

Fructose 2,6-Bisphosphate Hydrolyzing Enzymes in Higher Plants¹

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ABSTRACT

The phosphatases that hydrolyze fructose 2,6-bisphosphate in a crude spinach (*Spinacia oleracea* L.) leaf extract were separated by chromatography on blue Sepharose, into three fractions, referred to as phosphatases I, II, and III, which were further purified by various means. Phosphatase I hydrolyzed fructose 2,6-bisphosphate, with a K_m value of 30 micromolar, to a mixture of fructose 2-phosphate (90%) and fructose 6-phosphate (10%). It acted on a wide range of substrates and had a maximal activity at acidic pH. Phosphatase II specifically recognized the osyl-link of phosphoric derivatives and had more affinity for the β -anomeric form. Its apparent K_m for fructose 2,6-bisphosphate was 30 micromolar. It most likely corresponded to the fructose-2,6-bisphosphatase described by F. D. Macdonald, Q. Chou, and B. B. Buchanan ([1987] *Plant Physiol* 85: 13–16). Phosphatase III copurified with phosphofructokinase 2 and corresponded to the specific, low- K_m (24 nanomolar) fructose-2,6-bisphosphatase purified and characterized by Y. Larondelle, E. Mertens, E. Van Schaftingen, and H. G. Hers ([1986] *Eur J Biochem* 161: 351–357). Three similar types of phosphatases were present in a crude extract of Jerusalem artichoke (*Helianthus tuberosus*) tuber. The concentration of fructose 2,6-bisphosphate decreased at a maximal rate of 30 picomoles per minute and per gram of fresh tissue in slices of Jerusalem artichoke tuber, upon incubation in 50 millimolar mannose. This rate could be accounted for by the maximal extractable activity of the low- K_m fructose-2,6-bisphosphatase. A new enzymic method for the synthesis of β -glucose 1,6-bisphosphate from β -glucose 1-phosphate and ATP is described.

Fru-2,6- P_2 ² is a regulatory metabolite present in most eukaryotic cells (reviewed in Van Schaftingen [23]). It is synthesized from Fru-6-P and ATP by a PFK 2, also called phosphofructokinase 2, and is hydrolyzed back to Fru-6-P and Pi by a specific FBPase 2. Two types of FBPase 2, which greatly

differ in their affinity for their substrate, are known. A low (below 1 μM) K_m enzyme is present in most eukaryotic cells, including those from higher plants (4, 12, 22, 27, 28), and is easily detected, even in crude preparations, by its ability to form a [³²P]phosphoenzyme intermediate when incubated in the presence of 1 μM [2-³²P]Fru-2,6- P_2 (12, 23). A high (100–300 μM) K_m FBPase 2 is present in yeast (8) and in higher plants (2, 11, 13, 14, 18), with the reservation, however, that the specificity of this enzyme has only been carefully investigated by François *et al.* (8), and MacDonald *et al.* (13, 14). The biphasic substrate saturation curve reported by several groups working with plants (4, 19) can probably be attributed to the use of a mixture of the low- and high- K_m enzymes. The respective role of the two types of enzyme in the hydrolysis of Fru-2,6- P_2 *in vivo* has not been defined. Another complication is that, in the liver (23) and muscle (23) and in higher plants (12), but not in yeast (8) or in *Trypanosoma brucei* (26), PFK 2 and the low- K_m FBPase 2 are part of a single bifunctional protein. We recently described (12) the extensive purification and characterization of the bifunctional protein from spinach leaves and also reported the presence of a large excess of other phosphatase(s) displaying much less affinity for Fru-2,6- P_2 (12). On the other hand, MacDonald *et al.* (13, 14) reported that the high- K_m FBPase 2 from spinach leaf and from other plants could be separated from PFK 2 on the basis of either its charge or mol wt and that only a negligible amount of FBPase 2 was associated with PFK 2.

The purpose of the present work was to further characterize the several phosphatases acting on Fru-2,6- P_2 in higher plants and to estimate their relative role in the hydrolysis of Fru-2,6- P_2 *in vivo*.

