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MULTIPLE FORMS OF STARCH PHOSPHORYLASE FROM *SORGHUM* LEAVES

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Key Word Index—*Sorghum vulgare*; Gramineae; leaves; starch phosphorylase; multiple forms; kinetic studies.

Abstract—Two major and three minor peaks of starch phosphorylase activity from leaves of *Sorghum vulgare* have been separated by chromatography on DEAE-cellulose and further purified using Sephadex G-200 chromatography. The initial velocity patterns of the reaction between glucose-1-phosphate and various primers, viz. starch, glycogen, amylopectin and amylose, determined as a function of substrate concentration are characteristic of a sequential reaction mechanism. The K_m values of starch phosphorylases III, IV and V in the direction of polysaccharide degradation for starch, glycogen, amylopectin and amylose are greater than those of starch phosphorylases I and II. All the multiple forms have shown similar affinity for orthophosphate. The K_m values of starch phosphorylases III, IV and V for glucose-1-phosphate are much greater than those of starch phosphorylases I and II. M_rs of starch phosphorylases I, II, III, IV and V determined by gel filtration chromatography are 180 000, 210 000, 180 000, 210 000 and 330 000, respectively. Aromatic amino acids, phenolic compounds and heavy metal ions inhibit the phosphorylase reaction.

INTRODUCTION

In plants, starch phosphorylase (EC 2.4.1.1; α-1,4-D-glucan: orthophosphate, α-glucosyl transferase) plays a dynamic mediatory role between starch synthesis and degradation [1]. Although starch synthesis takes place exclusively in chloroplasts, the enzyme has been shown to be located in the cytosol as well as chloroplasts of the same cell [2, 3]. The cytosolic phosphorylase in higher plants remained an enigma since no suitable assay substrate was available. Cytosolic and chloroplastic phosphorylases differ greatly in their substrate affinity for various primers [4–6] and in their immunological cross-reactivity [7, 8]. Among C_4 plants, in corn leaves [9] one form is restricted to mesophyll and the other to bundle sheath cells [10, 11]. In the present study, multiple form patterns and characterization of phosphorylase have been investigated in *Sorghum*, a C_4 plant.

RESULTS AND DISCUSSION

The summary of purification of multiple forms of phosphorylase from sorghum leaves is given in Table 1. DEAE-cellulose chromatography of the 30–65% $(NH_4)_2SO_4$ fraction of the initial homogenate revealed two major and three minor peaks of phosphorylase activity (Fig. 1). These five forms, I, II, III, IV and V, from their order of elution, were eluted at 0.136, 0.176, 0.208, 0.296 and 0.328 M NaCl, respectively, as determined according to Morris and Morris [12]. PAGE of the crude extract followed by enzyme activity stain also showed the presence of five isoenzymes. None of the purified isoenzymes showed any interfering enzyme activity tested for phosphoglucomutase, phosphohexoisomerase, unspecific phosphatases, ATPase, amylases, and branching and debranching enzymes. The reaction product of each isoenzyme was glucose-1-phosphate (g-1-p). The stoichiometry of the reaction for g-1-p formation and orthophosphate (Pi) utilization was also confirmed.

Earlier, two phosphorylase isoenzymes were shown to be present in a variety of plant tissues [2, 6, 9, 11, 13, 14] including prokaryotes [15]. No change in the elution profile of the five isoenzymes of phosphorylase, even in the presence of 1 mM *p*MSF, rules out the possibility of proteolysis of the enzyme during extraction.

The M_rs of forms I, II, III, IV and V determined by Sephadex G-200 gel filtration chromatography are 180 000, 210 000, 180 000, 210 000 and 330 000, respectively. These M_rs are in the same range as described for various multiple forms of phosphorylase from C_3 and C_4 plants [11, 16].

