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Research Note

Isolation and Partial Purification of Phytase from *Vicia faba* Minor

ABSTRACT

Phytase was partially purified from small fababeans by ammonium sulphate fractionation and Sephadex gel filtration. Phytase activity coincided with phosphatase activity, suggesting it may be part of the general group of phosphatases. Kinetic studies showed that the properties of fababean phytase were similar in general to those of phytases from other sources. Fababean phytase was inhibited by the presence of substrate concentrations greater than 1·5 mM.

INTRODUCTION

Phytate is the major storage form of phosphorus in mature grains and legumes. Its hydrolysis is catalysed by the enzyme phytase (myoinositol hexaphosphate phosphohydrolase EC 3.1.3.8) to inositol and orthophosphate. Previous studies by Eskin & Wiebe (1983) identified an active phytase in two fababean cultivars (*Vicia faba* L. minor var. Ackerperle and Diana). The beans were germinated over a 10-day period with Ackerperle exhibiting a much greater phytase activity which reached a maximum 6 days after germination. This paper describes further studies to isolate and partially characterise phytase from *Vicia faba* L. minor var. Ackerperle and examine some of its properties. In addition to phytase activity, phosphatase was also monitored as most studies have tended to ignore its presence when studying this enzyme.

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EXPERIMENTAL

Sample preparation

Seed samples of fababean (*Vicia faba* L. minor var. Ackerperle) were provided by Dr Laurie Evans, Department of Plant Science, University of Manitoba. The dry seeds were germinated as described previously by Eskin & Wiebe (1983), removed after 6 days' germination, freeze-dried and ground in a Wiley Mill through a 30-mesh screen.

Enzyme extraction and partial purification

Crude phytase extracts were prepared using the procedure described by Goel & Sharma (1979) and the acetone powder extracted at pH 5·0. The acetone powder was then treated with polyvinylpyrrolidone (PVP) to remove polyphenols and then purified by ammonium sulphate fractionation. The fraction precipitating between 40 and 70% ammonium sulphate saturation was dialysed overnight at 3–4°C against 0·05M sodium acetate buffer, pH 5·0. The slight precipitate formed was removed by centrifugation. The supernatant was further purified by gel filtration on a 1×30 cm Sephadex G-100 column. A 0·5-ml aliquot of the dialysed 40–70% ammonium sulphate fraction was applied to the column and eluted with 0·05M sodium acetate buffer. The elution flow rate was approximately 12 ml h^{-1}. The effluent was collected in 2·0-ml fractions and monitored for phytase activity, phosphatase activity and protein.

Enzyme assay

Phytase activity was monitored using the procedure described by Lolas & Markarkis (1977). The reaction mixture consisted of 0·2 ml of 0·6M sodium acetate buffer, pH 5·0; 0·15 ml of 2 mM sodium phytate (Sigma Chemical Co. St. Louis, MO) 0·2 ml enzyme solution and 0·65 ml distilled water. The final volume was 1·2 ml with concentrations of buffer and phytate of 0·1M and 0·25M, respectively. The reaction mixture was held at 50 ± 1°C for 30 min in a water bath and the reaction terminated with 1·0 ml of cold 1·54M trichloroacetic acid. The mixture was then chilled in an ice bath for 15 min and aliquots were removed for determination of inorganic phosphorus by the method of Chen *et al.* (1956). Phytase activity was expressed as μmoles Pi/mg protein/30 min.

Phosphatase enzyme activity was assayed using the method described for phytase but substituting 0·15 ml of 20 mM *p*-nitrophenylphosphate for the sodium phytate as substrate and reducing incubation time from 30 to 10 min.

Protein determination

Protein content was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

RESULTS

Partial purification

Only a slight increase in enzyme activity was evident following treatment with PVP (Table 1). A 4·4-fold purification with a 67% yield of fababean phytase was obtained from the 40–70% ammonium sulphate fraction. Gel filtration of this fraction on the Sephadex column resulted in a 15·8-fold purification of the enzyme with a yield of 18%. A single peak exhibiting phytase activity was observed following separation on Sephadex G-100 which coincided with a somewhat larger single peak for phosphatase activity (Fig. 1).

