Phytase activity in the human and rat small intestine

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Abstract
Phytate is the major storage form of phosphorus in seeds and so is a common dietary constituent. Excessive ingestion of undegraded phytates can cause mineral deficiencies in humans. In addition, phytic acid is antineoplastic in animal models of both colon and breast carcinoma. There have been no previous studies quantifying phytase activity in the human small intestine although it is present in animals. Small intestinal phytase and alkaline phosphatase activity and distribution was measured in vitro in mucosal homogenates from two human small intestinal specimens obtained from transplant donors. Rat intestine was also studied for comparison. Phytase activity was found in human small intestine at low values (30 times less than that in rat tissue and 1000-fold lower than alkaline phosphatase in the same tissue). The activity was greatest in the duodenum and lowest in the ileum. In conclusion, the normal human small intestine has very limited ability to digest undegraded phytates. Although this may have adverse nutritional consequences with respect to metabolic cation imbalances, the presence of undigested phytate in the colon may protect against the development of colonic carcinoma.

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Phytate (myo-inositol hexaphosphate) is the major storage form of phosphorus in seeds and as such forms a variable large component of the diet of many animals and humans.[1] Phytic acid is a strong chelator of cations and has been shown to cause deficiencies in minerals such as calcium, iron, zinc, and magnesium when present in excess in the human diet.[2]

Phytase (E.C.3.1.3.8.) is present in the small intestines of animals such as rats, calves, chickens, and pigs.[3-5] Dietary phytate intake and the ability of the intestine to break down phytic acid have been extensively studied in animals because of the importance of phytic acid in mineral balance and the potentially deleterious effect of high concentrations of undigested phosphates in livestock excreta.[6 7]

Despite the fact that humans consume large amounts of phytates, which can cause mineral deficiencies,[8] published works on the ability of the human intestine to degrade phytic acid are surprisingly sparse. There has only been one previous study on phytase activity in the human small intestine, which found it to be present but made no mention of the activity of

the enzyme or its distribution in the intestine.[5] Metabolic balance studies in human volunteers have suggested that the human intestine is poor at metabolising phytic acid.[9]

Our aim was to measure human intestinal phytase activity in vitro on fresh small intestinal material and to compare this with the activity in the rat. As there has been controversy in the past as to whether phytase activity actually results from alkaline phosphatase,[10] we planned to measure alkaline phosphatase activity in the same tissues and to use phenylalanine, which is known to inhibit alkaline phosphatase but not phytase.[11]

Methods
Phytase activity is determined by measuring the liberation of inorganic phosphate when inositol hexaphosphate is broken down. Most methods for measuring phosphate are based on the colour formed by the reduction of phosphomolybdate complexes.[12] Various reducing agents have been used. The application of this principle to the determination of phytase activity has been complicated by the finding that phytate itself interferes in these colorimetric determinations of inorganic phosphate.[13] A published method for the determination of phytase in duodenal biopsy specimens entails the reduction of phosphomolybdate complexes with ascorbic acid after the extraction of these complexes from remaining phytates using isobutyl/heptane.[14]

In a preliminary trial of this method, however, we found that the variability at the extraction and reduction phases made the procedure too imprecise for the determination of the very small amounts of phosphate released by the human tissue.

We therefore used a method for phosphate measurement using malachite green as the chromogen.[15] This improved method does not entail phytate extraction before colour development. It also has the added advantage of not requiring protein precipitation as deproteination has been shown to release phosphate groups from cellular constituents.[15]

REAGENTS
The following reagents were used: sodium phytate (30 mmol/l), hydrochloric acid (5 mol/l); malachite green made up in acidified molybdate (340 mg/l); aqueous TWEEN solution (15 ml/l); ammonium molybdate in hydrochloric acid (34 mmol/l); aqueous urea solution (360 g/l), aqueous zinc chloride (4 mmol/l); aqueous magnesium chloride (40 mmol/l).

