Phytochemistry, 1972, Vol. 11, pp. 495 to 502. Pergamon Press. Printed in England.

ISOLATION, PURIFICATION AND CHARACTERIZATION OF PHYTASE FROM GERMINATING MUNG BEANS*

N. C. MANDAL, S. BURMAN and B. B. BISWAS
Bose Institute, Calcutta-9, India

(*Received* 21 *June* 1971)

Abstract—Phytase isolated from mung bean cotyledons was purified about 80-fold with a recovery of 28%. The enzyme is stable at 0°, has a pH optimum at 7·5 and optimal temperature of 57°. The energy of activation is approximately 8500 cal/mole between 37° and 57°. Inhibition by P_i has been found to be competitive, the K_i value being 0·40–0·43 $\times$ 10^{-3} M; the K_m value with phytate is 0·65 $\times$ 10^{-3} M. Divalent cations are not required for activity. Lower members of inositol phosphates are better substrates, as shown by their V_{max} and K_m values. When subjected to polyacrylamide gel electrophoresis two bands have been resolved; one (major) corresponds to phytase and the other (minor) to phosphatase and pyrophosphatase activity. Filtration through Biogel P-200 partially resolves phytase from phosphatase and pyrophosphatase. The molecular weight of phytase is approximately 160,000.

INTRODUCTION

THERE are a number of enzymes in biological systems which are known to dephosphorylate either free or bound inositol phosphates.[1] The dephosphorylation of free inositol phosphates is effected by phytase (mesoinositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8). It has a wide distribution in plant and animal tissues as well as in many species of fungi.[1] Many reports are now available on the characteristics of phytase, [2–8]and it is apparent that the metabolism of phytate plays an important role in germinating seeds. Phytase has not been detected in the cotyledons of ungerminated mung bean seeds, but it appears on germination.[9] In the present study an attempt has been made to purify and characterize the newly synthesized phytase in the cotyledons of germinated mung bean seeds.

RESULTS

Isolation and Purification of Phytase

The enzyme was isolated and purified by the procedures given in the Experimental section. The summary of the purification procedures is shown in the Table 1. It is seen that the phytase has been purified 80-fold with a recovery of 28% while the phosphatase has been purified 8-fold with a recovery of only 3% of the original activity. The phytase is stable at pH 7·0, at 0° without freezing, but freezing and thawing result in loss of the activity of the enzyme.

* Part III of the series "Metabolism of Inositol phosphates".

[1] D. J. COSGROVE, *Rev. Pure and Appl. Chem.* **16,** 209 (1966).
[2] J. COURTOIS, *Bull. Soc. Chim. Biol.* **29,** 944 (1947).
[3] F. G. PEERS, *Biochem. J.* **53,** 102 (1953).
[4] A. M. MAYER, *Enzymologia* **19,** 1 (1958).
[5] Y. NAGAI and S. FUNAHASHI, *Agri. Biol. Chem. Tokyo* **26,** 794 (1962).
[6] L. N. GIBINS and F. W. NORRIS, *Biochem. J.* **86,** 67 (1963).
[7] M. P. GREAVES, G. ANDERSON and D. M. WEBLY, *Biochim. Biophys. Acta* **132,** 412 (1967).
[8] V. K. POWER and V. JAGANATHAN, *Indian J. Biochem.* **4,** 184 (1967).
[9] N. C. MANDAL and B. B. BISWAS, *Plant. Physiol* **45,** 4 (1970).

TABLE 1. SUMMARY OF PURIFICATION PROCEDURE

	Treatment	Total volume (ml)	Total protein (mg)	Sp. Ac. (units/mg)		Total activity (Units)	
				Phosphatase	Phytase	Phosphatase	Phytase
1	Crude extract	450	2250	1·60	0·40	3646	900
2	Heat extract	415	1056	3·75	0·83	3960	880
3	First $(NH_4)_2SO_4$ (50–70%)	110	450	3·50	1·63	1575	733
4	Second $(NH_4)_2SO_4$ (45–70%)	24	300	5·00	2·06	1500	619
5	Acetone (50–80% v/v)	—	88	2·50	4·48	220	395
6	Bentonite	—	8	13·40	32·00	107	256

One unit of enzyme has been defined as that amount of protein which can liberate 1 μmole P_i/hr from respective substrate under experimental conditions. Phosphatase was assayed by using G-6-P (2 μ mole) as substrate.

pH Optimum

In tris-acid maleate–NaOH buffer, phytase exhibits its maximum activity at pH 7·5, while the phosphatase present in the phytase preparation shows its maximum activity at pH 6·0.

