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Alkaline β-Glycerophosphatase of Green Gram* (*Phaseolus radiatus*) †

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During the course of investigations on riboflavin 5′-phosphate and flavin adenine dinucleotide-hydrolyzing enzymes in green gram (*Phaseolus radiatus*), we observed that crude extracts possessed high phosphatase activity against a variety of phosphorylated compounds. Despite intensive studies on the animal phosphatases, plant phosphatases have received very little attention.

Pfankuch (1) purified phosphatase from potato and sugar beet by alcohol precipitation. Later Giri (2) obtained highly active preparations of phosphatase from soya bean (*Glycine hispida*). A nonspecific phosphomonoesterase has been reported in green gram by Giri (3); later Ramanarayanan and Giri (unpublished observations) made a detailed study of the effect of molybdenum on the acid β-glycerophosphatase and inorganic pyrophosphatase of green gram.

Previous attempts by several workers to demonstrate the presence of an alkaline glycerophosphatase in plants have not been successful. Naganna *et al.* (4, 5) have reported a magnesium-activated alkaline pyrophosphatase in potato. Racker and Schroeder (6) demonstrated the occurrence of a specific fructose diphosphatase with maximal activity at pH 8.5. This paper describes a method of partial purification of an alkaline β-glycerophosphatase of green gram which is activated by ferric ion, and also some of its general properties.

EXPERIMENTAL PROCEDURE

Materials

Sodium β-glycerophosphate was a product of the British Drug Houses, Ltd. All the amino acids used were products of the Nutritional Biochemicals Corporation, and the salts used were of analytical reagent grade. *p*-Chloromercuribenzoate was a product of Light Chemicals obtained as a gift from Dr. T. Ramakrishnan. Green gram seeds were obtained from the local market.

Methods

Enzyme Assay—The reaction mixtures, unless otherwise stated, contained 10 μmoles per ml of Veronal-acetate buffer pH 8.5; 20 μmoles per ml of sodium β-glycerophosphate; 2.9 μmoles per ml of ferric sulfate; and 0.5 ml of enzyme in a total volume of 2.5 ml. After temperature equilibration the reaction was started by the addition of the enzyme. The reaction mixture was incubated at 49° for 15 minutes and the reaction stopped by the addition of 1.0 ml of 20% trichloroacetic acid. The mixture was centrifuged at 2500 × *g* for 10 minutes, and 1.0-ml aliquots were used for phosphate estimation by the method of Fiske and SubbaRow (7). One unit of enzyme activity is defined as μg of P_i liberated in 2.5 ml of the reaction mixture in 15 minutes and at 49°. Protein was estimated by the biuret method (8).

Purification of Enzyme—A total of 20 g of freshly powdered green gram seeds (passing 40 mesh) was extracted with 200 ml of water for 6 hours in the cold (0–4°) and centrifuged at 4000 × *g* for 15 minutes. The extract (Step 1, Table I) was dialyzed against water in the cold for 18 hours with repeated changes of water, and the precipitate obtained on centrifugation at 4000 × *g* for 15 minutes was rejected. The supernatant was fractionated by the addition of solid ammonium sulfate; the precipitate appearing between 0.40 and 0.55 saturation was dissolved in 25 ml of water and dialyzed free of ammonium sulfate in the cold. The dialyzed extract was centrifuged at 4000 × *g* for 20 minutes and made up to 50.0 ml (Step 2). To the supernatant was added 5.0 ml of 0.02 M acetic acid in the cold, and the precipitate obtained on centrifugation at 4000 × *g* for 10 minutes was rejected. The supernatant was dialyzed against water in the cold for 18 hours, and the clear dialyzed extract was used as the enzyme (Step 3). An 80-fold purification with 81% recovery was achieved, and this partially purified enzyme was used in all further investigations.

