Purification and Physicochemical Properties of Starch Phosphorylase from Young Banana Leaves†

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ABSTRACT: Starch phosphorylase from young banana leaves has been purified to homogeneity, as tested by disc polyacrylamide gel electrophoresis at various pHs and gel concentrations, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunodiffusion, and immunoelectrophoresis, using the techniques of ammonium sulfate fractionation, DEAE-cellulose chromatography, and filtration through Sephadex G-100 and Sephadex G-200. The molecular weight of the enzyme is found to be 450 000 as determined by gel filtration chromatography over Sephadex G-200. The enzyme showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis having the molecular weight 55 000. The enzyme contains eight SH groups per mol of the enzyme. Unlike other 1,4-α-glucan phosphorylases, no evidence is found for the presence of pyridoxal 5′-phosphate as a prosthetic group of the enzyme. Of the various amino acids tested, only aromatic amino acids inhibited the enzyme activity. ADP, AMP, and 3′,5′-AMP did not produce any effect on the enzyme activity whereas ATP and UDP-glucose proved to be inhibitors. The enzyme utilized starch, amylose, and glycogen as primers with equal efficiency whereas dextrin, amylopectin, maltotriose, and maltose were less effective as primers. Schardinger dextrin, cellulose, or sucrose could not be utilized as a primer. The enzyme showed absolute specificity for glucose 1-phosphate as a substrate, and this could not be replaced by glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, or ribose 5-phosphate.

Starch phosphorylase (EC 2.4.1.1; 1,4-α-glucan:orthophosphate glucosyltransferase) has been extensively studied and purified from tubers (Nakamura, 1952; Phillips & Averill, 1953; Lee, 1960a; Ariki & Fukui, 1975a), seeds (Bliss & Naylor, 1946; Porter, 1950; Hobson et al., 1950; Matheson & Richardson, 1978), and leaves (Defekete, 1968; Khanna et al., 1971, Preiss et al., 1980; Steup et al., 1980a,b; Steup, 1981; Shimomura et al., 1982). In the present study, the enzyme has been purified to homogeneity from young banana leaves, and some physicochemical properties are studied. An interesting finding is the absence of pyridoxal 5′-phosphate as the prosthetic group of the enzyme.

Materials and Methods

Chemicals. Soluble starch (Analar grade) and glycogen from BDH, amylose from Koch Light Laboratory Ltd., amylopectin from Biochemical Units, V.P. Chest Institute, Delhi, India, dextrin from Fluka, A.G. Buchs, maltotriose (grade II), Schardinger α-dextrin, bovine serum albumin, and glucose-6-phosphate dehydrogenase (grade II) from Sigma Chemical Co., maltose from E. Merck, phosphoglucomutase from Boehringer Mannheim, and cellulose from W and R Boloton Ltd. were obtained. Glucose 1-phosphate from E. Merck was purified according to the method of Kamogawa et al. (1968) by using a charcoal column. Potato phosphorylase was purified according to the method of Kamogawa et al. (1968). All the other chemicals used were of analytical grade.

Leaf Tissue. Young tender leaves preceding the flower were collected immediately before use from banana plants (*Musa paradisiaca* species) grown in the departmental garden.

Enzyme Assays. Starch Phosphorylase. Starch phosphorylase assay in the direction of polysaccharide synthesis was carried out according to the method of Green & Stumpf (1942) with slight modifications. The assay system consisted of 0.2 mL of tris(hydroxymethyl)aminomethane (Tris)–maleate buffer, pH 6.0 (0.1 M), 0.1 mL of freshly prepared soluble starch (3%), 0.1 mL of sodium fluoride (0.2 M), and 0.5 mL of enzyme preparation and water. The reaction was started by the addition of 0.1 mL of glucose 1-phosphate (0.05 M) and incubated at 30 °C. At the end of 30 min, the reaction was stopped by the addition of 1.0 mL of 10% trichloroacetic acid. In case of crude preparation, containing Triton X-100, a pinch of Norite A was also added. The precipitate was removed by centrifugation in the cold and inorganic phosphate in the clear supernatant determined according to the method of Fiske & Subbarow (1925). A control which received glucose 1-phosphate after deproteinization was simultaneously run. In some experiments the enzyme was assayed by determining the polysaccharide formed using maltose as a primer. The enzyme assay was carried out as above, but the reaction was terminated by keeping the tubes in a boiling water bath for 1 min, and polyglucoside formed was determined in 0.5 mL of the clear supernatant by adding 7.5 mL of iodine reagent (10 mg of I_2 plus 100 mg of KI plus 3 mL of 1 N HCl in 100 mL volume) and measuring the color in a Klett Summerson photocolorimeter using a red filter.

In the direction of polysaccharide degradation, the assay system consisted of 0.2 mL of Tris–maleate buffer, pH 7.5 (0.2 M), 0.1 mL of freshly prepared soluble starch (5%), 0.1 mL of sodium fluoride (0.2 M), and 0.5 mL of enzyme preparation and water. The reaction was started by the addition of 0.1 mL of inorganic phosphate, pH 7.5 (0.5 M), and incubated at 30 °C for 30 min. The reaction was stopped by keeping the tubes in a boiling water bath for 1 min, and the precipitate, if formed, was removed by centrifugation in the cold. Glucose 1-phosphate formed was measured in the clear supernatant by a coupled enzyme assay using phosphoglucomutase and glucose-6-phosphate dehydrogenase (Bergmeyer & Klotzsch, 1965).

Enzyme Unit. One unit of the enzyme (nkat) is the amount of the enzyme that causes the conversion of 1 nmol of the substrate into product in 1 s under the experimental conditions.