MATERIALS AND METHODS

Materials

Mono S, Mono Q, and Superose 6 HR 10/30 columns, blue Sepharose, and gel filtration calibration standards were from Pharmacia-LKB (Uppsala, Sweden). Dowex AG-1 was from Bio-Rad (Richmond, CA), α -Glc-1-P from Fluka (Buchs, Switzerland) and DTT from Janssen Chimica (Beerse, Belgium). Bovine γ -globulin, bovine serum albumin (fraction V), antipain, Fru-1-P, Fru-6-P, Fru-1,6- P_2 , β -Glc-1-P, NADP⁺, glycerol 3-P, *p*-nitrophenylphosphate, as well as rabbit muscle PFK 1 (type III) and α -phosphoglucosyltransferase were from Sigma (St Louis, USA). Other enzymes and biochemicals were from Boehringer (Mannheim, FRG). Glycerol 2-P and chemicals were from Merck (Darmstadt, FRG) and were

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² Abbreviations: Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; PFK 2, 6-phosphofructo 2-kinase (EC 2.7.1.105); FBPase 2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-1-P, fructose 1-phosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; α - or β -Glc-1-P, α - or β -glucose 1-phosphate; PFK 1, 6-phosphofructo 1-kinase (EC 2.7.1.11); α - or β -Glc-1,6- P_2 , α - or β -glucose 1,6-bisphosphate; Fru-2-P, fructose 2-phosphate; Glc-6-P, glucose 6-phosphate; Man-6-P, mannose 6-phosphate; $I_{0.5}$, concentration of inhibitor giving 50% inhibition.

of analytical grade. Fru-2,6-P₂ (24), [2-³²P]Fru-2,6-P₂ (25), and the dye-reagent for the Bradford protein assay (3) were prepared as described. The extract of *Euglena gracilis* containing β -phosphoglucumutase was a kind gift of Dr. F. Opperdoes from this Institute. Spinach leaves (*Spinacea oleracea*) and Jerusalem artichoke tubers (*Helianthus tuberosus*) were obtained from a local market.

Synthesis of β -Glc-1,6-P₂

In the knowledge that, in the presence of ATP, PFK 1 converts α -Glc-1-P slowly into α -Glc-1,6-P₂ (6), we attempted to make β -Glc-1,6-P₂, using β -Glc-1-P as substrate. We observed that, in the general conditions described below, rabbit muscle PFK 1 was, respectively, 25- and 200-fold less active on 4 mM β - and α -Glc-1-P than on 4 mM Fru-6-P.

The reaction mixture contained 0.2 mM ATP, 1 mM β -Glc-1-P, 0.5 mM AMP, 1.1 mM phosphoenolpyruvate, 2 mM MgCl₂, 0.33 mM NADH, 2 μ g of pyruvate kinase, 1 μ g of lactate dehydrogenase, 35 μ g of PFK 1, and 50 mM Tris/ acetic acid (pH 7.8) in a total volume of 1 mL. AMP was used, instead of Fru-2,6-P₂, to stimulate PFK 1, because the bisphosphoric ester would have been difficult to separate from β -Glc-1,6-P₂. The reaction was started by the addition of PFK 1 and run at 37°C. It was followed by the oxidation of NADH, thanks to the pyruvate kinase-lactate dehydrogenase coupled system. This system had the advantage of regenerating ATP and, thus, allowed the use of a noninhibitory concentration of the latter nucleotide. The reaction reached near completion after 3 h and was then stopped by adding 50 μ L of 2.0 N NaOH and heating at 60°C for 5 min. β -Glc-1,6-P₂ was purified on a Dowex AG-1 column, by a procedure similar to that described for Fru-2,6-P₂ (25), and identified by its ability to be converted to Glc-6-P under acidic conditions (16). In agreement with Posternak (16), it was more acid-labile than α -Glc-1,6-P₂. It stimulated β -phosphoglucumutase in an extract of *Euglena gracilis* but had no effect on the commercial α -phosphoglucumutase from rabbit muscle.

Assay of Enzymes

All assays were performed at 30°C. The enzymic hydrolysis of Fru-2,6-P₂ was measured either in a radiochemical assay by the release of [³²P]Pi or [³²P]Fru-2-P from [2-³²P]Fru-2,6-P₂ or spectrophotometrically by the production of Fru-6-P in a coupled enzyme assay. The radiochemical assay mixture contained 20 mM potassium acetate, 1 mM EDTA, 5 mM DTT, 2 mg/mL bovine serum albumin, 0.25 mM Pi, 100,000 cpm of [2-³²P]Fru-2,6-P₂ at the indicated concentration and 50 mM Hepes (pH 7.0) in a total volume of 0.25 mL. The assay was stopped after various times ranging from 2 to 20 min by the addition of 1 volume of 0.1 N NaOH containing 5 mM Pi. The alkaline mixture was heated at 80°C for 5 min, cooled, and chromatographed on Dowex AG-1 to separate [³²P]Pi and [³²P]Fru-2-P from [2-³²P]Fru-2,6-P₂ as described earlier (25). [³²P]Pi and [³²P]Fru-2-P were eluted together from the Dowex AG-1 column by 0.15 M NaCl but could be separated afterward by precipitation of [³²P]Pi as an ammonium-magnesium salt by a modification of the procedure described in Friedkin (9). One mL of the 0.15 M NaCl eluate

