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Purification and characterization of sorbitol-6-phosphate phosphatase from apple leaves

Rui Zhou [a], Lailiang Cheng [a,*], Randy Wayne [b]

[a] Department of Horticulture, Cornell University, Ithaca, NY 14853, USA
[b] Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA

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Abstract

Sorbitol-6-phosphate phosphatase (SorPP; EC 3.1.3.50) catalyzes the final step in sorbitol biosynthesis in sorbitol-synthesizing plant species, but its kinetic and regulatory properties have not been characterized. In this study, the enzyme was purified 1727-fold to apparent homogeneity from apple leaves with a maximal specific activity of 89.8 μmol min^{-1} mg^{-1} protein measured at 2 mM sorbitol-6-phosphate (sorbitol-6-P). The enzyme is a monomer with a molecular mass of 61 kDa. The enzyme is highly specific for sorbitol-6-P with a Km of 0.85 mM and is unable to cleave other phosphate esters at a significant rate. The activity is absolutely dependent on Mg^{2+} with a Km of 0.29 mM at an optimal pH of 6.8. Fluoride, vanadate, molybdate, and inorganic phosphate inhibit SorPP activity. Sorbitol is a competitive inhibitor for SorPP with a Ki of 109 mM. The possible feedback mechanism for the regulation of sorbitol biosynthesis is also discussed.

Keywords: Sorbitol-6-phosphate phosphatase; Sorbitol; Apple

1. Introduction

In addition to sucrose and starch, sorbitol is a major photosynthetic product in many plant species, including some economically important deciduous tree fruits in the two subfamilies of Rosaceae, Pomoideae and Prunoideae, such as apple, pear, peach, and cherry [1,2]. About 70% of the newly fixed carbon is partitioned into sorbitol in mature apple leaves [3]. Sorbitol is also the primary translocatable carbohydrate and storage carbohydrate in these plants [2]. Sorbitol synthesis shares a common precursor, glucose 6-phosphate (Glc6P), with sucrose biosynthesis in the cytosol. Sorbitol formation in mature apple leaves is catalyzed by the sequential action of aldose-6-phosphate reductase (A6PR) (Glc6P + NADPH → sorbitol-6-P + NADP) and sorbitol-6-phosphate phosphatase (SorPP) (sorbitol-6-P → sorbitol + Pi) to yield free sorbitol in the cytosol [2].

A6PR has been purified from loquat and apple, and well characterized [4–6]. However, to fully understand the biochemical regulatory mechanism of sorbitol biosynthesis in vivo, it is necessary to investigate the other enzyme, SorPP, in the pathway. Currently, only one report indicates the existence of SorPP in apple leaves [7], but its physical, catalytic, and regulatory properties remain unknown. As a part of our efforts to understand the regulation of sorbitol biosynthesis, we purified SorPP to homogeneity from apple leaves and characterized its properties.

2. Materials and methods

2.1. Plant materials

Fully developed leaves were harvested from 9-year-old apple (*Malus domestica*, Borkh. cv. Liberty) trees grown at Cornell Experimental Orchards.

* Corresponding author. Tel.: +1-607-255-1779; fax: +1-607-255-0599.
E-mail address: lc89@cornell.edu (L. Cheng).

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2.2. Enzyme assay

SorPP activity was mainly determined by measuring the release of inorganic phosphate (Pi) [8]. Five to twenty μl of the enzyme preparation was mixed with 320 μl of assay mixture containing 50 mM MES-NaOH (pH 6.8), 10 mM $MgCl_2$, and 2 mM sorbitol-6-P, kept at 30 °C for 15 min, then 160 μl of 15% (w/v) trichloroacetic acid was added to terminate the reaction, and 500 μl of the ascorbic acid–ammonium molybdate reagent was added and kept at 40 °C for 20 min before recording the absorbance at 660 nm [8]. To determine the effect of Pi on SorPP activity, the release of sorbitol was measured. Twenty μl of enzyme preparation was added to the assay mixture containing 50 mM MES-NaOH (pH 6.8), 2 mM sorbitol-6-P, 10 mM $MgCl_2$, 1 mM NAD^+, and various amount of Pi. Sorbitol dehydrogenase (10 Units) was added to initiate the reaction, and the change of absorbance at 340 nm was recorded.