† From the Department of Biochemistry, Lucknow University, Lucknow, India. *Received January 14, 1982.* This work was supported by grants from the University Grants Commission, New Delhi, under the U.G.C. Special Assistance Programme. This department received generous grants from UNESCO/UNDP.

0006-2960/82/0421-4152$01.25/0

Specific activity is described as nanokatals per milligram of protein.

Other Enzymes. Phosphoglucomutase and phosphohexoisomerase were assayed according to Gibbs & Turner (1964), unspecific phosphatase was assayed according to Heppel (1955) by using glucose 1-phosphate as the substrate, ATPase was assayed according to Kielley (1955), branching enzyme was assayed according to Larner (1955) by using amylose or amylopectin as a substrate but the reaction was stopped by adding an aliquot of the reaction mixture into I_2 reagent instead of using perchloric acid, amylases were assayed according to Bernfeld (1955) based on reducing sugar estimation, and debranching enzyme (EC 3.2.1.41) was assayed according to Brown & Brown (1966) by using amylopectin as a substrate but measuring the release of oligosaccharide chains of glucose units by virtue of their reducing power according to Somogyi (1952). Ribulosebisphosphate carboxylase was assayed according to the procedure of Racker (1962) by using a coupled enzyme assay method.

Protein determination was done according to the procedure of Lowry et al. (1951) as modified by Khanna et al. (1969), using bovine serum albumin as a standard.

Enzyme Purification. Unless otherwise stated, the following steps were carried out at 0–4 °C.

(*1*) *Initial Extract.* After the midrib was removed, 35 g of leaf tissue was cut into small pieces and blended for 30 s at low speed and 50 s at high speed with 350 mL of the medium consisting of 0.01 M Tris-HCl buffer, pH 7.5, 0.02 M 2-mercaptoethanol, and 0.05% Triton X-100. The homogenate was filtered through two layers of muslin and centrifuged at 1600*g* for 30 min.

(*2*) *Ammonium Sulfate Fractionation.* To the supernatant, was added slowly ammonium sulfate (61.6 g) with constant stirring to give 0–0.3 salt saturation, maintaining the pH at neutrality by the addition of dilute ammonia. The suspension was centrifuged at 1600*g* for 1 h, and the supernatant was treated with ammonium sulfate (75 g) to get 0.3–0.6 salt saturation. After the suspension was kept overnight, it was centrifuged at 1600*g* for 1 h, and the precipitate was collected and suspended in buffer A (0.01 M Tris-HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol), using pestle and mortar. The suspension on centrifugation at 15000*g* for 30 min yielded a clear supernatant which was desalted by passing through a column of Sephadex G-25.

(*3*) *DEAE-cellulose Chromatography.* The desalted enzyme fraction was loaded onto a DEAE-cellulose column (20 × 1.4 cm), previously equilibrated with buffer A. The column was washed with 3 bed volumes of the above buffer and the enzyme eluted by using a linear NaCl gradient (0–1 M). The active fractions, constituting a single peak, were pooled. To this was added solid ammonium sulfate to 0.8 saturation and the suspension kept overnight for precipitation. The precipitate was collected by centrifugation at 15000*g* for 30 min and dissolved in 3 mL of buffer A.

(*4*) *Sephadex G-100 Chromatography.* A Sephadex G-100 column (80 × 1.4 cm) was prepared and equilibrated with buffer B (buffer A containing 0.05 M NaCl). The enzyme fraction after step 3 was passed through the column and washed with buffer B. The enzyme, obtained immediately after the void volume, was concentrated by precipitation with ammonium sulfate as described in step 3 and dissolved in 1 mL of buffer A.

(*5*) *Sephadex G-200 Chromatography.* A Sephadex G-200 column (80 × 1.4 cm) was equilibrated with buffer B. The enzyme fraction after step 4 was loaded onto the column and subsequently chromatographed with buffer B. Fractions (1 mL) were collected at a flow rate of 5 mL/h and the active fractions pooled.

(*6*) (*a*) *Affinity Chromatography.* Starch-bound Sepharose 4B was prepared according to Matheson & Richardson (1976). A starch-bound Sepharose 4B column (15 × 1.4 cm) was equilibrated with 0.02 M sodium citrate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol. Sephadex G-200 effluent (after concentrating as described in step 3 of the purification procedure and subsequently desalting) was loaded onto the column and washed with 0.02 M sodium citrate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol followed by 1 M NaCl–60 mM sodium citrate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol. The enzyme was eluted by using a starch gradient (0–2%) in 0.06 M sodium citrate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol and 1 M NaCl.

(*b*) *Crystallization of the Purified Enzyme.* Ammonium sulfate was added to 0.4 saturation to the pooled Sephadex G-200 effluent when slight turbidity was observed. Complete crystallization of the enzyme occurred in 4 days on storing the fraction at 0–4 °C.

Polyacrylamide gel electrophoresis was carried out according to the method of Davis et al. (1967) with some modifications. In each case, the spacer gel contained 3% acrylamide and 0.75% methylene bis(acrylamide) in 0.05 M, pH 6.5, sodium citrate buffer. In the separating gel, the concentration of methylenebis(acrylamide) was kept 0.2%, but the concentration of acrylamide varied to 5, 6, or 7.5%; the buffer was either 0.05 M Tris-HCl or sodium citrate. The electrode buffer was the same as the buffer in the separating gel. A current of 8 mA/tube was used. Bromophenol blue, used as a front indicator, reached the other end of the gel in about 1.5–2 h. At this stage there was little movement of the protein. The time of electrophoresis run was, therefore, fixed in each case at 3.5 h. In a typical experiment, electrophoresis was run in each case (different pHs and gel concentrations) for different times between the time of reaching the bromophenol blue at another end and 3.5 h at 15-min intervals to check the presence of any low molecular weight protein. The amount of the protein applied was 80–100 μg. After electrophoresis, gels were stained with Amido Black, and excess dye was removed by immersing gels overnight in 7% acetic acid. For the enzyme activity, the gels, after electrophoresis, were incubated for 3 h in a mixture consisting of 0.03% soluble starch (freshly prepared), 0.02 M NaF, 0.02M Tris–maleate buffer, pH 6.0, and 5 mM glucose 1-phosphate and then stained with I_2 reagent (Krisman, 1962). Place(s) showing starch phosphorylase activity became dark blue with light background, as described earlier (Kumar & Sanwal, 1977). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was done in 7% gel at pH 7.5 according to the method of Weber & Osborn (1969) as modified by Stoklasa & Latz (1974). For denaturion, the protein solution was kept at 100 °C for 5 min with 1% sodium dodecyl sulfate and 0.2 M 2-mercaptoethanol (Pringle, 1970). Commassie brilliant blue was used for staining protein bands.

