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Sorbitol-6-Phosphate Dehydrogenase from Loquat Fruit[1]

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ABSTRACT

Sorbitol-6-phosphate dehydrogenase was found in flesh tissue of mature fruit of the loquat (*Eriobotrya japonica* Lindl. var. Tanaka). The enzyme was purified about 30-fold from the crude extract of the fruit, and was demonstrated to catalyze sorbitol-6-phosphate + NADP ⇌ glucose-6-phosphate + NADPH. The optimal pH values for sorbitol 6-phosphate oxidation and glucose 6-phosphate reduction were 9.8 and 9.1, respectively.

Sorbitol is recorded from many fruit trees in the Rosaceae (7, 10), and was also found in loquat fruit (unpublished results). It is known to be an important translocatory carbohydrate in these plants (14–16). Metabolism of sorbitol is interesting from the viewpoint of the physiology of woody plants as well as that of pomology. From labeling studies, sorbitol is assumed to be formed by reduction of hexose phosphate rather than that of hexose (3). In addition, S6P[2] was found in leaves of Rosaceae (11). Despite this, the enzymes involved in this pathway have not been studied as yet.

In this paper, we report the occurrence of S6P dehydrogenase in loquat fruit, and also report some properties of the enzyme.

MATERIALS AND METHODS

Extraction and purification of the enzyme. Extraction medium was composed of 0.4 M Tris-HCl buffer (pH 8.0), 10 mM isoascorbate, 1 mM DTT, and 0.15% Triton X-100. Before use, the medium was deoxygenated by bubbling N_2 through it. Flesh tissue of mature loquat fruit (*Eriobotrya japonica* Lindl. var. Tanaka) was homogenized with 1.5 ml/g tissue weight of the above medium using a mortar and pestle under air. The homogenate was filtered through nylon cloth, and the filtrate was centrifuged at 8,000*g* for 10 min. The resulting supernatant was fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 40 and 60% saturation was dissolved in 0.02 M Tris-HCl buffer (pH 8.0), 1 mM DTT (column medium). The dissolved protein (5 ml) was passed through a Sephadex G-25 column (29 × 120 mm) equilibrated with column medium. The slightly colored fraction of the eluate (10–15 ml) was loaded on a DE52 column (15 × 110 mm) equilibrated with column medium. The column was washed with column medium, then the enzyme was eluted with 0.05 M KCl in column medium. The initial fraction (13.5 ml) of the eluate was discarded and the following active fraction was collected (DE52 fraction). The fraction was concentrated with a collodion bag by evaporation and applied to a Sephadex G-150 column (12 × 450 mm) equilibrated with 0.1 M Tris-HCl buffer (pH 8.0), 1 mM DTT, at a flow rate of 7 ml/h. Fractions of 1.4 ml were collected. The active fractions (fractions 22–24) of the eluate were gathered (G-150 fraction). All of these procedures were carried out at 4 C.

Assay of Enzymes, Products and Protein. For S6P dehydrogenase, the standard assay mixture was composed of 0.04 M Tris-HCl buffer (pH 9.1), 0.5 mM NADP, 5 mM S6P (obtained from Sigma), and enzyme in a total volume of 0.5 ml. For the reverse reaction of S6P dehydrogenase, the assay mixture was composed of 0.04 M Tris-HCl buffer (pH 9.1), 120 μM NADPH, 20 mM hexose-P, and enzyme in a total volume of 0.5 ml. In the case of crude extract, the extract was passed through a Sephadex G-25 column equilibrated with column medium, then used as enzyme. The change in *A* at 340 nm was recorded. The reaction was linear for 5 min or more in both directions, except for using F6P with NADPH. Glucose-P isomerase (EC 5.3.1.9) was assayed using G6P dehydrogenase from yeast (Boehringer Mannheim GmbH) (1). For each enzyme, one unit of activity is defined as the activity that catalyzes the reaction of 1 μmol of substrate per min at 30 C.

