EXPERIMENTAL

Seeds of *Vicia faba* L. (var. Triple White) were purchased from the Tyneside Seed Company, Gateshead, Co. Durham, U.K. Commercial soya flour (S200W) was obtained from Central Soya Chicago, U.S.A. Purification of legumin and separation of its subunits was performed as described previously [4]. A reasonably pure glycinin preparation was obtained as the cold-insoluble fraction from a total H_2O extract (incorporating 0.5 mM dithiothreitol and 0.02% (w/v) NaN_3 of soya flour. This was purified further by zonal isoelectric precipitation [4] using modified conditions. The basic subunits of glycinin were prepared by the method used for the separation of legumin subunits, except that the protein was not carboxymethylated and 10 mM 2-mercaptoethanol was included in all buffers. *N*-Terminal amino acid sequences were determined using a Beckman 890c automatic sequencer. Methods were as those described in ref. [8].

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FURTHER CHARACTERIZATION OF PHYTASE FROM *PHASEOLUS AUREUS**

I. B. MAITI† and B. B. BISWAS
Department of Biochemistry, Bose Institute, Calcutta 700 009, India

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Key Word Index—*Phaseolus aureus*; Leguminosae; mungbean; phytase; myoinositol phosphates.

Abstract—Phytase purified to homogeneity from germinated mungbean cotyledons was inhibited by EDTA although it did not show any absolute requirement for divalent cations. Sodium fluoride, sodium citrate, mercaptoethanol and *p*CMB also inhibit the phytase activity but L-phenylalanine has no effect on activity. The phytase has a low affinity for inositol monophosphate. The relative rate of dephosphorylation of myo-inositol-1-phosphate and myo-inositol-5-phosphate by phytase is 6 and 18% respectively of that of myo-inositol-hexaphosphate. Mungbean phytase cannot cleave myo-inositol-2-phosphate, 1,2-cyclic inositol phosphate, Na-β-glycerophosphate or *p*-nitrophenylphosphate. The relative rates of hydrolysis of different isomers of inositol hexaphosphate are in the following order: myo-IP_6 > neo-IP_6 > scyllo-IP_6 = D-chiro-IP_6 > L-chiro-IP_6. This enzyme seems to be most active with myo-inositol hexaphosphate.

INTRODUCTION

During the germination of seeds, dephosphorylation of phytate is mediated through phytase (myo-inositol-hexaphosphate phosphohydrolase, EC 3.1.3.8) [1–4]. The mode of action of phytase from other sources had already been partly elucidated [5, 6]. We have purified a phytase from germinating mungbean seeds and shown that the dephosphorylation of myo-inositol-hexaphosphate is sequential [7]. The present paper deals with the further characterization of the enzyme and its specificity towards different substrates and the end product of dephosphorylation reaction.

RESULTS AND DISCUSSION

Effect of divalent and monovalent ions on phytase activity

Activity was increased to *ca* 30–50% above the control

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† Present address: Department of Agricultural Chemistry, Washington State University, Pullman, WA 99164, U.S.A.

Abbreviations: *p*CMB, *p*-chloromercuribenzoate; IP, IP_2, IP_3, IP_4, IP_5, IP_6, Inositol mono-, di-, tri-, tetra-, penta-, and hexaphosphate respectively.

in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} at 2 mM, but at higher concentration the activity was inhibited. This is due to the precipitation of phytate as insoluble metal salts. There does not seem to be an absolute requirement for these cations. However, this phytase like animal phytase [8] is inhibited by EDTA *ca* 85% at 5 mM. This is in contrast to *Aspergillus ficuum* phytase [6]. The monovalent cations, Na^+, K^+ and NH_4^+ have no effect on mungbean phytase activity.

Effect of NaF, Na-*citrate*, L-*phenylalanine, mercaptoethanol and* p*CMB on phytase activity*

L-Phenylalanine is without any appreciable effect. This is in contrast to animal phytase [8]. NaF, Na-citrate and mercaptoethanol cause inhibition. *p*CMB inhibits this enzyme, and the Lineweaver–Burk plot showed two straight lines with the same intercept ($1/V_{max}$), indicating that the inhibition is competitive. The K_i was *ca* 0.5×10^{-4} M.

