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PURIFICATION AND CHARACTERIZATION OF STARCH PHOSPHORYLASE FROM *CUSCUTA REFLEXA* FILAMENTS

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Key Word Index—*Cuscuta reflexa*; Convolvulaceae; dodder; starch phosphorylase; purification; characterization.

Abstract—Starch phosphorylase (EC.2.4.1.1.) from the filaments of *Cuscuta reflexa* has been purified using $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography using DEAE cellulose and gel filtration through Sephadex G-200. The enzyme preparation is estimated to be about 85–90% pure as revealed by native polyacrylamide gel electrophoresis. The native M_r of the enzyme as determined by gel filtration through Sephadex G-200 is $400\,000 \pm 5000$. Subunit M_r of the enzyme as determined by SDS polyacrylamide gel electrophoresis is $100\,000 \pm 3000$. Unlike other starch phosphorylases, the present enzyme is not inhibited by aromatic amino acids. The kinetic data are characteristic of a sequential reaction mechanism. The optimum temperature for starch phosphorylase enzyme activity is 45°, the energy of activation being 12.2 kcal $(°mol)^{-1}$ and energy of activation for denaturation is 32.94 kcal $(°mol)^{-1}$. Maximum activity occurred at pH 5.7 with half-maximum activity at pH 5.2 and 7.8.

INTRODUCTION

Starch phosphorylase (SP) (EC.2.4.1.1; α-1,4-glucan: orthophosphate, glucosyl transferase) is an important enzyme during carbohydrate degradation. SP has been extensively studied and purified from tubers [1], banana fruits [2], banana leaves [3] and seeds [4]. The presence of SP has been reported in *Loranthus* leaves, an angiospermic parasite and its properties have also been studied [5]. The presence of SP in *Cuscuta reflexa*, reported earlier [6] provided evidence that it is a partial parasite capable of carrying out photosynthesis. SP from tapioca leaves, as well as young banana leaves, is inhibited by aromatic amino acids. The present study shows no inhibition of SP purified from *Cuscuta reflexa* by aromatic amino acids.

RESULTS AND DISCUSSION

Enzyme purification

A summary of the purification of SP from *C. reflexa* filaments is given in Table 1. Fractionation with $(NH_4)_2SO_4$ of the initial extract, followed by chromatography on DEAE cellulose and gel filtration through Sephadex G-200 resulted in a 138-fold purification of SP with 45% recovery from the initial extract. Elution profiles of the enzyme on DEAE cellulose chromatography and Sephadex G-200 chromatography are shown in Fig. 1. The specific activity of the enzyme is 116 nkats mg^{-1} protein which is similar to that in chloroplastic spinach leaf, four times that of banana leaf enzyme and a half to one-third that of potato, banana fruit and cytoplasmic spinach leaf enzymes [2,3,7–9].

Purity of enzyme preparation

The enzyme, after Sephadex G-200 chromatography, was found to be 85–90% pure on polyacrylamide gel electrophoresis using 7% gel at pH 8.3. On protein staining with Coomassie Brilliant Blue, a single major band, along with 3–4 minor bands, appeared (Fig. 2). Upon activity staining, the major band corresponded to the enzyme activity band.

The purified enzyme preparation was found to be free from other interfering enzymes, namely, phosphoglucomutase, phosphohexoseisomerase, unspecific phosphatase, ATPase, amylase, ribulose bisphosphate carboxylase, branching and debranching enzymes. The contamination was tested with 100 μg of enzyme protein. Starch phosphorylase assays were usually carried out with 15–20 μg (*ca* 2 nkats) of the enzyme protein.

Evidence for purified enzyme protein as SP

In the absence of primer, no phosphate (Pi) was liberated from glucose-1-phosphate (g-1-p), showing the ab-

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Table 1. Purification of starch phosphorylase from *Cuscuta reflexa* filaments

S. No.	Purification step	Enzyme activity (nkats)	Protein (mg)	Specific activity (nkats mg^{-1})	Fold enrichment	Recovery %
1	Initial extract	2138	2541	0.84		100
2	$(NH_4)_2SO_4$ fraction (30–70%)	2000	430	4.65	5.5	93
3	DEAE cellulose	1306	45	29	34.5	61
4	$(NH_4)_2SO_4$ fraction (0–90%)	1198	40	30	36	56
5	Sephadex G-200	953	8.2	116	138	45