2.3. Enzyme purification

All purification steps were performed at 4 °C and the chromatography was conducted in AKTA chromatography system (Amersham, Sweden). Approximately, 100 g of freshly harvested mature apple leaves were homogenized at full-line voltage in a Waring blender with 600 ml extraction buffer containing 0.1 M Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), and 0.01% (v/v) Triton X-100. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at $12000 \times g$ for 10 min. Solid ammonium sulfate was slowly added to the supernatant to attain 40% saturation with constant stirring. After stirring for 30 min the suspension was centrifuged at $12000 \times g$ for 10 min. The supernatant was slowly brought to 60% saturation with solid ammonium sulfate as described above. The sample was centrifuged as above and the 40–60% pellet was resuspended in 30 ml extraction buffer minus PVPP and Triton X-100. The resultant suspension was clarified by centrifugation at $12000 \times g$ for 5 min and the supernatant was desalted using ten PD10 columns (Amersham, Sweden) with 10 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT (buffer A). The desalted preparation was then applied directly to a DEAE cellulose column (25 mm $\times$ 30 cm) equilibrated with buffer A. After washing the column with two bed volumes of buffer A, the enzyme was eluted with 500 ml buffer A in a linear gradient of KCl from 0 to 1 M at a flow rate of 1.2 ml min^{-1}. Fractions containing SorPP activity were pooled and condensed by dialysis against PEG8000. An aliquot of the desalted enzyme preparation was loaded to a Mono Q HR5/5 anion-exchange column (Amersham, Sweden). The column was rinsed with 4 ml buffer A and the enzyme was then eluted at 1 ml min^{-1} with 20 ml buffer A in a linear gradient of KCl from 0 to 1 M. Fractions with the SorPP activity were desalted with PD10 columns as described above and were passed through a Blue Sepharose column (20 mm $\times$ 5 cm). The flow-through was loaded on to a Mono Q HR5/5 column equilibrated with 20 mM Tris–HCl (pH 7.0) containing 5 mM $MgCl_2$, 0.5 mM EDTA, 0.2 mM DTT (buffer B). The column was washed with 4 ml of buffer B and then eluted with 40 ml buffer B using a linear gradient of 0–0.5 M KCl. The fraction with the highest activity was loaded onto a Superose 6 column (Amersham, Sweden) equilibrated with 2 $\times$ buffer A. The enzyme was eluted with column buffer at a flow rate of 0.15 ml min^{-1} with a fraction size of 0.5 ml. Active fractions containing purified SorPP were pooled and stored at −80 °C.

2.4. Electrophoresis

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7×10 cm^2 mini-gels prepared with a 12% (w/v) acrylamide resolving gel according to Laemmli [9]. Samples were mixed with an equal volume of buffer containing 0.3 M Tris–HCl (pH 6.8), 3.3% SDS, 0.15 M DTT and 33% glycerol, and were denatured in a boiling H_2O bath for 2 min. Electrophoresis was performed for 80 min at 100 V. The bands were located by staining with Silver Staining Kit (Sigma) according to the manual.

2.5. Protein assay

Protein concentration was measured according to Bradford [10] using bovine serum albumin as the protein standard.

2.6. Estimation of the molecular mass of SorPP

Gel filtration was carried out using the purified SorPP. The enzyme was loaded on to a Superose 6 column (10 mm $\times$ 30 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The molecular mass was determined using the standard curve derived from standard proteins. Molecular mass of SorPP was also determined by SDS-PAGE (12%) using the standard curve derived from standard proteins.

