THE RELATIONSHIP OF PHYTASE TO ALKALINE PHOSPHATASE ACTIVITIES IN THE RAT, CALF, CHICKEN AND HUMAN INTESTINAL MUCOSA

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THE RELATIONSHIP OF PHYTASE TO ALKALINE PHOSPHATASE ACTIVITIES IN THE RAT, CALF, CHICKEN AND HUMAN INTESTINAL MUCOSA

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A C K N O W L E D G M E N T
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LIST OF ABBREVIATIONS

DEAE-cellulose	-	Diethyl amino ethyl cellulose
Tris-Base	-	Tris (hydroxymethyl) amino methane
Ph	-	Phytase
AP	-	Alkaline phosphatase
NaPh	-	Sodium phytate
PNPP	-	p-nitrophenyl phosphate (disodium)
Naβgl.	-	β-glycerophosphate (disodium)
x g	-	Times gravitation

CHAPTER I

INTRODUCTION

A. Scope and Purpose

Phytic acid (myo-inositol hexaphosphoric acid) exists in large amounts as phytin, its calcium and magnesium salt, in cereal grains (1). It has attracted attention recently because of its potential for interference with absorption of calcium, iron, magnesium, zinc, and perhaps other trace metals. It is believed that phytin is converted to phytic acid by the action of HCl in the stomach. Phytic acid is attacked in the gastrointestinal tract by phytase, an enzyme which hydrolyzes phytic acid into its inositol and phosphoric acid components (2). This hydrolysis decreases the formation of poorly soluble metal phytates, and diminishes interference with absorption of calcium and other metals.

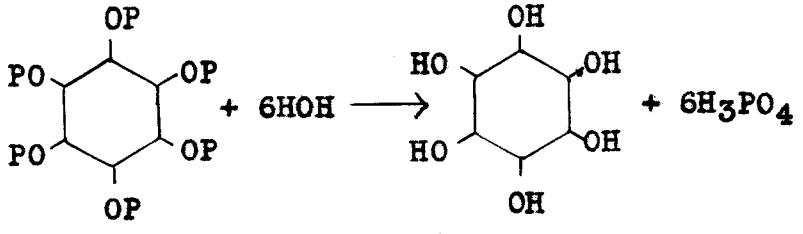
There is a scarcity of information concerning the behavior and identity of phytase and its role in trace metal metabolism. Although phytase (EC 3:1:3:8) is listed as an orthophosphoric monoester phosphohydrolase distinct from alkaline phosphatase (EC 3:1:3:1), there are some who question its identity as a separate enzyme in the intestinal tract. It is maintained that both activities are properties of a single enzyme (3, 4). This present study describes

experimental work undertaken to clarify this relationship and to investigate certain other aspects of phytase and alkaline phosphatase activities in the human, rat, calf, and chicken intestinal mucosa.

B. Nature and Mode of Action of Phytase and Alkaline Phosphatase

Phytase hydrolyzes phytic acid to give inorganic phosphate (measured to determine rate of hydrolysis) plus a sequence of intermediate phosphate esters of inositol. These have been identified by paper chromatography (5).

The overall reaction may be illustrated as follows:



Phytic acid Inositol

Tomlinson and Ballou (6) proposed a scheme for the enzymatic hydrolysis of phytic acid in wheat bran, adapted from that of Courtois (7), in which dephosphorylation occurred stepwise.

Phosphomonoesterases are grouped into two main classes: One exhibiting optimum activity in the region of pH 9-10, and the other at pH values near 5, designated as alkaline and acid phosphatases respectively (8). In addition, a few phosphatases have optimum activity around pH 7.0 (9). Various workers have demonstrated the existence of several electrophoretically distinct alkaline phosphatase components in human blood serum (10, 11), human intestinal mucosa (12, 13), human placenta (14), as well as in calf intestine (15, 25). However, it has been suggested that the action of intestinal alkaline phosphatase upon a variety of phosphomonoesters may be due to a single enzyme with a broad specificity rather than a family of isoenzymes (16).

Engström (17) was able to isolate the peptide Asp-Ser ^{32}P-Ala, by incubating ^{32}P with calf intestinal alkaline phosphatase. He suggested that the active center contains a serine residue. A plausible mechanism for the hydrolysis of phosphate esters by alkaline phosphatase was proposed by Schwartz (18) in which orthophosphate phosphorylates the enzyme at a particular serine OH group of the active center (See Appendix A).

Two types of reactions are catalyzed by phosphatases (19):

(1) $R(Ar)O - PO_3H_2 + HOH \longrightarrow ROH + H_3PO_4$

(2) $R(Ar)O - PO_3H_2 + R'OH \longrightarrow ROH + R'-OPO_3H_2$

where R and Ar are alkyl and aryl groups respectively.

In (1), the enzyme acts as a hydrolase that cleaves the O-P bond (20), with the subsequent removal of a phosphoryl group to liberate orthophosphate.

In (2), a transphosphorylation occurs in which the enzyme acts as a phosphotransferase. The latter does not involve the formation of inorganic phosphate in the transmission of the phosphoryl group to the acceptor, or require the presence of ATP or high energy phosphate compounds (21).

The type of reaction catalyzed by alkaline phosphatase depends upon (a) competition between water and other hydroxyl compounds for sites at the enzyme donor complex, as well as (b) the relative concentrations of the participating compounds, and (c) the velocity constants of the reactions (21).

The non-specific alkaline phosphatases are known to hydrolyze orthophosphoric monoesters of a wide variety of phenolic, alcoholic, sugar and other compounds (22). They have no effect on metaphosphate or diphosphoric esters such as diphenyl phosphate (23). In general acid and alkaline phosphatases which can bring about both hydrolysis and transfer of the phosphate moiety of the substrate molecule, are specific for the phosphate group, and comparatively non-selective for the remainder of the molecule (24). This is in contrast, for example, to D-fructose 1,6-diphosphate 1-phosphohydrolase, and 5'-ribonucleotide phosphohydrolase,

examples of phosphatases which are specific for the substrate molecule as a whole (21).

C. Role of Zinc in Phytase and Alkaline Phosphatase Activities

Chicken intestinal phytase was found to be activated by zinc ions in vitro (3). Although intestinal alkaline phosphatases are known to be zinc metallo-lipoproteins (25, 26), the mode of action of zinc is not yet established.

In the phosphatase of E. coli, zinc is necessary for the dimerization of two unfolded monomers (27, 28). This phosphatase may contain up to four zinc atoms, two of which are essential for activity (29). Co^{++} can replace the zinc of the native phosphatase (30) to form a new active enzyme, and its spectral and magnetic properties render it an intrinsic probe of the active site (29).

D. Distribution of Phytase and Alkaline Phosphatase

Phytase is widely distributed among plants. Other sources include certain fungi, yeasts, bacteria, and most animal tissues (31).

Alkaline phosphatases have been found widely distributed among some strains of yeasts (32), and bacteria (33), molluske (34), echinoderms (35), insect larvae (36), annelids (37), teleosts (38), elasmobranchs (39), amphibia (40), reptiles (41), birds (42), and most mammalian tissues. The presence of alkaline phosphatase in plants is not yet

established (43). However, some phosphatases such as 3'-ribonucleotide-phosphohydrolase and 2-phosphoglycerol phosphohydrolase are present mainly in plant tissues (44).

Often the mammalian tissues with highest alkaline phosphatase activity are those concerned with active transport, notably the kidney (proximal convoluted tubules) (45), intestine (46, 47), and placenta (48). However, bile canaliculi (49), osteoblasts (50), and active mammary glands (51) are also rich in alkaline phosphatase. The enzymes in the small intestinal mucosa differ from some non-intestinal alkaline phosphatases by their substrate specificity and electrophoretic mobility (52), inhibition by L-phenylalanine (53), and resistance to neuraminidase (10, 54). Serum alkaline phosphatases are derived mainly from the skeleton and hepatobiliary system (55) although some are of intestinal origin especially in the rat (52-54).

The organ distribution of phytase in the rat parallels that of alkaline phosphatase. However, brain shows a higher specific activity with respect to phytase. This seems to be related to the abundance of inositol phosphates which are potential natural substrates for phytase (56). Chicken intestinal phytase and alkaline phosphatase were reported to be most abundant in the microsomal fraction (3). In the present study, the same has been found to be true for the intestinal enzymes of the rat.

E. Functions of Phytase and Alkaline Phosphatase

The hydrolysis of phytic acid by intestinal phytase would have two useful effects. It may:

a. Decrease the interference with absorption of calcium and other divalent metals by diminishing the formation of the poorly soluble phytate salts (57, 58).

b. Make available inorganic phosphate to supplement phosphate from other sources. This effect was shown clearly in chicken (59). A similar function was reported by Albaum et al (60) in grain where the inorganic phosphate liberated from phytic acid was utilized in the initial stages of germination.