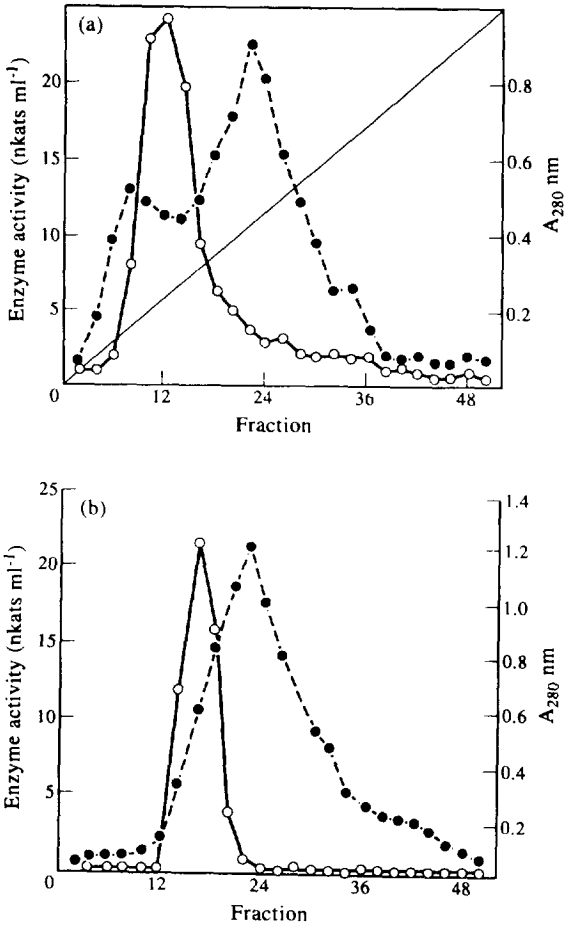

Fig. 1. Elution profiles of *Cuscuta reflexa* starch phosphorylase from: (a) DEAE cellulose column, ○—○, enzyme activity; ●----●, absorbance at 280 nm; diagonal line shows 0–1 M NaCl gradient. (b) Sephadex G-200 column, ○—○, enzyme activity ●----●, absorbance at 280 nm.

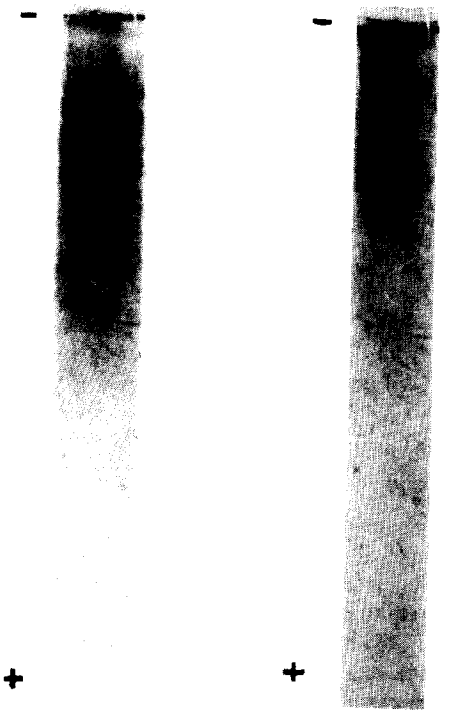

Fig. 2. Native polyacrylamide gel electrophoresis showing degree of purity of *Cuscuta reflexa* starch phosphorylase.

sence of phosphatase activity. The primer content was unchanged in the absence of g-1-p as revealed by I_2 colour estimation, showing the absence of amylase, as well as branching and debranching enzymes. The enzyme exhibited activity only when g-1-p as well as starch were present in the reaction mixture. When assayed in the direction of polysaccharide degradation, one of the products was identified as g-1-p, using a spectrophotometric assay [10]. In the presence of starch, Pi utilized is stoichiometrically equal to the g-1-p produced, further confirming the identity of the enzyme.

Optimum pH

The enzyme preparation exhibited optimum activity at pH 5.7 in the polysaccharide synthesis direction with half-maximum activity at pH 5.2 and 7.8; this is in the same range as reported for other plant SPs [3].

Optimum temperature

The maximum enzyme activity in the direction of polysaccharide synthesis was found to be at 45°, when the incubation time was fixed at 30 min. Other plant SPs

have been shown to have optimum temperature around 40°. This suggests that the present enzyme is more thermostable compared to other reported SPs. The energy of activation, as determined by the Arrhenius plot, is 12.2 kcal (° mol)$^{-1}$ and the energy of activation for denaturation is 32.94 kcal (° mol)$^{-1}$.