Determination of Sulfhydryl Groups. The purified enzyme sample was dialyzed in the cold for 45 h against 0.01 M Tris-HCl buffer, pH 7.5, with several changes to remove 2-mercaptoethanol. In the dialyzed sample (0.9 mg of protein/mL), the number of –SH groups was determined by titration against 5,5′-dithiobis(2-nitrobenzoic acid) according to the method of Ellman (1959). The reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was also performed in the presence of 1% sodium dodecyl sulfate.

Table I: Purification of Starch Phosphorylase from Young Banana Leaves

fraction	total vol (mL)	total act. (nkat)	total protein (mg)	sp act. (nkat/ mg of protein)	fold enrichment	recovery (%)
(1) initial extract	350	138.9	545	0.25	1	100
(2) 30–60% $(NH_4)_2SO_4$	58	83.5	168	0.50	2	60
(3) DEAE-cellulose chromatography	50	56.5	18.1	3.12	12	41
(4) Sephadex G-100 chromatography	6	42.1	3.8	11.08	44	30
(5) Sephadex G-200 chromatography	3	38.9	1.4	27.79	111	28
(6) (a) affinity chromatography	4	34.9	1.35	25.85	103	25
(b) crystallization	2	24.8	1.0	24.80	99	18

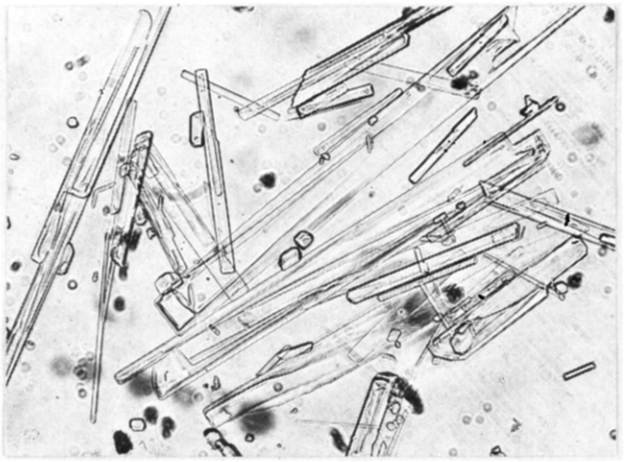

FIGURE 1: Photomicrograph of crystals of starch phosphorylase purified from young banana leaves (280×).

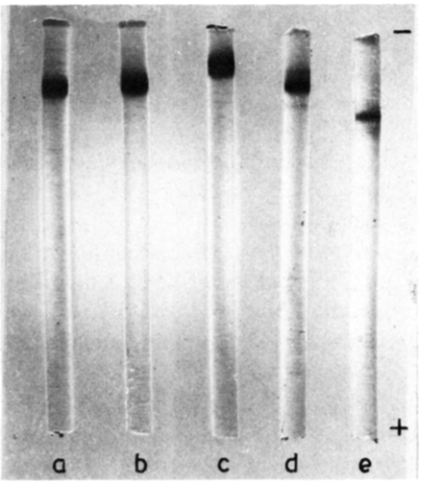

FIGURE 2: Polyacrylamide gel electrophoresis showing homogeneity of starch phosphorylase. Electrophoresis was carried out at pH 7.5 by using (a) 5% (b) 6%, and (c) 7.5% gel concentration and at pH (d) 6.5 and (e) 8.5 by using 6% gel concentration. At pH 6.5, 0.05 M sodium citrate buffer was used, and at pH 7.5 and 8.5, 0.05 M Tris-HCl buffer was used.

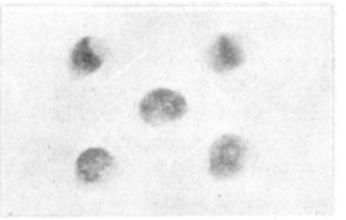

FIGURE 3: Immunodiffusion showing cross-reaction of starch phosphorylase against its antiserum. The central well contains antiserum. The surrounding wells contain purified enzyme protein (antigen).

Preparation of Antiserum. A 1.5-mg sample of purified enzyme protein in 1 mL of 0.01 M Tris-HCl buffer, pH 7.5, was mixed with an equal volume of Freund's complete adjuvant and was injected subcutaneously at several sites in a rabbit. After 2 weeks, another injection containing 2 mg of enzyme protein mixed with an equal volume of Freund's complete adjuvant was given to the same rabbit. After the last injection, blood was taken weekly by puncturing the heart and was tested for the antibodies. After 6 weeks of the last injection, antibodies were present in the blood. Antiserum was prepared from the blood containing antibodies.

Immunodiffusion. Immunodiffusion was done according to the method of Ouchterlony (1949) by using 1.5% agar gel.