Effect of Increasing Temperature

Phytase activity was assayed by incubation at different temperatures for 1 hr. The release of phosphorus from phytate increases with the temperature up to 57°, then decreases rapidly on further rise of temperature, and at 65°, the enzyme shows approximately one third of the activity of that at 57°. The energy of activation for the phytase mediated reaction has been calculated to be approximately 8500 cal/mole between 37° and 57°.

Effect of Different Time of Heating at 57° on the Enzyme Activity

Twelve tubes containing 25 μg enzyme and 100 μmoles buffer, pH 7·5, in a total volume of 0·8 ml, were incubated at 57°. Two tubes were taken out after 10, 20, 30, 40 and 60 min of incubation and cooled in ice. To one tube from each set was added 1·3 μmole phytate, and to the other was added 2 μmoles of glucose-6-phosphate, and the total volume was made up to 1 ml in each tube. These were then incubated at 37° for 1 hr with appropriate controls minus heat treatment for the respective substrate. The phytase and the phosphatase activities fall off exponentially, the rate of inactivation of phytase being greater. After 1 hr heat treatment the inactivation reached about 90% in both the cases.

Both phytase and phosphatase activity were protected almost 81–85% when heated in the presence of phytate, glucose-6-phosphate or P_i. The protection seems to be mediated through P_i.

Effect of Phytate Concentration on the Release of P_i

The assay was conducted with different concentrations of phytate at pH 7·5 with 25 μg of protein at 37° and at 57°. The K_m value, calculated from a double reciprocal plot, was $0{\cdot}65 \times 10^{-3}$ M at 37° while at 57° it was $0{\cdot}606 \times 10^{-3}$ M. When phytate is replaced by

other homologues of inositol phosphates different K_m values are obtained. The V_{max} and K_m values for different inositol phosphates are recorded in Table 2.

TABLE 2. V_{max} AND K_m VALUES FOR DIFFERENT INOSITOL PHOSPHATES*

Substrate	V_{max} (μmole P_i/hr/mg)	K_m ($M \times 10^3$)
IP_6	44·4	0·65
IP_5	68·3	0·29
IP_4	88·4	0·24
IP_3	88·4	0·24
IP_2	88·0	0·20

V_{max} and K_m values were obtained from a double reciprocal plot for each substrate.

* IP_6, IP_5, IP_4, IP_3, IP_2 and IP represent inositol hexa-, penta-, tetra-, tri-, di- and monophosphate respectively.

Differential Inhibition by Inorganic Phosphate

Inorganic phosphate was found to inhibit both phytase and phosphatase activity, and the inhibitory effects of the different amounts of P_i on the activity of the two enzymes were studied. The phosphatase activity was more susceptible to inhibition by P_i and was completely inhibited at a concentration of 2 μmoles P_i/ml. at which phytase activity has been inhibited by only 49% (Fig. 1). This was verified using ^{32}P-labelled phytate as substrate. The pattern of inhibition was the same as that obtained by the colorimetric assay system. It is also evident that 16% of the phytase activity has been retained by the enzyme when the P_i concentration is above 4 μmole/ml, whereas at 2 μmole/ml the inhibition is 61% in contrast to 49% with the colorimetric assay under similar condition. This might be due to low concentration of substrate used in the radioactive assay system.

The Nature of Inhibition by P_i

The nature of the inhibition by P_i was studied using ^{32}P-labelled phytate as substrate. The experiment was performed as follows: Incubation mixture contained the following in a total volume of 0·25 ml: Buffer pH 7·5, 25 μmoles; ^{32}P-labelled phytate, in different amounts; enzyme, 7 μg protein. After 1 hr incubation at 37° the reaction was stopped by adding 0·5 ml of cold 0·5 M $HClO_4$. The liberated P_i was separated by chromatography on a short Dowex-1-Cl column (0·5 × 8·0 cm) by elution with 20 ml of 0·05 N HCl. The amount of phosphorus released was then calculated in μmoles per millilitre of incubation mixture from the specific radioactivity of phosphorus in the labelled phytate. The inhibition was determined with two fixed concentrations of P_i (i.e. 1 and 2 μmoles/ml) (Fig. 2). The K_i value was calculated to be 0·40–0·43 $\times 10^{-3}$ M.

