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PHYTASE AND PHYTATE METABOLISM IN THE RAT

by

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A DISSERTATION

Presented to the Faculty of

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In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Nutrition Interdepartmental Area

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TITLE

PHYTASE AND PHYTATE METABOLISM IN THE RAT

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P.D.R.

TABLE I

ABBREVIATIONS

CM-Sephadex	carboxymethyl-Sephadex
DEAE-Sephadex	diethyl amino ethyl-Sephadex
Elon (Metol)	methyl-p-amino phenol sulphate
NRRL	National Regional Research Laboratory
Pi	inorganic phosphorus
psi	pounds per square inch
rpm	revolutions per minute
TCA	Trichloracetic acid
TRIS	tris (hydroxymethyl) amino methane
SE-Sephadex	sulpho ethyl-Sephadex

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INTRODUCTION AND LITERATURE REVIEW

Phytic acid is an ester formed by the combination of the six alcoholic groups of myo-inositol (cyclohexanol), with six molecules of orthophosphoric acid. It is unique in having six phosphate ester linkages in one molecule of small molecular weight; hitherto a highly phosphorylated compound such as phytic acid has not been known. As it has several phosphoric acid radicals, it can form simple salts (with one metal) or mixed salts (with several metals in the same molecule) as well as complexes with metals and proteins. The alkali salts of phytic acid are soluble in water while the salts resulting from combination with calcium, magnesium, iron, zinc, copper and lead are practically insoluble. The mixed calcium-magnesium salt of phytic acid is known as phytin while the term phytate has been used to include phytic acid, its simple salts and the mixed calcium-sodium salt. In literature phytin and phytates have sometimes been used interchangeably. Phytic acid is also thought of as a chelating agent but whether it can rank as a true chelator like EDTA, for example, is open to question.

Phytate occurs widely in plants and particularly high amounts are found in seeds of cereals, dried legumes, oilseeds and nuts. Analytical data compiled so far indicate that the phytate phosphorus in these sources may range anywhere from 40 to 90 percent (1, 2, 3, 4, 5, 6) of the total phosphorus. Phytate is considered as a primary source of phosphorus and inositol and to a lesser extent of calcium, magnesium, iron and potassium in seeds, the stores being rendered available during germination. In animals, the situation is different, particularly in

monogastrics, where phytate is considered to be poorly utilized. A study of the mechanism of its breakdown and factors involved in this process whereby the stored nutrients may be released for utilization by the animal, therefore, assumes great biological importance. Phytase (myo-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8), an enzyme which catalyzes the hydrolysis of phytic acid to inositol and inorganic phosphorus, could play a key role in the utilization of the nutrients stored in the phytate.

Utilization of Phytate Phosphorus

In rats, it has been shown that phytate phosphorus can be utilized. Lowe and Steenbock (7) fed phytin from wheat bran with and without inorganic phosphate and calcium carbonate and found that rats were able to utilize phytate phosphorus in the presence of adequate amount of vitamin D, but calcium carbonate and carbonates of other bivalent alkali metals depressed phytate utilization when used at 3 percent level in the diet. A similar finding was reported by Boutwell and coworkers (8) using wheat bran containing 80 percent total phosphorus as phytate phosphorus. These workers considered that phytate phosphorus utilization was as efficient as inorganic phosphate in the rat. Pileggi (9) and Roberts and Yudkin (10) also found that rats could utilize phytate phosphorus if calcium and vitamin D content of the diet was normal. On the other hand, Krieger et al. (11) reported that rats were not able to utilize phytate phosphorus when vitamin D was low and even on replenishing vitamin D, phytate utilization compared unfavorably with inorganic phosphate. The general view as emerging from the literature seems to

hold that phytate phosphorus is not completely unavailable to the rat, but it compares less favorably with inorganic phosphate.

In chicks and turkeys, utilization of phytate phosphorus is much less than with rats. However, the extent to which phytate phosphorus is utilized is a controversial subject as pointed out by Taylor (12) and Nelson (13) in their reviews. Nelson concludes that chicks are not able to utilize phytate phosphorus as efficiently as inorganic phosphorus.

In the case of pigs, Hart and coworkers (14) showed that phytate phosphorus could be utilized. On the other hand, Chapman and coworkers (15) showed in young pigs that a phytate-rich diet caused faulty bone mineralization and their balance studies indicated reduced intestinal absorption and retention of both phosphorus and calcium compared to inorganic phosphate. Plumlee et al. (16) using P^{32} labeled natural corn phytate and inorganic phosphorus in a 7-day feeding trial, showed that swine were able to absorb only about 39 percent of the phytate phosphorus compared to the 98 percent absorption of inorganic phosphorus.

