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Purification and Characteristics of Sorbitol-6-phosphate Dehydrogenase from Loquat Leaves[1]

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MASASHI HIRAI
Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Okitsu, Shizuoka, 424-02, Japan

ABSTRACT

To study the role of sorbitol-6-phosphate dehydrogenase in sorbitol synthesis in leaves of Rosaceous plants, properties of the enzyme and its presence in several plants in the family was investigated. The activity of the enzyme, which catalyzes an NADP-dependent oxidation of the substrate to glucose-6-phosphate, was detected in leaves of *Prunus mume*, *Prunus persica*, *Rhaphiolepsis indica*, *Sorbus aucuparia*, *Cydonia oblonga*, *Photinia glabra*, *Sorbaria kirilowii*, and *Spiraea thunbergii*.

The enzyme was purified about 60-fold from leaves of loquat (*Eriobotrya japonica*) using affinity chromatography with Blue Sepharose. Neither mannitol-1-phosphate nor fructose-6-phosphate served as substrate. Molecular weight of the enzyme was calculated to be 65,000 at pH 8.0 by gel filtration. Since sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a peptide of 33,000 daltons, the enzyme was assumed to be a dimer at pH 8.0. K_m values for sorbitol-6-phosphate, glucose-6-phosphate, NADP, and NADPH were 2.22 millimolar, 11.6 millimolar, 13.5 micromolar, and 1.61 micromolar, respectively. Equilibrium constant for sorbitol-6-phosphate oxidation was 5.12×10^{-10}. Optimal pH for sorbitol-6-phosphate oxidation was 9.8. The enzyme showed its maximum activity within a broad pH range between 7 and 9 for glucose-6-phosphate reduction. The enzyme was more effective in the direction of glucose-6-phosphate reduction than in the reverse direction at neutral pH. Thus, it is suggested that the enzyme catalyzes sorbitol synthesis from glucose-6-phosphate during photosynthesis in leaves of Rosaceous plants.

Sorbitol is known to be a product of photosynthesis and is a translocated sugar in some plants of the Rosaceae family (10, 14), plus being a component of their fruits (11). Studies on sorbitol metabolism may provide fundamental information on these crops.

At least two enzymes which metabolize sorbitol have been reported in higher plant tissues, *i.e.* sorbitol dehydrogenase (EC 1.1.1.14) (9) and S6P[2] dehydrogenase (7). From studies by Bieleski and Redgwell (1), S6P is assumed to be a precursor of sorbitol synthesis from photosynthate in leaves of apricot. Therefore, S6P dehydrogenase in loquat fruit could participate in the synthesis of sorbitol during photosynthesis. However, properties of the enzyme were reported only briefly, and the enzyme was not reported from photosynthetic tissue. This paper reports the purification procedure and properties of S6P dehydrogenase from loquat leaves and the presence of the enzyme in several Rosaceae species.

[1] This paper is Contribution B-63 Fruit Tree Research Station.

[2] Abbreviations: S6P, sorbitol-6-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate.

MATERIALS AND METHODS

Mature leaves of loquat (*Eriobotrya japonica* Lindl. cv. Tanaka) were obtained from our orchard as an enzyme source for purification. Leaves of *Sorbus aucuparia* and *Cydonia oblonga* were obtained from the Fruit Tree Research Station, Yatabe, Ibaraki. The other plant leaves were collected from local gardens. S6P was prepared by reduction of G6P with potassium borohydride by the method of Wolf and Kaplan (16). The enzyme activity and protein content were assayed as described previously (7). Mannitol-1-P was purchased from Sigma. The polyacrylamide gel electrophoresis was conducted as described (4). To detect enzyme activity, the gel was incubated in a mixture containing 0.1 mg/ml *N*-methylphenazonium methosulfate, 0.2 mg/ml nitroblue tetrazolium, 7.7 mM KCN, 0.24 mM NADP, 2 mM S6P, and 20 mM Tris-HCl buffer (pH 7.5) in darkness at 30 C for 5 min.