RESULTS

pH-Optima—The effect of pH on the activity of the crude extract with and without Fe^{+++} is shown in Fig. 1*A* and of the purified enzyme in Fig. 1*B*. In the absence of Fe^{+++}, the extract was active only at acid pH, with a maximum at pH 4.3. In the presence of Fe^{+++}, however, the acid phosphatase activity was inhibited and a new peak at pH 7.65 appeared. In the case of the purified enzyme, acid phosphatase activity remained unaffected in the presence of Fe^{+++}, and the optimal pH of the alkaline phosphatase was shifted to 8.5. Tris(hydroxymethyl)-aminomethane chloride, glycine-NaOH, boric acid-borax, and Veronal-acetate buffers were tried in the alkaline range in the presence of Fe^{+++}. It was found that Veronal-acetate was the best buffer, as maximal activity was obtained in it and there was no change in pH optimum.

Substrate Specificity—The substrate specificity of the purified enzyme is given in Table II. The preparation was found to catalyze the hydrolysis of β-glycerophosphate, inorganic pyrophosphate, hexametaphosphate, ATP, 3′-AMP, 5′-AMP, ribo-

* Synonymous with "mung bean."

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flavin 5′-phosphate, and glucose 1-phosphate at pH 5.0 in the absence of metal ion. The rate of hydrolysis of these substrates was, however, low. In the presence of Fe^{+++} all these activities were inhibited. The alkaline Fe^{+++}-requiring phosphatase showed an absolute specificity for sodium β-glycerophosphate. In the presence of Mg^{++}, there was no activity against β-glycerophosphate but sodium pyrophosphate, glucose 6-phosphate, hexose 1,6-diphosphate, fructose 6-phosphate, 5′-AMP, 3′-AMP, and ATP were hydrolyzed.

Influence of Temperature—The effect of temperature on the rate of hydrolysis was examined over the range 20 to 100°. The optimal temperature was 49° with rapid inactivation at higher temperatures (Fig. 2).

Effect of Substrate Concentration—The Michaelis-Menten constant, K_m, was calculated from Lineweaver-Burk plots to be 6.25 μmoles per ml, and the optimal substrate concentration was 17.5 μmoles per ml at a ferric ion concentration of 2.9 μmoles per ml.

Effect of Ferric Ion Concentration—The effect of Fe^{+++} concentration is depicted in Fig. 3. It is clear that it was a critical factor. The optimal concentration was 2.9 μmoles per ml, higher concentrations being inhibitory.

Effect of Various Substances—The effect of various substances is presented in Table III. There was no activity at pH 8.5, in the absence of Fe^{+++}, but in its presence, heavy metal ions like Mn^{++}, Cu^{++}, Zn^{++}, and Fe^{++}, were highly inhibitory. Mg^{++}, which is a known activator of the alkaline phosphatase of animal tissues, was slightly inhibitory. The well known sulfhydryl in-

TABLE I

*Purification of Fe^{+++}-requiring alkaline β-glycerophosphatase**

Step†	Volume	Protein	Total activity	Specific activity	Yield
	ml	*mg*	*units*	*units/mg protein*	%
1. Extract	180	3840	15,750	4	
2. $(NH_4)_2SO_4$ fractionation	50	330	13,500	41	86
3. Supernatant from acetic acid treatment	55	40	12,810	320	81

* With the use of 20 g of powdered seeds.
† For details of steps, see text.

TABLE II

Substrate specificity

Standard assay with 0.18 mg of enzyme protein and the indicated concentrations of substrate. Activity expressed in units.

Substrate*	pH 5.0		pH 8.6		
	In absence of metal ions	In presence of Fe^{++}	In absence of metal ions	In presence of Fe^{++}	In presence of Mg^{++} (1 μmole/ml)
	units		*units*		
Sodium β-glycerophosphate (20)	71	66	Nil	70	Nil
Sodium pyrophosphate (20)	61	56	Nil	Nil	53
Sodium hexametaphosphate (20)	42	28	Nil	Nil	Nil
Riboflavin 5′-phosphate	14	7	Nil	Nil	Nil
Glucose 6-phosphate (6)	Nil	Nil	Nil	Nil	22
Fructose 6-phosphate (6)	Nil	Nil	7	Nil	19
Glucose 1,6-diphosphate (6)	Nil	Nil	6	Nil	26
Glucose 1-phosphate (6)	54	24	Nil	Nil	Nil
ATP (6)	39	7	6	Nil	23
5′-AMP (6)	19	Nil	3	Nil	15
3′-AMP (6)	12	Nil	Nil	Nil	14

* Concentration (in μmoles per ml) given in parentheses.