In man, McCance and Widdowson (17) found that almost one-half of the phytate phosphorus of cereals was unavailable. On the other hand, Hoff-Jorgensen (18) found that ingestion of phytate caused increased absorption and retention of phosphorus in infants as well as growing children though calcium retention was impaired. They considered that about 70 percent of the phytate phosphorus was utilized.

Effect of Phytate on Calcium Utilization

Phytate is considered to exert a rachitogenic effect particularly if the diet has high calcium and low phosphorus. The net effect is not

mere deficiency of phosphorus but also unavailability of calcium. By maintaining the contents of vitamin D, calcium and total phosphorus at constant levels and varying only the intake of phytates, it was found that phytate caused increased losses of these elements through feces and decreased retention of calcium (19, 20). In experiments with young rats fed a diet containing adequate amounts of vitamin D, it was found that phytate reduced incorporation of orally administered radioactive calcium in the skeleton and teeth (21).

In man, phytate was shown to cause reduced calcium absorption on a low-calcium diet by McCance and Widdowson (17) and Hoff-Jorgensen (18). Bronner and coworkers (22) studied the effect of phytate from oats on the absorption of radioactive calcium in children and adolescents and showed that it reduced the absorption and retention of calcium, the effect being weakened by tripling the calcium and lowering the phytate in the diet.

Effect of Phytate on Availability of Zinc

Phytate has been shown to decrease availability of zinc in animal diets. Decrease in zinc availability due to phytate was reported by O'Dell and Savage in chicks (23) and by Oberleas and coworkers (24, 25) in swine and rats. The effect of high dietary calcium and plant proteins believed to be rich in phytates in reducing zinc availability has been investigated by Likuski et al. (26, 27) and Smith et al. (28). The effect of calcium, protein and phytate on zinc availability has been reviewed by O'Dell (29) leading to the conclusion that phytate by itself or in association with plant proteins reduces zinc availability.

Effect of Phytate on Iron Absorption

In rats, it has been shown that dietary phytate reduces iron absorption and retention as well as efficiency of hemoglobin production compared to inorganic iron. In man, phytic acid has been shown to decrease absorption and retention of iron (30, 31, 32, 33).

Role of Phytase in the Metabolism of Phytate

Phytases, the enzymes capable of catalyzing the hydrolysis of inositol hexaphosphoric acid (phytic acid) to inositol and free orthophosphoric acid, are widely distributed in the plant kingdom and are usually found in the foodstuffs containing phytate. Suzuki et al. (34) first discovered the enzyme in rice bran and named it phytase. Other studies followed demonstrating the presence of this enzyme in plants and animals. McCance and Widdowson (35) and Mellanby (36) indicated the presence of phytase in cereals. Courtois and Joseph (37) studied the phytase from wheat bran. Powar and Jagannathan (38) isolated and purified a phytase from Bacillus subtilis.

In the animal kingdom, McCollum and Hart (39) demonstrated phytase activity in the blood and liver of calves and Rapoport et al. (40) found phytase activity in the plasma of goose, pigeon, turtle and frog. Patwardhan (41) first reliably demonstrated phytase activity in the rat intestinal mucosa. The enzyme was further studied by Spitzer and Phillips (42) in the rat and Pileggi (9) studied its distribution in the rat and some of its properties. In chicks, the enzyme has been studied by Steenbock et al. (43) and Davies et al. (44).

Phytases from microorganisms have been subjected to only a few studies (45, 46, 47). Recently, Yamada et al. (48) isolated Aspergillus terreus as a potent strain for phytase production and studied the purification and properties of the enzyme (49). Shieh and Ware (50) after surveying several bacteria and molds found that Aspergillus ficuum NRRL 3135, a soil isolate, produced the highest phytase activity. These mold phytases are characterized by being extra-cellular, stable over a wide pH and temperature range and easy to produce. Nelson et al. (51, 52) have used Aspergillus ficuum as the source for supplemental phytase in their study with chicks.