In the direction of polysaccharide synthesis, maximal activities of phosphorylases I, II, III, IV and V are found at pH 6.0, 5.8, 6.0, 6.4 and 5.8, respectively. However, in the direction of g-1-p synthesis, maximal activities are found at pH 7.0, 7.2, 7.0, 7.4 and 7.6, respectively. In the direction of polysaccharide synthesis, maximal activities of the five multiple forms are found at 40°, 40°, 35°, 45°

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Table 1. Purification of starch phosphorylase from *Sorghum* leaves

Fraction	Total* activity (nkat)	Total protein (mg)	Sp. act. (nkat mg^{-1} protein)	Fold enrichment	Recovery (%)
Initial extract	2300	743	3.1	—	100
$(NH_4)_2SO_4$ (30–65%)	2070	265	7.8	2.5	89
DEAE cellulose chromatography					
Form I	433	8.0	54.2	17	18
Form II	133	3.2	41.6	13	5
Form III	133	3.6	27.0	12	5
Form IV	633	11.2	56.5	18	27
Form V	67	1.9	35.3	11	3
Sephadex G-200 chromatography					
Form I	300	1.9	157.9	51	13
Form II	83	0.9	92.6	30	3
Form III	67	0.6	111.1	36	3
Form IV	483	2.6	185.9	62	21
Form V	33	0.3	111.0	36	1

*40 g of tissue was used.

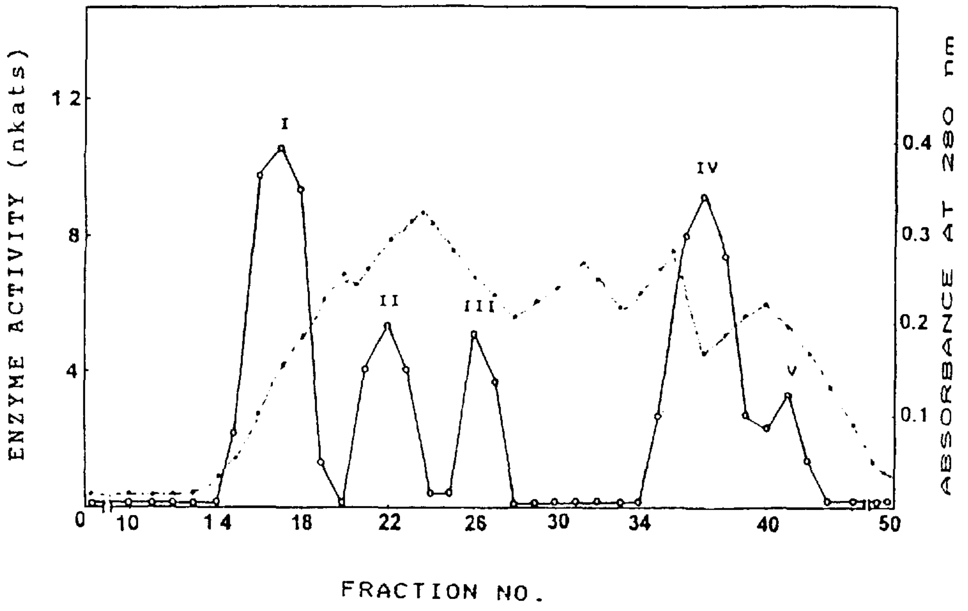

Fig. 1. Elution profile of starch phosphorylase on DEAE-cellulose.

Table 2. Cleland's constants for starch phosphorylase from *Sorghum* leaves

Kinetic coefficient	Values				
	Form I	Form II	Form III	Form IV	Form V
K_a	10.00	1.54	2.10	2.00	0.53
K_b	0.75	0.50	3.55	4.00	1.68
K_{ia}	4.45	3.08	3.00	1.00	1.88
K_{ib}	0.33	1.00	5.07	2.00	6.00
V_1	16.70	7.69	7.89	10.00	5.26

1 g l^{-1} starch has been taken as equivalent to 6.2 mM.