Immunoelectrophoresis. Immunoelectrophoresis was done according to the method of Garber & Williams (1955). Borate buffer (0.05 M), pH 8.8, was used as the electrode buffer during electrophoresis.

Results

Enzyme Purification. The summary of the purification of starch phosphorylase from banana leaves is given in Table I.

Ammonium sulfate fractionation of the initial extract followed by chromatography on DEAE-cellulose and gel filtration through Sephadex G-100 and Sephadex G-200 resulted in 110-fold purification of starch phosphorylase with 28% recovery from the initial extract. The specific activity of the enzyme (27.8) was about $^1/_{14}$ that of the potato enzyme (Kamogawa et al., 1968), about $^1/_{14}$ of banana fruit (Singh & Sanwal, 1973), about $^1/_{10}$ of the cytoplasmic spinach leaf enzyme (Steup et al., 1980a), about $^1/_4$ of the chloroplastic spinach leaf enzyme (Steup, 1981), and about $^1/_7$ of pea seed (Matheson & Richardson, 1978) phosphorylase. The further purification of the enzyme by affinity chromatography using starch-bound Sepharose-4B did not increase the specific activity of starch phosphorylase. The specific activity of the enzyme also did not increase on crystallization of the enzyme protein. A photomicrograph of the crystals is shown in Figure 1.

Purity of the Enzyme Preparation. The enzyme after Sephadex G-200 chromatography was found homogeneous on polyacrylamide gel electrophoresis using 6% gel at pH 6.5, 7.5, and 8.5 and at pH 7.5 using 5% and 7.5% gel (Figure 2). The protein band also showed starch phosphorylase activity, confirming that the protein band is of starch phosphorylase only.

The purified enzyme preparation was free from phosphoglucomutase, phosphohexoisomerase, unspecific phosphatase, ATPase, amylases, ribulosebisphosphate carboxylase, and branching and debranching enzymes. The contamination was tested with 100 μg of enzyme protein. Ribulosebisphosphate carboxylase contamination was also tested with 300 μg of enzyme protein. Starch phosphorylase assays were usually carried out with 20–30 μg of enzyme protein.

On immunodiffusion, only one precipitin line was present, giving further evidence of homogeneity of the enzyme preparation (Figure 3). On immunoelectrophoresis also, only one precipitin arc was present (Figure 4).

FIGURE 4: Immunoelectrophoresis of starch phosphorylase against its antiserum showing homogeneity of the enzyme preparation.

Presence of 0.1 mL of the antiserum in the assay system resulted in loss of 70–80% of the enzyme activity.

Enzyme Activity Linearity. Enzymic activity was proportional to protein up to 50 μg of the enzyme protein.

Optimum pH. Tris–maleate buffer was used in the pH range 5.2–7.5 and Tris-HCl buffer in the pH range 7.5–9.0. The enzyme preparation exhibited optimum activity at pH 6.0 in the direction of polysaccharide synthesis and at pH 7.5 in the direction of polysaccharide degradation.

Effect of Incubation Time. Enzyme activity showed linearity with time at least up to 1 h in the direction of polysaccharide synthesis and up to 3 h in the direction of polysaccharide degradation.

Effect of Temperature. In these experiments (temperature range 10–60 °C), the control tubes containing glucose 1-phosphate were also incubated at these temperatures, but the enzyme was added after the reaction was stopped. The optimum temperature was 40 °C when the incubation period was fixed at 30 min. Linearity in the enzyme activity was tested at each temperature up to at least 45 min. By use of the data, a plot of 1/absolute temperature vs. log of the velocity was drawn. From the slope of the plot, the energy of activation of the enzyme was found to be 11.3 K·cal/(°C·mol) according to the Arrhenius equation.

Effect of Ions. The metal chelating agent ethylenediaminetetraacetic acid (EDTA) and ions such as F^-, ClO_4^-, and SO_4^{2-} tested in 10 mM concentration did not affect the enzyme activity, but Ag^+, Hg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} tested in 2 mM concentration inhibited 78, 100, 100, 73, and 73%, respectively.

Effect of Sulfhydryl Group Binding Agent. The sulfhydryl group binding agent *p*-(chloromercuri)benzoate was inhibitory to the enzyme activity (0.5 mM Pcmb produced 60% inhibition) which could be almost completely reversed by 10 mM 2-mercaptoethanol.

Effect of Glucose, Glycolytic and Krebs Cycle Intermediates, Pyridoxal 5′-Phosphate and Amino Acids. Glucose, the glycolytic intermediates, namely, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, phosphoenolpyruvate, and 3-phosphoglycerate, the Krebs cycle intermediates, namely, succinate, α-oxoglutarate, and malate, and pyridoxal 5′-phosphate, each tested at 10 mM concentration, did not produce any significant effect at pH 5.5, 6.0, and 7.0.

Various amino acids, namely, glycine, L-alanine, L-cysteine, L-isoleucine, L-lysine, DL-serine, L-serine, L-methionine, L-valine, L-proline, L-threonine, DL-leucine, L-leucine, L-hydroxyproline, L-arginine, L-histidine, L-aspartic acid, DL-aspartic acid, and L-glutamic acid, each tested at 10 mM concentration at pH 6.0, did not show any effect on the enzyme activity. In contrast, aromatic amino acids considerably inhibited the enzyme activity. L-phenylalanine tested at 3 mM concentration produced 41, 44, and 54% inhibition at pH 5.5, 6.0, and 7.0, respectively. L-Tyrosine tested at 3 mM concentration caused 51, 59, and 68% enzyme inhibition at pH 5.5, 6.0, and 7.0, respectively. DL-Tryptophan tested at 3 mM concentration caused 44, 49, and 64% inhibition at pH 5.5, 6.0, and 7.0, respectively.