The role of alkaline phosphatase in metabolism is not yet defined. However, it is believed that its functions include the transport of phosphate or phosphorylated compounds across cell walls (61). The absorption of phospholipid is accompanied by resynthesis of phospholipid in the mouse intestinal mucosa as well as a simultaneous increase in alkaline phosphatase activity (26). It is supposed that the latter might include the phosphatidic acid phosphatase whose role in phospholipid biosynthesis had been well established (62). Recently, it was shown that the rise in the activity of alkaline phosphatase in the rat intestinal mucosa when fat is being absorbed may be due to an increased requirement for this enzyme in the resynthesis of triglycerides (63).

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Chemicals

Sodium phytate was prepared from calcium phytate (Calbiochem) by the method of Peers (2). The concentration and purity of solutions were determined by measuring total phosphate and free inorganic phosphate.

p-Nitrophenylphosphate (disodium), sodium β-glycerophosphate (disodium), L-phenylalanine, tris (hydroxymethyl) amino methane, succinic acid, and diethyl amino ethyl cellulose were obtained from the Sigma Chemical Co. Ammonium sulfate used was either analytical reagent grade from British Drug Houses Ltd., Hopkins and Williams Ltd. (Analar), or specially purified for enzyme analysis, Nutritional Biochemicals Corporation.

Sephadex G-200 (particle size 40-120 μ) was purchased from Pharmacia, Uppsala.

n-butanol, a product of Hopkins and Williams Co., was distilled through a fractionating column before use.

2. Laboratory Animals

Rat intestinal phytase and alkaline phosphatase were

prepared from the mucosae of the small intestine of albino rats of a Sprague-Dawley strain obtained from the colony of the Medical School of the American University of Beirut. They weighed between 200 and 350 g and were fasted for 24 hr. before an experiment. Mucosas of 10 to 20 rats were combined, and processed within an hour after killing with ether.

Calf intestines were obtained from the Beirut Slaughter House shortly after death. The intestines were thoroughly washed with tap water and frozen until used. The mucosa of one calf was processed at a time.

Chicken (White Leghorn) intestines were obtained shortly after death from a farm near Beirut, and treated in the same manner as the calf intestines. Mucosas of 40 to 60 chickens were combined.

Human intestines were obtained at autopsy by courtesy of the Pathology Department of the American University of Beirut.

B. Assay of Intestinal Phytase and Alkaline Phosphatase Activities

The assay systems consisted of sodium phytate,[1]

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1. Substrate used to assay intestinal phytase activity.

disodium p-nitrophenyl phosphate,[1] or disodium β-glycerophosphate,[2] Mg^{++}, Tris-succinate[3] and a suitably diluted enzyme preparation[4] in a final volume of 5 ml unless otherwise stated. The reaction was run for 15 minutes at 37° C, and was stopped by the addition of 5 ml 10 % trichloroacetic acid. A suitable aliquot of the protein free filtrate was used to determine the liberated phosphate by the method of Fiske and SubbaRow (64). The pH values and the final composition of the reaction mixtures which differ for each species are included in the legends for the figures. The initial rates of hydrolysis were maintained during the reaction interval.

The pH and substrate concentration at which phytase showed maximum activity were also used to assay alkaline

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1. Substrate used to assay rate, chicken and human intestinal alkaline phosphatase.

2. Substrate used to assay calf intestinal alkaline phosphatase.

3. A stock solution of 1 M tris-succinate was prepared by dissolving 118 g of succinic acid and 121 g of Tris-base in water. The pH of solutions prepared from the stock solution were adjusted by titration with 0.1 N NaOH.

4. In all experiments 0.5 ml of the enzyme sample was used unless otherwise stated.

phosphatase activity. This insured identical experimental conditions for the comparison of both activities where the substrate was the only variable.

All experiments were done at least twice. In the event of uncertainty concerning the results, additional experiments were carried out. Total activity is expressed as μmoles PO_4 hydrolyzed/min. at 37° C. Protein concentration was measured by the biuret reaction (65), or spectrophotometrically at 260 and 280 mμ using Kalckar's formula (66)

$$(O.D._{280\ m\mu} \times 1.45 - O.D._{260\ m\mu} \times 0.74) = \text{mg protein/ml}$$

for colorless solutions.

Specific activity is expressed as activity/mg of protein.

C. Partial Purification of Rat, Calf and Chicken Intestinal Phytase and Alkaline Phosphatase

Both phytase and alkaline phosphatase were purified by a procedure adapted from Morton (67) and Behal et al (15).

1. Crude Extract

The rat intestinal mucosa was removed by scraping the washed intact intestine with the edge of a glass slide. Cold 0.25 M sucrose[1] was then added to give a dilution of

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1. Except for the purification procedure, all other rat homogenates were made in cold distilled water.

1:10. The suspension, adjusted to pH 7.5 with 0.1 N NaOH, was stirred mechanically for 30 minutes, then macerated in a glass homogenizer equipped with a teflon pestle. The homogenate was filtered through washed cotton wool.

Calf, chicken and human intestinal mucosa were suspended in equal volumes of cold distilled water, and the pH was adjusted to pH 7.5 with 0.1 N NaOH. The suspension were stirred mechanically for 30 minutes, then further macerated in a waring blendor for 1 minute. The human intestinal homogenate was filtered through cotton wool and examined without further purification. The chicken and calf intestinal homogenates were centrifuged at 1000 x g for 30 minutes at 5° C, and filtered through cotton wool on a Buchner funnel.

2. Acid Precipitate

The homogenate was cooled to 5° C and adjusted to pH 5.0 by the slow addition of 2 M acetate buffer pH 4.0. The suspension was held for 45 minutes at 5° C and centrifuged at 1800 x g for 30 minutes. The grey precipitate was then evenly dispersed in 0.14 M NaCl, adjusted to pH 7.5 with 0.5 M Na_2CO_3 and stirred gently overnight at 5° C. The preparation was re-precipitated as before, and the precipitate collected by centrifugation. It was washed in the centrifuge cups with cold distilled water, and immediately centrifuged.

The precipitate was dispersed in cold distilled water at 5° C, adjusted to pH 7.5 with 0.5 M Na_2CO_3, and stirred until homogeneous. The enzyme solution at this stage was free of mucoid and pigments.

3. Butanol Treatment

0.4 volume of n-butanol was added slowly at room temperature with mechanical stirring. The suspension was centrifuged at 1800 x g for 30 minutes, and the lower aqueous layer removed by aspiration. This layer was dialyzed against water overnight at 5° C, and then passed through a 4-5 cm layer of Hyflo Super Cel on a Buchner funnel to remove remaining insoluble proteins. The solution was now clear and colorless.

4. Ammonium Sulfate Fractionation

The pH was adjusted to 7.0 with NH_4OH (1:5 v/v) and maintained at this pH during the slow addition of solid ammonium sulfate until ≈ 45 % saturation was attained (68). The mixture was centrifuged and the precipitate discarded. Additional solid ammonium sulfate was added to the supernatant until 70 % saturation was attained. Both phytase and alkaline phosphatase activities precipitated between 45-70 % ammonium sulfate saturation.

5. Chromatography on DEAE-Cellulose

A portion of the precipitate so obtained was dissolved in a minimum volume of 0.05 M Tris-HCl adjusted to pH 7.8 (69), and dialyzed exhaustively against the same buffer. The sample was applied to a DEAE-cellulose column (20 x 1 cm) that had been packed by gravitation and equilibrated with the buffer at 5° C. The sample was eluted by applying to the column 10 ml portions of increasing NaCl concentrations (0, 0.1, 0.15 and 0.2 M) in 0.05 M Tris-HCl adjusted to pH 7.8. 2 ml effluent samples were collected automatically. Pressure was kept constant by the use of a Mariot bottle.

6. Gel Filtration on Sephadex G-200

Another portion of the precipitate obtained from ammonium sulfate fractionation was dissolved in 0.01 M Tris-HCl adjusted to pH 7.8, and dialyzed against the same buffer. The sample was applied to a Sephadex G-200 column (20 x 1 cm) that had been washed thoroughly with the buffer. Elution of the sample was effected by the same buffer, and 2 ml effluent samples were collected automatically.

D. Separation of Subcellular Fractions From the Rat Intestinal Mucosa

Nuclei and mitochondria were isolated by centrifugation of the crude homogenate in 0.25 M sucrose at 1000 x g and 1500 x g respectively in a Sorvall SS centrifuge at 5° C.

A Spinco Model L centrifuge was then used to separate the microsomes as shown in the flowsheet (Figure 1).

E. Thermal Inactivation at 50° C and 60° C

Partially purified rat enzyme preparations were heated to 50° C and 60° C in a water bath for 10, 20 and 30 minutes. At each time interval, 1 ml aliquot was removed and assayed at 37° C for both phytase and alkaline phosphatase activities.