Table 3. K_m and V_{max} values of starch phosphorylase from *Sorghum* leaves determined in the direction of polysaccharide synthesis

Enzyme	Primer used	Conc. of primer ($g l^{-1}$)	Conc. of g-1-p (mM)	K_m for g-1-p (mM)	V_{max} (nkat mg^{-1})
Phosphorylase I	Starch	0.5	var	0.40	73
	Starch	1.5	var	0.53	104
	Starch	3.0	var	0.80	208
Phosphorylase II	Starch	0.5	var	0.66	95
	Starch	1.5	var	0.60	111
	Starch	3.0	var	0.54	128
Phosphorylase III	Starch	0.5	var	3.03	81
	Starch	1.5	var	3.28	101
	Starch	3.0	var	3.50	119
Phosphorylase IV	Starch	0.5	var	3.56	119
	Starch	1.5	var	3.64	132
	Starch	3.0	var	4.00	157
Phosphorylase V	Starch	0.5	var	2.33	72
	Starch	1.5	var	1.82	79
	Starch	3.0	var	1.72	83

var: Variable; g-1-p; glucose-1-phosphate.

Table 4. K_m and V_{max} values of starch phosphorylase from *Sorghum* leaves determined in the direction of g-1-p synthesis at 10 mM Pi

	Primers							
	Starch		Glycogen		Amylopectin		Amylose	
	K_m ($\mu g ml^{-1}$)	V_{max} (nkat mg^{-1})	K_m ($\mu g ml^{-1}$)	V_{max} (nkat mg^{-1})	K_m ($\mu g ml^{-1}$)	V_{max} (nkat ml^{-1})	K_m ($\mu g ml^{-1}$)	V_{max} (nkat mg^{-1})
Phosphorylase I	5	67	37	42	13	53	9	47
Phosphorylase II	6	60	43	42	12	67	11	45
Phosphorylase III	5	60	39	45	12	54	9	48
Phosphorylase IV	11	58	133	64	27	54	22	43
Phosphorylase V	13	55	118	52	20	56	20	46

and 35°, respectively, and Q_{10} values from the activity at 25° and 35°, are 1.67, 1.62, 1.59, 1.74 and 1.54, respectively.

Kinetic studies

Kinetic studies were carried out under initial velocity conditions. The reciprocal plots obtained for g-1-p in the presence of starch were found to be characteristic of a sequential reaction mechanism. As described by Gold *et al.* [17], from replots of vertical intercepts and slopes, various Cleland kinetic constants were calculated and recorded in Table 2.

The K_m and V_{max} values in the direction of polysaccharide synthesis indicated that phosphorylases I and II have a greater affinity for g-1-p than phosphorylases III, IV and V (Table 3). In the direction of polysaccharide degradation, the K_m values of phosphorylases I, II, III for starch, glycogen, amylopectin and amylose are higher than those of phosphorylases IV and V (Table 4). Similar affinity for Pi has been shown by all the multiple forms (Table 5). None of the multiple forms showed activity with maltotriose.

Table 5. K_m and V_{max} values of starch phosphorylase from *Sorghum* leaves for Pi using 3% soluble starch as primer

Enzyme	K_m (mM)	V_{max} (nkat mg^{-1})
Phosphorylase I	0.67	19.0
Phosphorylase II	0.69	22.1
Phosphorylase III	0.77	19.9
Phosphorylase IV	1.25	15.8
Phosphorylase V	1.45	19.4