was mixed with 10 μ L of 100 mM Pi, 25 μ L of 200 mM glycerol 2-P (to avoid adsorption of small amounts of other phosphoric esters on the MgNH₄PO₄ precipitate), 20 μ L of 2.5 M magnesium chloride, 100 μ L of 5 M ammonium chloride, and 75 μ L of 15 N ammonium hydroxide, and this mixture was kept at 0°C for 2 h. The MgNH₄PO₄ precipitate was then discarded by centrifugation and 0.25 mL of the supernatant was counted. Standards made either with [³²P]Pi or [U-¹⁴C]Glc-1-P confirmed that more than 99% of the Pi precipitated by this method, whereas more than 99% of the sugar phosphates remained in the supernatant. Thus, the radioactivity remaining in the supernatant corresponded to a [³²P]monophosphate derivative formed from [2-³²P]Fru-2,6-P₂, i.e. [³²P]Fru-2-P. The amount of [³²P]Pi was calculated by the difference between the radioactivity present in the solution before and after precipitation.

The formation of Fru-6-P from Fru-2,6-P₂ or Fru-1,6-P₂, as well as that of Glc-6-P from α - or β -Glc-1,6-P₂, was assayed spectrophotometrically at 340 nm in a mixture (0.8 mL) containing 20 mM potassium acetate, 1 mM EDTA, 0.25 mM NADP⁺, 5 μ g of Glc-6-P dehydrogenase, 10 μ g of phosphoglucose isomerase (for Fru-6-P formation only), the substrate at the indicated concentration and 50 mM Hepes (pH 7.0). The enzymic hydrolysis of α -Glc-1-P, β -Glc-1-P, and phosphoenolpyruvate was assayed spectrophotometrically at 340 nm in a mixture (0.8 mL) containing 20 mM potassium acetate, 1 mM EDTA, 0.5 mM substrate, and 50 mM Hepes (pH 7.0), plus 0.25 mM NADP⁺, 2 mM ATP-Mg, 10 μ g of hexokinase, and 5 μ g of Glc-6-P dehydrogenase for the hexoses-P, or 0.25 mM NADH and 2 μ g of lactate dehydrogenase in the case of phosphoenolpyruvate. The changes in A₃₄₀ were followed in a Perkin-Elmer 555 UV-VIS spectrophotometer or in an Aminco DW 2 spectrophotometer in the dual-wavelength mode (340–400 nm).

The hydrolysis of *p*-nitrophenylphosphate was measured by the change in A₄₀₀ in the presence of 1 mM Pi, 5 mM DTT, 20 mM substrate, and 100 mM Hepes (pH 7.0). The hydrolysis of inositol(1,4,5)trisphosphate was measured as in Erneux *et al.* (5). PFK 2 was assayed as in Larondelle *et al.* (12).

Purification of the Phosphatases Acting on Fru-1,6-P₂

Spinach leaves (35 g) washed with deionized water were homogenized twice for 30 s with a Waring Blendor in 1 volume of an ice-cold solution containing 50 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 1 mM iodoacetate, 2.5 mg/L antipain, and 50 mM Tris/acetic acid (pH 7.8) (buffer A). All subsequent steps were performed at 0 to 4°C. The homogenate was filtered through cheesecloth and the resulting filtrate was made 6% in PEG by addition of 0.16 volume of a 44% PEG 6000 solution prepared in buffer A. The mixture was allowed to stand for 5 min and was then centrifuged for 10 min at 15,000g. The resulting supernatant (40 mL) was poured, at a rate of 20 mL/h, on a blue Sepharose column (12 $\times$ 1.5 cm), equilibrated with buffer A. The column was washed with 20 mL of buffer A and a discontinuous NaCl gradient (80 mL, 0–0.5 M, followed by 40 mL, 0.5–1.6 M, and 25 mL, 2 M) made in the same buffer was then applied, at the same flow rate. Three peaks of activity were separated by this chromatography (Fig. 1).