F. Inhibition Studies

The behavior of rat intestinal phytase and alkaline phosphatase was compared by incubation with 0.005 M, 0.01 M and 0.02 M solutions of L-phenylalanine, sodium fluoride and potassium cyanide.

In separate experiments, kinetic constants were determined by assaying activities at various concentrations of sodium phytate and p-nitrophenylphosphate. The results were evaluated in the presence and absence of 2.2×10^{-3} M. L-phenylalanine by constructing a Lineweaver-Burk plot (70).

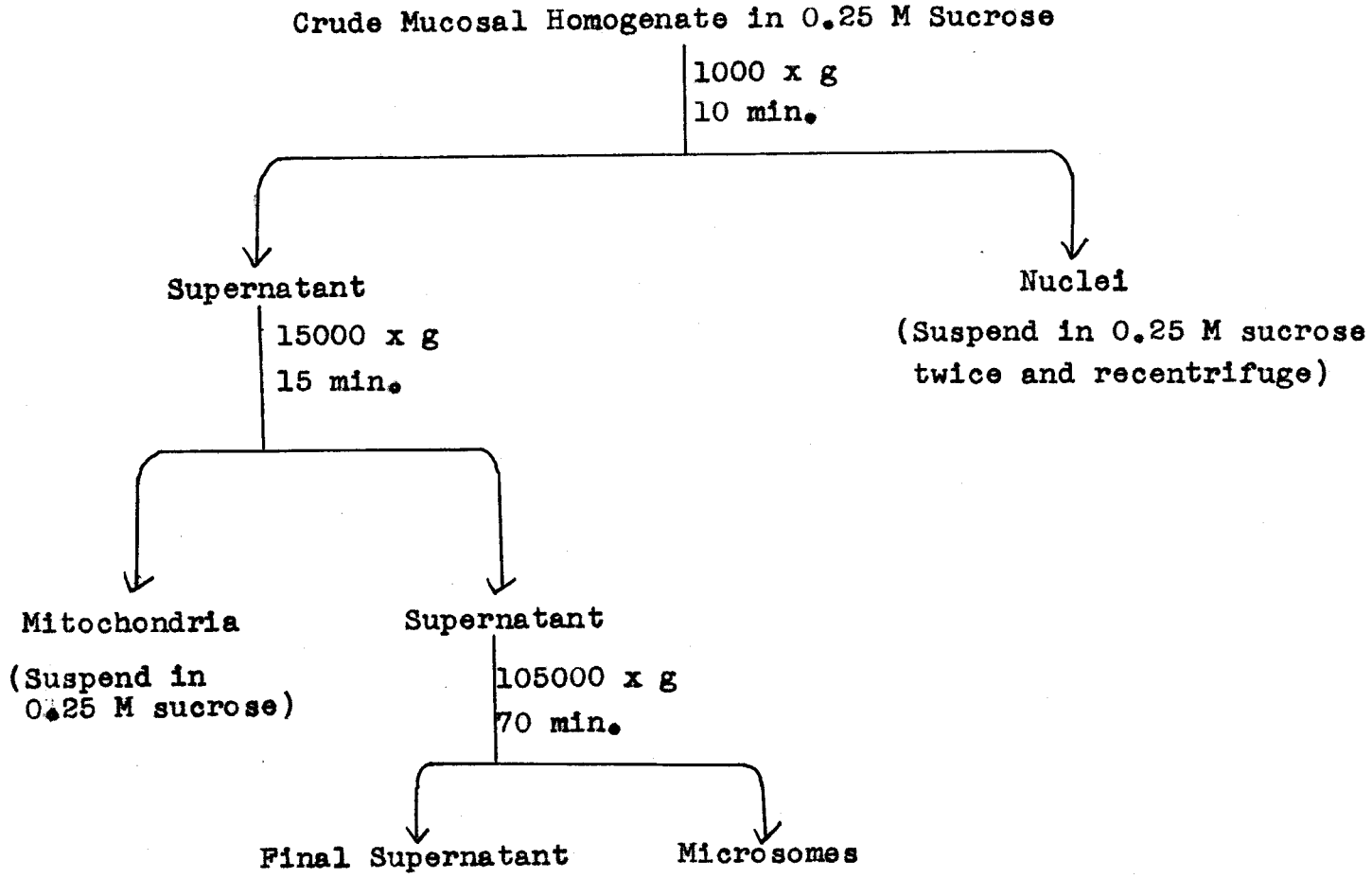


FIGURE 1. FLOW SHEET FOR THE ISOLATION OF THE SUBCELLULAR FRACTIONS FROM RAT INTESTINAL MUCOSA.

CHAPTER III

RESULTS

A. Comparison of the Specific Activities of Phytase and Alkaline Phosphatase in the Whole Homogenate

The specific activities of phytase and alkaline phosphatase, measured to provide a preliminary evaluation of relative activities, differed in the whole homogenates (Table I). In all cases alkaline phosphatase showed a higher specific activity. In addition, the results suggest that variation between species may exist.

B. Partial Purification of Rat, Calf and Chicken Intestinal Phytase and Alkaline Phosphatase

Tables 2, 3 and 4 summarize the results obtained during the partial purification of the rat, calf and chicken intestinal enzymes.

During the different steps of the purification procedure, the ratio of specific activities (AP/Ph) either increased (rat, Table 2, and Calf, Table 3) or decreased (Chicken, Table 4).

The partial purification of the rat, calf and chicken intestinal phytase resulted in 51, 86 and 31 fold gain of specific activity; for alkaline phosphatase 85, 130 and 11 respectively.

TABLE 1

The Relative Specific Activities[1] of Phytase and Alkaline Phosphatase in the Homogenates of Intestinal Mucosas of Four Species

	Human	Rat	Chicken	Calf
pH	7.4	7.0	8.3	8.6
Alkaline Phosphatase	0.430	0.270	0.110	0.058
Phytase	0.240	0.230	0.053	0.026

[1] Results are expressed in terms of μmoles P_i/min./mg protein.

Activities are compared at the optimal pH of the phytase of each species.

TABLE 2

Partial Purification of Rat Intestinal Phytase and Alkaline Phosphatase

Stage[1]	Volume (ml)	Total Activity		Total Protein (mg)	Specific Activity x 10^{-2}		Ratio of Specific Activities
		Ph[2]	AP[2]		Ph	AP	(AP/Ph)
1	290	28.60	33.80	1820.00	1.57	1.86	1.1
2	360	13.00	20.40	54.00	24.10	37.80	1.5
3	20	2.01	3.56	5.60	35.80	63.50	1.7
4	25	0.55	1.04	0.68	81.00	153.00	1.8

[1] Stage 1: Whole homogenate in 0.25 M sucrose. 2: Filtered aqueous layer from butanol treatment. 3: Precipitate obtained from 40-60 $(NH_4)_2SO_4$ saturation after dialysis. 4: Eluate obtained from DEAE-Cellulose at 0.1 M NaCl in 0.05 M Tris-HCl pH 7.8.

[2] Ph: Phytase, AP: Alkaline phosphatase.

The reaction mixtures contained 1.57×10^{-3} M sodium phytate or p-nitrophenyl phosphate, 1×10^{-3} M Mg^{++}, 4×10^{-2} M Tris-succinate pH 7.0.

TABLE 3

Partial Purification of Calf Intestinal Phytase and Alkaline Phosphatase

Stage[1]	Volume (ml)	Total Activity		Total Protein (mg)	Specific Activity x 10^{-2}		Ratio of Specific Activities (AP/Ph)
		Ph[2]	AP[2]		Ph	AP	
1	550	26.60	58.60	7910.00	0.33	0.74	2.24
2	355	13.70	36.60	4540.00	0.31	0.80	2.58
3	295	1.51	4.95	82.50	1.80	6.00	3.33
4	18.50	0.21	0.76	0.74	28.60	102.00	3.60

[1] Stage 1: Supernatant in water after centrifugation of the crude enzyme preparation for 30 min. at 1800 x g. 2: pH 7.5 precipitate dispersed in distilled water after acid precipitation. 3: Filtered aqueous layer from butanol treatment. 4: Precipitate obtained from 45-70 % $(NH_4)_2SO_4$ saturation after dialysis.

[2] See Footnote 2 of Table 2.

The reaction mixtures contained 4.4 x 10^{-4} M sodium phytate or sodium β-glycerophosphate, 1 x 10^{-3} M Mg^{++}, 4 x 10^{-2} M Tris-HCl pH 8.6. In Stages 1 and 2 the preparation was further diluted in a proportion of 1:5.