To determine the equilibrium constant of the reaction, a mixture containing 1.20 μmol NADP (sodium salt), 1.20 μmol S6P, 90 μmol Tris-HCl buffer (pH 8.96), and 400 units (287 μg) purified enzyme at a final volume of 1.80 ml was incubated at 30 C for 70 min. At the end of the reaction, the pH of the mixture was checked. The mixture was placed in a boiling-water bath for 3 min and centrifuged, and the products were assayed as described previously (7).

RESULTS

Purification of S6P Dehydrogenase from Loquat Leaf. The purification procedure for the fruit enzyme was modified for the leaf enzyme. Mature leaves of loquat were homogenized and the homogenate was centrifuged as described previously (7). Buffer concentration in the extraction medium was 0.1 M instead of 0.4 M, and Polyclar-AT (0.1 g/g tissue weight) was added. The resulting supernatant was fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 40 and 80% saturation was dissolved in 20 mM Tris-HCl buffer (pH 8.7), containing 1 mM DTT (medium A). The dissolved protein was passed through a Sephadex G-25 column (34 × 240 mm) equilibrated with medium A. The colored fraction of the eluate then was applied to a column of Whatman DE52 (34 × 90 mm) equilibrated with medium A. The active fraction was not retained on the resin but passed through it. The fraction was collected, adjusted to pH 8.0 with 0.1 N HCl, and then loaded on a column of Blue Sepharose CL-6B (22 × 55 mm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT (medium B). The column was washed with medium B containing 0.14 M KCl, and then the enzyme was eluted with medium B containing 0.25 M KCl. The initial fraction (15.5 ml) was discarded and the subsequent active fraction was collected. The fraction was passed through a Sephadex G-25 column and then purified further by column chromatography with DE52 and Sephadex G-150, as described previously (7), except that column size used for Sephadex G-150 was altered to 12 × 820 mm. The

enzyme was eluted from the Sephadex G-150 column in a peak coinciding with protein profile. A typical result was shown in Table I. The enzyme was purified about 60-fold. Purity of the final enzyme preparation was checked electrophoretically. Polyacrylamide gel electrophoresis of the purified enzyme showed two protein bands, stained in almost equal densities (Fig. 1). The bands were both active. SDS-polyacrylamide gel electrophoresis revealed one main band. A few additional bands were also stained slightly.

Molecular Weight of S6P Dehydrogenase. The mol wt was calculated to be 65,000 by gel filtration at pH 8.0 in the presence of DTT (Fig. 2). Omission of DTT did not alter the molecular weight (data are not shown). SDS-polyacrylamide gel electrophoresis showed one peptide of 33,000 daltons (Fig. 3). Molecular weight of the two proteins which appeared in the polyacrylamide gel electrophoresis were almost identical and roughly calculated to be 125,000 by the method of Hedrick and Smith.

Kinetic Properties of S6P Dehydrogenase from Loquat Leaf. Specificities for substrates and cofactors were checked. The enzyme could not utilize F6P, glucose-1-P, or mannitol-1-P as substrate and was specific to G6P and S6P. NAD did not replace NADP as a cofactor. Activity as G6P reduction by the purified enzyme was 2620 units/g protein in 40 mM Hepes-NaOH buffer (pH 7.5), 120 μM NADPH, and 20 mM G6P. In the direction of G6P reduction, the maximum activity was observed within a broad pH range from 7 to 9 (Fig. 4). Mg^{2+} and Mn^{2+} did not stimulate the enzyme. The optimum pH for S6P oxidation was 9.8. Kinetic constants of the enzyme were shown in Figures 5 and 6. The K_m value for G6P also was determined at pH 9.1 (13.9 mM) and was found to be similar to that obtained at pH 7.5. No allosteric features were observed.