Fractions 10 to 14 eluted from the blue Sepharose column were pooled. A portion (3 mL) of this solution was brought to pH 5.5 by the addition of 1 mL of 200 mM Mes (pH 5.5) and 5 mL of a solution (buffer B) containing 25 mM Mes (pH 5.5) and 5 mM DTT. The resulting mixture was poured on a cation-exchanger column (Mono S; 5.0×0.5 cm) previously equilibrated with buffer B. A continuous NaCl gradient (40 mL, 0–0.3 M) made in buffer B and programmed in a fast protein liquid chromatography system (Pharmacia-LKB) was applied and phosphatase I was eluted at 0.1 M NaCl (Fig. 2).

Fractions 31 and 32 from the blue Sepharose column were pooled. Four mL of this mixture were added to 12 mL of 50 mM Tris/acetic acid (pH 7.8) containing 5 mM DTT (buffer C) and poured on an anion-exchanger column (Mono Q; 5.0×0.5 cm) equilibrated with buffer C. A discontinuous NaCl gradient (25 mL, 0–0.32 M followed by 10 mL, 0.5 M) made in buffer C and programmed in the fast protein liquid chromatography system was applied (see Fig. 3).

Molecular mass was determined by gel filtration of a 0.2 mL sample of enzyme on a Superose 6 HR 10/30 column (30×1 cm) equilibrated with 25 mM Hepes (pH 7.0) containing 0.2 M NaCl and 5 mM DTT. Fractions of 0.2 mL were collected. M_r was calculated by comparison with protein standards as described previously (12).

A 6% PEG supernatant of Jerusalem artichoke tuber was prepared as described for spinach leaf, except that the peeled tubers were homogenized in 2 volumes of buffer A, and that 0.5% PVP was added to the filtrate before the PEG fractionation.

Other Methods

Man-6-P (10) was measured as described. Other methods, including the measurement of ATP, Glc-6-P, and Fru-2,6-P₂ were as in Mertens *et al.* (15). Protein was determined according to Bradford (3) with bovine γ -globulin as a standard.

RESULTS

Phosphatases Acting on Fru-2,6-P₂ in an Extract of Spinach Leaf

When a 6% PEG supernatant prepared from a spinach leaf homogenate, was loaded on a blue Sepharose column, the capacity to cause the disappearance of Fru-2,6-P₂, measured by the release of either [³²P]Pi or [³²P]Fru-2-P from [2-³²P]Fru-2,6-P₂ separated into three peaks. A first peak, referred to here for convenience as phosphatase I, corresponded to the activity not retained on the gel; a second peak (phosphatase II) was eluted at 0.25 M NaCl, and the third one (phosphatase III) at 1.4 M NaCl (Fig. 1).

Phosphatase I

Phosphatase I was easily measured by the formation of radioactive monophosphate derivatives upon incubation with 0.1 μ M [2-³²P]Fru-2,6-P₂. More than 90% of this radioactive product could not be precipitated as an ammonium-magnesium salt and was therefore [³²P]Fru-2-P (see fractions 22–25 in Fig. 2). Phosphatase I could also be detected by the spec-