TABLE 4

Partial Purification of Chicken Intestinal Phytase and Alkaline Phosphatase

Stage[1]	Volume (ml)	Total Activity		Total Protein (mg)	Specific Activity x10^{-2}		Ratio of Specific Activities
		Ph[2]	AP[2]		Ph	AP	(AP/Ph)
1	875	230.00	485.00	21300	1.08	2.28	2.12
2	590	199.00	416.00	14000	1.42	2.96	2.09
3	550	31.40	55.80	723	4.35	7.70	1.79
4	20	6.80	4.90	20	34.00	24.41	0.72

[1] The same stages of purification as in Table 3.

[2] See Footnote 2 of Table 2.

The reaction mixtures contained 4.4×10^{-4} M sodium phytate or p-nitrophenyl phosphate, 1×10^{-3} M Mg^{++}, 4×10^{-2} M Tris-succinate pH 8.3. In Stages 1 and 2 the enzyme was further diluted in a proportion of 1:5.

C. pH Activity Curves of Phytase and Alkaline Phosphatase

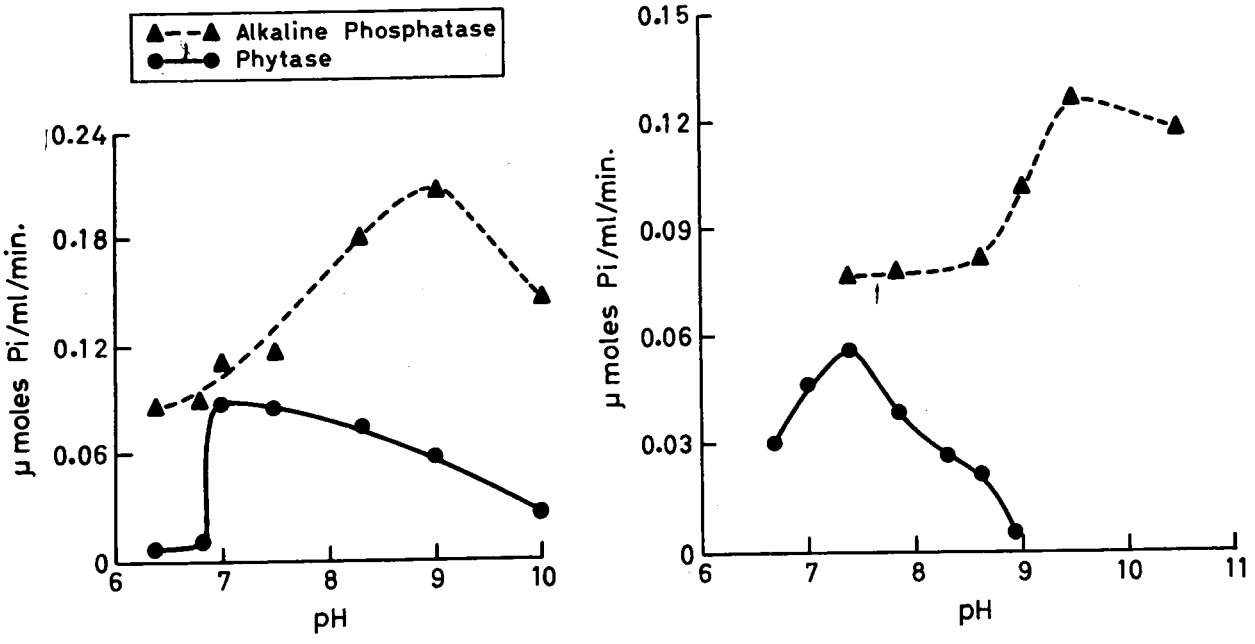
The pH optima for the rat, human, chicken and calf intestinal phytase differed considerably (Figs. 2a, b, 3a, b). These were respectively 7.0, 7.4, 8.3 and 8.6. In contrast alkaline phosphatase exhibited pH optima that were higher and sharper with less variability (Figs. 2a, b, 3a, b). These were at 9.0, 9.5, 9.0, and 9.5 respectively.

D. Effect of Substrate Concentration on Phytase and Alkaline Phosphatase Activities

The substrate concentrations at which rat and human intestinal phytase and alkaline phosphatase showed maximum activity were at about 1.76×10^{-3} M and 1.5×10^{-3} M (Figs. 4a, b). An S shaped curve was obtained in the case of the human preparation. In the calf and chicken, phytase activity was maximal at about 4.4×10^{-4} M. The substrate activity curves for these two species, in contrast to that of the rat, did not follow the Michaelis-Menten kinetics.

E. Elution Patterns from DEAE-Cellulose and Sephadex G-200

In the elution from DEAE-cellulose or Sephadex G-200 (Figs. 6-9), the activity peaks of both phytase and alkaline phosphatase coincided with the exception of one peak of the chicken preparation (Fig. 8). However, the ratio of both activities changes as they were eluted from the column. An exception occurred when the chicken preparation was passed through Sephadex G-200 (Fig. 9). Here, the ratio remained almost constant.



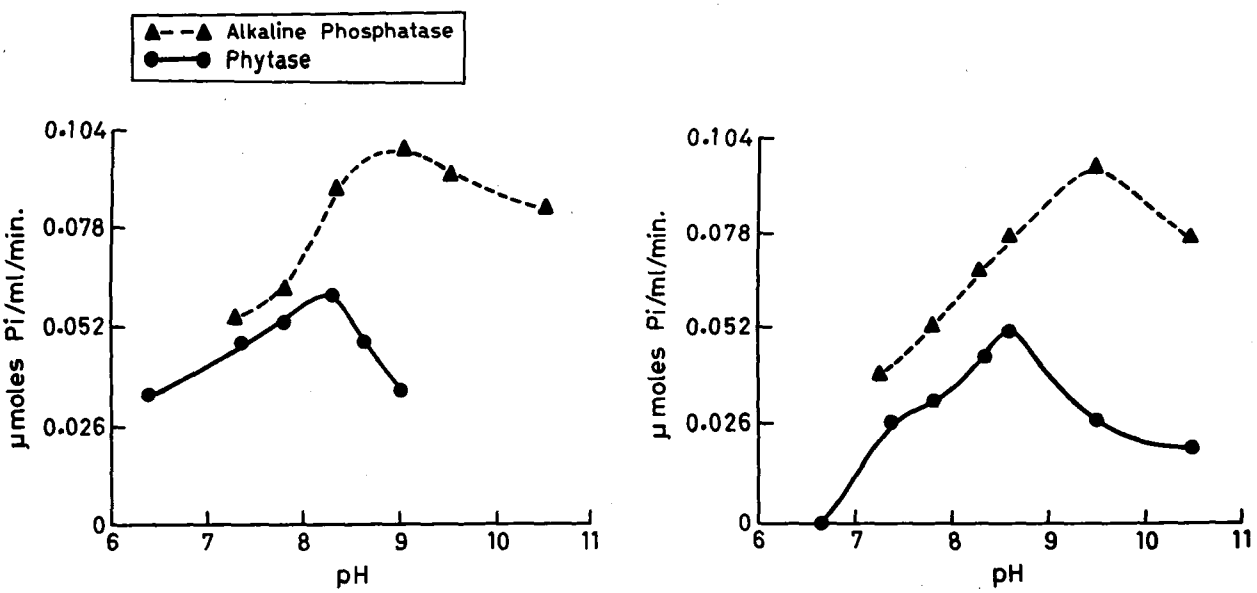
2a. 2b.

FIGURE 2 a. pH ACTIVITY CURVES OF RAT INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE

The reaction mixtures contained 1.56 x 10^{-3} M NaPh or PNPP, 1 x 10^{-3} M Mg^{++}, and 4 x 10^{-2} M of the respective buffer (pH 6.4 to 8.3 tris-succinate; pH 9.0 tris-HCl, pH 10.0 to 10.5 glycine buffer). pH values indicated were adjusted by titration with 0.1 M NaOH.

b. pH ACTIVITY CURVES OF HUMAN INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE

