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THE PHYTASES

I. LYSOLECITHIN-ACTIVATED PHYTASE FROM WHEAT BRAN

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SUMMARY

1. *myo*-Inositol hexaphosphate phosphohydrolase (EC 3.1.3.8, phytase) from wheat bran may be separated by DEAE-cellulose into two fractions (F_1 and F_2) which yield different substrate degradation patterns.

2. Fraction F_1 contains a lipid activator whose properties are consistent with those of lysolecithin. After butanol extraction of F_1 to remove the activator, enzyme activity may be restored by substituting synthetic 1-*O*-palmitoyl glyceryl phosphoryl choline or naturally occurring lysolecithins from wheat endosperm or egg yolk. The following compounds were inactive as substitutes for lysolecithin: Glyceryl phosphoryl choline; 1,2-di-*O*-acetyl glyceryl phosphoryl choline; synthetic lecithin (1,2-di-*O*-palmitoyl glyceryl phosphoryl choline); phosphatidic acid; oleic acid; stearic acid and palmitic acid.

INTRODUCTION

Phytic acid, or more correctly *myo*-inositol hexakis (dihydrogen phosphate), is the major phosphorus constituent of cereal grains. Its enzymic degradation by *myo*-inositol hexaphosphate phosphohydrolase (EC 3.1.3.8, phytase) has engaged the attention of chemists and biochemists for more than 60 years and has been the subject of several reviews[1-3]. Despite the ease with which this enzyme may be extracted from bran, even the most active preparations[4] have exhibited heterogeneity in physical properties.

In a classic paper describing the stepwise enzymic degradation of phytic acid, TOMLINSON AND BALLOU[5] showed that a partially purified phytase preparation degraded phytic acid by two distinct pathways. The application of paper electrophoresis in oxalate buffer[6,7] to the separation of inositol polyphosphates has provided a rapid method for characterisation of the products of phytase degradation. COSGROVE[8,9] has extensively employed anion-exchange chromatography for the same purpose and the two procedures are complementary. It was therefore logical to enquire and feasible

to examine whether or not the existence of multiple substrate degradation pathways, *i.e.* the formation of isomeric inositol polyphosphates, could indicate the presence of more than one phytase.

The present paper describes the separation on DEAE-cellulose of two peaks of phytase activity, F_1 and F_2, which differ in their substrate degradation pathways. A search for an enzyme modifier which might control the degradation pathway led to the isolation of a phospholipid activator for Fraction F_1 which was identified as lysolecithin. Some of the structural requirements for the activation of F_1 phytase have been determined. Apart from activation of phytase, no evidence was found that lysolecithin modified the degradation pathway.

MATERIALS AND METHODS

Wheat bran was obtained locally from Whiting and Chambers (Adelaide).

The ascomycete *Neurospora crassa* (wild type S.T.A. 4) was obtained from the Fungal Genetics Stock Centre (Dartmouth College, U.S.A.) and was grown on a minimal-growth[10] medium containing phytate instead of inorganic phosphate for the production of *N. crassa* phytase.

Corn (*Zea maize*) sodium phytate grade V, the cadmium salt of glyceryl phosphoryl choline, phosphatidic acid and snake venom (*Crotalus adamanteus*) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.); bovine serum albumin and synthetic 1,2-di-*O*-palmitoyl glyceryl phosphoryl choline from Koch-Ligjt Colnbrook (Bucks., England); *n*-butanol, palmitic, stearic and oleic acids and egg lecithin from British Drug Houses (Poole, England). The methyl esters of fatty acids used as chromatographic standards were obtiaed from Supelco, Inc. (Bellafonte, Pa., U.S.A.).

All standard inorganic reagents were of analytical grade.

Sodium phytate was recrystallised from corn phytic acid according to the method of JOHNSON AND TATE[11]. Protein was determined by the Folin method, as described by LOWRY *et al.*[12], using bovine serum albumin as a standard or simply by taking the absorbance at 280 nm.

Phytase determination

Phytase activity was assayed by measuring the increase in inorganic phosphorus by the ascorbic acid method[13]. The reaction mixture was essentially that of NAGAI AND FUNAHASHI[4]. Incubation was normally carried out at 37° for 30–60 min, and enzyme activity was terminated by adding cold 10% trichloroacetic acid. Enzyme activity was expressed in international units, *i.e.* one unit of enzyme activity is the amount of enzyme which liberates 1 μmole of inorganic phosphorus per min.