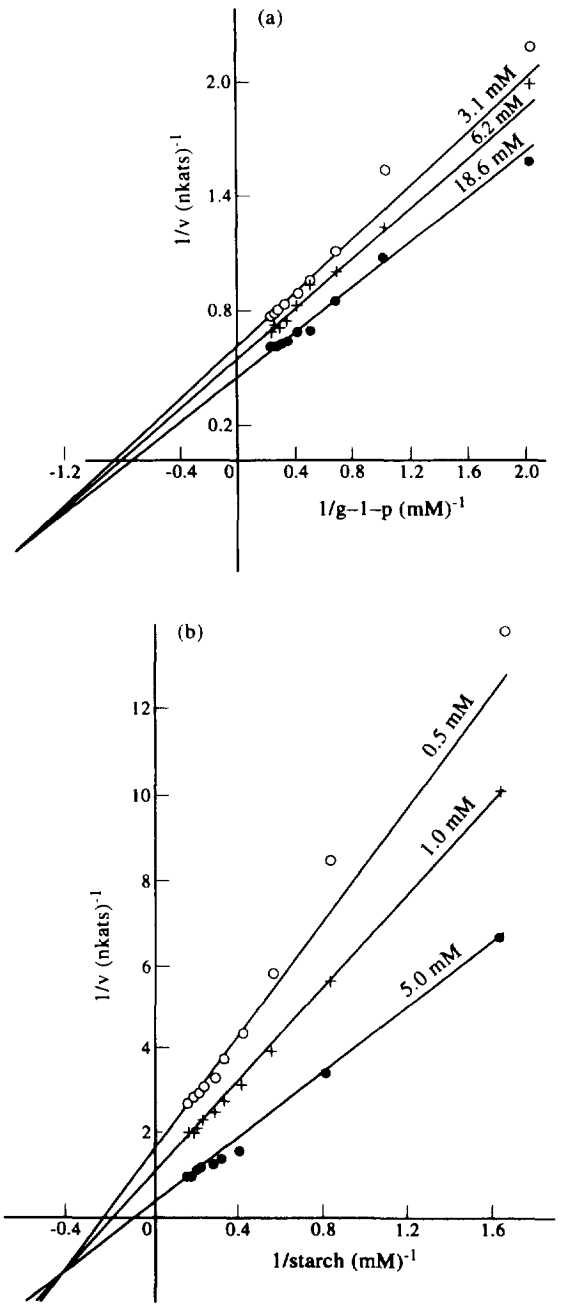

Fig. 3. Lineweaver–Burke plots of: (a) velocity of *Cuscuta reflexa* starch phosphorylase as a function of [g-1-p] at three levels of [starch]: ○—○, 3.1 mM starch: ×—×, 6.2 mM starch and ●—●, 18.6 mM starch. (b) Velocity of starch phosphorylase as a function of [starch] at three levels of [g-1-p]: ○—○ 0.5 mM g-1-p: ×—×. 1.0 mM g-1-p and ●—●, 5.0 mM g-1-p.

Substrate specificity

SP from *C. reflexa* is specific for g-1-p as a substrate. When g-1-p 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. As determined by a Lineweaver–Burke plot, the 'K_m' of the enzyme for g-1-p was found to be 1.19, 1.25 and 1.38 mM at 3.1, 6.2 and 18.6 mM (as glucosyl residues) of starch (Fig. 3a). The 'K_m' value of starch determined at 0.5, 1.0 and 5.0 mM of g-1-p was found to be 4.4, 5.6 and 15.5 mM, respectively (Fig. 3b). The 'K_m' values of SPs for g-1-p have been reported in the range 0.6–5 mM [2,11,12]. A large difference in the 'K_m' values of SPs for the primer has been reported. The 'K_m' values of starch for three different forms of banana fruit SPs have been reported to be 0.24, 0.95 and 1.21 g l^{-1}, respectively [2]. This indicates that the 'K_m' value of *C. reflexa* SP is of the same magnitude as reported earlier. In both cases, the intersection of the lines at a point to the left of the vertical axis, characteristic of a sequential reaction mechanism, is observed indicating that both substrate and primer should be added to the enzyme before any product is released. The Hill coefficient, both in the case of g-1-p, as well as starch, is calculated to be 1, indicating that the enzymatic reaction follows classical Michaelis–Menten kinetics.

Effect of aromatic amino acids

Unlike other plant SPs, the present enzyme from *C. reflexa* is not inhibited by aromatic amino acids even at 5 mM. This can be considered to be an important characteristic of the *C. reflexa* SP, which distinguishes it from other plant SPs [13, 14].