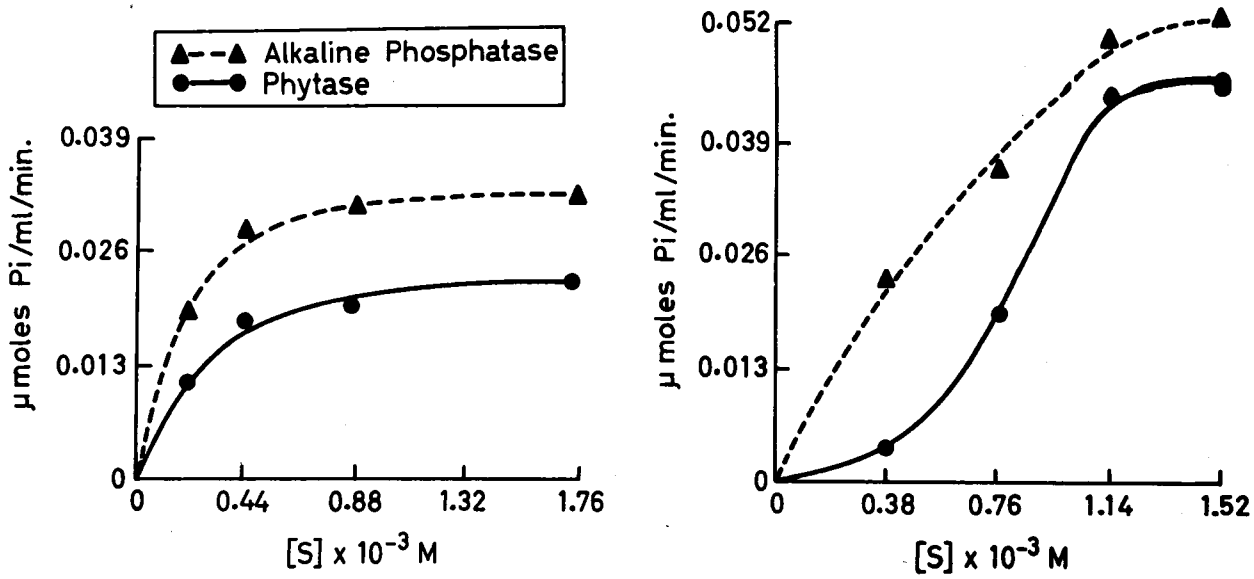
The reaction system was that described for Fig. 2a.



3a. 3b.

FIGURE 3 a. pH ACTIVITY CURVES OF CHICKEN INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE
The reaction mixtures contained 4.4 x 10^{-4} M NaPh or PNPP, 1 x 10^{-3} M Mg^{++}, and 4 x 10^{-2} M of the respective buffer (as described in Fig. 2a). pH values indicated were adjusted by titration with 0.1 M NaOH.

b. pH ACTIVITY CURVES OF CALF INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE
The reaction system was that described for Fig. 3a, except that NaβGl was used instead.



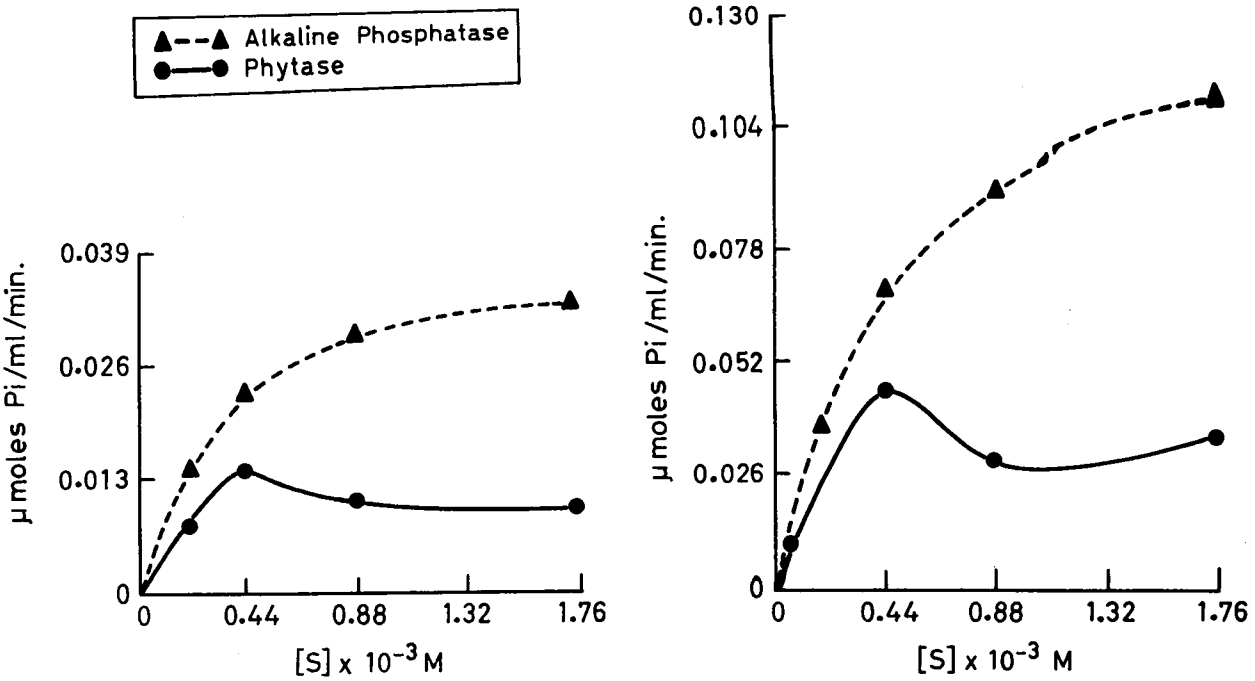
4a. 4b.

FIGURE 4 a. EFFECT OF SUBSTRATE CONCENTRATION ON RAT INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES

The reaction mixtures contained NaPh or PNPP, 1 x 10^{-3} M Mg^{++}, 4 x 10^{-2} M Tris-succinate pH 7.0.

b. EFFECT OF SUBSTRATE CONCENTRATION ON HUMAN INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES

The reaction system was that described for Fig. 4a, except that Tris-succinate pH 7.4 was used instead.



5a. 5b.

FIGURE 5 a. EFFECT OF SUBSTRATE CONCENTRATION ON CALF INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES

The reaction mixtures contained NaPh or NaβGl., 1 x 10^{-3} M Mg^{++}, 4 x 10^{-2} M Tris-succinate pH 8.6.

b. EFFECT OF SUBSTRATE CONCENTRATION ON CHICKEN INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES

The reaction system was that described for Fig. 5a, except that PNPP and Tris-succinate pH 8.3 were used instead.

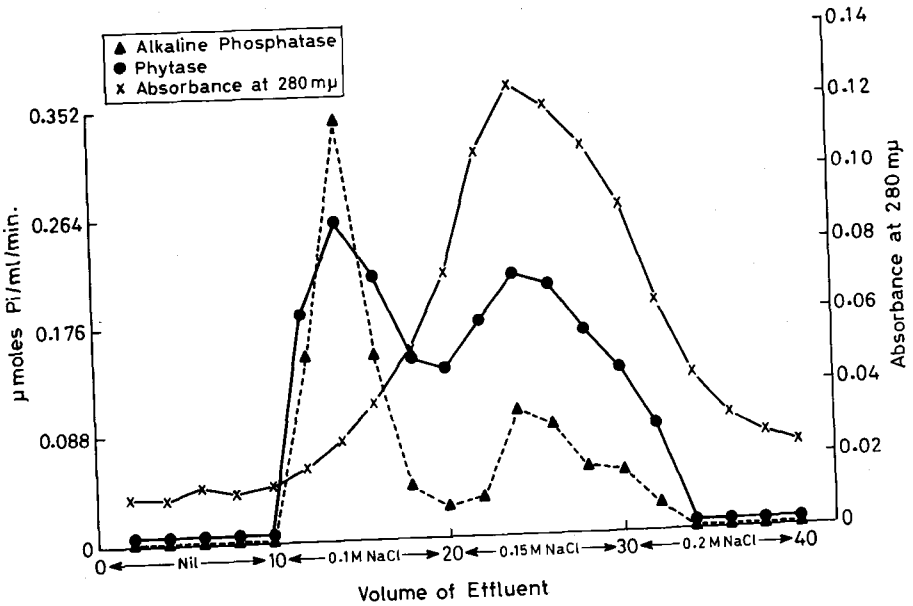


FIGURE 6. ELUTION PROFILES FROM DEAE-CELLULOSE OF A PARTIALLY PURIFIED RAT INTESTINAL PREPARATION

The reaction mixtures contained 1.5×10^{-3} M NaPh or PNPP, 1×10^{-3} M Mg^{++}, 4×10^{-2} M tris-succinate pH 7.0 made up with H_2O to 4.5 ml. Flow rate was 2 ml/6 minutes.

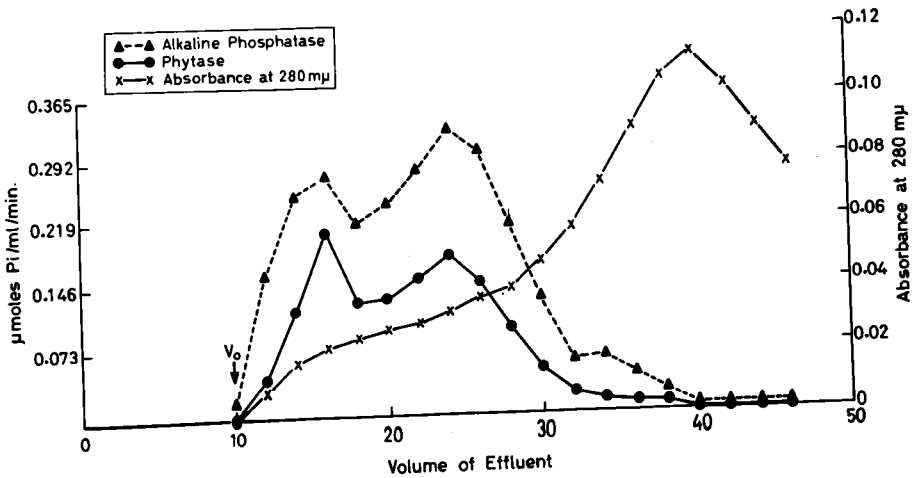


FIGURE 7. GEL FILTRATION ON SEPHADEX G-200 OF A PARTIALLY PURIFIED RAT INTESTINAL PREPARATION

The reaction mixture was that described for Fig. 6. Flow rate was 2 ml/30 minutes.