The products were assayed by the methods of Bernt *et al.* (2) using enzymes from yeast (Boehringer Mannheim GmbH). The assay mixture was composed of 0.25 M triethanolamine buffer (pH 7.5), 2.5 mM $MgSO_4$, 1.1 mM ATP, 0.8 mM NADP, and the sample in a total volume of 0.6 ml. To the above medium, 3.3 μg each of G6P dehydrogenase, glucose-P isomerase, and hexokinase were added in this order. Increases in *A* at 340 nm caused by each emzyme were recordéd for G6P, F6P and glucose plus fructose, respectively.

Protein was assayed by the methods of Lowry *et al.* (9).

RESULTS AND DISCUSSION

The enzyme was purified about 30-fold from the crude extract of the fruit. A typical purification is shown in Table I. Using the DE52 fraction as the enzyme, G6P was the main product of the reaction, but a certain amount of F6P was also formed. The ratio of the products (G6P/F6P) was increased on further purification of the enzyme (Table II). The reverse reaction of the enzyme was studied in order to see if the primary product was G6P or F6P.

Table I. Purification of S6P dehydrogenase from loquat fruit.

Fraction	Vol.	Protein	Activity	Specific activity
	ml	mg	munits	units/g prot.
Crude extract	295	87.5	1130	12.9
$(NH_4)_2SO_4$ 40-60% sat.	10.9	46.0	1170	25.4
DE-52	5.3	2.59	639	247
G-150	5.6	0.470	211	449

[1] Contribution B-43 of Fruit Tree Res. Stn.

[2] Abbreviations: S6P: sorbitol 6-phosphate; G6P: glucose 6-phosphate; G1P: glucose 1-phosphate; F6P: fructose 6-phosphate.

The elution pattern from Sephadex G-150 showed that glucose-P isomerase was present in the peak containing S6P dehydrogenase (Fig. 1). Fraction 21 of the eluate catalyzed the reaction of F6P with NADH. However, the rate increased with time. In the case of fraction 24, the reactivity of F6P was very low and the reaction rate did not increase with time (Table III). We conclude that

Table II. Products of S6P oxidation by S6P dehydrogenase from loquat fruit.

NADPH was assayed by the change in absorbance at 340 nm. G6P,F6P and hexoses were assayed enzymatically.

Enzyme	Incubation time	NADPH	G6P	F6P	Glucose+ Fructose
	min	n moles/reaction mixture			
DE-52 fraction	20	79.5	78.6	3.5	0.3
	40	136	136	13.5	0
G-150 fraction	10	---[1]	56.6	1.1	0
	40	---[1]	133	7.2	0

[1] the assay was not performed

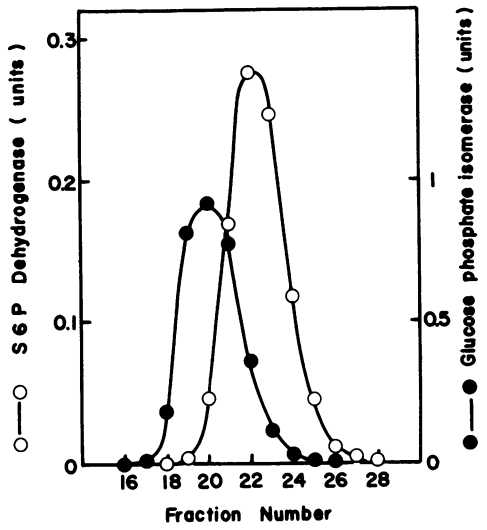

FIG. 1. Sephadex G-150 chromatography of S6P dehydrogenase. Column dimensions were 12 × 450 mm, eluting buffer was 0.1 M Tris-HCl (pH 8.0), containing 1 mM DTT. Fractions of 1.4 ml were collected. S6P dehydrogenase was assayed by G6P reduction, and glucose-P isomerase was assayed using G6P dehydrogenase.

Table III. Reduction of sugar phosphate by various fraction of G-150 eluate

One hundred microliters of each fraction were used as enzyme.

