the dephosphorylation scheme proposed by Tomlinson and Ballou[18] for their relatively unfractionated enzyme. The main differences reside in the presence of the symmetrical pentaphosphates Va and Vc and the absence of the dephosphorylation pathway incorporating the asymmetrical D-*myo*-inositol 1,2,5,6 tetrakis (dihydrogen phosphate) and D-*myo*-inositol 1,2,6 trikis (dihydrogen phosphate).

As with all enzymic[1,8,16] and chemical[28] hydrolysates of *myo*-inositol hexakis (dihydrogen phosphate) (phytic acid) VI, the axial *myo*-inositol-2-phosphate Ia is the

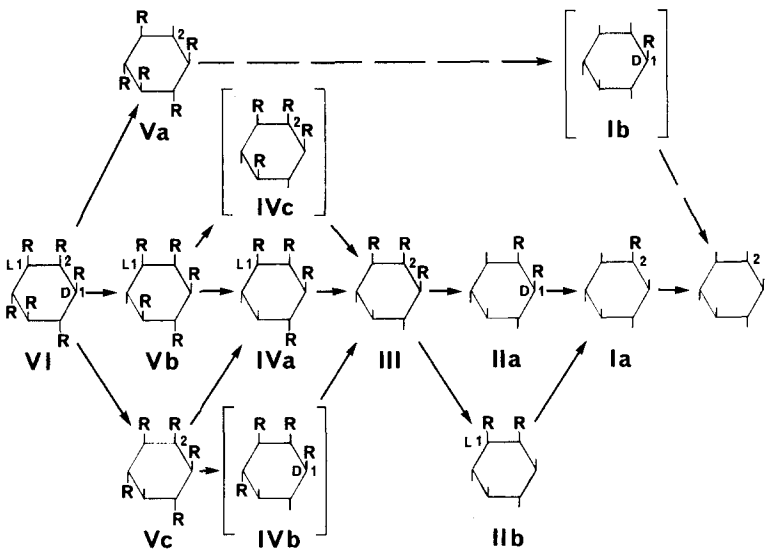


Fig. 2. Phytic acid dephosphorylation scheme for enzyme fraction F_2. An unsubstituted vertical stroke on the cyclohexane ring denotes a free hydroxyl, those terminating in 'R' represent the dihydrogen phosphate ester group (R = OPO_3H_2).

main penultimate product. However, it is clear from this work and others[18,16,15] that there are several pathways whereby compound Ia is produced. In the case of the F_2 enzyme fraction, it arises through the stepwise hydrolysis of both the asymmetrical L-*myo*-inositol 1,2,3,4,5 pentakis (dihydrogen phosphate) Vb and the symmetrical *myo*-inositol 1,2,3,4,6 pentakis (dihydrogen phosphate) Vc to give L-*myo*-inositol 1,2,3,4 tetrakis (dihydrogen phosphate) IVa; this tetraphosphate is dextrorotatory in acid solution[15,19] and corresponds to the minor tetraphosphate component of the crude wheat bran phytase hydrolysates[15,18,19,29].

The data do not exclude the possibility that the tetraphosphate fraction F_2P_4 also contains D-*myo*-inositol 1,2,3,4 tetrakis (dihydrogen phosphate) IVb which is the enantiomorph of IVa and could be derived from the pentaphosphate Vc. Nor does it exclude the symmetrical *myo*-inositol 1,2,3,5 tetrakis (dihydrogen phosphate) IVc which may be derived from the pentaphosphate Vb as first postulated by Tomlinson and Ballou[18]. As shown in Fig. 2, all of the possible tetraphosphate components are potential precursors of the symmetrical *myo*-inositol 1,2,3 trikis (dihydrogen phosphate) III.

Hydrolysis of the symmetrical triphosphate III proceeds mainly to yield the levorotatory D-*myo*-inositol 1,2 dikis (dihydrogen phosphate) IIa, although the magnitude of the rotation suggests that some of its enantiomer IIb may also be present. D-*myo*-Inositol 1,2 dikis (dihydrogen phosphate) is also a component in crude bran phytase reactions[15,18] as well as *Neurospora crassa*[15] and *Pseudomonad* phytases[19].

It can be seen that for the F_2 phytase 9 of the 12 phosphates in Fig. 2 represent one of the two pathways in the dephosphorylation scheme proposed by Tomlinson and Ballou[18] for a crude bran phytase. However, the detection and identification of the two additional pentaphosphates Va and Vc provide two hitherto unrecognised routes

for the enzymic degradation of phytic acid and probably indicate the presence of at least three phytases in fraction F_2.