Enzyme preparation

Crude supernatant fraction. The crude enzyme extract from wheat bran (30 g/100 ml water) was obtained by the osmotic shock treatment of NAGAI AND FUNAHASHI[4]. The supernatant fraction left after centrifuging at 7500 × *g* for 20 min was used for the butanol extraction.

Water-saturated n-butanol extraction. Just prior to extraction of the enzyme, *n*-butanol was saturated with cold double-distilled water. The saturated butanol was

stirred into the crude enzyme (1:1, v/v) and then the mixture was centrifuged at 7500 × *g* for 5 min. The butanol layer was siphoned off and the aqueous layer was further extracted (9 ×) with water-saturated *n*-butanol. The final aqueous phase was used as the butanol-extracted phytase.

DEAE-cellulose (DE-11) chromatography. DE-11 cellulose was pretreated according to the manufacturer's recommendation (Whatman Column Chromedia, W and R Balston Ltd., England). It was washed till the pH of the effluent was neutral and then packed into a column (1.8 cm × 18 cm). Excess bovine serum albumin (20–30 mg) in water was passed through the column to saturate irreversible adsorption sites. Excess protein was then eluted with 1 M Tris–HCl, pH 7.3. The column was washed thoroughly with water before the enzyme material was loaded. For subsequent reuse, the DE-11 cellulose was washed with 0.1 M NaOH and then with water.

The pH of the enzyme solution was adjusted to 7 before loading. The first enzyme (F_1) was eluted with 0.02 M Tris–HCl, pH 7.3 (200 ml). A linear gradient of 0.02 M Tris–HCl (150 ml) to 0.5 M Tris–HCl (150 ml), pH 7.3, was then applied and a second enzyme peak (F_2) was eluted. Each fraction (5 ml) was collected in the cold by an automatic fraction collector, examined at 280 nm for protein absorbance and assayed for phytase activity.

Thin-layer chromatography with Merck Silica gel H

Preparative thin-layer chromatograms were carried out on activated (110°/17 h) Silica gel H plates (0.25 mm thick), and Eastman Chromagram strips were used for rapid qualitative examination of extracts. These were developed in an ascending manner using chloroform–methanol–water (65:30:5, by vol.) and were examined for the following compounds: (a) Unsaturated and saturated carbon chains using iodine vapour; (b) phospholipids by the method of VASKOVSKY AND KOSTETSKY[14]; (c) choline residues with the choline spray of WAGNER *et al.*[15]; (d) free amino groups with 0.3% ninhydrin in acetone.

Gas–liquid chromatography

Methyl esters of fatty acids were prepared with diazomethane. They were then analysed in a Perkin–Elmer 801 Gas Chromatograph using 10% ECNSS on chromosorb W as the polar support. Both polar and non-polar phase separations were temperature programmed at 5°/min from 100 to 220°.

Separation and identification of inositol polyphosphates

Lower inositol polyphosphates were separated in 0.1 M oxalate buffer, pH 1.5, on Whatman 3 MM paper using the high-voltage electrophoresis procedure described by TATE[7]. The phosphate compounds were detected by the phosphomolybdate test[16].

Preparation of synthetic and related compounds of lysolecithin

References to the methods used to prepare compounds for this study are as follows: 1,2-di-*O*-acetyl glyceryl phosphoryl choline, KOGL *et al.*[17]; 1-*O*-palmitoyl glyceryl phosphoryl choline (snake venom), prepared from synthetic lecithin by the method of HANAHAN *et al.*[18]; 1-*O*-alkenyl glyceryl phosphoryl choline (ox heart), PANGBORN[19] and HARTREE AND MANN[20].

Egg lecithin and lysolecithin

The thin-layer chromatographic technique was adapted to a column procedure using silica H for the large-scale separation of egg lecithin and employing the solvent system of WAGNER *et al.*[15]. Each fraction collected was examined by thin-layer chromatography for lysolecithin and lecithin and for their total phosphorus content; the latter method was that of BARTLETT[21]. The determination of fatty acid ester groups in the phospholipids was that of GODDU *et al.*[22] and RAPPORT AND ALONZO[23], using methyl stearate as the standard.

Determination of phosphorus to myo-inositol ratio

A known quantity of L-inositol (as internal standard) and the *myo*-inositol polyphosphate (under examination) were acetylated using acetic anhydride and pyridine (1:1, v/v) at 110° for 2 h. The acetylated products were analysed at a programmed rate of 17°/min from 140 to 220° in a Perkin–Elmer 801 Gas Chromatograph. The weights of the area of the two peaks and the ratio of phosphorus to *myo*-inositol for the particular polyphosphate were calculated.