Fraction	Substrate	Time[1]	Activity
		min	units
No. 21 (see Fig.1)	G6P	0	12.8 (100)
	F6P	0	0.5 (3.9)
		5	1.6 (12.5)
		10	2.6 (20.5)
No. 24 (see Fig.1)	G6P	0	9.08(100)
	F6P	0	0.12(1.3)
		5	0.12(1.3)
		10	0.12(1.3)

[1] time interval between substrate addition and the assay

Table IV. Reaction of S6P dehydrogenase from loquat fruit

For exp.A and B, 40 and 100 µl of G-150 fraction was used as enzyme respectively.

Substrate	Coenzyme	Activity	
Exp. A		units/g prot.	%
S6P	NADP	449	100
Sorbitol	NADP	1	< 1
None	NADP	1	< 1
S6P	NAD	1	< 1
Exp. B			
G6P	NADPH	1700	100
F6P	NADPH	36	2.1
		60[1]	3.5
		129[2]	7.6
G1P	NADPH	17	1.0
Glucose	NADPH	8	0.4
Fructose	NADPH	2	0.2
G6P	NADH	5	0.3
F6P	NADH	2	0.2

[1] activity at 5 min after substrate addition

[2] activity at 10 min after substrate addition

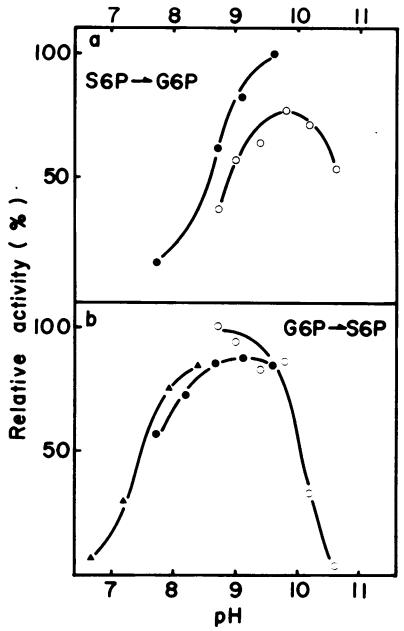

FIG. 2. Effect of pH on activity of S6P dehydrogenase. DE52 fraction (50 µl) was used as enzyme. Concentration of each buffer was 0.06 M. Other conditions were same as standard mixture. a: S6P oxidation; b: G6P reduction. ▲: Hepes-NaOH buffer; ●: Tris-HCl buffer; ○: glycine-NaOH buffer.

glucose-P isomerase in our preparation converts the product to F6P, and also causes the time-dependent increase in reactivity with F6P in the reverse reaction. Considering the cofactor specificity (Table IV), we conclude that the S6P dehydrogenase catalyzes the following reaction:

$$NADP + S6P \rightleftharpoons NADPH + G6P$$

The optimal pH values for S6P oxidation and for G6P reduction were 9.8 and 9.1, respectively (Fig. 2).

In vertebrates, sorbitol is metabolized in its free form by sorbitol dehydrogenase (13). In some microorganisms (8, 12) and an insect (5), it is metabolized as the phosphate ester as well. S6P dehydrogenases have been reported to be present in these organisms. In

contrast with our results, the product in the microorganisms is not G6P but F6P (8, 12). On the other hand, G6P was reduced by an enzyme in silkworm blood to a product thought to be S6P. This enzyme has not been purified and its nature is obscure (5).

In higher plant tissue, there are only two reports of enzymes of polyol metabolism. Both enzymes catalyze polyolketose conversion (4, 6). No sorbitol-P dehydrogenase has been reported. From tracer experiments with apricot leaves, Bieleski *et al.* (3) assumed that sorbitol was synthesized from primary photosynthate by the way of F6P rather than G6P; but their data can also be explained if we assume that the sorbitol is formed directly by the way of G6P. In addition, Lewis and Smith (7) suggested the possibility of sorbitol synthesis from aldoses. Although we have no data on the enzyme in plant tissue other than loquat fruit, it is possible that the S6P dehydrogenase reported here plays an important role in sorbitol synthesis in Rosaceae.

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