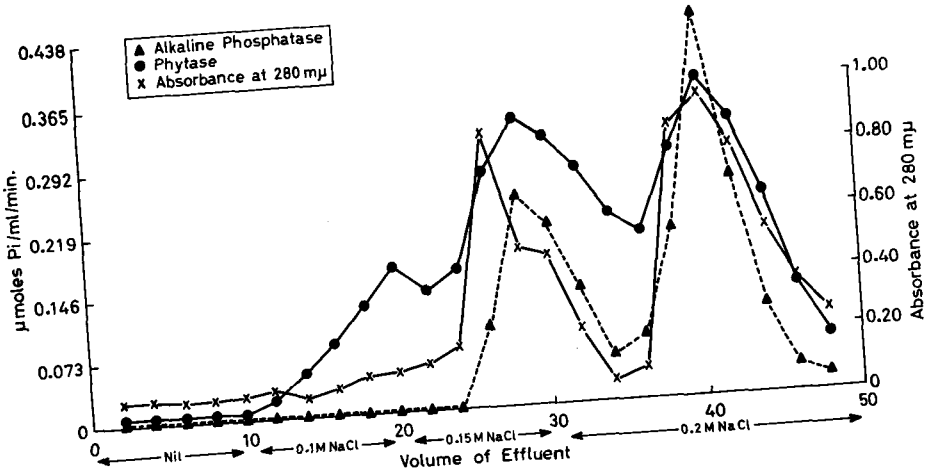


FIGURE 8. ELUTION PROFILES FROM DEAE-CELLULOSE OF A PARTIALLY PURIFIED CHICKEN INTESTINAL PREPARATION

The reaction mixtures contained 4.4×10^{-4} M NaPh or PNPP, 1×10^{-3} M Mg^{++} and 4×10^{-2} M Tris-succinate pH 8.3 made up with H_2O to 4.5 ml. Flow rate was 2 ml/6 minutes.

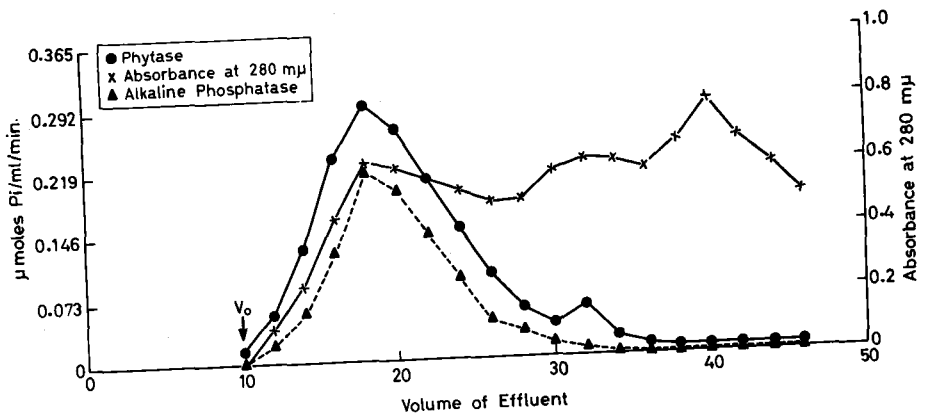


FIGURE 9. GEL FILTRATION ON SEPHADEX G-200 OF A PARTIALLY PURIFIED CHICKEN INTESTINAL PREPARATION

The reaction system was that described for Fig. 8. Flow rate was 2 ml/30 minutes.

Figure 6 shows the presence of two main activity peaks for both rat phytase and alkaline phosphatase in eluates of a DEAE-cellulose column. The first shows higher specific activities with respect to both enzymes. These appeared well before the major protein peak.

Two main activity peaks for rat phytase and alkaline phosphatase activities were eluted from a Sephadex G-200 column as illustrated in Figure 7, again before the major protein peak appeared.

Figure 8 illustrates the elution pattern of chicken intestinal phytase and alkaline phosphatase from DEAE-cellulose. Phytase exhibited three main activity peaks, two of which coincided with the two peaks of alkaline phosphatase at the same salt concentrations in the eluent.

The ratios of phytase to alkaline phosphatase activities eluted from a Sephadex G-200 column remained almost constant as illustrated in Figure 9 in the chicken preparation. Both phytase and alkaline phosphatase exhibited one major activity peak in which phytase had the higher specific activity.

F. Inhibition Studies

Rat phytase was competitively inhibited by L-phenyl alanine (Fig. 10). By contrast alkaline phosphatase was uncompetitively inhibited (Fig. 11). The apparent K_m for phytase activity increased from 1.66×10^{-3} M to 2.85×10^{-3} M

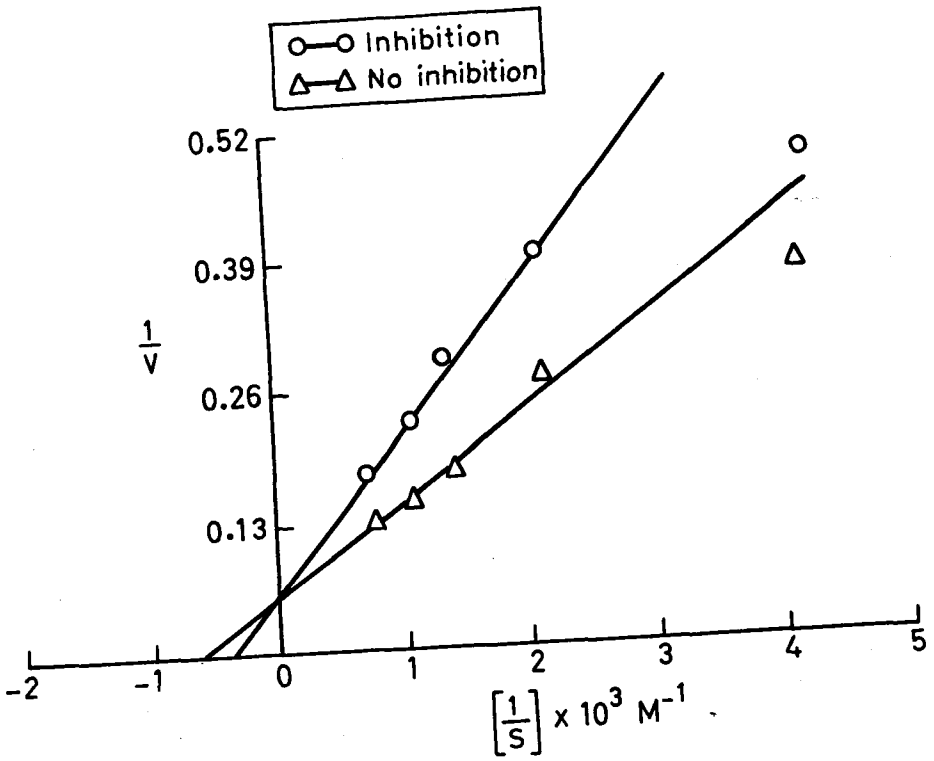


FIGURE 10. INHIBITION OF RAT INTESTINAL PHYTASE BY L-PHENYLALANINE

The reaction mixtures contained NaPh, 1 x 10^{-3} M Mg^{++}, 4.4 x 10^{-2} M Tris-succinate pH 7.0, 2.2 x 10^{-2} M L-phenylalanine or water. The sample was partially purified to 60 fold. (Eluate obtained from DEAE-cellulose at 0.1 M NaCl in 0.05 M Tris-HCl pH 7.8; 0.027 mg protein/ml).