Effect of Nucleotides, Nucleosides, and Free Bases. The effect was tested at two levels of glucose 1-phosphate, namely, 0.5 (unsaturated concentration) and 5 mM (saturated concentration). 5′-AMP, ADP, 3′,5′-AMP, GTP, GMP, adenosine, guanosine, and cytosine, each tested at 5 mM concentration, did not produce any effect on the enzyme activity. Adenine at 5 mM concentration inhibited the enzyme activity by 13 and 81% at pH 5.5 and 7.0, respectively. Guanine at 1 mM concentration produced 19 and 54% inhibition at pH 5.5 and 7.0, respectively.

ATP proved to be a powerful inhibitor when tested in the presence of a nonsaturating level of glucose 1-phosphate. There was about 50% inhibition at 1 mM ATP. However, no inhibition by ATP was observed when tested at saturating levels of glucose 1-phosphate. The inhibition by 1 mM ATP was not reversed by AMP and ADP tested in 10 mM concentration. The inhibition by ATP and L-tyrosine, tested at pH 6.0, was found to be additive. ATP was equally inhibitory at pH 5.5 (about 50% inhibition at 1 mM ATP) but less inhibitory at pH 7.0 (about 20% inhibition at 1 mM ATP). Like ATP, UDP-glucose was also inhibitory only at nonsaturating levels of glucose 1-phosphate. At pH 6.0, 10 mM UDP-glucose caused 44% inhibition, whereas at pH 5.5 and 7.0, 2.5 mM UDP-glucose caused 24 and 18% inhibition, respectively.

Thermal Stability. Heating the enzyme preparation at 55 °C for 5 min resulted in 42% loss in the enzyme activity. The heated preparation was found more sensitive to tyrosine inhibition compared to native enzyme. Tyrosine, tested at 4.5 mM concentration, completely inhibited the heated enzyme, whereas the native enzyme was inhibited only 43%.

Effect of Phenolic Compounds. Various phenolic compounds, namely, resorcinol, *p*-cumaric acid, cinnamic acid, 2,4-dinitrophenol, caffeic acid, gallic acid, phloroglucinol, phlorizin, phenol, dihydroxyphenylalanine, and vanillin, each tested at 1 mM concentration, and tannic acid (10 μg/mL), did not exert any effect on the enzyme activity, but at 10 mM concentration, most of the phenolic compounds inhibited the enzyme activity significantly.

Effect of Organic Acids. Organic acids, namely, malonic acid, oxalic acid, and oxaloacetic acid, tested at 1 and 10 mM concentrations, did not exert any effect on the enzyme activity, whereas fumaric acid and maleic acid, tested at 10 mM concentration, increased the enzyme activity by 20 and 27%, respectively.

Effect of Plant Hormones. Indoleacetic acid and gibberellic acid, tested at 10 mM concentration, did not cause any effect on the enzyme activity.

Primer Requirement. At saturated concentration of the substrate and the primer, the enzyme utilized starch, glycogen, and amylose with equal efficiency. Amylopectin was about 30% and dextrin about 63% active compared to starch, and Schardinger dextrin, cellulose and sucrose proved ineffective as primers. Maltose and maltotriose could be used as a primer but with low efficiency. Maltose and maltotriose were respectively 10 and 14% active compared to starch. The kinetic data suggested a sequential type of reaction mechanism (unpublished data). In this manner, it differs from sucrose phosphorylase which has been shown to have a ping-pong type of reaction mechanism (Silverstein et al., 1967).

Substrate Specificity. Enzyme was specific for glucose 1-phosphate as a substrate. When glucose 1-phosphate was replaced by other phosphoglycosides, e.g., glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, and ribose 5-phosphate, no enzyme activity was detected.

The K_m value, in the polysaccharide synthesis direction, for glucose 1-phosphate was 0.66 mM at a fixed concentration of starch (3 g/L) and for starch 0.19 g/L at a fixed concentration of glucose 1-phosphate (5 mM). In the polysaccharide degradation direction, the K_m value for inorganic phosphate was 10.87 mM at a fixed concentration of starch (1 g/L) and for starch 0.13 g/L at a fixed concentration of inorganic phosphate (25 mM).

Evidence for the Purified Enzyme Protein as Starch Phosphorylase. In the absence of the primer, no inorganic phosphate was liberated from glucose 1-phosphate, showing absence of phosphatase activity. The primer content was unchanged in the absence of glucose 1-phosphate as evidenced by I_2 color estimation, showing the absence of amylases as well as branching and debranching enzymes. The enzyme exhibited activity when both glucose 1-phosphate and starch were present together. Low activity was observed when starch was replaced with maltose or dextrin as a primer. In case of maltodextrin phosphorylase, low molecular weight maltodextrins are preferred compared to glycogen (Chen & Segel, 1968). The enzyme was active with 1,4-α-glucans as the primers. Assayed in the direction of polysaccharide degradation, one of the reaction products was identified as glucose 1-phosphate, by spectrophotometric technique.

Stoichiometry of the Reaction. In the presence of starch, orthophosphate utilized was stoichiometrically equal to glucose 1-phosphate produced, further confirming the identity of the enzyme.

Molecular weight was determined by gel filtration through Sephadex G-200 column (40 × 1.4 cm). Ferritin (M_r 540 000), catalase (M_r 240 000), and aldolase (M_r 147 000) were used as reference proteins. The molecular weight of the enzyme was found to be 450 000 as calculated by the method of Whitaker (1963). Upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the enzyme yielded a single protein band with a subunit molecular weight of 55 000 ± 5000. Ferritin (M_r 135 000), catalase (M_r 60 000), and aldolase (M_r 36 750) were used as reference proteins.