3. Results

3.1. Purification of SorPP

SorPP was purified to homogeneity from apple leaves following the purification steps presented in Table 1. A final purification of about 1727-fold was achieved with a

Table 1
The purification of SorPP from apple leaves

Purification step	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1} protein)	Purification (fold)	Yield (%)
Crude extraction	1386	0.052	1	100
$(NH_4)_2SO_4$ (40–60%)	516	0.12	2.3	86
DEAE cellulose	111	0.35	6.7	54
First Mono Q	22	1.38	26.5	42
Blue Sepharose	9.5	2.58	49.6	34
Second Mono Q	0.66	16.9	325	15
Superose 6	0.078	89.8	1727	9.7

specific activity of 89.8 μmol min^{-1} mg^{-1} protein measured at 2 mM sorbitol-6-P. SorPP activity appeared in one of the major protein peaks eluted from the Superose 6 column, which corresponded, to a molecular mass of 61 kDa. This fraction showed a single protein band on the SDS-polyacrylamide gel with a molecular mass of 61 kDa (Fig. 1). These results indicate that the native apple leaf SorPP is a monomer with a molecular mass of 61 kDa.

3.2. Substrate specificity

The purified SorPP preparation from apple leaves demonstrated the highest activity with sorbitol-6-P in comparison with other sugar phosphates and phosphate esters. Less than 4% of the activity was observed when the enzyme was assayed with *p*-nitrophenyl-phosphate, hexose phosphates, and sucrose phosphate and other phosphate esters as substrates compared with that of sorbitol-6-P as substrate (Table 2).

3.3. Kinetic properties of SorPP

The dephosphorylation of sorbitol-6-P by apple leaf SorPP showed Michaelis–Menten kinetics in the presence of 10 mM $MgCl_2$. The apparent Km for Sorbitol-6-P was 0.85 mM with a maximum activity of 137.4 μmol min^{-1} mg^{-1} protein (Fig. 2). The optimal pH for SorPP activity was between pH 6.0 and 7.0 and the highest activity was shown at pH 6.8 (Fig. 3).

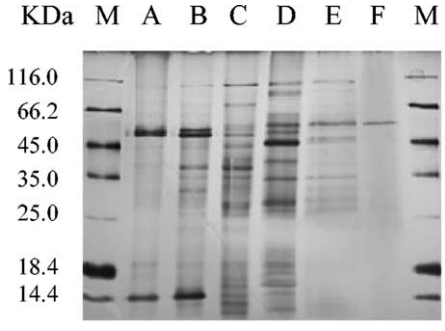

Fig. 1. SDS-PAGE of samples from each stage in the purification of SorPP from apple leaves. Lane M shows the molecular mass standards. Amounts of protein loaded were as follows: lane A, crude extract (1 μg); lane B, DEAE cellulose fraction 35 (2 μg); lane C, First Mono Q fraction 6–11 (2 μg); lane D, Blue Sepharose (2 μg); lane E, Second Mono Q fraction 18 (0.5 μg); lane F, Superose 6 fraction 30 (0.1 μg).

Table 2
Substrate specificity of SorPP

Substrate	Relative activity (%)
Sorbitol-6-phosphate	100
Glucose-6-phosphate	1.2
Glucose-1-phosphate	0
Fructose-6-phosphate	1.4
Fructose-1,6-bisphosphate	3.5
Sucrose-6-phosphate	3.1
Phosphoglyceric acid	1.4
Ribulose-1,5-bisphosphate	2.3
p-Nitrophenyl-phosphate	0.9

The SorPP activity of 91.3 μmol phosphate release min^{-1} mg^{-1} protein determined with 50 mM MES-NaOH (pH 6.8), 10 mM $MgCl_2$, and 2 mM sorbitol-6-P was calculated as 100%. Each value is the mean of three determinations. All the standard errors are less than 10% of the mean.