Hydrolysis of Inositol Phosphates and Other Phospho-Compounds at Different pH values

Since the bentonite preparation contains phosphatase and pyrophosphatase activity it was thought pertinent to test different phospho-compounds at different pH values in order to differentiate those activities. It is observed that inositol phosphates are hydrolysed faster at pH 7·5 than at pH 6·0. However, other phospho-compounds are hydrolysed faster at pH 6·0 than at pH 7·5 (Table 3).

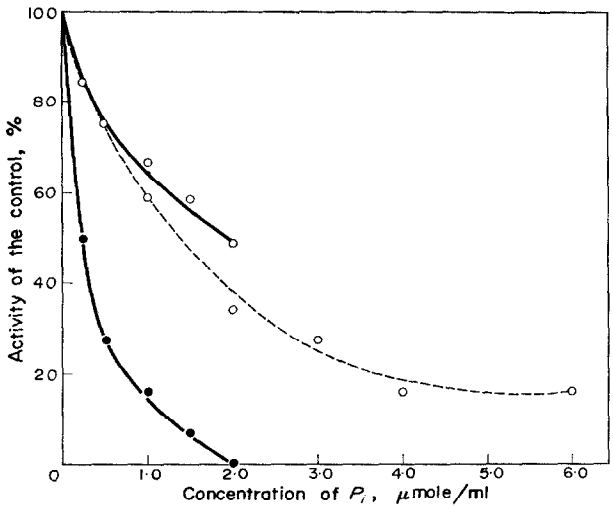

FIG. 1. EFFECT OF INITIAL CONCENTRATION OF P_i ON THE ENZYME ACTIVITY.
—●— Phosphatase by colorimetric method; —⊙— phytase by colorimetric assay; – –⊙– – phytase assay using ^{32}P-phytate as substrate (1 μmole/ml). In colorimetric assay the amount of phosphorus liberated was calculated by determining the total P_i at the end of the reaction and then subtracting from this amount the quantity of P_i added.

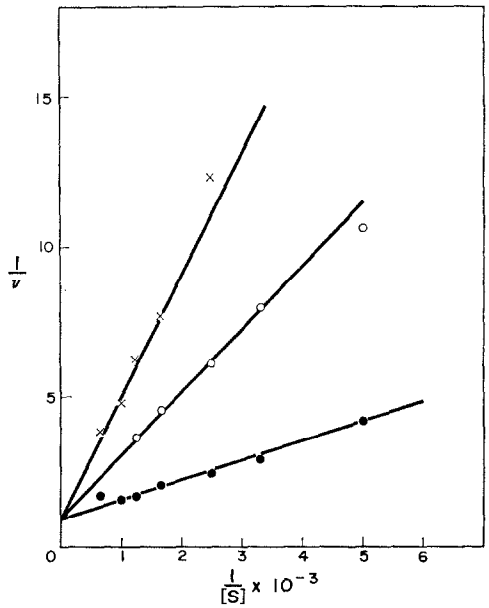

FIG. 2. EFFECT OF P_i ON PHYTASE ACTIVITY AT VARYING SUBSTRATE CONCENTRATIONS.
—●— Without P_i; —⊙— 1 μmole P_i/ml and —×— 2 μmole P_i/ml.

Product Analysis of Phytase at Intervals of Time

^{32}P-labelled IP_6 (see Table 2 for explanation of abbreviations) was incubated with phytase and aliquots taken at intervals were subjected to analysis for different inositol phosphates. It is seen that as the incubation progresses the radioactivity released as $^{32}P_i$ is increased proportionately (Table 4). The hexaphosphate does not disappear even after 150 min incubation. About one-fifth of the amount of IP_6 added originally remains unhydrolysed after 150 min. It is also interesting to note that IP_5 appears less in amount during the periods of incubation, even in 30 min, all other lower inositol phosphates have been detected; the level of IP_4 decreases rapidly, but that of IP_3 slowly; the level of IP_2 remains almost unaltered and that of IP increases.

Electrophoretic Behaviour of the Phytase on Polyacrylamide Gel

Polyacrylamide gel electrophoresis of the phytase preparations (20 μg protein) gave only two bands. It was found that the major band corresponds to phytase activity and the minor band predominantly the phosphatase and pyrophosphatase activities.