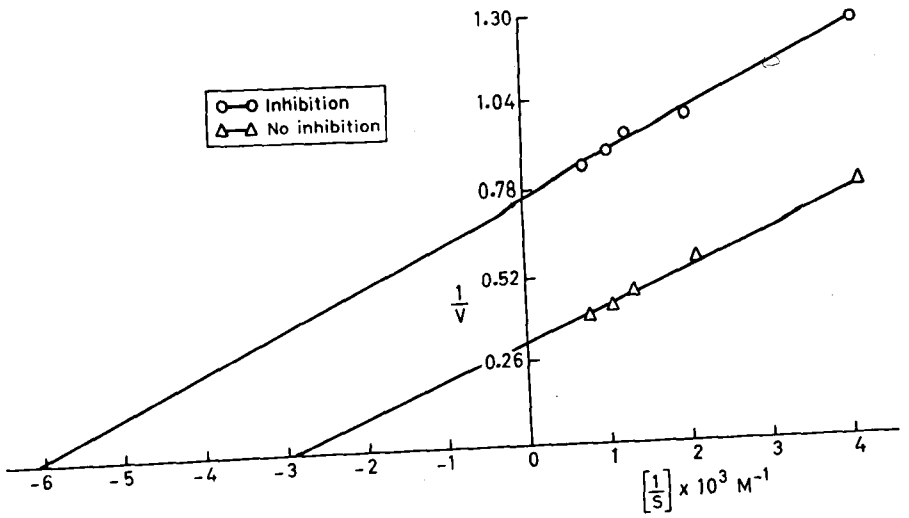


FIGURE 11. INHIBITION OF RAT INTESTINAL ALKALINE PHOSPHATASE BY L-PHENYLALANINE

Reaction system was that described for Fig. 10.

in the absence and presence of 2.2×10^{-3} M L-phenylalanine respectively. That of alkaline phosphatase decreased from 3.5×10^{-4} to 1.65×10^{-4} M in the presence of the same concentration of the inhibitor and same experimental conditions.

Table 5 summarizes the behavior of the two rat enzyme activities toward three inhibitors: L-phenylalanine, sodium fluoride, and potassium cyanide. Both enzyme activities were totally inactivated by concentrations of KCN >0.01 M. Phytase was more sensitive to inhibition at several different concentrations of L-phenylalanine and sodium fluoride.

G. Thermal Inactivation of Rat Phytase and Alkaline Phosphatase Activities

Both enzymes were inactivated slightly at 50° C and to the same extent (Fig. 12). Inactivation was more extensive at 60° C. The percentage of residual activity decreased as time of heating increased. About 30 % of activity remained after heating the enzyme sample at 60° C for 30 minutes.

H. Subcellular Localization of Rat Intestinal Phytase and Alkaline phosphatase

Phytase and alkaline phosphatase activities were found mainly in the microsomes. The specific activities of both enzymes in several fractions was in the sequence: microsomes >mitochondria >nuclei >supernatant. Moreover,

TABLE 5

Effect of L-phenylalanine, Sodium Fluoride, and Potassium Cyanide on Rat Intestinal Phytase and Alkaline Phosphatase[1]

Inhibitor	nil		0.005 M		0.01 M		0.02 M	
	Ph	AP	Ph	AP	Ph	AP	Ph	AP
L-phenylalanine	1.22	1.55	1.17	1.51	0.91	1.42	0.64	1.42
Sodium Fluoride	1.10	1.16	1.08	1.16	1.03	1.16	0.70	1.16
Potassium Cyanide	1.22	1.49	1.05	1.14	0	0	0	0

[1] Results are expressed in terms of μmoles P_i/ml/min. x 10^{-2}.

The reaction mixtures contained 1.56×10^{-3} M sodium phytate or p-nitrophenylphosphate, 1×10^{-3} M Mg^{++}, 4×10^{-2} M Tris-succinate pH 7.0 (Filtered aqueous layer from butanol treatment).

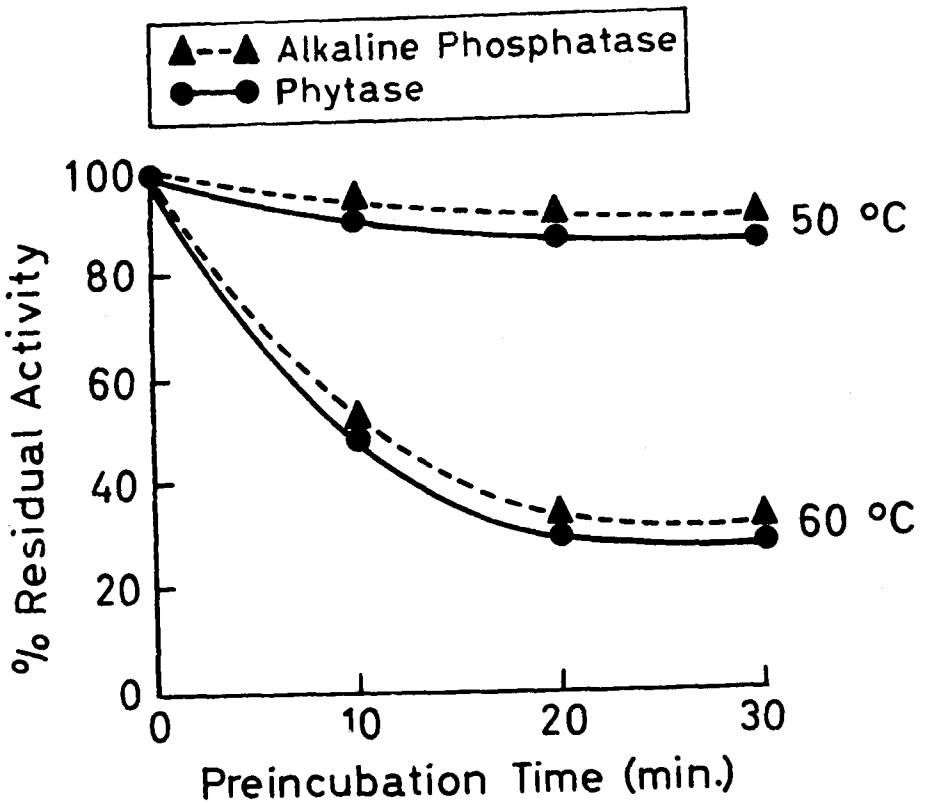


FIGURE 12. THERMAL INACTIVATION OF RAT INTESTINAL PHYTASE AND ALKALINE PHOSPHATASE AT 50° AND 60° C
The reaction mixtures contained 1.87×10^{-3} M NaPh or PNPP, 1×10^{-3} M Mg^{++}, and 4×10^{-2} M Tris-succinate pH 7.0. Sample was partially purified to 50 fold. (Eluate obtained from DEAE-cellulose at 0.1 M NaCl in 0.05 M Tris-HCl pH 7.8; 0.10 mg protein/ml).

the ratios of specific activities (AP/Ph) were almost constant in the various subcellular fractions (Table 6).

TABLE 6

Subcellular Localization of Rat Intestinal Phytase and Alkaline Phosphatase

Fraction	Volume (ml)	Total Activity		Total Proteins (mg)	Specific Activity x 10^{-2}		Ratio of Specific Activities (AP/Ph)
		Ph	AP		Ph	AP	
Whole Homogenate	65.00	5.80 (100%)[1]	7.26 (100%)	278.20	2.00	2.60	1.30
Nuclei	12.50	0.53 (9.15)	0.62 (8.53)	41.50	1.28	1.49	1.10
Mitochondria	12.00	1.14 (19.70)	1.63 (22.41)	45.12	2.52	3.62	1.40
Microsomes	11.00	1.49 (25.70)	2.01 (27.70)	32.56	4.60	6.20	1.30
Final Supernatant	43.00	0.64 (11.00)	0.64 (8.80)	108.36	0.59	0.59	1.00

The reaction mixtures contained 1.76×10^{-3} M sodium phytate or p-nitrophenyl phosphate, 1×10^{-3} M Mg^{++}, 4×10^{-2} M Tris-succinate pH 7.0.

1. Numbers in brackets indicate % recovery.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The relative specific activities of intestinal phytase differed in the four species studied, but paralleled those of alkaline phosphatase. In descending order, specific activities were as follows: human > rat > calf > chicken. Human and rat intestinal phytase also exhibited similar pH optima and substrate activity curves that differed considerably from those of the calf and chicken. The S shaped curve obtained for the human preparation could indicate a bimolecular reaction or two enzymes acting on the same substrate. The findings bring out a resemblance in phytases with respect to ionization behavior and other properties in the human and rat as compared with those of the calf and chicken. Additional studies are needed in order to clarify the effect of age and other factors on relative activities.