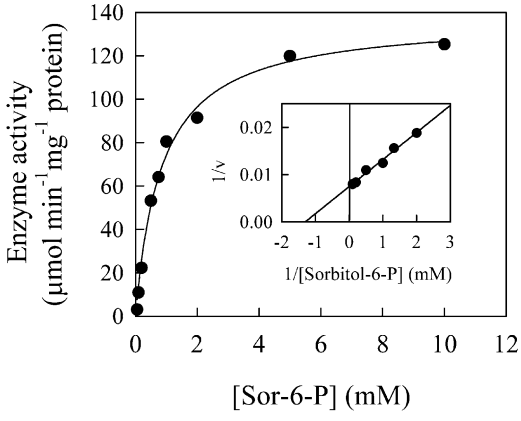

Fig. 2. The activity of purified SorPP in response to sorbitol-6-P concentration. Inset: Lineweaver–Burk plot. The activity was determined with 50 mM MES-NaOH, pH 6.8, 10 mM $MgCl_2$, and varying concentrations of sorbitol-6-P indicated in the figure. Each point is the mean of three determinations. All the standard errors are less than 5% of the mean.

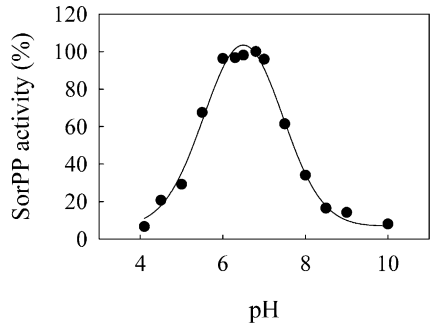

Fig. 3. Effects of pH on the apple leaf SorPP activity. The enzyme activity was assayed with 2 mM sorbitol-6-P and 10 mM $MgCl_2$ at the different pHs indicated in the figure. Three buffer systems were used for the pH range from 4.0 to 10.0: 50 mM citrate–NaOH buffers for pH 4.0–5.0, 50 mM MES-NaOH buffers for pH 5.0–8.0, and 50 mM glycine–NaOH buffers for pH 8.0–10.0. Each point is the mean of four independent measurements. All the standard errors are less than 5% of the mean.

3.4. Effect of Mg^{2+}

The activity of SorPP was absolutely dependent on the presence of Mg^{2+} in the reaction mixture. The activity of purified SorPP was not detectable without the inclusion of Mg^{2+}. The reaction rate of SorPP showed a hyperbolic response to Mg^{2+} concentration with a Km of 0.29 mM for Mg^{2+} (Fig. 4).

3.5. Effects of soluble carbohydrates

Sorbitol, glucose, fructose, and sucrose, the main soluble carbohydrates in apple leaves, were tested for the effect on apple leaf SorPP activity. Sorbitol was found to be an inhibitor for this enzyme. SorPP activity decreased linearly as sorbitol concentration increased from 0 to 150 mM in the assay mixture. At a concentration of 150 mM, sorbitol resulted in 40% inhibition of the enzyme activity measured at 2 mM

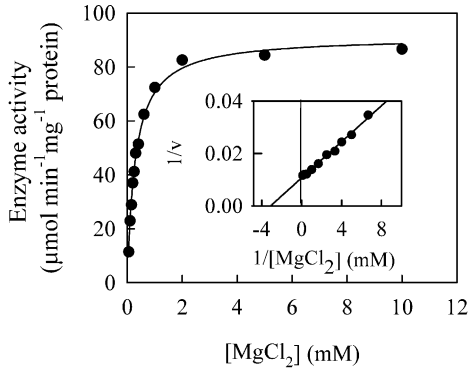

Fig. 4. Effect of Mg^{2+} on the activity of SorPP from apple leaves. Inset: Lineweaver–Burk plot. The enzyme activity was assayed with 50 mM MES-NaOH (pH 6.8), 10 mM $MgCl_2$, 2 mM sorbitol-6-P and various concentrations of $MgCl_2$ indicated in the figure. Each point is the mean of four independent measurements. All the standard errors are less than 5% of the mean.