Although no further intermediates in the degradation of *myo*-inositol 1,3,4,5,6 pentakis (dihydrogen phosphate) Va were observed in this study, it is possible that the D- and/or L-*myo*-inositol 1-dihydrogen phosphate (Ib and its enantiomer) detected by Tomlinson and Ballou[18] could be derived from the symmetrical pentaphosphate Va instead of the diphosphates IIa and IIb. From a consideration of the ion-exchange behaviour of the minor pentaphosphate in bran phytase dephosphorylation mixture noted by Schormuller[30], and also depicted in the second figure of a paper by Uehara *et al.*[29], it seems likely that this minor pentaphosphate also corresponds to Va, which has previously been isolated from chicken blood[2].

It is now clear that no characterization of a phytase can be considered complete until the structure of the pentaphosphate produced has been determined. The results of the present study show that the F_2 enzyme fraction attacks the phytate molecule at the 2, D-4 and 5 positions. It is also known that the *Pseudomonad* phytase[16] and *Neurospora crassa* phytase[15] both attack the L-1 position. Hence, in biological systems four of the six possible loci for hydrolysis of the phytate molecule have now been identified, thereby providing another valuable criterion for homogeneity of these phosphatases.

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REFERENCES

1 Lim, P. E. and Tate, M. E. (1971) *Biochim. Biophys. Acta* 250, 155–164
2 Johnson, L. F. and Tate, M. E. (1969) *Can. J. Chem.* 47, 63–73
3 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
4 Watanabe, F. S. and Olsen, S. R. (1965) *Soil Sci. Soc. Am. Proc.* 29, 677–678
5 Nagai, Y. and Funahashi, S. (1962) *Agric. Biol. Chem. (Japan)* 26, 794–803
6 Graham, J. S. D. (1963) *Aust. J. Biol. Sci.* 16, 342–348
7 Brewer, G. J. and Sing, C. F. (1970) *Introduction to Isoenzyme Techniques*, p. 92, Academic Press, New York and London
8 Tate, M. E. (1968) *Anal. Biochem.* 23, 141–149
9 Seiffert, U. B. and Agranoff, B. W. (1965) *Biochim. Biophys. Acta* 98, 574–581
10 Harrap, F. E. G. (1960) *Analyst* 85, 452
11 Anet, E. F. L. J. and Reynolds, T. M. (1954) *Nature* 174, 930
12 Oades, J. M. (1967) *J. Chromatogr.* 28, 246–252
13 Steward, J. H. and Tate, M. E. (1969) *J. Chromatogr.* 45, 400–406
14 Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466–468
15 Johnson, L. F. and Tate, M. E. (1969) *Ann. N.Y. Acad. Sci.* 165, 526–532
16 Cosgrove, D. J. (1970) *Aust. J. Biol. Sci.* 23, 1207–1220
17 IUPAC/IUB Committee (1968) *Eur. J. Biochem.* 5, 1–12
18 Tomlinson, R. V. and Ballou, C. E. (1962) *Biochemistry* 1, 166–171
19 Cosgrove, D. J. (1969) *Ann. N.Y. Acad. Sci.* 165, 677–686
20 Fletcher, H. G., Anderson, L. and Lardy, H. (1954) *J. Org. Chem.* 16, 1238–1246
21 Peers, F. G. (1953) *Biochem. J.* 53, 102–110
22 Luers, H. and Silbereisen, K. (1927) *Wochenschr. Brau.* 44, 263–268
23 Luers, H. and Silbereisen, K. (1927) *Wochenschr. Brau.* 44, 273–278

24 Hoff-Jorgensen, E. (1947) *Nature* 159, 99–100
25 Yoshida, A. (1950) *J. Chem. Soc. (Japan)* 71, 115–117
26 Eastwood, D. and Laidman, D. L. (1971) *Phytochemistry* 10, 1275–1284
27 Mayer, A. (1958) *Enzymologia* 19, 1–8
28 Pizer, F. L. and Ballou, C. E. (1959) *J. Am. Chem. Soc.* 81, 915–921
29 Uehara, K., Kometani, Y., Kogami, J. and Kiyotani, J. (1962) *Seikagaku* 34, 351–354
30 Schormuller, J. (1960) *Ber. Getreidechem.-Tag., Detmold*, 57–63
31 Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666
32 Andrews, P. (1965) *Biochem. J.* 96, 595–606