Equilibrium Study. As concluded in a previous paper (7), the enzyme catalyzes the following reaction: S6P + NADP ↔ G6P + NADPH. At the end of the reaction in an equilibrium study, the pH was 8.66. The NADPH and G6P produced were 395 and 388 nmol, respectively. The ratio of the products was close to unity. The results support the above conclusion. The K value was calculated to be 5.12×10^{-10}, based on the following equation:

$$K = \frac{[G6P]\,[NADPH]\,[H^+]}{[S6P]\,[NADP]}$$

Table I. *Purification of S6P Dehydrogenase from Loquat Leaf*

Fraction	Volume	Activity	Protein	Specific Activity
	ml	*units*	*mg*	*units/g protein*
Crude homogenate	240	3.26	131	24.9
$(NH_4)_2SO_4$ fractionation	95	2.83	110	25.7
First DE52	113	3.06	25.8	119
Blue Sepharose	15.3	1.95	3.34	589
Second DE52	9.1	1.03	0.719	1430
Sephadex G-150	11.2	0.568	0.384	1480

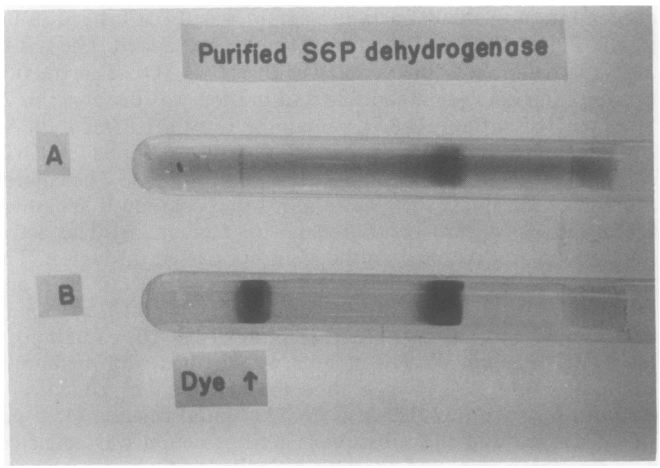

FIG. 1. Polyacrylamide gel electrophoresis of the purified S6P dehydrogenase from loquat leaf. A, stained with Amido black 10 B; B, stained by enzyme activity.

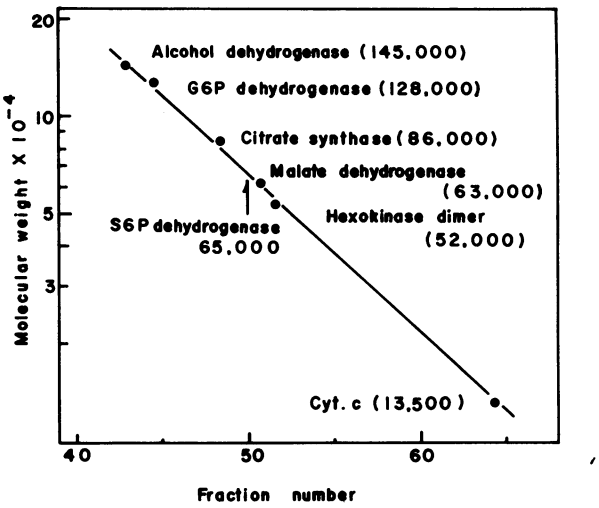

FIG. 2. Determination of molecular weight of S6P dehydrogenase from loquat leaf. Condition of the gel filtration with Sephadex G-150 was the same as that used for the enzyme purification. The purified enzyme and the other standard enzymes were mixed and then loaded on the column.