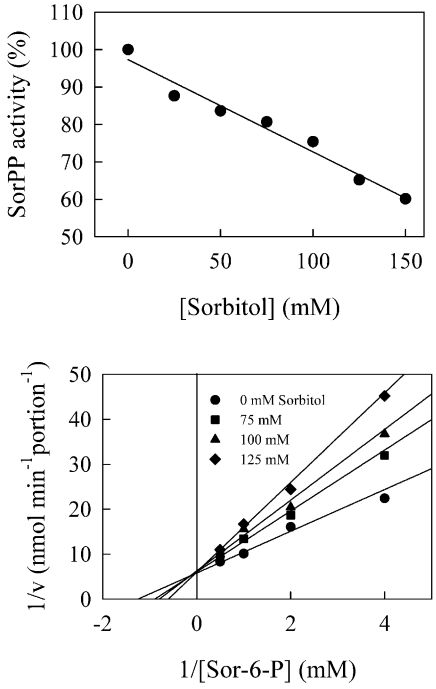

Fig. 5. The inhibitory effect of sorbitol on SorPP activity. Upper: The dose effect of sorbitol on SorPP activity. The enzyme activity was assayed with 50 mM MES-NaOH (pH 6.8), 10 mM $MgCl_2$, 2 mM sorbitol-6-P and various concentration of sorbitol indicated in the figure. Bottom: Lineweaver–Burk plot. Each point is the mean of four independent measurements. All the standard errors are less than 5% of the mean.

sorbitol-6-P (Fig. 5). Sorbitol is a competitive inhibitor of apple leaf SorPP with a Ki of 109 mM (Fig. 5).

Apple leaf SorPP activity was reduced by less than 10% with the inclusion of glucose, fructose, or sucrose up to 200 mM in the assay mixture (data not shown).

3.6. Effects of other substances and ions

A wide variety of substances were tested for their effects on the purified enzyme using 2 mM sorbitol-6-P as substrate. Fluoride, molybdate, vanadate, and Pi are well known non-specific inhibitors for phosphatases. Apple leaf SorPP was extremely sensitive to vanadate and molybdate. Vanadate at 50 μM or molybdate at 0.5 mM decreased the enzyme activity by more than 99%. Fluoride was also a potent inhibitor. Only 4 and 0.3% of the enzyme activity remained in the presence of 2 and 5 mM fluoride, respectively. Pi, a product of the SorPP catalyzed reaction, showed modest inhibitory effect on SorPP activity. At the maximum concentration (10 mM) used in this study, Pi caused an 82% inhibition (Fig. 6).

NaCl, KCl, glutamine, glutamic acid, asparagine, aspartic acid, ATP, ADP, AMP, reduced glutathione, oxidized glutathione, ascorbic acid at 2 mM had little effect (±15% control activity) on SorPP activity. Pre-incubation of the enzyme with 1 mM *N*-ethylmaleimide

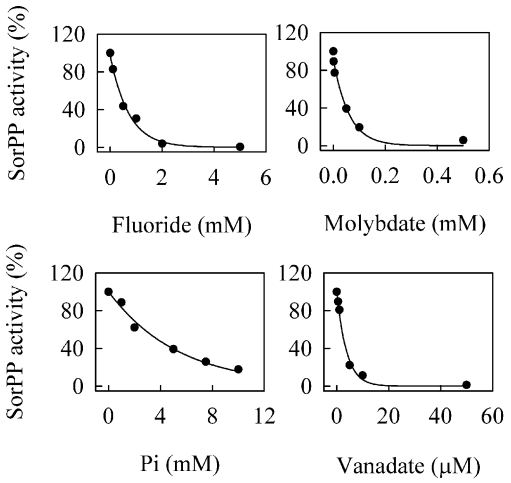

Fig. 6. Effects of various common phosphatase inhibitors on apple leaf SorPP. Each point is the mean of four independent measurements. All the standard errors are less than 5% of the mean.

for up to 2 min at 25 °C also had no effect on the enzyme activity (data not shown).