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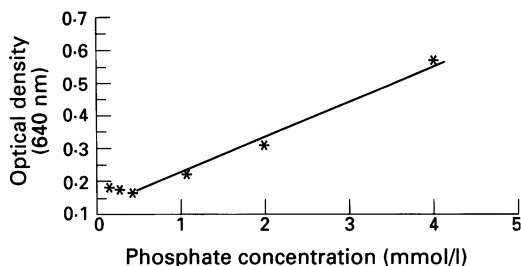Accepted for publication
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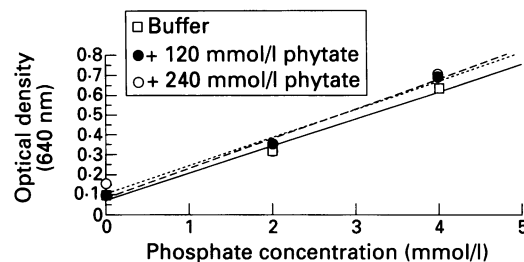Figure 1: *Working range for malachite green method.*

Figure 2: *Effect of phytate on malachite green method for phosphate.*

SPECIMENS

Complete human small intestinal specimens were obtained from two previously fit subjects during organ harvesting for transplantation purposes. One was a 50 year old woman who died suddenly from a subarachnoid haemorrhage; the other a 40 year old man who died suddenly in a road traffic accident.

Rat small intestine was harvested from two Wistar male rats killed by cervical dislocation.

All tissue samples were immediately frozen in liquid nitrogen. Subsequently the rat and human donor specimens were kept at −70°C.

PHYTASE DETERMINATION

For the determination of phytase activity the brush borders were scraped off underlying tissue with a glass microscope slide before homogenisation.

The tissue (approx 200 mg wet weight) was homogenised in 45 ml cold 50 mmol sucrose plus 2 mmol TRIS HCl buffer (pH 7·1) with 20 strokes of a Griffiths tube homogeniser. The homogenate was centrifuged at 6000 rpm for three minutes and the supernatant separated from the pellet, which was discarded. An aliquot was taken for alkaline phosphatase activity, which was estimated on the 'Cobas-Bio' automated enzyme analyser using paranitrophenol phosphate as substrate,[16] and for protein determination by the method of Bradford.[17]

Homogenate (2 ml) was combined with $MgCl_2$ solution (100 μl) and zinc chloride solution (100 μl). Sodium phytate substrate (100 μl) was then added to the tests and water added to the blanks. Reagent blanks consisting of buffer with or without phytate were included. The tubes were then incubated at 37°C for two hours after which an aliquot (100–500 μl) was taken for determination of inorganic phosphate content. Urea solution (1 ml) was added to this to prevent protein precipitation and then malachite green reagent (4 ml) containing acidified molybdate. After allowing 10 minutes for colour development, the samples were read at 640 nm on a Pye Unicam PU8610 UV/Visible spectrophotometer.

For the measurements on rat tissue we found that 100 μl aliquots from the incubation mixture gave good colour formation. The much smaller phytase activity in the human intestine, however, required 500 μl of incubate

and at this concentration there is a contribution from undigested phytate to the final colour. This could be compensated for by reagent blanks consisting of buffer with and without phytate.

The concentrations of phosphate in the incubates and blanks were derived from the standard curve.

The phytase activity expressed as μmolP/min/mg tissue was calculated as:

Phytase activity (μmolP/mg protein/min) =
(Tp−T)−(Bp−B)/(protein conc×time)

where Tp: tissue+phytate, T: tissue blank, Bp: buffer+phytate, B: reagent blank.

Results

WORKING RANGE OF MALACHITE GREEN METHOD

By plotting the absorbance of varying concentrations of phosphate standards (Fig 1) we derived a linear working range of between 1 and 5 mmol/l.

FREEDOM FROM INTERFERENCE BY PHYTATE

Phosphate standards with added phytate up to 400 times that used in the final colour reaction product had negligible effect on the absorbance (Fig 2).