The bulk of experimental data reported in the literature seems to indicate that monogastrics are not able to utilize phytate as efficiently as inorganic phosphorus. There is no general agreement on the extent to which phytate is utilized. The limited utilization of phytate observed in balance studies is generally attributed to the hydrolytic action of either the dietary phytase or the intestinal phytase or both. McCance and Widdowson (35) attribute the partial utilization of bread phytate to the presence in wheat of a phytase that is heat-stable. Some workers have suggested that the ingestion of phytase with food may be necessary for efficient utilization of phytate (7, 53). On the other hand, Courtois and Valentino (54) reported that the ingestion of a phytase purified from wheat bran did not prove beneficial in the utilization of phytate in the rat.

Recently, Nelson et al. (51) reported that they have used a mold phytase to hydrolyze the phytate in soybean meal prior to ingestion by

chicks and that the phytate was utilized by the chicks as efficiently as inorganic phosphorus. In their next experiment (52), they used the same mold phytase as a diet supplement with chicks and reported that the enzyme was active in the alimentary tract of the chick and was able to render the phytate of the soybean meal in the diet available for growth and bone mineralization.

It seems, therefore, that the unavailability of phytate has perhaps been overemphasized in literature, though one must recognize that the situation is made complicated by dietary factors such as vitamin D content, calcium content, calcium-phosphorus ratio, the natural state of dietary phytate and presence of other minerals in the diet. Nevertheless, in a situation where the inter-playing effect of the above dietary factors is kept to the minimum or constant, the limited availability of phytate to the monogastrics then raises the following questions:

1. Is the limited availability of phytate due to the absence of natural dietary phytase or its possible inactivation in the alimentary tract?
2. How efficient is the intestinal phytase in rendering the phytate available? If the secretion of phytase by the intestine is considered critical for phytate utilization, which one may assume to be the logical safeguard provided by nature in monogastrics, would the ingestion of phytate induce phytase production by the intestine?
3. What would be the overall effect of supplementing the diet with a phytase? Would it suppress or complement the effect

of intestinal phytase? What effect would the proteolytic enzymes of the alimentary tract have on the dietary or supplemental phytase? Is the intestinal phytase activity in any way correlated to bone mineralization? Is phytase supplementation of a phytate-rich diet of such practical value to the monogastric as to warrant its wholesale application?

Literature does not seem to provide a satisfactory explanation of these questions. The research reported here has, therefore, the following objectives:

1. Purification and study of the properties of phytase from Aspergillus ficuum.
2. Determination of the effect of this mold phytase as a diet supplement on the utilization of phytic acid by the rat.
3. Determination of the effect of phytate ingestion on the activities of intestinal phytase and phosphatase and bone mineralization in the rat.

CHAPTER I

PARTIAL PURIFICATION AND PROPERTIES OF PHYTASE FROM *ASPERGILLUS FICUUM*

Introduction

The purpose of this study was to purify phytase from the mold *Aspergillus ficuum*, to study some of its properties and reaction characteristics and to extend our knowledge concerning its susceptibility to digestion by the gastrointestinal proteolytic enzymes leading to its assessment as a diet supplement for the rat.

Phytases of plant and animal origin have been subjected to several studies, but scant work has been reported on the phytase from microorganisms. Dox and Golden (45) first demonstrated that three species of *Aspergilli* contained phytase. Their study gave no indication of the pH-activity relationship for the enzyme or of its prevalence among the fungi. *Aspergilli* are common inhabitants of soil and can be conveniently isolated. Casida (46) studied several soil fungi as possible sources of phytase and found that quite a few members of the genus *Aspergillus* contained phytase. An enzyme preparation from *Aspergillus niger* NRRL 67 showed broad substrate specificity in dephosphorylating not only phytate but also organic phosphates such as DNA and RNA as well as inorganic metaphosphates. The enzyme had a pH optimum of 2.5 for phytate and 4.5 for DNA. He also found that the enzyme from this mold did not require phytate in the growth medium for activity or as a stimulus to production.

Yamada and coworkers (49) obtained phytase from Aspergillus terreus No. 9 A-1 using a rice bran medium and purified it 520-fold by a combination of ammonium sulphate precipitation, acetone precipitation and chromatography on SE-Sephadex C-50 and Sephadex G-200 columns. The purified preparation had a pH optimum of 4.5, a temperature optimum of 70°C and pH stability range of 1.2 to 9.0. They found that the presence of phytate in the screening medium was indispensable for phytase production and on the basis of this observation suggested phytase from Aspergillus terreus to be an induced enzyme. Interestingly, rice bran used as a phytate source produced the highest phytase activity, whereas soybean meal produced none in comparison. They found that ammonium salts (nitrate, sulphate, phosphate) used as source of nitrogen in the culture medium for the mold at 0.3% level produced the highest activity, whereas potassium nitrate produced none in comparison.