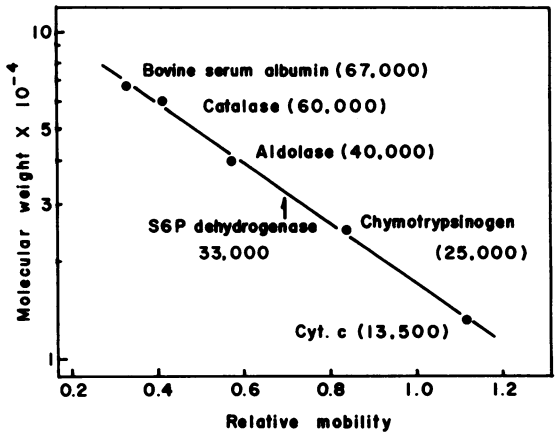

FIG. 3. Estimation of the molecular weight of S6P dehydrogenase subunit by SDS-polyacrylamide gel electrophoresis. The electrophoresis was conducted according to Weber and Osborn (13), using 7% acrylamide gels. Protein was dissolved in 100 μl 10 mM sodium phosphate buffer (pH 7.2) containing 1% SDS and 50 mM DTT heated at 50 C for 30 min, and then subjected to electrophoresis.

S6P Dehydrogenase Activity in Other Plants of Rosaceae. The occurrence of the enzyme in some Rosaceous plants was briefly surveyed (Table II). S6P-dependent NADP reduction by crude homogenates of these plants was observed. But, in contrast to loquat, the activity was rapidly lost in a few minutes incubation. Glycerol was effective in preventing the inactivation. The activity was, therefore, assayed in the presence of 1 M glycerol. Using crude homogenates, the product of the reaction was not detected in some cases. In these cases, the enzyme was partially purified by DE52 column chromatography as described previously (7). For some plants, the enzyme was eluted with medium B containing 50 mM KCl. But for other plants, if the activity was not found in the

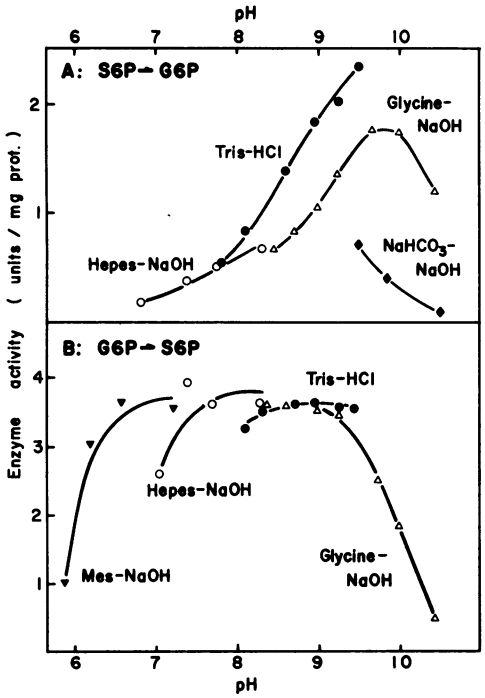

FIG. 4. Effect of pH on activity of S6P dehydrogenase from loquat leaf. Concentration of each buffer was 60 mM. Amount of the purified enzyme was 4.66 milliunits (3.15 μg)/mixture. The other conditions were same as standard assay. A, for S6P oxidation; B, for G6P reduction.