RESULTS

Phytate degradation pathways

Fig. 1 shows the separation of an aqueous extract of wheat bran into enzyme fractions F_1 and F_2 after elution from a DEAE-cellulose column. The phytate degradation pathways, as determined by paper electrophoresis (0.1 M oxalate, pH 1.5) of these two fractions, are compared with that for a phytase from *Neurospora crassa*[24] in Fig. 2. The pattern for F_1 cannot be easily differentiated from the pattern of the crude enzyme. The markedly different pattern of F_2 is obscured in the crude enzyme pattern but, as will be detailed in a later paper, the products formed by this enzyme

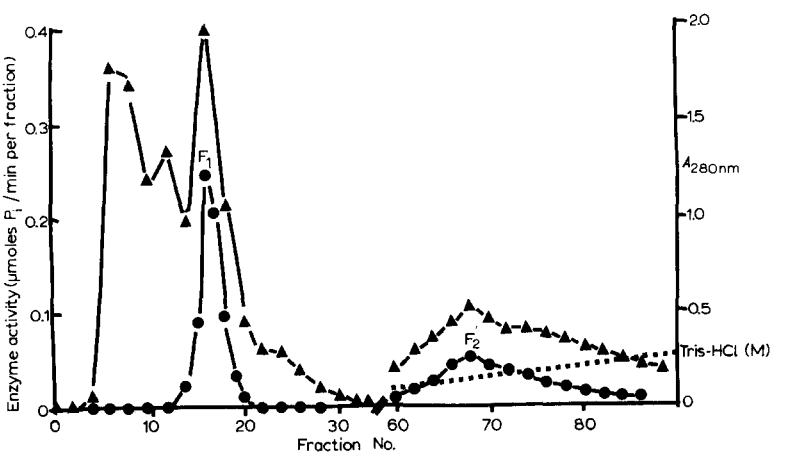

Fig. 1. DE-11 cellulose chromatography of wheat bran phytase. The protein concentration in the fractions was determined at $A_{280\ nm}$. The reaction mixture was incubated at 37° and contained, in a total of 1 ml, 0.02 M sodium phytate (pH 5.0) as substrate, 0.25 M sodium acetate buffer and enzyme solution (0.2–0.5 ml) and water. Phytase activity was assayed as given in MATERIALS AND METHODS. ▲—▲, absorbance at 280 nm; ●—●, phytase activity in I. U.; ■—■, Tris–HCl (molarity).