Purification of Phytase by Gel Filtration

Since the phytase preparation after electrophoresis on polyacrylamide gel yielded two protein bands, gel filtration technique was tried to purify phytase from the contaminant

TABLE 3. HYDROLYSIS OF INOSITOL PHOSPHATES AND OTHER PHOSPHO-COMPOUNDS

Substrate used	μmole P_i liberated at pH 6·0	7·5
IP_6	0·42	0·60
IP_5	1·16	1·41
IP_4	1·05	1·60
IP_3	1·30	1·76
Glucose-6-phosphate	0·33	0·22
Glucose-1-phosphate	0·96	0·70
β-Glycerophosphate	0·40	0·20
Pyridoxal phosphate	1·40	0·60
* Pyrophosphate	1·38	1·30
* ATP	0·85	0·76

* The data with these substrates represent the liberation in 10 min instead of 1 hr as in other cases. Assay procedure as given under experimental. 2 μmole of each substrate was used.

phosphatase. About 2–2·5 mg protein was applied to a Bio-gel P-200 column (1·5 × 25 cm) and filtration started with a flow rate of 1 ml/10 min using 0·05 M Tris HCl buffer, pH 7·0 and collecting 1 ml fractions. The phytase and the phosphatase activity were assayed with 0·1 ml aliquots from each fraction, as also from the void volume. Though the two enzyme activities were not fully resolved, there was an indication of the two enzymes being separated partially. Maximum activity of the phytase was found in fraction 11, and in fraction 8 for the phosphatase activity.

TABLE 4. ANALYSIS OF THE REACTION PRODUCTS OF PHYTASE

Fractions	Counts/min recovered in different fractions after 0 min	30 min	90 min	150 min
IP_6	461,000	295,000	151,800	91,000
IP_5	70	1700	1080	580
IP_4	45	32,700	12,900	7040
IP_3	40	33,200	21,800	12,360
IP_2	35	2975	2690	2500
IP	15	11,200	7,2000	27,000
P_i	15	78,000	242,000	318,000

The incubation mixture contained the following in a total volume of 1·5 ml. Tris acid maleate–NaOH buffer pH 7·5, 150 μmoles; ^{32}P-phytate, 1·5 μmoles ($4·62 \times 10^5$ counts/min) enzyme 4·7 units. Out of 4 such sets, 3 were incubated at 37° for different periods as indicated, the fourth one was kept as control at 0 time. The reaction was stopped by adding 0·1 ml of cold 70% perchloric acid; the acidified mixture was neutralized with KOH and the $KClO_4$ was spun down. Different inositol phosphates from the supernatant were isolated as described.[15]

DISCUSSION

Phytase has been isolated from the cotyledons of mung beans soaked for 72 hr. By isolating phytase from this stage a 200-fold purification, in comparison to 6 hr soaked cotyledons, has been achieved.[9] This was further purified through different procedures and about 80-fold purification from the crude extract has been obtained (Table 1). When this preparation was subjected to polyacrylamide gel electrophoresis one major and one minor band were detected. The minor band has been found to have a nonspecific acidic phosphatase and pyrophosphatase whereas the major band, phytase activity. From Table 1 it is also apparent that these activities are differentially purified and in fact, before acetone fractionation the specific activity of the phosphatase has been higher than that of phytase. After bentonite treatment 28% of the original phytase activity and 3% of phosphatase activity have been recovered. Only 8·4-fold purification has been obtained in the case of phosphatase. This, coupled with the gel electrophoretic profile, suggests that two enzymes are present even after bentonite treatment. This observation has further been substantiated by the optimal pH of those two systems. Phytase activity when assayed shows a distinct pH optimum at 7·5 whereas that of phosphatase is around 6·0.