FREEDOM FROM SPONTANEOUS HYDROLYSIS OF PHYTATE

Reagent blanks comprising buffer with and without phytate showed negligible effects on the final absorbance from phytate showing that under the experimental conditions there was no spontaneous hydrolysis of phytic acid.
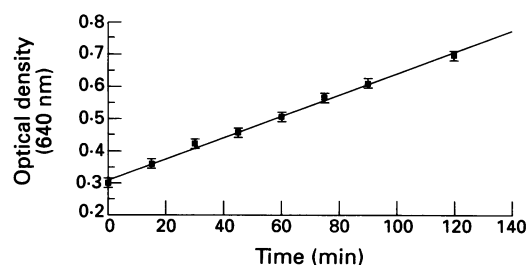

Figure 3: *Rate of phosphate liberation during incubation of rat duodenal homogenate with phytate.*

TABLE I   *Rat duodenal phytase and alkaline phosphatase activity and kinetics. Mean (SD) on six replicates shown*

	Activity	Vmax	Km (mmol/l)
Phytase (umolP/mg/min)	270 (4·8)×10⁻⁴	0·83	4·21
Alkaline phosphatase (umolP/mg/min)	2·75 (0·02)		
Alkaline phosphatase phytase ratio	102		

TABLE II   *Human phytase and alkaline phosphatase. Mean (SD) on six replicates shown*

	Phytase (umolP/mg/min)	Alkaline phosphatase (umolP/mg/min)	Ratio (alkaline phosphatase/phytase)
Duodenum:			
Subject 1	9·33 (0·8)×10⁻⁴	0·966 (0·07)	1083
Subject 2	6·50 (0·19)×10⁻⁴	0·203 (0·01)	363
Jejunum:			
Subject 1	3·67 (0·5)×10⁻⁴	0·614 (0·02)	1702
Subject 2	4·50 (0·12)×10⁻⁴	0·647 (0·01)	1526
Ileum:			
Subject 1	3·83 (0·4)×10⁻⁴	1·11 (0·02)	2938
Subject 2	3·00 (0·6)×10⁻⁴	1·75 (0·01)	6089

Figure 5: Effect of phenylalanine (Phe) on human enzymes. Bars represent means of three replicates.

RECOVERY EXPERIMENT

Known concentrations of phosphate standards (1–6 mmol/l) were added to rat tissue homogenates in the absence of phytate substrate. Recoveries ranged from 90% at a concentration of 1 mmol/l to 83% at 6 mmol/l.

RAT DUODENUM

Linearity of reaction with time

Rat homogenate (2 ml) was incubated with phytic acid for two hours and 100 μl samples were taken every 15 minutes for immediately assay of phosphate content. Figure 3 shows the rate of phosphate production obeys first order kinetics.

Phytase activity, Km, and Vmax for rat duodenum

Table 1 shows the results of phytase activity, alkaline phosphatase activity, and kinetics for phytase. The results are in good agreement with those found by other workers.[11]
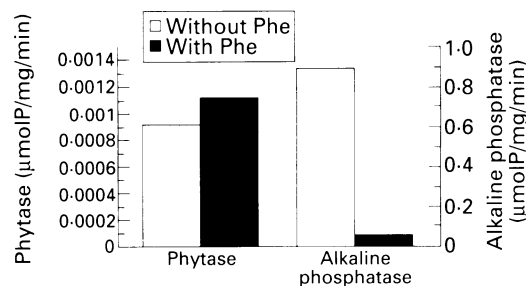
HUMAN INTESTINE

Human intestinal phytase activity

Table II shows the activity of phytase and alkaline phosphatase at different sites of the human small intestine.

It can be seen that there is much less phytase activity in the human small intestine compared with the rat and that this activity diminishes lower down the intestine.

Figure 4 shows the distribution of phytase and alkaline phosphatase activities in the two human intestines investigated. Determination of the kinetics of human phytase was beyond the sensitivity of the method because of the low phytase activity found in the human tissue.

Phenylalanine inhibition

We estimated phytase activity in three human duodenal homogenate replicates with and without the addition of phenylalanine (50 mmol/l). Alkaline phosphatase was estimated on the same homogenate with and without the addition of phenylalanine in the same concentration. Figure 5 shows that while the alkaline phosphatase activity was reduced by 95% by phenylalanine, there was no effect on the phytase activity.