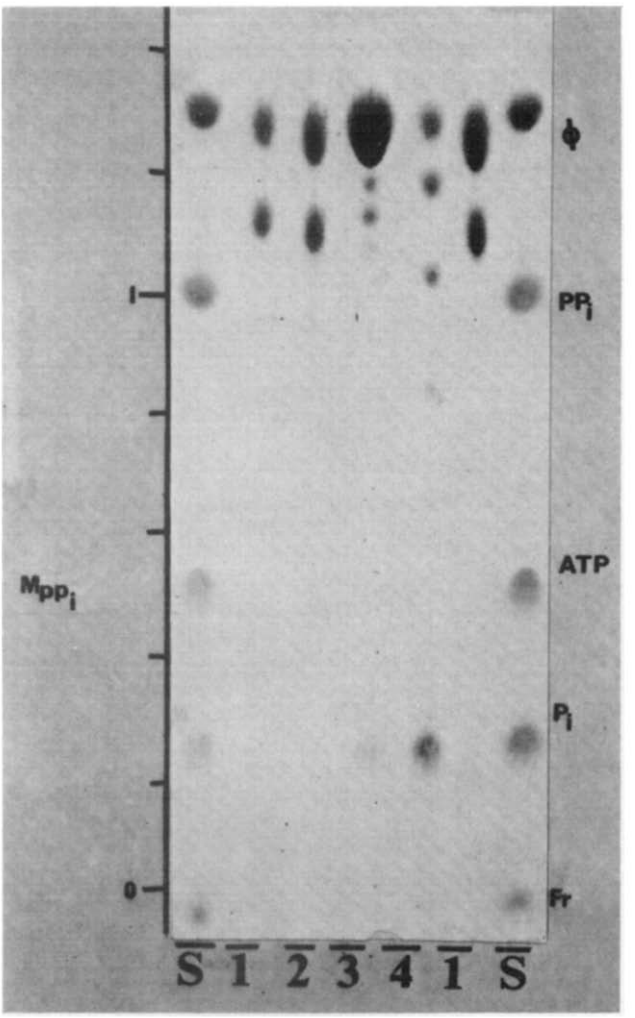

Fig. 2. Electrophoretogram of the enzymic degradation of phytic acid. The incubation mixture contained, in a total of 1 ml, 0.25 M sodium acetate buffer (pH 5.0), 0.02 M sodium phytate (pH 5.0), enzyme solution (0.2–0.5 ml) and water. The mixture was incubated at 37° until the percentage of total phosphorus liberated as inorganic phosphate was between 10 and 20%. The electrophoretogram was obtained using 0.1 M oxalate buffer, pH 1.5. Phosphate compounds were developed by the phosphomolybdate reagent. Lane 1, crude bran phytase; Lane 2, bran phytase F_1; Lane 3, bran phytase F_2; Lane 4, *Neurospora crassa* phytase. Lane S, standard, with φ, *myo*-inositol hexaphosphate; PP_i, inorganic pyrophosphate; ATP, adenosine triphosphate; P_i, inorganic phosphate; and Fr, fructose. MPP_i, mobility with reference to PP_i.

account for some of the observations made by TOMLINSON AND BALLOU[5]. The pattern for *Neurospora crassa* phytase has already been outlined by JOHNSON AND TATE[24] and is presented in Fig. 2 as a third example of a phytase degradation pattern which is discernably different at the pentaphosphate stage.

The electrophoretic and paper chromatographic mobilities (relative to inorganic pyrophosphate) of the detectable inositol phosphate intermediates of enzyme frac-

TABLE I

ELECTROPHORETIC AND CHROMATOGRAPHIC MOBILITIES OF INOSITOL POLYPHOSPHATES PRODUCED BY ENZYME FRACTIONS F_1 AND F_2 (P_i RELEASE = 50% SUBSTRATE P_t*)

The electrophoretic mobilities (MPP_i) at pH 1.5 and paper chromatographic mobilities (RPP_i) using isopropanol–ammonia–water (60:10:30, by vol.) were calculated with reference to inorganic pyrophosphate. The first two symbols (F_1 or F_2) denote the enzyme fraction, the second two symbols (P_n) denote the degree of phosphorylation of the inositol ring and the last letter is used to differentiate isomeric polyphosphates in decreasing order of electrophoretic mobility. The number of phosphate residues to the inositol molecule (*i.e.* P/I ratio) is as given in MATERIALS AND METHODS

Substance	*MPP_i Electrophoretic mobility (0.1 M oxalate, pH 1.5)*	*RPP_i Chromatographic mobility (Isopropanol–ammonia–water, 6:1:3, by vol.)*
PP_i	1.00	1.00
F_1P_6	1.27	0.2
F_1P_5	1.14	0.74
F_1P_4A	1.04	0.77
F_1P_4B	0.99	0.77
F_1P_3	0.86	1.01
F_1P_2	0.71	1.55
F_1P_1	0.48	—
F_2P_6	1.27	0.20
F_2P_5A	1.23	0.50
F_2P_5B	1.14	0.74
F_2P_5C	1.06	0.52
F_2P_4	0.99	0.77
F_2P_3	0.86	0.82
F_2P_2	0.71	1.55
F_2P_1	0.48	—
P_1	0.30	1.70

*P_t, total phosphorus.

tions F_1 and F_2 are listed in Table I. In this table each observable spot is given a name, in which the first two symbols (F_1 or F_2) denote the enzyme fraction, the second two symbols (P_n) denote the degree of phosphorylation of the inositol ring and the last letter is used to differentiate isomeric polyphosphates in decreasing order of mobility. It should be noted that the pentaphosphate F_2P_5C and the tetraphosphate F_1P_4A are unique to each pathway and are the basis for the electrophoretic assignments of degradation pathways and that the existence of isomeric polyphosphates such a F_2P_5A, F_2P_5B and F_2P_5C probably indicates that at least three phytases are present.