The phytase preparation was subjected to gel filtration in an attempt to eliminate the acid phosphatase activity. It has been noted that the enzymes are excluded through a column of Biogel P-100 whereas these are retained in Biogel P-200. The elution profile shows that even with this treatment, phosphatase activity cannot be completely eliminated. However, this indicates that the phytase preparation contains both enzymes and the molecular weight would be higher than 100,000 but lower than 160,000. Further characterization of this phytase was done with the bentonite preparation. A number of reports occur in the literature on phytase where the pH optimum has been recorded at about 5·0.[2–7] The phytase studied in the present case records a pH optimum at 7·5. In the case of bacteria where phytase can be induced in presence of phytate, the pH optimum has also been recorded at 7·5.[8] However, the bacterial enzyme is solely dependent on Ca^{2+} while the present system has no absolute requirement for any cations. The present enzyme alone, without the substrate, could not withstand any temperature higher than 30° and even glucose-6-phosphate or inorganic phosphate can protect the phytase and phosphatase at a temperature as high as 57°. Inhibition of the enzyme activity has been recorded by *p*CMB, but the extent of inhibition is different in the case of phytase and phosphatase reported here, the former being more susceptible to *p*CMB. Similar observations have been reported by Preece and Grav with phenylmercuricacetate.[10] At 2 μmoles P_i/25 μg enzyme the phosphatase activity seems to be completely inhibited when phytase activity is depressed by about 50%; at 4 μmoles P_i per 25 μg enzyme, however, phytase activity is inhibited by 84–85%. This inhibition by P_i had already been suggested as early as 1927 by Luers and Silbereisen[11] and subsequently demonstrated by others.[7, 12, 13] Analysis of the products of phytase reaction at different intervals with IP_6 as substrate shows that all the lower inositol phosphates appear at the initial stage. The levels of IP_4 and IP_3 have been found to be higher than those of IP_2 and IP. The presence of a low amount of IP_5 at any stage of hydrolysis might be explained by the fact that its rate of dephosphorylation is much higher than that of its formation from IP_6. In fact, IP_5 can be hydrolysed faster than IP_6 and the rate of hydrolysis of lower inositol phosphates is even higher than that of IP_5 (Tables 2 and

[10] I. A. PREECE and H. J. GRAV, *J. Inst. Brewing* **68**, 66 (1962).
[11] H. LUERS and K. SILBEREISEN, *Wschr. Brau.* **44**, 263, 273 (1927).
[12] E. HOFF-JORGENSEN, *Nature, Lond.* **159**, 99 (1947).
[13] M. SUGUIRA and Y. SUNOBE, *Bot. Mag. Tokyo* **25**, 63 (1962).

3). This is in contrast to the phytase from wheat bran, reported by Nagai and Funahashi,[5] which can hydrolyse IP_6, IP_5 and IP_4 with almost equal rate. The K_m for IP_6 with this enzyme has been calculated to be $0{\cdot}65 \times 10^{-3}$ M and the inhibition by P_i has been found to be of the competitive type, with a K_i value of $0{\cdot}43 \times 10^{-3}$ M. The K_m value has been reported by other workers to be almost in this range, i.e. from $0{\cdot}3 \times 10^{-3}$ M to $0{\cdot}57 \times 10^{-3}$M with the phytase from wheat bran.[3–6]

The most highly purified phytase preparation studied so far was that of Nagai and Funahashi.[5] They obtained a 1500-fold purified phytase from wheat bran with a very high specific activity which is about 100 times greater than that of the present phytase. The present phytase is quite different from that of Nagai and Funahashi as can be inferred by the fact that their enzyme has the optimum pH at 5·0 for all the phospho-compounds studied including phytate. Their enzyme thus might not be compared with the present enzyme. Recently phosphatase and pyrophosphatase have been purified from phytase which record their optimal activity at pH 6·0 and 5·5 respectively.[14]

EXPERIMENTAL

Isolation of inositol phosphates and purification of phytase. Inositol phosphates were synthesized chemically and different inositol phosphates were separated as described earlier.[15] About 100 g of mung bean seeds were allowed to germinate for 72 hr at 35°[9] and cotyledons were separated and collected. About 200 g of cotyledons was homogenized with 2 vol. 0·05 M Tris HCl buffer pH 7·0 for about 10 min with a pestle and mortar. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 10,000 *g* for 25 min. The supernatant was collected and designated as *crude extract.*

Heat treatment. The crude extract (450 ml) containing the enzyme, was subjected to heat treatment at 60° for 6–7 min, cooled and then centrifuged at 10,000 *g* for 20 min. The supernatant was collected and designated as the *heat treated supernatant.*

Ammonium sulphate fractionation. The heat treated supernatant (415 ml) was 50% saturated with $(NH_4)_2SO_4$ and then centrifuged at 10,000 *g* for 20 min. The supernatant fraction was then made 70% saturated with $(NH_4)_2SO_4$ and the pellet after centrifugation was dissolved in 110 ml of homogenizing buffer. This was designated as *first ammonium sulphate fraction.* This fraction was again subjected to ammonium sulphate fractionation, the precipitate obtained between 45–70% saturation was designated as *second ammonium sulphate fraction.* This was dialysed against 0·001 M Tris HCl buffer pH 7·0, overnight.