Shieh and Ware (50) screened various microorganisms to determine the most potent producers of phytase and found that Aspergillus ficuum NRRL 3135, a soil isolate, produced the highest extracellular phytase activity. In their study, corn starch proved to be a better source of carbon than glucose or sucrose in the culture medium for phytase production. Phosphate decreased phytase production in almost all the yeasts and molds capable of producing phytase. Presence of phytate in the culture medium was not necessary for phytase production. The most active phytase was produced when the inorganic phosphate concentration was less than 0.004% (w/v) in a medium containing 8% (w/v) corn starch. This led them to conclude that an increased carbon to phosphorus ratio along with a limiting inorganic phosphorus concentration in the medium

induces phytase production by A. ficuum. However, these workers made no attempts at purification or characterization of the enzyme beyond a preliminary butanol precipitation.

Phytases of plant origin have generally displayed broad substrate specificities, and it is not clear if this is due to the presence of other phosphatases in the phytase preparations or due to lack of inherent specificity. Since Casida (46) did obtain broad substrate specificity for phytases from Aspergilli, it is logical to ask if the phytase from A. ficuum has any phosphatase activity and conversely if the known phosphatases exhibit any phytase activity. If the mold enzyme is to be used as a diet supplement for a diet rich in phytate, the knowledge of its reaction towards the common proteolytic enzymes such as pepsin and trypsin assumes importance. In the research work reported herein, these aspects were, therefore, studied along with the purification and characterization of the enzyme.

Experimental Procedures

A. Culture Techniques and Enzyme Production

Maintenance of the mold. Parent culture of the mold Aspergillus ficuum NRRL 3135 was obtained from the Northern Regional Research Laboratory, Peoria, Illinois. Routine cultures were maintained on nutrient agar slants by periodic transfers from the parent culture. To do this, 1.0 g dehydrated nutrient broth (Difco), 2.0 g agar and 2.0 g glucose were dissolved in warm water. Ten ml volumes of the mixture were taken in tubes and sterilized by autoclaving at 121°C for 20 min at

15 psi. The tubes were allowed to cool at room temperature in a slanting position for 2 days. A loop of the parent mold was transferred aseptically to the agar slants and the mold was allowed to grow at room temperature for 3 days by which time profuse growth of the mold occurred. These routine cultures were kept refrigerated and used for the inoculation of the growth culture medium.

Preparation of the growth medium. The standard growth medium (Table II) was based on corn starch and was similar to the one used by Shieh and Ware (50). The pH of the medium was adjusted to 5.0. Aliquots of 10 ml or 25 ml of the medium were taken in 50 ml Erlenmeyer flasks plugged with cotton and sterilized by autoclaving at 121°C for 20 min at 15 psi. In another set of 500 ml Erlenmeyer flasks, 115 ml of the medium was sterilized in the same manner. The smaller flasks were used for primary inoculation of the mold culture.

Growth of the mold and enzyme production. A loop of the mold from the agar slants was transferred aseptically into the standard medium contained in the 50 ml flasks, the contents mixed and incubated at room temperature (22°-26°C) for 3 days. The contents were next transferred into the 50 ml Erlenmeyer flasks containing the standard medium and incubated at room temperature (22°-26°C) for 7 days on an oscillating shaker (90 cycles per min). At the end of this period, the contents were filtered through several folds of surgical gauze. The filtrate containing the extracellular enzyme was preserved for further purification and was termed the crude enzyme preparation.

TABLE II

COMPOSITION OF THE STANDARD GROWTH MEDIUM

Component	g/liter
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.1
$NaNO_3$	8.6
$K_2HPO_4 \cdot 3H_2O$	0.2
Glucose	30.0
Corn starch	40.0

Several variations of the standard growth medium were studied in an effort to increase enzyme production. This included varying the carbon to phosphorus ratio and the contents of corn starch, glucose, sodium nitrate and phosphate (Table III).