TABLE 1
Partial Purification of Phytase from *Vicia faba* Minor

Fraction	*Specific activity* ($\mu g\ P_i/mg\ protein/30\ min$)	*Yield* (%)	*Purification*
Acetone powder	1·50	100	1·0
PVP treatment	1·87	92	1·3
Ammonium sulphate fraction (40%–70%)	6·64	67	4·4
Sephadex G-100	23·7	18	15·8

Kinetic studies

A linear relationship was observed between enzyme concentration and the amount of inorganic phosphorus liberated in all determinations completed. The K_m value for fababean phytase was 0·017 mM with optimum enzyme activity at pH 5·0 over 1·5 pH units. Fababean phytase exhibited optimum activity at 50°C with a 25% loss after heating for 10 min at 55°C. The enzyme was completely inactivated following exposure at 70°C for 10 min.

Substrate inhibition

Phytase activity was inhibited in the presence of phytate levels greater than 1·5 mM under optimum conditions of temperature and pH (Fig. 2).

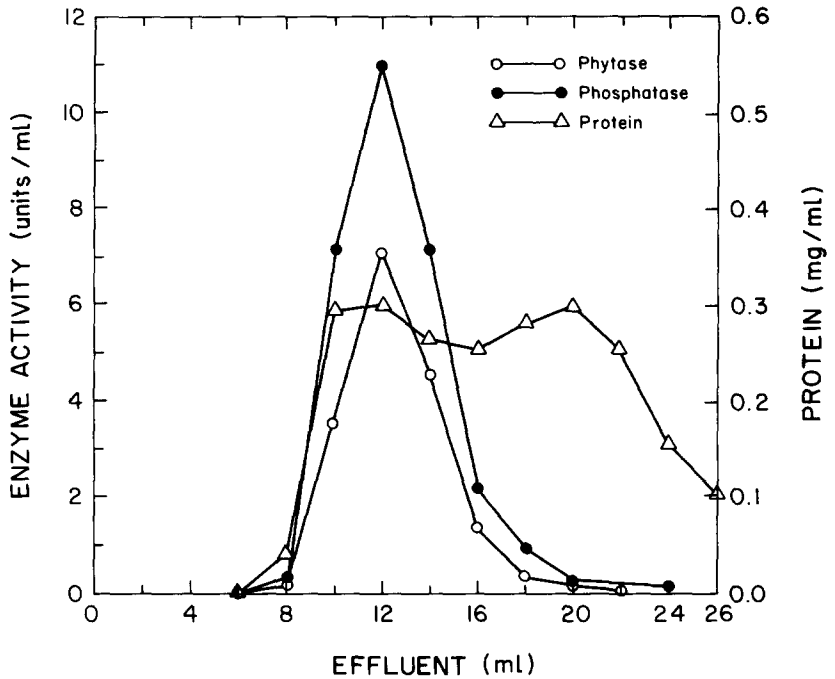

Fig. 1. Gel-filtration of phytase and phosphatase on Sephadex G-100. Unit of phytase activity = μmoles Pi/mg protein/30 min. Unit of phosphatase activity = μmoles Pi/mg protein/10 min.

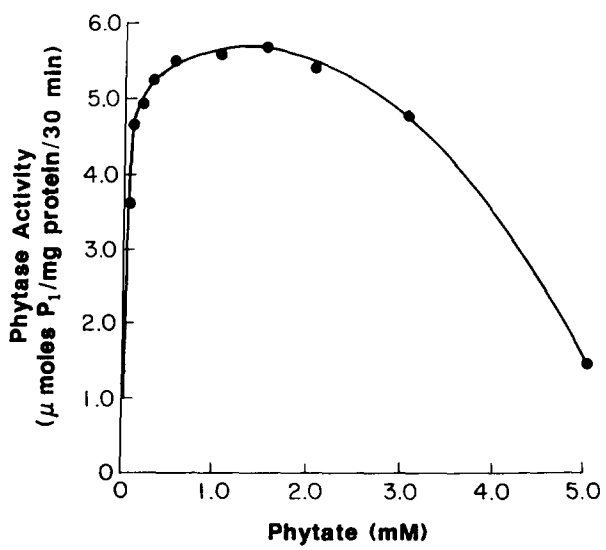

Fig. 2. Effect of substrate concentration on fababean phytase.