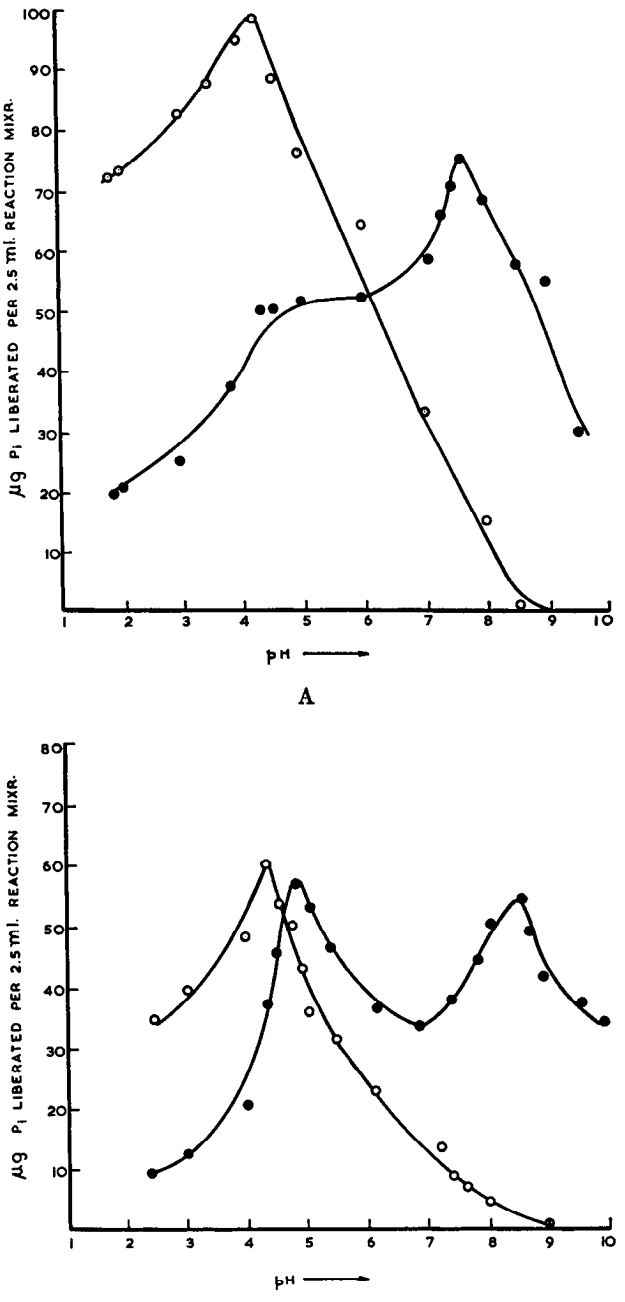

FIG. 1. Effect of pH on *Phaseolus radiatus* phosphatase: *A*, crude extract (10.6 mg of protein) and *B*, partially purified enzyme (0.18 mg of protein). Conditions of standard assay except for pH. Ten μmoles of Veronal-acetate-HCl buffer per ml were used. Sumbols: O——O, in the absence of Fe^{+++}; ●——●, in the presence of Fe^{+++}.

hibitors, *p*-chloromercuribenzoate and iodoacetate, were without effect.

The effect of sodium molybdate and sodium tungstate on acid and alkaline β-glycerophosphatase activity was studied at the levels listed in Table IV. Alkaline phosphatase was more resistant to the action of molybdate and tungstate than acid phosphatase activity.