The phospholipid activator for Enzyme 1

The activity of the crude enzyme was reduced with successive butanol extractions but the restoration of activity was achieved by recombining the treated enzyme with the concentrated butanol extract. No detectable alteration in degradation pattern was observed by this reconstituted enzyme or by the extracted enzyme. Thin-layer chromatography on silica gel H of the concentrated butanol extract showed the presence of at least seven compounds detectable by iodine vapour, but only two of these compounds gave strong reactions with the phospholipid reagent. Preparative thin-layer chromatography of the concentrated butanol extract showed that restoration of enzyme activity was associated only with the slowest moving phospholipid. The two phospholipids had mobilities which were similar to lecithin and lysolecithin respectively.

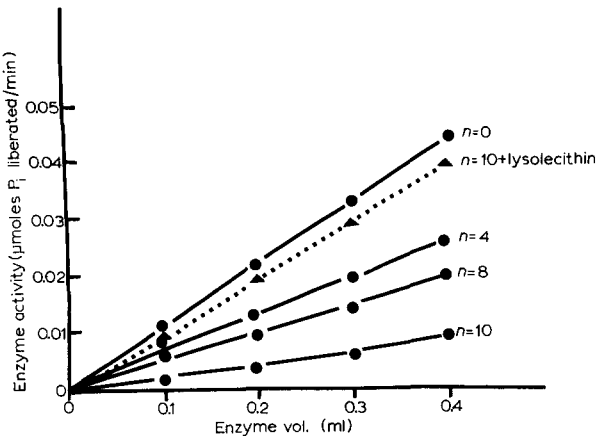

Fig. 3. Phytase activation by lysolecithin. Aliquots of the enzyme were assayed for phytase activity after n sequential extractions with water-saturated butanol. The reaction mixture (total 1 ml) contained 0.02 M sodium phytate (pH 5.0), 0.25 M sodium acetate buffer (pH 5.0), enzyme solution and water. ●—●, phytase activity after n extractions; ▲----▲, phytase activity with the addition of lysolecithin (15 μg P_t/ml) after the 10th extraction.

After mild de-O-acylation, both phospholipids gave glyceryl phosphoryl choline, which was characterised by its chromatographic and electrophoretic properties; this product was further degraded by acid hydrolysis to give choline and a mixture of α- and β-glycerophosphate, which were also identified by their electrophoretic and

TABLE II

EFFECT OF LIPIDS AND PHOSPHOLIPIDS ON THE ACTIVITY OF THE BUTANOL-TREATED ENZYME (10 EXTRACTIONS)

The enzyme reaction mixture contained, in a total of 1 ml, 0.25 M sodium acetate buffer (pH 5.0), 0.02 M sodium phytate (pH 5.0), 0.2 ml of enzyme solution (4–5 mg protein/ml) and water, with or without the lipid (15–20 μg P_t). Conditions of incubation and assay are given in MATERIALS AND METHODS. Phytase activity was expressed as a percentage of the original activity of the bran phytase which had not been subjected to butanol extraction. 100% activity is equivalent to 0.053 international unit.

Enzyme reaction mixtures	*Activity (%)*
Crude	100
Butanol-extracted (10×) enzyme	50
Synthetic 1-*O*-acetyl glyceryl phosphoryl choline	50
Synthetic 1,2 di-*O*-acetyl glyceryl phosphoryl choline	51
Synthetic lecithin (1,2 di-*O*-palmitoyl glyceryl phosphoryl choline)	51
Egg lecithin	57*
1-*O*-Palmitoyl glyceryl phosphoryl choline (snake venom)	70
1-*O*-Palmitoyl glyceryl phosphoryl choline (commercial)	70
Egg lysolecithin	75
Wheat endosperm lysolecithin	85
Phosphatidic acid	50
Oleic acid	50
Stearic acid	50
Palmitic acid	50
1-*O*-Alkenyl glyceryl phosphoryl choline (ox heart)	50
Glyceryl phosphoryl choline	50

* A small amount of lysolecithin was detectable by thin-layer chromatography in this sample.