TABLE 2
Effect of Metal Ions on Fababean Phytase Activity

Compound	*Relative enzyme activity* (%)		
	10^{-3}M	10^{-4}M	10^{-5}M
$CaCl_2$	58	123	104
$CoCl_2$	13	107	103
$MgSO_4$	40	100	102
$FeSO_4$	4	6	77
$CuSO_4$	0	6	44
$ZnSO_4$	0	16	28

Metal ions

The effect of different metal ions on fababean phytase activity is summarised in Table 2. An inhibitory effect was observed in the presence of 10^{-3}M concentrations of Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+} and Ca^{2+} ranging from complete inhibition to a 42% loss of activity. Decreasing the levels of Mg^{2+}, Co^{2+} and Ca^{2+} to 10^{-4}M and 10^{-5}M completely restored the enzyme activity. With respect to Fe^{2+}, Cu^{2+} and Zn^{2+} only a slight increase in activity was seen in the presence of 10^{-4}M of these ions as compared to the activity observed with 10^{-3}M concentrations. The addition of 10^{-5}M of Fe^{2+}, Cu^{2+} and Zn^{2+} only partially restored the activity of phytase, accounting for 77%, 44% and 28% of the original activity, respectively.

DISCUSSION

Partial purification of an active phytase enzyme in *Vicia faba* L. minor has been achieved which accounts for the rapid degradation of phytate during the germination of fababeans (Eskin & Wiebe, 1983). The considerable loss of activity during the purification of fababean phytase may be explained in part by instability of the preparation. It is evident from this study that phytase is contaminated by considerable phosphatase activity. Most studies examining phytase have tended to ignore the presence of phosphatases but it is evident that these enzyme activities could not be separated by gel filtration on Sephadex G-100. This study suggests that phytase could be part of a general group of phosphatases differing by its ability to hydrolyse phytic acid. Further research will be needed to delineate these differences more precisely.

Based on its action on phytate, the K_m value observed for fababean

phytase of 0·017 mM is similar to that reported for Navy beans (0·018 mM) by Lolas & Markarkis (1977). This is considerably less than the K_m values of 0·22 mM reported for small white beans (Chang & Schwimmer, 1977), 0·15 mM for Navy beans (Gibbins & Norris, 1963), and 0·21 mM for yeast phytase (Nayini & Markarkis, 1984). The pH optimum of 5·0 for fababean phytase is within the pH range 5·0–5·6 reported for phytases from other plant sources (Peers, 1953; Nagai & Funahashi, 1962; Chang, 1967; Chang & Schwimmer, 1977; Lolas & Markarkis, 1977; Singh & Sedeh, 1979). The optimum temperature for fababean phytase was within the range reported for Navy beans (Lolas & Markarkis, 1977) and corn seed (Chang, 1967).

Fababean phytase activity is inhibited by substrate concentrations greater than 1·5 mM. This is in agreement with earlier studies on Navy beans (Lolas & Markarkis, 1977) and wheat phytase (Gibbins & Norris, 1963).

The effect of metal ions on fababean phytase differed from that observed with yeast phytase by Nayini & Markarkis (1977). Similar effects were evident in the presence of 10^{-4}M and 10^{-5}M concentrations of Ca^{2+} and Co^{2+} ions for both fababean and yeast phytase. In the case of fababean phytase, however, the presence of 10^{-3}M of these ions caused substantial decreases in activity of 32% and 87%, respectively. Marked differences were also apparent in the presence of both Cu^{2+} and Zn^{2+} ions resulting in substantial decreases in fababean phytase activities with increasing concentrations of these ions compared to only slight decreases for yeast phytase. It is difficult to explain the reasons for the different metal ion effects although possible interaction with the substrate cannot be ignored.

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