Activation by Amino Acids—A characteristic property of alkaline phosphatase of animal origin is the marked activation by amino acids (9–12). It was therefore desirable to test the effect of amino acids on the plant alkaline β-glycerophosphatase both in the presence and absence of Fe^{+++}. The results are presented in Table V. It may be seen that in the absence of Fe^{+++}, the

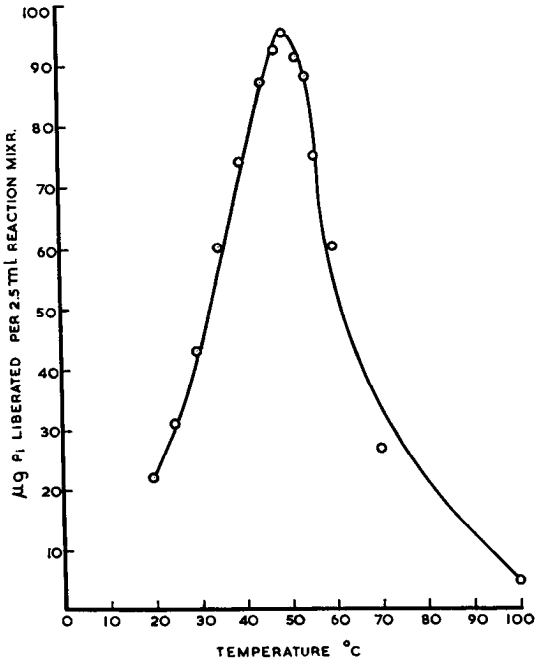

FIG. 2. Effect of temperature. Standard assay conditions except for temperature, with 0.18 mg of enzyme protein.

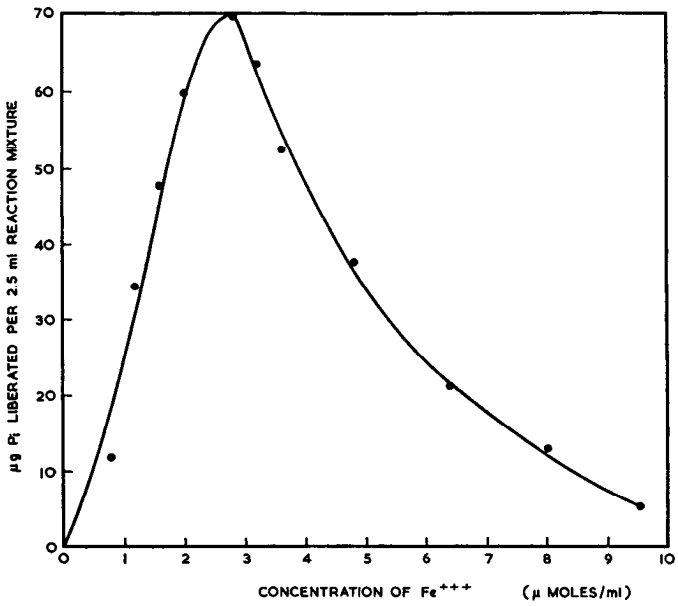

FIG. 3. Effect of Fe^{+++} concentration. Standard assay, except for Fe^{+++}, with 0.18 mg of enzyme protein.

TABLE III

Effect of various substances on alkaline β-glycerophosphatase

Conditions of standard assay with 0.18 mg of enzyme protein and 10 µmoles per ml of each compound tested.

Substance	Inhibition
	%
Manganese sulfate	33
Copper sulfate	100
Magnesium sulfate	11
Cobalt acetate	21
Mercuric chloride	87
Zinc sulfate	100
Sodium fluoride	75
Ferrous sulfate	82
Potassium dichromate	67
Potassium permanganate	100
Iodine	50
p-Chloromercuribenzoate	Nil
Iodoacetate	Nil

TABLE IV

Effect of molybdate and tungstate on alkaline β-glycerophosphatase

Conditions of standard assay with 0.18 mg of enzyme protein.

Concentration	Inhibition			
	Sodium molybdate		Sodium tungstate	
	pH 5.0	pH 8.6	pH 5.0	pH 8.6
µmoles/ml	%		%	
1.0	98	85	100	94
0.1	94	67	98	75
0.01	82	25	91	55
0.001	42	14	75	30

TABLE V

Effect of various amino acids on alkaline β-glycerophosphatase

Conditions of standard assay with 0.18 mg of enzyme protein and 10 µmoles of amino acid per ml.