M_r determination

The M_r of the enzyme, as determined by gel filtration through Sephadex G-200, was 400 000 ± 5000. The native SP from banana leaves has a M_r of 450 000 [3]. Sweet potato SP is reported to have a native M_r of 210 000 [15]. In spinach leaves, a M_r of 194 000 for cytoplasmic SP and 203 800 for chloroplastic SP has been reported [16]. On SDS polyacrylamide gel electrophoresis, *C. reflexa* starch phosphorylase showed one major band having a M_r of 100 000 ± 3000, together with three minor bands.

EXPERIMENTAL

Tissue. Young filaments of *C. reflexa* which parasitized *Chlerodendrum enermi* were collected from the University Botanical Garden.

Chemicals. Glucose-1-phosphate, DEAE cellulose and Sephadex G-200 were purchased from Sigma. All other chemicals used were of high quality analytical grade purchased locally.

Enzyme extraction. Young filaments (10 % by wt) were homogenized in a chilled Waring blender for 30 s at low speed and 1 min at high speed using 0.05 M Tris–HCl

buffer, pH 7.6 containing 0.02 M 2-mercaptoethanol and 0.2% Triton X-100, filtered through 8-fold muslin cloth and centrifuged at 5000 *g* for 30 min. The supernatant obtained was treated as the initial extract.

Enzyme assay. Assay in the direction of polysaccharide synthesis, as well as degradation, was performed as described in ref. [3]. However, incubation was at 40°, instead of 30°.

Protein determination. Concn was determined according to ref. [17], as modified in ref. [18], using BSA as standard. Sp. act. was defined as nkats mg^{-1} protein.

Other enzymes. Phosphoglucomutase and phosphohexoseisomerase were assayed as described in ref. [19], unspecific phosphatase as given in ref. [20] using g-1-p as substrate. ATPase as in ref. [21], branching enzyme as in ref. [22] using amylose or amylopectin as a substrate, but the reaction was stopped by adding an aliquot of the reaction mixt. into I_2 reagent instead of using perchloric acid, amylases as in ref. [23] and debranching enzyme according to ref. [24] using amylopectin as substrate, but measuring the release of reducing power as described in ref. [25]. Ribulose bisphosphate carboxylase was assayed according to ref. [26] using a coupled enzyme assay method.

Enzyme purification. The entire procedure was carried out at 0–4°. To the crude extract (770 ml) finely ground solid $(NH_4)_2SO_4$ was added with constant stirring in order to bring 30% satn. After storage for 5 hr, the suspension was centrifuged at 5000 g for 30 min and the supernatant further subjected to 70% satn. After centrifugation, the ppt. was dissolved in 0.02 M Tris–HCl, pH 7.6 containing 5 mM 2-mercaptoethanol (Buffer A), dialysed against the same buffer for 14 hr and loaded onto a DEAE cellulose column (2.8 cm × 13 cm). The enzyme was eluted from the column at 0.2–0.28 M NaCl in a linear gradient elution in the form of a single peak. The frs were pooled and concd by 0–90% $(NH_4)_2SO_4$ and further purified by gel filtration over Sephadex G-200 (1.2 cm × 55 cm) pre-equilibrated with buffer A. SP activity was obtained in the form of a single peak, the most specifically active frs of which were pooled and concd using a Speed-vac system. This was used for the determination of the physiochemical properties of the enzyme.

M_r *determination.* Native M_r of the enzyme was determined by the method of ref. [27], using Sephadex G-200 (1.2 cm × 55 cm). Thyroglobulin (M_r 668 000), apoferritin (M_r 443 000), β-amylase (M_r 200 000), alcohol dehydrogenase (M_r 150 000), albumin (M_r 66 000) and carbonic anhydrase (M_r 29 000) were used as ref. proteins. The subunit M_r of the enzyme was determined by SDS–PAGE according to ref. [28], using myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 97 400), bovine albumin (M_r 66 000) and egg albumin (M_r 45 000) as markers.

Polyacrylamide gel electrophoresis. This was performed as described in ref. [28], but without SDS. Tris–glycine buffer (0.025 M, pH 8.3) was used as the tank buffer. A 7% separating gel was prepd and a current of 20 mA applied. Bromophenol Blue was used as the indicator dye and the gel stained using 0.2% Coomassie Brilliant Blue R-250, prepd in MeOH–HOAc–H_2O (3:1:6). In order to stain for enzyme activity, the gel, after electrophoresis, was incubated for 3 hr in a mixt. consisting of 0.03% freshly prepd starch, 0.02 M NaF, 0.02 M Tris–maleate buffer (pH 5.7) and 5 mM g-1-p, and then stained with I_2 reagent [29]. The position on the gel showing enzyme activity became dark blue [30].

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