4. Discussion

SorPP catalyzes the final step in sorbitol biosynthesis in sorbitol-synthesizing species. Our data show that the apple leaf SorPP is highly specific to sorbitol-6-P. This enzyme also possesses some regulatory properties.

Our finding that the purified SorPP is highly specific to sorbitol-6-P is in contrast to that of Grant and ap Rees' [7]. They showed that the partially purified SorPP had a relatively high activity with hexose phosphate and more than 40% activity was demonstrated with fructose-1,6-bisphosphate as a substrate in comparison with that of sorbitol-6-P. This may have been caused by the difference in purity of the enzyme preparations. Grant and ap Rees used an enzyme preparation of 40–60% $(NH_4)_2SO_4$ precipitation from crude extract, in which other phosphatases may have not been completely removed. The difference in purity of enzyme preparations may also be responsible for the discrepancy of the optimal pH observed between the two studies. Two peaks of activity were reported with sorbitol-6-P at pH 5.0 and 7.1 by Grant and ap Rees [7], but our data indicate that the enzyme activity is very low at pH 5.0. However, we cannot exclude the possibility that another form of SorPP might be present in the enzyme preparation used by Grant and ap Rees [7].

Our results indicate that SorPP activity is absolutely dependent on Mg^{2+}. It is interesting to note that Mg^{2+} also regulates the activity of A6PR, another enzyme in the pathway of sorbitol biosynthesis. Mg^{2+} increases the affinity of A6PR for glucose-6-P, a substrate for that enzyme [11].

The feedback inhibition of SorPP by sorbitol observed in this study suggests that the reaction catalyzed by SorPP might be a regulatory step in sorbitol biosynthesis. The carbon partitioning in sorbitol synthesizing plants is influenced by developmental and environmental factors [2], but the mechanism is poorly understood. A shift of photoassimilate partitioning away from sorbitol to starch takes place as sorbitol accumulates in apple leaves during the day in a diurnal cycle [12] and at elevated CO_2 [13]. In peach leaves the carbon partitioning between sorbitol, sucrose and starch is related to net photosynthetic rate [13]. At low photosynthetic rate, most of the newly fixed carbon is channeled into sorbitol, but as photosynthetic rate increases, carbon partitioning into starch and sucrose is favored with concomitant accumulation of sorbitol [14]. Although sorbitol inhibits the hydrolysis of sorbitiol-6-P with a relatively high Ki of 109 mM (Fig. 5), high concentrations of sorbitol may still lead to a significant inhibition of sorbitol formation. Currently, we do not know the exact concentration of sorbitol in the cytosol of apple leaves, but as a primary photosynthetic end product and predominant storage carbohydrate, sorbitol may accumulate to a very high level in the leaves of sorbitol-synthesizing species. The sorbitol concentration in apple leaf sap is higher than 300 mM in well-watered plants [3]. Peach leaf blade tissues accumulate as high as 415 mM sorbitol [15]. Celery, a mannitol synthesizing species, accumulates mannitol up to 300 mM in the cytosol of the leaves [16]. If the accumulation of sorbitol in the cytosol reaches a high level, it is possible that feedback inhibition of SorPP activity by sorbitol occurs, leading to the buildup of sorbitol-6-P, which, in turn, induces changes in the photosynthetic carbon partitioning in the cells. Zhou et al. [17] demonstrated that sorbitol-6-P alters the activity of apple leaf sucrose phosphate synthase, a key enzyme for sucrose synthesis, as a competitive inhibitor. Accumulation of sorbitol in the cytosol, therefore, may eventually lead to a decrease in carbon partitioning to both sorbitol and sucrose in the cytosol and an upregulation of starch synthesis in the chloroplast.

Acknowledgements

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