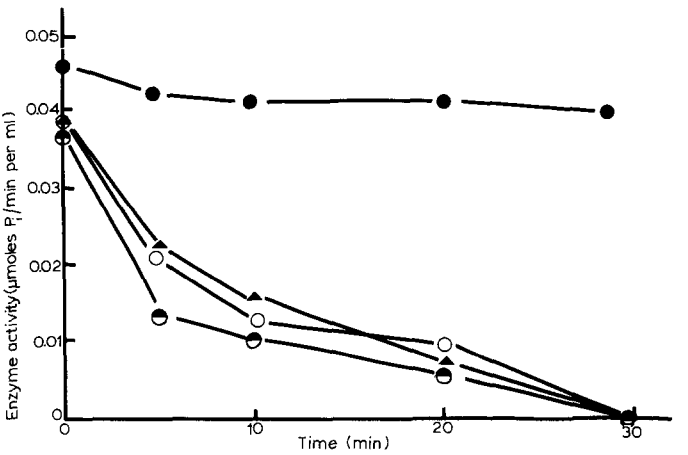

Fig. 4. Thermal inactivation of butanol enzyme. The enzyme solution (4–5 mg protein/ml) was heated at 60°. At the time specified in the figure, aliquots of the enzyme solution were removed and assayed for phytase activity with or without the addition of lipid. The reaction mixture (incubated at 37° for 30 min) contained, in a total volume of 1 ml, 0.02 M sodium phytate (pH 5.0) as substrate, 0.25 M sodium acetate buffer (pH 5.0) and 0.2 ml enzyme and water. ●—●, enzyme before butanol extraction; ▲—▲, enzyme after butanol extraction; ○—○, enzyme with lysolecithin; ◓—◓ enzyme with lecithin. Readdition of the combined and concentrated butanol extracts to the enzyme after butanol extraction did not detectably alter the curve (▲—▲).

paper chromatographic mobilities. The fatty acids from the mild de-O-acylation were characterised by gas chromatography and were predominantly $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ straight-chain fatty acids. From these results it was deduced that the activator is a lysolecithin. Experimental result using egg lysolecithin as the enzyme activator is shown in Fig. 3. The substitution of a series of lysolecithin preparations, both synthetic and naturally occurring, and of related compounds for the concentrated butanol extract in the butanol-extracted enzyme preparation is shown in Table II.

From butanol-extraction studies on enzyme fractions F_1 and F_2 from the DEAE-cellulose separation (Fig. 1), it was found that only fraction F_1 was associated with a lysolecithin activator. The thermal stability of enzyme fraction F_1 was considerably reduced by butanol extraction and this stability was not restored by lysolecithin, lecithin or the butanol extract. These results are shown in Fig. 4.

DISCUSSION

In attempting to follow the phytase purification procedure of NAGAI AND FUNAHASHI[4], two problems were encountered: First, the methanol-precipitation step invariably led to a large loss in total activity with little or no increase in specific activity; secondly, the DEAE-cellulose step resulted in almost complete adsorption of the enzyme. Although no alterations to the methanol procedure were successful, it was found that good recovery of activity and protein from the DEAE-cellulose were obtained after irreversible adsorption sites had been covered with bovine serum albumin. In the light of the experiments reported here, this loss in activity with methanol was attributed to the removal or decomposition of an unidentified stabilising factor, coupled with the removal of the activator, lysolecithin. The fact that

NAGAI AND FUNAHASHI[4] failed to observe a second peak of activity from their DEAE-cellulose column is probably because the ionic strength of their elution buffer was not strong enough to elute fraction F_2. IKAWA *et al.*[25] observed a multiplicity of peaks in elution of rice bran phytase from DEAE-cellulose but did not investigate the degradation patterns of their fractions.

The specific activities of fractions F_1 and F_2 are much lower than that achieved by NAGAI AND FUNAHASHI[4], who reported a 1500-fold increase in activity. Our inability to remove all activity from F_1 by exhaustive butanol extraction, together with the complexity of the degradation pathway (*i.e.* the appearance of isomeric inositol polyphosphates), suggests that both F_1 and F_2 are still relatively crude enzyme fractions with much inactive protein and that each fraction contains more than one enzyme.

The most interesting aspect of this study is the apparently specific requirement of lysolecithin for activation of the main enzyme fraction F_1. With the exception of minor alterations in the degree of unsaturation in the chain and small variations in chain length (C_{16}–C_{18}), all other changes to the lysolecithin molecule failed to significantly restore activity to the enzyme.

Lysolecithin is now known to be a major phosphorus constituent of cereal endosperm[26] and is second only to phytic acid as a store of phosphorus. The water-miscible lysolecithin is known to form strong complexes with starch[26,27] and the work presented here is equally indicative of a strong interaction with an enzyme. If the interaction is extrapolated to storage proteins of wheat endosperm in general, the possible importance of lysolecithin as a modifier of rheological properties of dough is apparent.

The role of lysolecithin as an enzyme modifier does not appear to have hitherto been reported, but the chemical and physical properties are such that further investigations with this water-miscible phospholipid are warranted.

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