Discussion

This is the first investigation to quantify the activity of human small intestinal phytase. In our study the proximal human small intestine was found to contain phytase activity at very low values, which declined distally along the intestine. Human duodenal phytase activity was roughly 1000 times lower than that of alkaline phosphatase and 30 times less than that seen in the rat duodenum. This measured activity did not result from alkaline phosphatase as shown by the lack of inhibition of phytase activity under conditions where alkaline phosphatase is almost completely inhibited. Other workers have found that phytate itself inhibits alkaline phosphatase.[18 19] We found phytase activity in the rat small intestine comparable with that found by other workers using methods requiring phytate extraction.[11] The only previous study on human phytase activity showed the enzyme in the human small intestine but did not report the levels of activity.[5] The authors implied that they saw activity similar to that seen in the rat.
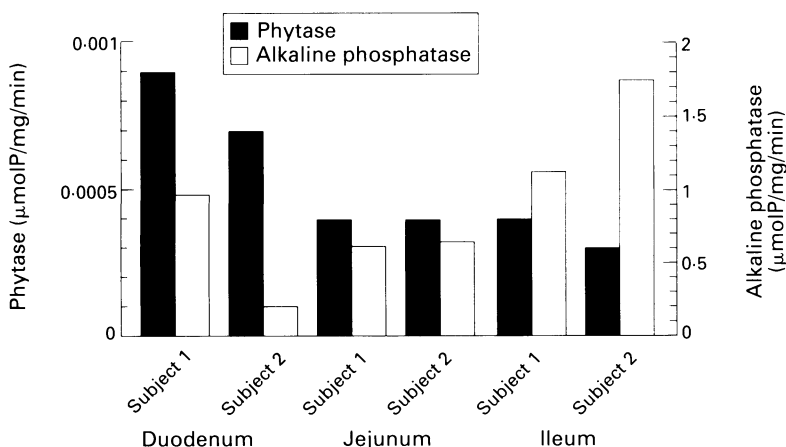
Figure 4: Distribution of human intestinal phytase and alkaline phosphatase in two subjects.

This is at variance with the much lower activity found in this study.

Nutritionists have estimated human phytase activity indirectly by studying the breakdown of various foodstuffs by the whole gut in normal controls and in patients with ileostomies and have suggested that intestinal phytase activity is very low.[9][20] Our direct measurements of human intestinal phytase activity in vitro would support these findings.

The ability of the small intestine to degrade phytic acid is of considerable nutritional importance as evidenced by the mineral deficiencies seen in human populations consuming large quantities of undegraded phytates.[20-23] Phytates in the diet are probably hydrolysed mainly by endogenous phytase in food (for example, yeast phytase) and this breakdown probably occurs largely in the acid environment of the stomach.[9] This would explain why most people taking diets containing modest amounts of phytates do not become deficient in minerals.

An additional recent development in phytate metabolism has been the hypothesis that phytate may be protective against colonic carcinogenesis.[24] Phytic acid has recently been found to be anti-neoplastic in both mammary and colon carcinogenesis models in vivo in the rat.[25][26]

The low level of phytase activity in the intestine dictates that, from the nutritional point of view, foodstuffs comprising high concentrations of phytic acid should undergo processing before human consumption.[27] In view of the recent evidence, however, that phytic acid is anti-neoplastic[26] it may be that consumption of undegraded phytates should be encouraged notwithstanding the nutritional implications.