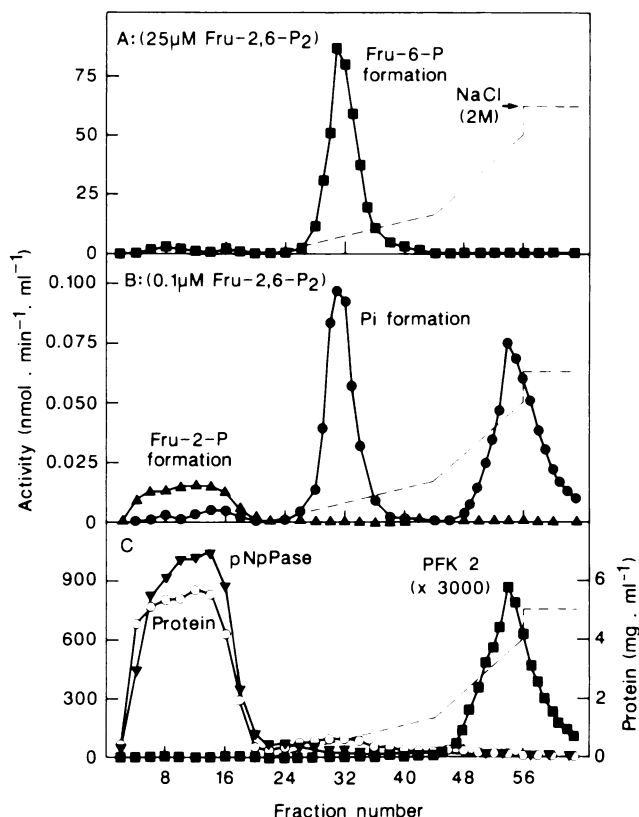


Figure 1. Elution profile of spinach leaf phosphatases from the blue Sepharose column. A portion (40 mL) of a 6% PEG supernatant of spinach leaf was loaded on the column, and fractions of 3.5 mL were collected. The hydrolysis of Fru-2,6-P₂ was measured directly by the spectrophotometric assay at 25 μ M substrate (panel A) and, after storage of the fractions at -80°C , by the radiochemical assay at 0.1 μ M substrate (panel B). Because of a loss of activity during the freezing and thawing procedure and of a 40% inhibition by Pi in the radiochemical assay, the activity of phosphatase II measured at 0.1 μ M Fru-2,6-P₂ was only about one-third of that expected from the known K_m value. The hydrolysis of 20 mM *p*-nitrophenylphosphate (pNpPase) and the activity of PFK 2 were also measured (panel C).

trophotometric assay thanks to the formation of a small proportion of Fru-6-P from 25 μ M Fru-2,6-P₂.

Phosphatase I was not retained on an anion exchanger (Mono Q) at pH 7.0 but could be purified more than 150-fold by chromatography on a cation exchanger (Mono S), on which it was adsorbed at pH 5.5 and from which it was eluted at 0.1 M NaCl (Fig. 2). By this procedure, phosphatase I was separated from other phosphatases displaying very little activity on Fru-2,6-P₂ and which were not retained on the gel. The fractions active on Fru-2,6-P₂ also catalyzed the formation of Fru-6-P from Fru-1,6-P₂, of *p*-nitrophenol from *p*-nitrophenylphosphate (Fig. 2), of pyruvate from phosphoenolpyruvate, of glucose from α - and β -Glc-1-P, and of Glc-6-P from α - and β -Glc-1,6-P₂ (not shown); the activity on α -Glc-1,6-P₂ was increased twofold by the addition of α -phosphoglucosyltransferase to the assay system, indicating that both α -Glc-1-P and Glc-6-P were formed.

At pH 7.0, the K_m values for Fru-2,6-P₂, Fru-1,6-P₂, phosphoenolpyruvate, and *p*-nitrophenylphosphate were 20 μ M,

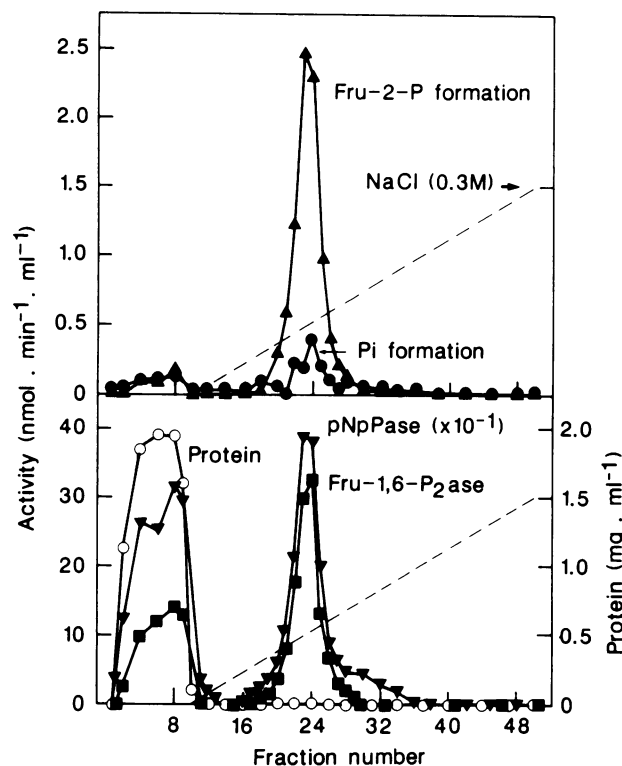


Figure 2. Elution profile of spinach leaf phosphatase I from the Mono S column. Three mL of the pool of fractions 10 to 14 from the blue Sepharose column (Fig. 1) were loaded on the column and fractions of 1 mL were collected. The activity on Fru-2,6-P₂ was measured by the radiochemical assay at 25 μ M substrate (upper panel). The activities leading to the production of Fru-6-P from 100 μ M Fru-1,6-P₂ (called here Fru-1,6-P₂ase) or of *p*-nitrophenol from 20 mM *p*-nitrophenylphosphate (pNpPase) were measured spectrophotometrically (lower panel).