Amino acid	In presence of Fe^{+++}*	In absence of Fe^{+++}
	%	*units*
Glycine	0	Nil
DL-α-Alanine	+8	Nil
DL-β-Alanine	+18	Nil
DL-α-Aminobutyric acid	+14	Nil
DL-β-Aminobutyric acid	0	Nil
DL-γ-Aminobutyric acid	0	Nil
DL-Aspartic acid	0	33
L-Glutamic acid	0	Nil
DL-Histidine	−31	56
L-Proline	0	Nil
DL-Methionine	0	Nil
L-Cysteine	+40	42
DL-Glutathione	+80	24
L-Cystine	−16	Nil

* Symbols: (+) = activation; (−) = inhibition.

enzyme was activated by aspartic acid, histidine, cysteine, and glutathione. The other amino acids tried were without effect. In the presence of Fe^{+++}, however, the pattern of amino acid activation was entirely different. Whereas α-alanine, β-alanine, α-aminobutyric acid, glutathione, and cysteine showed low but definite activation, aspartic acid showed no activation, and cystine and, surprisingly enough, histidine were inhibitory.

DISCUSSION

An alkaline β-glycerophosphatase activity as reported here had not previously been observed in plants. The optimal pH of the green gram enzyme (8.5 in the presence of 2.9 μmoles per ml of Fe^{+++}) is similar to that of bone alkaline phosphatase which has an optimal range of 8.6 to 9.0. Our enzyme is not identical with the plant alkaline pyrophosphatase of Naganna *et al.* (4, 5) because it is completely inactive against pyrophosphate in the presence of Fe^{+++}, and Mg^{++} has no activating effect.

Williams and Watson (13) showed that sulfhydryl groups were not involved in bone phosphatase activity. In the case of the green gram enzyme, —SH reagents, like *p*-chloromercuribenzoate and iodoacetate, were also without effect. Iodine, permanganate, and heavy metal ions were, however, inhibitory. The inactivation of the enzyme by strong oxidants can be explained as the result of the oxidation of the constituent amino acids such as tyrosine in the enzyme molecule (14).

It should be noted that Mg^{++} which activates the animal alkaline phosphatase and plant alkaline pyrophosphatase was completely inactive in our system. The enzyme displayed a high degree of specificity both with regard to the substrate in the alkaline range and to the requirement for Fe^{+++}. The difference in susceptibility to molybdate and tungstate of the acid and alkaline phosphatase activities of green gram is quite striking and is in agreement with the results obtained by Naganna *et al.* (4) with the acid and alkaline pyrophosphatase of potato.

Our results on activation by amino acids are anomalous. No definite conclusions could be drawn as to the effect of chain length and number of carboxyl groups. β-Alanine was more effective than α-alanine, but, whereas α-aminobutyric acid showed an activating effect, β- and γ-aminobutyric acids were completely inactive. The activating effect of histidine and aspartic acid in the absence of Fe^{+++} is remarkable and may be due to the removal of inhibitory metal ions by chelation.

The present findings stress the importance of metal ions like Fe^{+++} in plant metabolism and also emphasize the need for using a variety of experimental conditions when looking for the presence of a particular enzyme in biological systems.

SUMMARY

The occurrence of an alkaline β-glycerophosphatase has been observed for the first time in plants. This enzyme is activated by ferric ions. Partial purification of the enzyme from extracts of green gram seeds (*Phaseolus radiatus*) has been achieved by ammonium sulfate fractionation and acid precipitation. The enzyme functioned optimally at a pH of 8.5, at 49°, and at a ferric ion concentration of 2.9 μmoles per ml. The Michaelis-Menten constant, K_m, was found to be 6.25 μmoles per ml. Heavy metal ions were inhibitory. In the absence of Fe^{+++}, the enzyme was activated by aspartic acid, histidine, cysteine, and glutathione. *p*-Chloromercuribenzoate and iodoacetate were without effect. In the presence of Fe^{+++} the enzyme was highly specific for β-glycerophosphate.

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