1 Maga JA. Phytate; its chemistry, occurrence, food interactions, nutritional significance and methods of analysis. *J Agric Food Chem* 1982; **30**: 1-9.
2 Torre M, Rodriguez AR, Saura-Calixto F. Effects of dietary fiber and phytic acid on mineral availability. *Crit Rev Food Sci Nutr* 1991; **1**: 1-22.
3 Patwardhan VN. The occurrence of a phytin-splitting enzyme in the intestines of the albino rat. *Biochem J* 1937; **31**: 560-3.
4 Davies MI, Ritcey GM, Motzok I. Intestinal phytase and alkaline phosphatase of chicks: influence of dietary calcium, inorganic and phytic phosphorus and vitamin D3. *Poult Sci* 1970; **49**: 1280-6.
5 Bitar K, Reinhold JG. Phytase and alkaline phosphatase activities in intestinal mucosa of rat, chicken, calf and man. *Biochim Biophys Acta* 1972; **268**: 442-52.
6 Pointillart A, Fourdin A, Fontaine N. Importance of cereal phytase activity for phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. *J Nutr* 1987; **117**: 907-13.
7 Simons PCM, Versteegh HAJ, Jongbloed AW, Kemme PA, Slump P, Bos KD, et al. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Br J Nutr* 1990; **64**: 525-40.
8 Erdman JW. Oilseed phytate. Nutritional implications. *J Am Oil Chem Soc* 1979; **56**: 736-41.
9 Sandberg AS, Andersson H. Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. *J Nutr* 1988; **118**: 469-73.
10 Davies NT, Flett AA. The similarity between alkaline phosphatase and phytase activities in rat intestine and their importance in phytate-induced zinc deficiency. *Br J Nutr* 1978; **39**: 307-16.
11 Rao RK, Ramakrishnan CV. Studies on inositolphosphatase in rat small intestine. *Enzyme* 1985; **33**: 205-15.
12 Varley H. Gowenlock AH, Bell M, eds. *Practical clinical biochemistry*. 5th ed. Vol 1. London: William Heinemann, 1980: 883-5.
13 Irving GCJ, Cosgrove DJ. Interference by myo-inositol hexaphosphate in inorganic orthophosphate determinations. *Anal Biochem* 1970; **36**: 381-8.
14 Cooper JR, Gowing HS. A method for estimating phosphate in the presence of phytate and its application to the determination of phytase. *Anal Biochem* 1983; **132**: 285-7.
15 Hohenwallner W, Wimmer E. The malachite green micromethod for the determination of inorganic phosphate. *Clin Chim Acta* 1973; **45**: 169-75.
16 Hausamen TU, Helger R, Rick W, Gross W. Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. *Clin Chim Acta* 1967; **15**: 241-5.
17 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-51.
18 Martin CJ, Evans WJ. Inactivation of intestinal alkaline phosphatase by inositol hexaphosphate-Cu(II) coordinate complexes. *J Inorg Biochem* 1991; **42**: 161-75.
19 Martin CJ, Evans WJ. Reversible inhibition of intestinal alkaline phosphatase by inositol hexaphosphate and its Cu(II) coordinate complexes. *J Inorg Biochem* 1991; **42**: 177-84.
20 McCance RA, Widdowson EM. Phytates in human nutrition. *J Biochem* 1935; **29B**: 2694-9.
21 Hussain R, Patwardhan VN. Influence of phytate on the absorption of iron. *Ind J Med Res* 1959; **47**: 676-82.
22 Berlyne GM, Ben-Ari J, Nord E, Shainkin R. Bedouin osteomalacia due to calcium deprivation caused by high phytic acid content of unleavened bread. *Am J Clin Nutr* 1973; **26**: 910-11.
23 Reinhold JG, Faradji B, Abadi P, Ismail-Beigi F. Decreased absorption of calcium, magnesium, zinc and phosphorus due to increased fiber and phosphorus consumption as wheat bread. *J Nutr* 1976; **106**: 493-503.
24 Graf E, Eaton JW. Dietary suppression of colonic cancer. Fiber or phytate? *Cancer* 1985; **56**: 717-8.
25 Jariwalla RJ, Sabin R, Lawson S, Bloch DA, Prender M, Andrews V, et al. Effects of dietary phytic acid (phytate) on the incidence and growth rate of tumors promoted in Fisher rats by a magnesium supplement. *Nutr Res* 1988; **8**: 813-27.
26 Wattenberg L, Lipkin M, Boone CW, Kellof G, eds. *Cancer chemoprevention*. Boca Raton, FL: CRC Press, 1992: 285-308.
27 Sandberg AS. The effect of food processing on phytate hydrolysis and availability of iron and zinc. *Adv Exp Med Biol* 1991; **289**: 499-508.