45 μ M, 80 μ M, and 7.1 mM, whereas the maximal activities with these substrates were respectively 6, 30, 150, and 400 nmol/min/mL in the most active fractions from the Mono S column (fractions 23 and 24 in Fig. 2). Remarkably, the rates of formation of glucose from 500 μ M β -Glc-1-P (10 nmol/min/mL) and of Glc-6-P from 100 μ M β -Glc-1,6-P₂ (12 nmol/min/mL) were, respectively, 20- and 4-fold higher than those from similar concentrations of the corresponding α -anomers. The activity on phosphoenolpyruvate was competitively in-

hibited by Fru-2,6-P₂ and Fru-1,6-P₂ with K_i values of 20 and 40 μ M.

The activity of phosphatase I on Fru-2,6-P₂ increased 3.5-, 5.5-, and 6.0-fold when the pH was decreased from 7.0 to 6.5, 6.0, and 5.5, respectively. It could not be assayed at lower pH values because of the acid lability of the substrate.

Phosphatase II

Phosphatase II hydrolyzed [2-³²P]Fru-2,6-P₂ into equimolar amounts of Fru-6-P, measured by the spectrophotometric method, and of [³²P]Pi, forming no [³²P]Fru-2-P. As summarized in Table I, phosphatase II was purified 50-fold by chromatography on blue Sepharose (Fig. 1) and an additional 6-fold by anion exchange chromatography on Mono Q (Fig. 3). The active fractions obtained by this procedure, as well as upon gel filtration on a Superose 6 HR 10/30 column (not shown), converted α - or β -Glc-1,6-P₂ into Glc-6-P and Pi, and the rate of this conversion was not affected by phosphoglucomutase, indicating that only the osyl-phosphate linkage was split. The same fractions also catalyzed the formation of glucose from β -Glc-1-P, but had not detectable activity (less than 2% of the activity on Fru-2,6-P₂) on Fru-1,6-P₂, α -Glc-1-P, phosphoenolpyruvate, or inositol(1,4,5)trisphosphate. Fru-1,6-P₂ and Pi were mixed inhibitors relative to Fru-2,6-P₂. Kinetic parameters of phosphatase II are shown in Tables II and III. Because of the presence of 0.25 mM Pi, the radiochemical assay underestimated by 40% the activity of phosphatase II (see Fig. 1). The Boehringer monosodium salt of phosphoenolpyruvate contained an undefined contaminant that behaved as an allosteric competitive inhibitor relative to Fru-2,6-P₂ and that transformed the saturation curve for that substrate from hyperbolic to sigmoidal (not shown). This effect was not observed either when 0.2% bovine serum albumin was added to the assay mixture or with a preparation of phosphoenolpyruvate that had been purified by chromatography on Dowex AG-1.

The effects of other phosphate derivatives, such as glycerate 2-P, glycerate 3-P, 2,3-bisphosphoglycerate, glycolate 2-P, dihydroxyacetone-P, glycerol 3-P, and pyrophosphate were also analyzed by the spectrophotometric assay, whereas the effect of Fru-6-P was tested by the radiochemical assay. All these phosphate derivatives had little inhibitory effect ($I_{0.5}$ greater than 2 mM) on the activity when the assays were performed at 25 μ M Fru-2,6-P₂ (not shown). Phosphatase II was also inhibited by all divalent cations tested; indeed, $I_{0.5}$ for Mg²⁺,

Table I. Purification of Phosphatase II from Spinach Leaves

Values shown are calculated by making the assumption that all the enzyme obtained at one step is used for the next step.