Search for the Presence of Pyridoxal 5′-Phosphate in Purified Enzyme. (a) Phenylhydrazine Method of Wada & Snell (1961). Samples of the purified enzyme protein (2.8 and 5.4 mg) were treated with perchloric acid (final concentration 7%); after 30 min at room temperature with occasional stirring, the precipitate was removed by centrifugation, the volume of the supernatant was made to 3.8 mL, and to this was added 0.2 mL of phenylhydrazine reagent (2% phenylhydrazine–HCl in 10 N H_2SO_4). After 10 min the absorption spectrum of the sample was taken in the range 250–450 nm. No absorption peak at or near 410 nm was observed, indicating the absence of pyridoxal 5′-phosphate. Under similar conditions, potato phosphorylase gave absorption peak at 410 nm.

(b) Absorption Spectrum. (i) The absorption spectrum of the native enzyme protein dissolved in 0.01 M Tris-HCl buffer, pH 7.5, showed a peak near 280 nm; no absorption peak near 330 nm, characteristic of pyridoxal 5′-phosphate containing phosphorylases, was found (Figure 5a). Potato phosphorylase, under identical conditions, showed a broad absorption peak near 333 nm in addition to a peak at 280 nm.

(ii) The enzyme protein dissolved in 0.1 N NaOH (final concentration) showed only one absorption peak near 280 nm (Figure 5b). Potato phosphorylase, tested simultaneously, showed an additional absorption peak near 388 nm, characteristic of enzyme protein containing pyridoxal 5′-phosphate (Peterson & Sober, 1954).

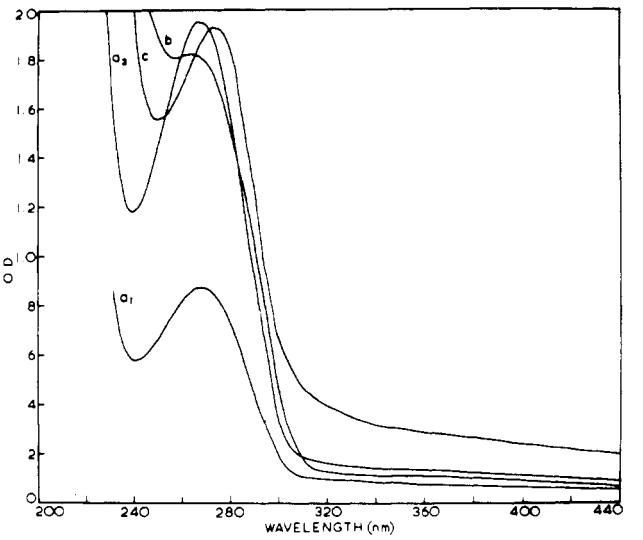

FIGURE 5: Absorption spectrum of banana leaf starch phosphorylase. (a_1) 0.64 mg of protein/mL in 0.01 M sodium citrate buffer, pH 6.5; (a_2) 2.0 mg of protein/mL in the same buffer; (b) 1.93 mg of protein/mL in 0.1 N NaOH; (c) 1.50 mg of protein/mL in 0.1 N HCl.

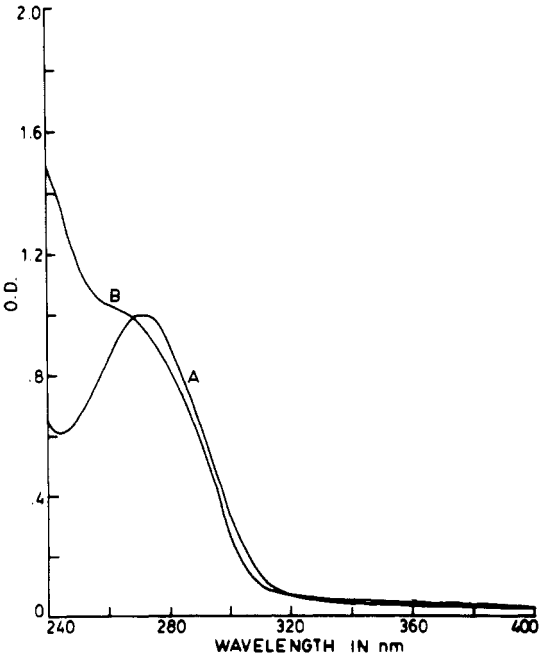

FIGURE 6: Absorption spectrum of banana leaf starch phosphorylase in (A) 6% perchloric acid and (B) 0.1 N NaOH. Amount of protein employed was 18.9 mg.

(iii) The enzyme protein was treated with HCl (0.1 N, final concentration), and the absorption spectrum was taken in the range 200–440 nm. Only one absorption peak near 280 nm was observed (Figure 5c). Potato phosphorylase, under identical conditions, showed absorption peaks at 295 and 330 nm.

On using a higher concentration of the purified enzyme (10 mg/mL) also, there was no evidence for the presence of pyridoxal 5′-phosphate.

(iv) A 18.9-mg sample of enzyme protein (7 mg/mL) was treated with 6% perchloric acid, and the absorption spectrum was taken in the range 240–400 nm. Only one absorption peak near 280 nm was observed (Figure 6). A 5.4-mg sample of potato phosphorylase (2 mg/mL), under identical conditions, showed a peak at 295 nm in addition to a broad peak near 330 nm which on addition of alkali shifted near 388 nm, charac-

teristic of pyridoxal 5′-phosphate containing enzyme protein (Peterson & Sober, 1954; Lee, 1960b).

(*c*) *Phosphate Estimation.* The purified enzyme (5 mg) was digested with concentrated H_2SO_4 and inorganic phosphate estimated by the method of Rathbun & Betlach (1969). No phosphate was detected in the enzyme protein, showing the absence of pyridoxal 5′-phosphate. Potato phosphorylase (3 mg), under similar conditions, gave a positive phosphate test. The phosphate color was developed in a total 3 mL volume, and absorbance was measured spectrophotometrically.