Acetone fractionation. To the dialysed second ammonium sulphate fraction (10–12 mg/ml) was added an equal vol. of cold acetone with thorough mixing and kept in ice for 5 min and centrifuged. The supernatant from 50% acetone treatment was then made 80% (v/v) with respect to acetone. The mixture was kept at 0° for 10 min, followed by centrifugation. The pellet thus obtained, was washed with cold acetone and then dried *in vacuo* in cold. This was designated *80% acetone fraction,* ground and dissolved in minimum vol. of 0·05 M tris HCl buffer pH 7·0.

Bentonite treatment. For every 30 mg of protein to be treated, 1 g of bentonite was suspended in dist. H_2O and kept cold over night. The suspension was centrifuged at 10,000 *g* for 15 min and the supernatant, with the loosely sedimented mass at the top of the sediment, was rejected. The pellet was suspended uniformly in 0·05 M Tris HCl buffer pH 7·0 and centrifuged again at 10,000 *g* for 20 min. The supernatant and the loose mass were again rejected. The packed sediment was used in enzyme purification.

The protein solution from the 80% acetone fraction was diluted with 0·05 M tris-buffer to have a protein concentration of 12–15 mg/ml. To this, the processed bentonite was added and thoroughly mixed by blending. It was then allowed to stand for 30 min followed by centrifugation at 30,000 *g* for 30 min. The clear supernatant thus obtained contained the enzyme. This enzyme preparation could be stored at 0° without freezing for months without appreciable loss of activity.

Assay procedure. The incubation mixture contained in a total vol. of 1 ml, the following; tris acid-maleate-NaOH buffer pH 7·5, 100 μmoles; phytate, 1·3 μmoles; enzyme, appropriate protein aliquot. The additions were made in cold and mixed thoroughly, then incubated for 1 hr at 37° with an appropriate blank (minus enzyme). After 1 hr the reaction was stopped by adding 1 ml of 0·4 M TCA (cold) and the mixture was chilled in ice. When assayed with the purified enzyme preparation, this arrested reaction mixture could be directly used for the determination of liberated phosphorus. But when working with crude preparation precipitated protein was removed by centrifugation; the supernatant was adjusted to pH 4·0 by adding 0·7

[14] S. BURMAN and B. B. BISWAS, *Plant Cell Physiol.* to be published.
[15] N. C. MANDAL and B. B. BISWAS, *Indian J. Biochem.* 7, 63 (1970).

vol. of 0·4 M sodium acetate and an aliquot from this mixture was used for the estimation of inorganic phosphorus.[16]

Polyacrylamide gel electrophoresis of phytase. The electrophoresis according to the method of Davis[17] was carried out in a 4° cold room for 120 min with the anode at the bottom at a current of 3 mA/tube. The tray buffer was made by dissolving 3·0 g of Tris, 14·4 g of glycine to make 1 l., the final pH being 8·3. After the run, the gels were removed from the tubes by rimming with H_2O from a hypodermic needle and then stained for 2 hr by immersion in a 1% solution of amido black in 7·5% HOAc. Destaining was carried out at room temp. at a current of 5 mA/tube with the anode at the bottom and 7·5% HOAc in the buffer trays. For detecting the enzyme activity the gels before staining were cut into 20 slices each of 2 mm thickness. Each slice was put into 0·5 ml of tris buffer pH 7·0 and homogenized carefully with a glass rod, kept over night at 0° and centrifuged. The supernatant from each fraction was assayed for phytase, phosphatase and pyrophosphatase.

Acknowledgements—Thanks are due to Dr. S. M. Sircar, Director, Bose Institute, for his encouragement during the progress of this work. The financial support from USDA Grant No. FG-In-321 is thankfully acknowledged.

[16] O. H. LOWRY and J. A. LOPEZ, *J. Biol. Chem.* **162,** 421 (1946).
[17] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).

Key Word Index—Phaseolus aureus; Leguminasae; mung beans; phytase; inositol.