Substrate specificity of phytase

Previously it was found by Mandal *et al.* [4] that the rate of hydrolysis of inositol phosphates by phytase tends to increase as the number of phosphate groups decreases. Since this preparation of phytase was freed from nonspecific phosphatase through polyacrylamide gel electrophoresis, it was tested with different phospho-compounds and with different inositol monophosphates. The inositol monophosphates show low affinity for phytase. Phosphate at the C-2 position of myoinositol (i.e. myo-inositol-2-P) has been found to be resistant to phytase action. The rate of hydrolysis of inositol-1-phosphate and inositol-5-phosphate is 6 and 18% respectively of that of myo-inositol hexaphosphate. It cannot cleave 1,2-cyclic inositol phosphate and *p*-nitrophenylphosphate.

Rate of dephosphorylation of different isomers of inositol hexaphosphate by mung bean phytase

This phytase is more active with myo-inositol hexaphosphate. The rate of dephosphorylation of different inositol hexaphosphate was measured under the same condition. The susceptibility of hydrolysis of different isomers is as follows: myo-IP_6 > neo-IP_6 > scyllo-IP_6 = D-chiro-IP_6 > L-chiro-IP_6. The progress of hydrolysis of different isomers of inositol hexaphosphate was also studied at 37° at an interval of 20 min. Myo-inositol hexaphosphate is hydrolysed most readily, L-chiro-inositol hexaphosphate is most resistant. The progress curve of D-chiro-IP_6 and scyllo-IP_6 is almost identical. Neo-IP_6 is hydrolysed linearly although the rate is much slower than that of myo-IP_6 but greater than that of L-chiro-, D-chiro and scyllo-IP_6. In the case of myo-inositol ester the rate of the reaction decreases after 20 min and a plateau is reached after 40 min. However, in case of *Aspergillus ficuum* phytase [6] the order of susceptibility of hydrolysis is as follows: L-chiro-IP_6 > D-chiro-IP_6 > myo-IP_6 > neo-IP_6 > Scyllo-IP_6 at pH 2.5 and neo-IP_6 > L-chiro-IP_6 > D-chiro-IP_6 > myo-IP_6 > Scyllo-IP_6 at pH 5.3.

Irving and Cosgrove [5] have proposed a model of an active centre satisfying the stereochemical requirement for the *Pseudomonas* phytase. According to them (i) the phosphate group hydrolysed first to yield pentaphosphate is usually a member of a pair of vicinal *trans*-equatorial group. Such a pair of phosphate groups can exist in one of two mirror images; (ii) the axially oriented C-2 phosphate group is important for the proper function of the enzyme; (iii) in the case of pentaphosphate and lower phosphate ester of inositol the phosphate group vicinal to hydroxyl group is preferentially cleaved by the enzyme. Mung bean phytase produces myo-inositol-1,2,3,4,5-pentaphosphate and 1,2,3,4-tetraphosphates [7]. In the case of this phytase the C5-C6 pair of phosphate groups seems to be involved in binding with the active site. Neo-inositol hexaphosphate has a single pair of phosphate groups C3-C4 with the required stereochemistry. The rate of hydrolysis of neo-IP_6 and myo-IP_6 is almost the same in the case of *Pseudomonas* phytase. In the case of mung bean phytase the rate of hydrolysis of neo-IP_6 is almost half of that of myo-IP_6. It appears that the mung bean phytase preferentially binds with the C5-C6 pair of phosphate group over the C3-C4 pair of phosphate as in neo-IP_6. The slow hydrolysis of scyllo-IP_6 may be due to its lack of an axially oriented C2 phosphate group which is largely essential for the proper function of the enzyme.

EXPERIMENTAL

Materials. Seeds of *Phaseolus aureus* cv B-1 were obtained from Seed Multiplication Farm, Berhampore, West Bengal. Various isomers of inositol hexaphosphate were kindly donated by Dr. D. J. Cosgrove, Division of Plants Industry, CSIRO, Australia; Dr. C. E. Ballou, University of California, Berkeley, U.S.A. kindly supplied inositol-1-phosphate and inositol-5-phosphate. 1,2 Cyclic inositol phosphate was obtained from Dr. A. Lahiri Majumder, National Institute of Health, U.S.A. Myo-inositol and inositol-2-phosphate were purchased from Sigma Chemical Co. U.S.A.

Purification of phytase. The isolation procedure has been outlined in refs. [4] and [7].

Assay was according to ref. [4]. Incubation was normally carried out at 37° for 30–60 min. The liberated Pi was estimated as in ref. [4]. The enzyme purified after the polyacrylamide gel electrophoresis step was used.