As previously mentioned, Aders et al (3) believed that intestinal phytase and alkaline phosphatase in the chicken are closely related. Maderich et al (4) claimed that a single enzyme hydrolyzed both phytate and β-glycerophosphate in the rat. However, an array of evidence summarized in Table 7 supports instead the existence of

TABLE 8

Behavior of Intestinal Phosphohydrolases

Experiment	Phytase	Alkaline Phosphatase
1. Order of relative specific activities in intestinal homogenate of four species	Human > Rat > Calf > Chicken	
2. Optimal pH		
a. Rat	7.0	9.0
b. Human	7.4	9.5
c. Chicken	8.3	9.0
d. Calf (β-glycerophosphate)	8.6	9.5
3. Substrate activity relation		
a. Rat	Follows Michaelis-Menten kinetics	
b. Human	S-shaped curve	
c. Chicken	Maximal activity at about 4.4×10^{-4} M	Michaelis-Menten kinetics
d. Calf		
4. Elution profiles from:		
a. Sephadex G-200, Rat	Partial separation of activities	

	Chicken	No separation of activities
b. DEAE cellulose, Rat	Partial separation of activities	
	Chicken	
5. Purification procedure		
a. Rat	Ratios of (AP/Ph) increased	
b. Chicken	Ratios of (AP/Ph) decreased.	
c. Calf	Ratios of (AP/Ph) increased.	
6. Inhibition studies on rat enzymes		
a. L-phenylalanine	Ratios of (AP/Ph) similar	
b. Type of inhibition by L-phenylalanine	Competitive	Uncompetitive
c. NaF	Ratios of (AP/Ph) similar	
d. KCN	Complete inhibition above 0.01 M	
7. Michaelis-Constant (Rat)	1.66×10^{-3} M	3.5×10^{-4} M
8. Thermal inactivation at 50 and 60°C (Rat)	Similar	
9. Subcellular localization (Rat)	Similar, predominantly microsomal	

TABLE 8

different enzymes or groups of enzymes. For example, the considerable difference in pH optima for the hydrolysis of phytate as compared with p-nitrophenylphosphate or β-glycerophosphate strongly suggest the existence of separate enzymes. The possibility that the formation of poorly soluble magnesium phytate in the reaction system at more alkaline pH depressed phytase activity must be taken into account (56). However, differences in the ionization of phytate as compared with that of p-nitrophenyl phosphate or β-glycerophosphate are excluded since phytase exhibited a wide range of pH optima extending from pH 7.0 (rat) to pH 8.6 (calf). Information concerning the pK values of the phosphate groups of phytate are needed to further evaluate the preceeding argument. Some of the results presented illustrate that hydrolysis of the phytate molecule occurs at a slower rate than that of the p-nitrophenyl phosphate or β-glycerophosphate molecules.

The patterns of elution from Sephadex G-200 and DEAE-cellulose demonstrate the presence of two major isoenzymes of both phytase and alkaline phosphatase in the rat and chicken. However, the ratios of AP/Ph activities undergo significant change throughout the elution patterns (Figs. 6-8). This further indicates that the isoenzymes of phytase are distinct from those of alkaline phosphatase.

As purification proceeded, a progressive change in AP/Ph ratios also supports separate origins of phytase and alkaline phosphatase activities. The fact that the ratio of specific activities (AP/Ph) increased (Tables 2 and 3) during the various stages as purification proceeded indicates that more phytase was lost than alkaline phosphatase. The decrease in ratios observed for the chicken (Table 4) is further evidence that the phytase of the latter differs from those of the calf and rat.

Inhibition of rat intestinal phytase and alkaline phosphatase by cyanide and fluoride may result from structural similarities, or from chelating Mg^{++} which is necessary for both activities. Inhibition of rat phytase by L-phenylalanine, a stereospecific inhibitor for intestinal alkaline phosphatase (71), is evidence of close relationship between the two enzymes. However, the type of inhibition of rat intestinal phytase by L-phenylalanine differs from that of alkaline phosphatase, being competitive in the former, uncompetitive for the latter. Fishman (72) proposed that in the inhibition of rat intestinal alkaline phosphatase by L-phenylalanine, a thermodynamically stable enzyme-inhibitor-substrate complex is formed which lowers the concentration of the enzyme-substrate complex capable of decomposing into products. Alternatively, a weakly dissociable enzyme-inhibitor-substrate complex may be formed.

Although most of the evidence supports the existence of separate sources of phytase and alkaline phosphatase activities, there is some that does not. The identical subcellular localization of the two in the rat supports the view that the two enzymes may be the same. Similar thermal inactivation curves suggest that rat phytase and alkaline phosphatase activities are due to proteins that, if not identical, share many structural features.

In conclusion, the most plausible interpretation of the results presented would be to ascribe intestinal phytase activity to one or more enzymes which are similar to but distinct from the intestinal alkaline phosphatases.

SUMMARY

1. Phytase and alkaline phosphatase activities were examined in homogenates of mucosas of the small intestine in the human, rat, calf and chicken. A partial separation of activities was effected when the three latter were partially purified by a procedure based on that of Morton (67), and Behal et al (15).

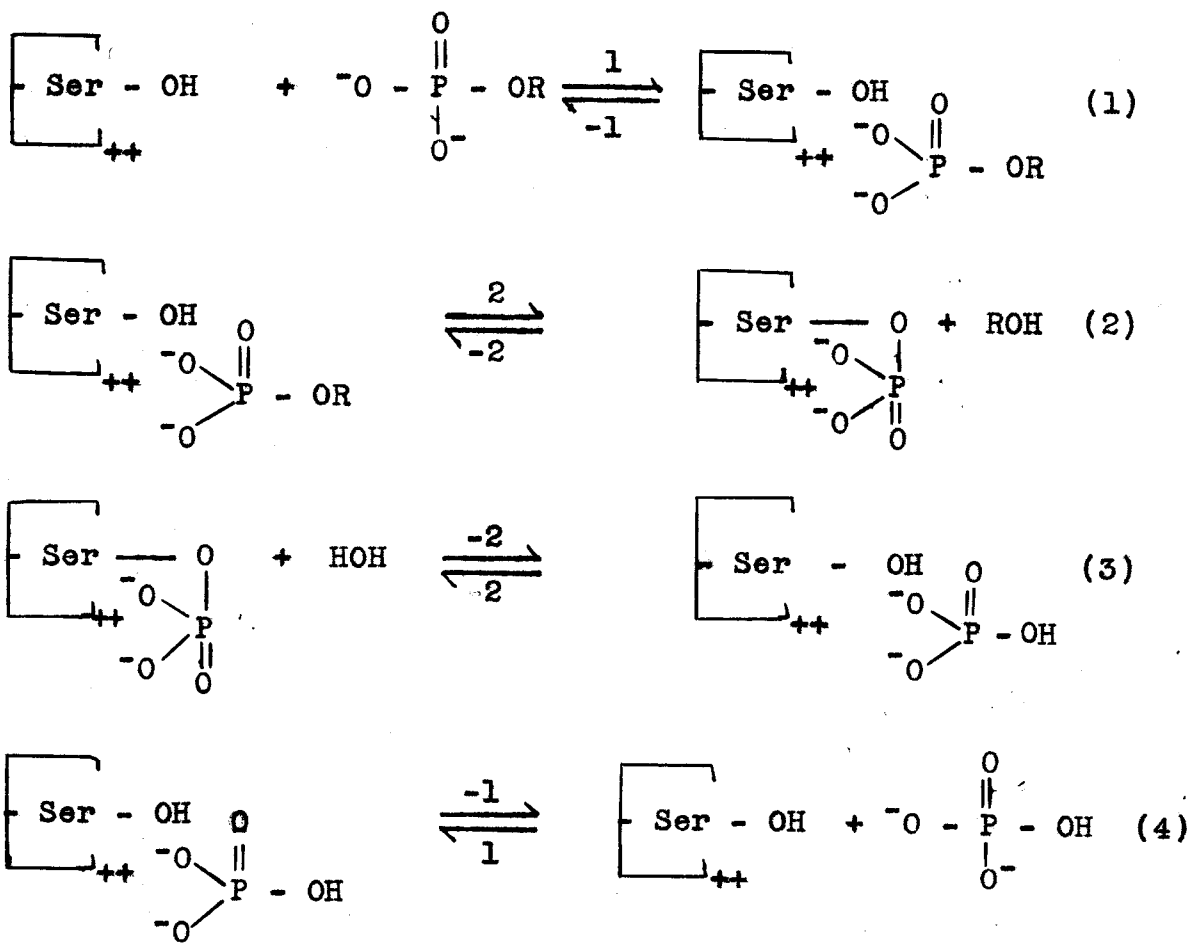
2. The behavior of phytase differed with the species studied. Phytases of human and rat were similar but differed from those of the calf and chicken.

3. The existence of two major isoenzymes of phytase and of alkaline phosphatase was demonstrated by chromatography on DEAE-cellulose and by gel filtration on Sephadex G-200 in preparations from rat and chicken.

4. An array of evidence was obtained indicating that phytase and alkaline phosphatase activities stem from two different sources, although they exhibit similarities in chemical properties.

APPENDIX A

A PLAUSIBLE MECHANISM FOR THE HYDROLYSIS OF MONOPHOSPHATE ESTERS BY ALKALINE PHOSPHATASE



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