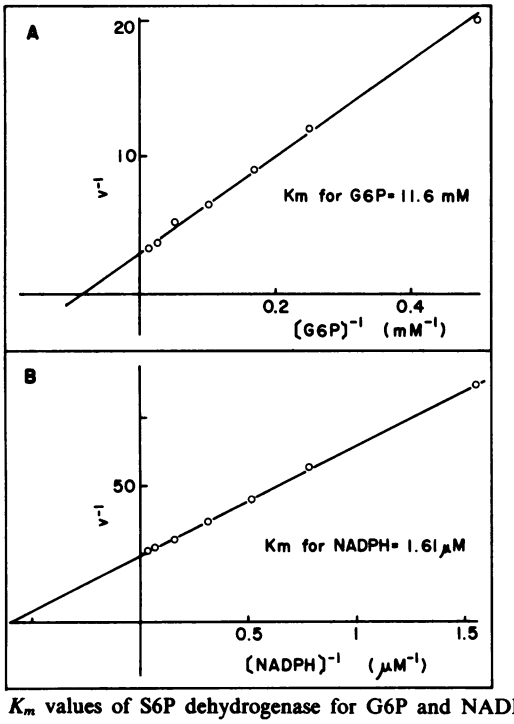

FIG. 5. K_m values of S6P dehydrogenase for G6P and NADPH. A, reaction mixture was composed of 40 mM Hepes-NaOH buffer (pH 7.5), 120 μM NADPH, 9.32 milliunits purified enzyme, and G6P in a total volume of 0.5 ml. B, reaction mixture was composed of the same buffer as in A, 80 mM G6P, 1.16 milliunits purified enzyme, and NADPH in a total volume of 0.5 ml.

eluate, then the column was followed by elution with medium B containing 100 mM KCl. The activity was found in this eluate. Using these partially purified enzymes, G6P was detected as a product of S6P oxidation in each sample.

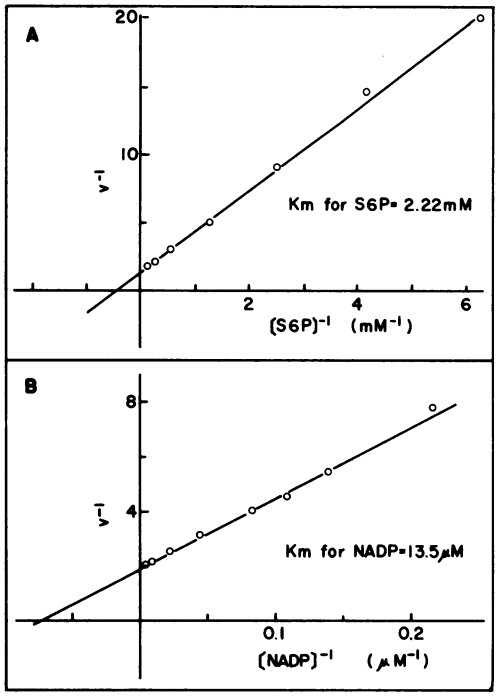

FIG. 6. K_m values of S6P dehydrogenase for S6P and NADP. A, reaction mixture was composed of 40 mM Tris-HCl buffer (pH 9.1), 0.5 mM NADP, 9.32 milliunits purified enzyme, and S6P in a total volume of 0.5 ml. B, reaction mixture was composed of the same buffer as in A, 10 mM S6P, 9.32 milliunits purified enzyme, and NADP in a total volume of 0.5 ml.

Table II. *S6P Dehydrogenase Activity in Leaves of Various Plants in Rosaceae*

The leaves were homogenized as loquat leaf, and then the homogenate was passed through a Sephadex G-25 column equilibrated with medium B. The eluate was used as crude homogenate. The eluate then was purified by a DE52 column (7). The activity was assayed in the presence of 1 M glycerol.

Subfamily and Species	Activity	
	In crude homogenate	In eluate from DE52
	units/g protein	
Prunoideae		
Prunus mume	1.41	3.03[a]
Prunus persica	5.49	11.9[a]
Pomoideae		
Photinia glabra	2.30	9.93[b]
Rhaphiolepis indica	0.69	2.22[a]
Sorbus aucuparia	3.38	11.8[b]
Cydonia oblonga	1.20	8.67[b]
Spiraeoideae		
Spiraea thunbergii	4.77	16.0[b]
Sorbaria kirilowii	1.00	4.23[b]

[a] Activity in eluate with medium B containing 50 mM KCl.
[b] Activity in eluate with medium B containing 100 mM KCl.

DISCUSSION

Some characteristics of S6P dehydrogenase from loquat fruit have been reported previously (7). Although the enzyme preparation from the fruit was not pure and although it utilized F6P as substrate at a low rate, the purified enzyme from leaves did not utilize F6P. Some properties of the leaf S6P dehydrogenase were

similar to those of the fruit enzyme. However, some differences were observed. For the fruit enzyme, the optimal range of pH was narrow and near pH 9 in the direction of G6P reduction. In contrast, the leaf enzyme showed its maximum activity over a broad pH range from 7 to 9. It is not known whether this difference is due to the enzyme molecule *per se* or to the impurity of the fruit enzyme sample.