Inhibition studies

Inhibition studies were carried out for phosphorylases I and IV only. Amino acids, viz. Gly, L-Ala, L-Lys, L-Met, L-Asp, DL-Asp and L-Glu, tested up to 10 mM at pH 6, showed no effect on either of the enzyme activities. In contrast, tested at 1 mM concentration, L-Phe showed 39 and 27%, L-Tyr showed 45 and 63%, and L-Trp showed 27 and 16% inhibition of phosphorylases I and IV activity, respectively. Gallic acid, phloroglucinol and 2,4-dinitrosalicylic acid, each tested separately at 10 mM, and tannic acid (5 μg ml^{-1}), caused 43 and 63%, 45 and 19%, 77 and 69%, and 39 and 27% inhibition of phosphorylases I and IV activity, respectively. None of the glycolytic intermediates, viz. 3-phosphoglycerate, glucose, glucose-6-phosphate, fructose-6-phosphate and phosphoenol pyruvate; ribulose bisphosphate, ADP glucose, EDTA and Mg^{2+}, each tested up to 5 mM, inhibited the activity of either of the multiple forms. *p*CMB and ATP, each at 5 mM, inhibited 68 and 82%, and 47 and 73% of phosphorylases I and IV activities, respectively. Silver ions, Cu^{2+}, Cd^{2+} and Hg^{2+}, each tested separately at 10 mM, caused 63 and 58%, 49 and 64%, 80 and 88%, and 80 and 88% inhibition of phosphorylases I and IV activity, respectively.

The overall studies indicate that phosphorylases I, II, III and IV, and V are similar to cytosolic and chloroplastic phosphorylases of C_3 plants and mesophyll and bundle sheath cell phosphorylases of C_4 plant leaves, respectively.

EXPERIMENTAL

Tissue. 15- to 20-day-old *Sorghum* {*Sorghum vulgare*; Pers. [Syn. *Andropogon sorghum* (L.) Brot.; *Holcus sorghum* L.] var. SPV 475} leaves were collected from the departmental garden.

Enzyme homogenate. Leaf tissue (40 g) was homogenized with 400 ml 0.05 M Tris–HCl buffer, pH 7.6, containing 0.02 M EDTA, 0.02 M $SH(CH_2)_2OH$ (2-ME), 1% Triton X-100 and 1 mM *p*MSF in a chilled waring blender for 1 min, filtered through 2-fold muslin cloth and centrifuged at 16 000 *g* for 30 min. The supernatant obtained was the crude extract.

Enzyme assay. This was performed in both the directions of polysaccharide synthesis, and degradation as described in ref. [18]. Protein was determined according to ref. [19] as modified in ref. [20] using BSA as standard. Sp. act. was defined as nkat mg^{-1} protein.

Other enzymes. Phosphoglucomutase and phosphohexoisomerase were assayed as described in ref. [21]; unspecific phosphatase as given in ref. [22] using g-1-p as substrate; ATPase as in ref. [23]; branching enzyme as in ref. [24] using amylose or amylopectin as a substrate, but the reaction was stopped by adding an aliquot of the reaction mixt. to I_2 reagent instead of PCA; amylases and debranching enzymes as in ref. [25] using amylopectin as substrate.

Enzyme preparation. The entire procedure was carried out at 0–4°. The 30–65% $(NH_4)_2SO_4$ ppt of the crude extract (400 ml) was suspended in 20 mM Tris–HCl buffer (pH 7.6) containing 5 mM 2-ME (buffer A) and dialysed against buffer A overnight. After centrifugation at 8000 *g* for 10 min, the supernatant was loaded on to a DEAE-cellulose column (3 x 18 cm). The enzyme was eluted from the column using a 0–1 M NaCl linear gradient. Pooled active frs were concd by 0–90% $(NH_4)_2SO_4$ and further purified by gel filtration over Sephadex G-200 (1.6 x 57 cm) pre-equilibrated with buffer A. Pooled active frs were concd in Speed Vac (Savant).

Native PAGE. Electrophoresis without SDS was performed in 1 mm thick 8% slab gel as described in ref. [26]. Enzyme activity stain was carried out as in ref. [13].

M_r was determined by the method of ref. [27] using Sephadex G-200 (1.6 x 57 cm), and carbonic anhydrase (M_r 29 000), albumin (M_r 66 000), β-amylase (M_r 200 000), apoferritin (M_r 443 000), thyroglobulin (M_r 669 000) were used as reference proteins.

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