(*d*) *Microbiological Assay.* The enzyme sample (5 mg) was autoclaved for 30 min at 15 lb of pressure with 2 N H_2SO_4 to hydrolyze vitamin B_6 from the enzyme protein (Siegel et al., 1943), and the sample was tested for pyridoxal 5′-phosphate by the microbiological assay of Ribonosowitz & Snell (1947) with *Streptococcus faecalis* as the test organism. A negative test was observed for the banana enzyme, but under similar conditions, potato phosphorylase (1-mg sample) gave a positive test.

Effect of Hydroxylamine. Hydroxylamine had no effect on the enzyme activity when tested up to 50 mM concentration in the assay system. Incubation of the enzyme preparation with 50 mM hydroxylamine at 0–4 °C for 1 h followed by exhaustive dialysis against buffer A for 24 h did not result in loss of or decrease in activity.

Sulfhydryl Groups. Titration of –SH groups against 5,5′-dithiobis(2-nitrobenzoic acid) revealed eight –SH groups per mol of the enzyme. No change in the number of –SH groups was observed when the reaction was performed in the presence of 1% sodium dodecyl sulfate.

Serine Phosphate. No inorganic phosphate was detected in the alkali-digested (1 N NaOH, 100 °C, 20 min) sample of the enzyme protein (10 mg sample), showing the absence of serine phosphate in the enzyme molecule.

Discussion

When the specific activity of banana leaf starch phosphorylase is compared with that of other α-glucan phosphorylases, it seems that the specific activity is comparatively low. There are reports for the variation in specific activity of phosphorylase isolated from different sources. The specific activities (nkat/mg of protein) of 1350 for rabbit muscle phosphorylase *a*, 984 for rabbit muscle phosphorylase *b*, 533 for lobster muscle phosphorylase *a*, 467 for lobster muscle phosphorylase *b*, 397 for potato phosphorylase, 583 for sweet potato phosphorylase, 200 for pea seed phosphorylase, 30 for *Physarum polycephalum* phosphorylase, 265 for cytoplasmic spinach leaf phosphorylase, and 96.4 for chloroplastic spinach leaf phosphorylase have been reported (Cori et al., 1955; Cowgill & Cori, 1955; Brown & Cori, 1961; Kamogawa et al., 1968; Ariki & Fukui, 1975a; Matheson & Richardson, 1978; Nader & Becker, 1979; Steup et al., 1980a; Steup, 1981). In the present case, although the specific activity of the enzyme is comparatively low, the preparation is homogeneous as tested by (a) polyacrylamide gel electrophoresis at various pHs and various gel concentrations, (b) identity of the protein band with starch phosphorylase activity, (c) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, (d) no increase in specific activity on affinity chromatography or crystallization of the enzyme, (e) immunodiffusion, and (f) immunoelectrophoresis. Although it is unexpected to get different specific activities of a homogeneous enzyme isolated from different sources, there may be many reasons for the low specific activity of banana starch phosphorylase. The low specific activity may itself be due to the absence of pyridoxal phosphate in the enzyme protein. Besides, it might be possible that as a result of extraction or purification only a fraction may be present in active conformation. The possibility of inactivation of the enzyme during purification seems remote since there is no unexpected decrease in enzyme activity at any step during the enzyme purification.

1,4-α-Glucan phosphorylases isolated from animals (Brown & Cori, 1961; Graves & Wang, 1972; Cowgill, 1959; Baranowski et al., 1957; Yunis et al., 1962; Appleman et al., 1966; Kent et al., 1958), microorganisms (Graves & Wang, 1972), pea seeds (Matheson & Richardson, 1978), sweet corn (Lee & Braun, 1973), spinach leaf (Steup et al., 1980a), potato tubers (Lee, 1960b), and sweet potato (Ariki & Fukui, 1975a) tubers are reported to contain pyridoxal 5′-phosphate as the prosthetic group. The removal of pyridoxal phosphate causes a loss of the enzyme activity (Illingworth et al., 1958), whereas reduction of the enzyme protein with $NaBH_4$ results in a material which is still 60% active (Fischer et al., 1958). Pfeuffer et al. (1972), on the basis of comparative studies on gel electrophoresis of the enzyme protein as well as studies with cross-linked phosphorylase using analogues of pyridoxal 5′-phosphate, emphasized the role of pyridoxal 5′-phosphate as a structural determinant. Weisshaar & Palm (1972) reported, on the basis of tritium-exchange experiments with rabbit muscle phosphorylase *b*, that pyridoxal phosphate is a major conformational determinant which affects the arrangements of motility of at least one-quarter of phosphorylase *b*. Johnson et al. (1970) reported that $NaBH_4$-reduced phosphorylase *b* is a catalytically active species; however, ultracentrifugal studies showed that native and $NaBH_4$-reduced enzyme have different conformations. Hedrick & Fischer (1965), on the basis of borohydride reduction, showed that if the cofactor was involved in catalysis, it would have to function in a way different than that observed for all other pyridoxal 5′-phosphate requiring enzymes. Feldmann & Hull (1977) demonstrated the involvement of pyridoxal 5′-phosphate in the allosteric transition of phosphorylase. Gani et al. (1978) presented a micellar model for the pyridoxal 5′-phosphate site of glycogen phosphorylase. Pyridoxal 5′-phosphate not only is described as the structural component but also is proposed to be directly involved in the catalytic event transfers. Palm et al. (1975) showed the presence of pyridoxal 5′-phosphate in maltodextrin phosphorylase of *Escherichia coli* but on the basis of stoichiometric studies proposed a different conformation around the active site than the one established for other phosphorylases. In contrast to the above observations, the present authors could not find any evidence for the presence of pyridoxal 5′-phosphate in the homogeneous preparation of α-glucan phosphorylase, isolated from young banana leaves, based on phosphate estimation, absorption spectrum in acid and alkaline medium, formation of phenylhydrazone, and microbiological assay. In this respect α-glucan phosphorylase from banana leaves appears to be similar to sucrose phosphorylase (Graves & Wang, 1972) and maltose phosphorylase (Kamogawa et al., 1973) which are reported to have no pyridoxal 5′-phosphate. In *Lactobacillus*, a pyruvoyl group attached with the phenylalanine in the enzyme protein is involved in the catalysis of histidine decarboxylase instead of pyridoxal 5′-phosphate (Riley & Snell, 1968). The formation of Schiff base with the carbonyl group of pyruvate and histidine has been established, and carbonyl group reagents inhibit the enzyme activity. The possibility of involvement of a pyruvoyl group in the present case is ruled out because banana starch phosphorylase activity is not inhibited by the carbonyl group reagent, hydroxylamine. In the case of monoamine oxidase, extracellular (plasma) enzyme has been reported to contain pyridoxal 5′-phosphate