Among the plants in Rosaceae, sorbitol is commonly found in three subfamilies: Prunoideae, Pomoideae, and Spiraeoideae; it is also found in a few species in Rosoideae (10). Here, the occurrence of S6P dehydrogenase in several plants of the former three subfamilies is shown. It is suggested, therefore, that the enzyme is common to the sorbitol-synthesizing plants in Rosaceae.

S6P dehydrogenase was found in photosynthetic tissue. From a tracer study, Bieleski and Redgwell (1) concluded that sorbitol is synthesized from primary photosynthate *via* S6P in the apricot leaf. The study presented here on effects of pH shows that the enzyme was more effective for G6P reduction than for S6P oxidation at neutral pH. Increases in S6P dehydrogenase activity coincided with sorbitol accumulation in loquat ripe fruit (unpublished data) and in apple seedlings (19). These findings suggest that this enzyme acts in the direction of G6P reduction *in vivo* and participates in sorbitol synthesis during photosynthesis in leaves of Rosaceae plants.

On the other hand, sorbitol may be oxidized to neutral sugar for carbon or energy sources in these plant tissues. Because the S6P dehydrogenase had little activity in the direction of S6P oxidation at neutral pH, the enzyme is not likely to participate in sorbitol oxidation in the tissue. Since sorbitol dehydrogenase (EC 1.1.1.14) from apple callus had a very high K_m value for fructose (9), the enzyme is not likely to act in sorbitol synthesis *in vivo*. Sorbitol dehydrogenase probably participates in sorbitol oxidation in plant tissue.

A S6P dehydrogenase which catalyzes the same reaction in silkworm as in loquat has been suggested (2, 5). It is interesting to compare sorbitol metabolism in plants with that in insects. Sorbitol is accumulated in diapausing eggs of silkworm (17). Breakdown of sorbitol at termination of the diapause is catalyzed by NAD-dependent sorbitol dehydrogenase (EC 1.1.1.14) (18); however the kind of enzyme that participates in the sorbitol synthesis at the beginning of the diapause is unknown. As well as NADP-dependent sorbitol dehydrogenase, the S6P dehydrogenase is a candidate for the synthetic enzyme (3). If this is true, sorbitol metabolism will have a common link between higher plants and insects.

Another type of S6P dehydrogenase was reported from microorganisms (8, 12). This enzyme catalyzes NAD-dependent conversion between S6P and F6P. The affinities of the loquat enzyme for substrates were similar to those of the NAD-dependent S6P dehydrogenase from *Aerobacter aerogenes* (8). But a lower K_m value for S6P was reported for the enzyme from *Lactobacillus casei* (12). Some similarities were observed. Both types of S6P dehydrogenase required no metal ion. Optimal pH values of the two types of enzyme were between 9 and 10 for S6P oxidation. Bicarbonate ion inhibited the activities of the enzyme from loquat and *Aerobacter*.

The equilibrium constant for the reaction catalyzed by sorbitol dehydrogenase (EC 1.1.1.14) was reported to be 2.05×10^{-9} (15), whereas that for NAD-dependent S6P dehydrogenase (F6P-forming) was reported to be 1.07×10^{-9} (12). The constant obtained in this study for S6P dehydrogenase from loquat is similar to those values.

Analysis of molecular weight indicates that the S6P dehydrogenase was a dimer at pH 8.0. The subunit of the enzyme was 33,000 daltons. Polyacrylamide gel electrophoresis at pH 9.4 showed a value about 4 times the molecular weight of the subunit. Although the value was not calculated, gel filtration with Sephadex G-150 at pH 9.0 showed a higher molecular weight than that obtained at pH 8.0. These findings suggest that dimer-tetramer conversion occurs between pH 8.0 and the higher pH.

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