B. Enzymatic Assays

Phytase activity. Phytase activity was measured routinely using phytic acid (General Biochemicals) as substrate. Calcium phytate (Calbiochem, Los Angeles, Calif.) was also tried as substrate, but it gave lower activity compared to phytic acid. A stock substrate solution of phytic acid was prepared and 1 ml of the substrate containing 1 mg was used in a total 5 ml reaction mixture so as to give a final substrate concentration of 1.5 mM. The reaction mixture consisted of 1 ml of the substrate, 1 ml of the enzyme preparation and 3 ml of 0.2 M glycine-HCl buffer, pH 3.0. The reaction was carried out at 37°C in a water bath for 10 min and terminated by the addition of 5 ml of 10% TCA. Blanks were prepared by adding TCA to the enzyme before adding the substrate.

Phosphorus determination. The inorganic phosphorus liberated by the enzymatic hydrolysis was determined by the method of Fiske and Subbarow (55). After the termination of the reaction, the mixture was filtered through Whatman #5 qualitative filter paper. A suitable aliquot (0.5 ml-1.0 ml) of the filtrate was diluted to 5 ml with water. Two ml of acid molybdic reagent (prepared by mixing 1 volume of 7.5% sodium molybdate, 1 volume of 10 N H_2SO_4 and 2 volumes of water) were added and after 2 min, 1 ml of Elon (metol) reducing reagent (1% solution of methyl-p-amino phenolsulphate in 3% sodium bisulphite) was added

TABLE III

EFFECT OF VARYING THE COMPOSITION OF CULTURE MEDIUM ON PHYTASE ACTIVITY

No.	$MgSO_4.7H_2O$ (g)	KCl (g)	$FeSO_4.7H_2O$ (g)	$NaNO_3$ (g)	$K_2HPO_4.3H_2O$ (g)	Glucose (g)	Corn Starch (g)	H_2O (ml)	Activity micromoles Pi/ml crude/hr
1	0.125	0.125	0.025	2.15	0.05	7.5	20	250	104.0
2	0.125	0.125	0.025	2.15	0.05	7.5	10	250	40.3
3	0.125	0.125	0.025	2.15	0.10	7.5	10	250	39.0
4	0.125	0.125	0.025	2.15	0.20	7.5	10	250	31.2
5	0.125	0.125	0.025	2.15	0.40	7.5	10	250	18.2
6	0.125	0.125	0.025	1.0	0.05	7.5	10	250	50.7
7	0.125	0.125	0.025	0.5	0.05	7.5	10	250	40.9
8	0.125	0.125	0.025	3.0	0.05	7.5	10	250	38.3
9	0.125	0.125	0.025	3.5	0.05	7.5	20	250	39.0
10	0.125	0.125	0.025	4.0	0.05	7.5	20	250	75.4
11	0.125	0.125	0.025	4.0	0.10	10.5	10	250	13.6
12	0.125	0.125	0.025	2.15	0.025	7.5	20	250	74.1
13	0.125	0.125	0.025	2.15	0.025	10.0	10	250	42.2
14	0.125	0.125	0.025	2.15	0.025	10.0	15	250	76.0

and the color allowed to develop for 30 min. At the end of this period, 5 ml water was added and the absorbance measured at 650 nm in a Bausch and Lomb Spectronic 20 Spectrophotometer. A standard phosphorus curve was prepared using a standard solution of KH_2PO_4 (80 μg P/ml) with appropriate dilutions to give a range of 5 μg to 50 μg P/ml.

Enzyme activity unit. A unit of enzyme activity was defined as the quantity of the enzyme required to liberate 1 micromole of inorganic phosphorus in 1 min under standard assay conditions.

C. General Properties of the Enzyme

pH-activity curve. The phytase activity of the crude enzyme preparation was measured as a function of pH using a series of buffers (Table IV) with a pH range of 1.0 to 10.0. Phytic acid substrate was prepared separately in each of these buffers. A plot of the pH against phytase activity appears in Figure 1.

Effect of varying substrate and buffer systems on phytase activity. This was studied using phytic acid (0.5 mg/ml) and calcium phytate (2.3 mg/ml) as substrates and 0.2 M HCl-glycine buffer, pH 3.0 and 0.2 M acetate buffer, pH 4.5. The reaction mixtures contained 1 ml each of the substrates, 1 ml crude phytase and 3 ml each of the buffers. Reaction was carried out for 15 min at 37°C and stopped with 5 ml 10% TCA. The liberated Pi was determined as in the standard phytase assay. The results appear in Table V.

Effect of temperature on phytase activity. The crude phytase preparation was heated in a water bath at various temperatures (37°, 45°, 55°, 65°, 73°C) for 1 hr. The enzyme preparation was then cooled,