PC of inositol phosphate was according to the method of ref. [9] using the solvent *iso* PrOH–NH_4OH–H_2O (7:1:2) with Whatman No. 1 paper. The chromatograms were developed (descending) at 35° for 48 hr. The compounds were detected by the modified phosphate spray of ref. [10] or [11]. The $AgNO_3$ reagent was used for glycol. Inositol phosphates were isolated, separated and characterized by the method of ref. [12].

The end product of mung bean phytase hydrolysis. Myo-inositol hexaphosphate was hydrolysed extensively with mung bean phytase and the reaction product was applied to a Dowex-1-Cl^- column (16 × 0.9 cm) as described to separate the different inositol phosphates. Inositol monophosphate was eluted and re-chromatographed on Dowex-1-Cl^- column (8 × 0.5 cm) to free it from any contamination of Pi. This short column was washed with 10 ml of 0.03 M HCl to elute Pi. Inositol monophosphate was eluted with 55 mM HCl. This was concd *in vacuo* in the cold and identified as inositol-2-phosphate by PC.

Disc electrophoresis in 5% polyacrylamide gel, performed according to the method of ref. [13], was carried out at pH 8 for 2 hr with a current of 3 mA/tube. One of the gels was stained with 0.2% Coomassie blue and another was used for elution of enzyme.

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PHOSPHOFRUCTOKINASE OF CARROT ROOTS

ROBERT A. CALDWELL and JOHN F. TURNER
Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia

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Key Word Index—*Daucus carota*; Umbelliferae; carrots; phosphofructokinase; monovalent cations; P-enolpyruvate; regulation of glycolysis; salt respiration.

Abstract—Phosphofructokinase was partially purified from carrot root extracts. Monovalent cations stimulated carrot phosphofructokinase activity. The enzyme was strongly inhibited by P-enolpyruvate and this inhibition was relieved by NaCl or KCl. Pi inhibited the enzyme at pH 7.9 but was stimulatory at pH 6.6.

INTRODUCTION

The properties of phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) have been studied with preparations from a variety of tissues. Phosphofructokinases from both mammalian tissues [1, 2] and higher plants [3] are inhibited by ATP and citrate and, in general, stimulated by Pi. A distinctive property of the plant enzyme is the strong cooperative inhibition given by low concentrations of P-enolpyruvate [4, 5] and by relatively low concentrations of 2-P-glycerate and 3-P-glycerate [6]. It is believed that the effects of metabolites on the activity of phosphofructokinase are of major significance in the control of glycolysis in plants [3, 7].

In view of the regulatory significance of phosphofructokinase, the study of the plant enzyme has been extended to include carrot roots which have been used extensively for physiological work. The present communication describes the effects of monovalent cations, P-enolpyruvate and Pi on carrot phosphofructokinase.

RESULTS

There was considerable activation of carrot phosphofructokinase by monovalent cations. The concentration of Na^+ in the assay mixture without added monovalent cation was 1.5 mM and the further addition of 12 mM (final concentration) LiCl, NaCl, KCl and RbCl increased phosphofructokinase activity by 40, 53, 59 and 47%, respectively. As most of the modifiers of phosphofructokinase activity used in this investigation were Na salts, the standard assays were performed at constant (24 mM) Na^+ ion concentration.

Carrot phosphofructokinase was subject to strong cooperative inhibition by low concentrations of P-enolpyruvate (Fig. 1). Increasing the concentration of Na^+ in the reaction mixtures relieved the inhibition and increased the degree of cooperativity shown in the P-enolpyruvate inhibition. Hill plots for P-enolpyruvate had slopes of 2.2, 2.5 and 3.1 with Na^+ ion concentrations 1.5, 12 and 24 mM, respectively. The addition of KCl also relieved the inhibition by P-enolpyruvate.

The effect of increasing concentrations of Pi on the activity of carrot phosphofructokinase at different concentrations of $MgCl_2$ is shown in Fig. 2. At pH 7.9 Pi inhibited the enzyme except at low concentrations of both $MgCl_2$ and Pi when a slight stimulation was observed. When the pH of the reaction mixtures was 6.6 there was significant stimulation by Pi.

DISCUSSION

This investigation has shown that carrot root phosphofructokinase is stimulated by monovalent cations and strongly inhibited by low concentrations of P-enolpyruvate. K^+ and NH_4^+ ions are known to stimulate phosphofructokinase from a number of animal tissues [2] and yeast [8]. Na^+ and K^+ ions also had a pro-