as a cofactor whereas the intracellular (mitochondrial) enzyme does not require pyridoxal 5′-phosphate as a cofactor (Kapeller-Alder, 1970; Blaschko, 1966; Rando & De Mairena, 1974; Dugal, 1977).

The native α-glucan phosphorylase from banana has a molecular weight of 450 000. Upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the enzyme yielded a single protein band with a molecular weight of 55 000. Sweet potato phosphorylase is also reported having subunits of molecular weight around 50 000 (Ariki & Fukui, 1975a). Iwata & Fukui (1973) showed that in the case of potato phosphorylase, each two regions of similar size with a molecular weight of 55 000 are linked by a peptide bond to produce the subunits of molecular weight 110 000. Recently, Preiss et al. (1980) reported a molecular weight of 194 000 with a subunit molecular weight of 92 000 for cytoplasmic starch phosphorylase and a molecular weight of 203 800 with a subunit molecular weight of 108 000 for chloroplastic starch phosphorylase from spinach leaves.

The K_m values of starch phosphorylases from various sources for glucose 1-phosphate have been reported in the range 0.6–5 mM and those for inorganic phosphate 1–10 mM (Tsai & Nelson, 1968; Gold et al., 1971; Lee & Braun, 1973; Singh & Sanwal, 1973, 1976; Ariki & Fukui, 1975b; Burr & Nelson, 1975; Preiss et al., 1980). A large difference in the K_m values of starch phosphorylases for the primer has been reported. Singh & Sanwal (1976) reported the K_m values for starch as 0.24, 0.95, and 1.21 g/L, respectively, for three different forms of banana fruit phosphorylase. Preiss et al. (1980) reported the K_m values for amylose as 0.165 and 0.122 g/L for cytoplasmic and chloroplastic phosphorylase, respectively. Matheson & Richardson (1978) reported the K_m values for amylopectin as 0.007 and 0.098 g/L for two different forms of pea seed phosphorylase. In the present case, the K_m values for glucose 1-phosphate and inorganic phosphate have been found as 0.66 and 10.87 mM, respectively. The K_m values for starch are 0.19 and 0.13 g/L, respectively, in the direction of polysaccharide synthesis and degradation. It seems that the K_m values of banana leaf phosphorylase are of the same magnitude as have been reported for other starch phosphorylases.

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Two pH Optima of Adenosine 5′-Triphosphate Dependent Deoxyribonuclease from *Bacillus laterosporus*†

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ABSTRACT: The various catalytic activities of the ATP-dependent deoxyribonuclease (DNase) of *Bacillus laterosporus* have pH optima at 6.3 and 8.3. Although the pH profile of ATP-dependent DNase activity on duplex DNA is bell shaped with a maximum at about pH 8.3, ATP-dependent DNase activity on single-stranded DNA has optima at pH 6.3 and 8.3. ATPase activities dependent on double-stranded and single-stranded DNA have a high bell-shaped peak with a maximum at pH 6.3 with a low and broad shoulder at about pH 8.3. ATP-independent DNase activity also has optima at pH 6.3 and 8.3. The ratio of the amount of ATP hydrolyzed per number of cleaved phosphodiester bonds in DNA increases with decrease in the pH value of the reaction. The ratios obtained at pH 8.3 and 6.3 were respectively about 3 and 22 with duplex DNA as substrate and 5 and 17 with single-stranded DNA as substrate. Formation of a single-stranded region of 15 000–20 000 nucleotides, which is linked to duplex DNA and about half of which has 3′-hydroxyl termini, was observed at about pH 6.3, but not at above pH 7.5. Furthermore, the optimum concentrations of divalent cations for the activity producing the single-stranded region and the activity hydrolyzing ATP were identical (3 mM Mn^{2+} or 5 mM Mg^{2+}). Thus the two activities are closely related. These results indicate that the enzyme has two different modes of action on duplex DNA which are modulated by the pH.

The general properties of the ATP-dependent DNase[1] from *Bacillus laterosporus* have been described in previous papers (Anai et al., 1975a,b). Similar DNases are widespread among bacterial species, and the involvement of this class of enzymes in genetic recombination and DNA repair has been proved (Buttin & Wright, 1968; Oishi, 1969; Barbour & Clark, 1970; Goldmark & Linn, 1970; Vovis & Buttin, 1970). These enzymes have a variety of in vitro catalytic activities (Whitehead, 1979). Apart from its postulated role in recombination, the ATP-dependent DNase has aroused considerable interest

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[1] Abbreviations: DNase, deoxyribonuclease; Tris, tris(hydoxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

0006-2960/82/0421-4159$01.25/0