Step	Protein	Total Activity	Specific Activity	Purification	Yield
	mg	μ mol · min ⁻¹	nmol · min ⁻¹ · mg ⁻¹	-fold	%
Homogenate	777	4.3	5.5	1	100
6% PEG supernatant	320	2.9	9.1	1.6	67
Blue Sepharose fractions (31–32)	4.4	1.17	266	48	27
Mono Q (fraction 35)	0.29	0.46	1586	288	11

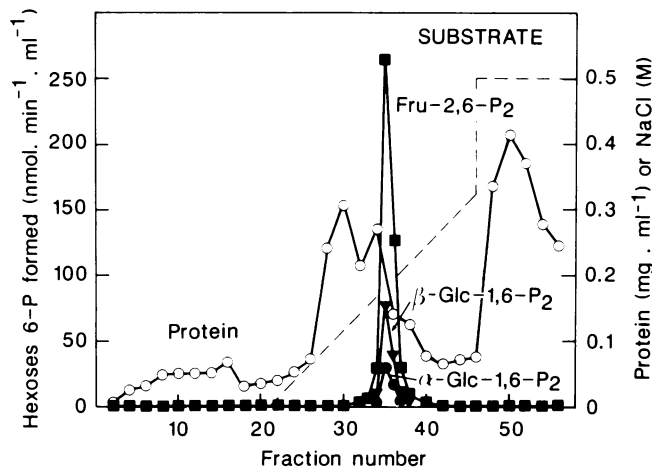


Figure 3. Elution profile of spinach leaf phosphatase II from the Mono Q column. Four mL of the pool of fractions 31 and 32 from the blue Sepharose column (Fig. 1) were loaded on the column and fractions of 1 mL were collected. The activities were measured spectrophotometrically, using 100 μ M Fru-2,6-P₂, α -Glc-1,6-P₂, or β -Glc-1,6-P₂ as substrate.

Table II. Kinetic Parameters of Phosphatase II

Substrates	K_m	V_{max}
	μ M	%
β -Fru-2,6-P ₂	30	100
α -Glc-1-P		ND ^a
α -Glc-1,6-P ₂	130	10
β -Glc-1-P	800	25
β -Glc-1,6-P ₂	30	25

^a Nondetectable (less than 2%).

Table III. Effect of Fru-1,6-P₂ and Pi on the Kinetic Properties of Spinach Leaf Phosphatase II

Enzymic preparation corresponded to fraction 31 from Figure 1.

Inhibitor		K_m for Fru-2,6-P ₂	V_{max} for Fru-2,6-P ₂
	<i>mM</i>	<i>μM</i>	<i>nmol · min⁻¹ · ml⁻¹</i>
None		30	220
Fru-1,6-P ₂	0.25	63	182
	0.50	105	155
	1.00	135	133
Pi	0.25	32	167
	0.50	40	143
	1.00	50	105

Ca²⁺, and Mn²⁺ were, respectively, 5.0, 6.0, and 0.5 mM, at 25 μ M Fru-2,6-P₂. These values were determined in the presence of 20 μ M EDTA, instead of 1 mM, in the assay mixture.

The activity of phosphatase II on Fru-2,6-P₂ was optimal between pH 6.5 and 7.0, and amounted to 50% of this value at pH 5.6 and 7.8. An M_r of 50,000 $\pm$ 7,000 (mean $\pm$ SEM, n = 3) was calculated for this enzyme by gel filtration on a Superose 6 column (not shown).

Phosphatase III

Phosphatase III copurified with PFK 2 and is the bifunctional PFK 2/low- K_m FBPase 2 described in Larondelle *et al.* (12). At 25 μ M substrate, the blue Sepharose preparation of the enzyme displayed a barely detectable activity on α - and β -Glc-1,6-P₂ (less than 5% of the activity on Fru-2,6-P₂).

Walker and Huber (27, 28) recently described a novel form of low- K_m FBPase 2 that was associated with PFK 2 and could be activated simultaneously with PFK 2 upon preincubation of the spinach leaf extract in the presence of ATP-Mg. Using the same experimental procedure (27), we were unable to reproduce this observation. The reason for this discrepancy is unknown.

Phosphatases Acting on Fru-2,6-P₂ in an Extract of Jerusalem Artichoke Tuber

The phosphatases acting on Fru-2,6-P₂ in a 6% PEG supernatant of Jerusalem artichoke tuber could be separated by chromatography on blue Sepharose, as in the case of the spinach leaf enzymes, but with two major differences (Fig. 4): first, the activity corresponding to phosphatase II separated into two peaks, called IIa and IIb; second, the relative activities of the phosphatases, measured at 0.1 μ M Fru-2,6-P₂, were very different from those of the spinach leaf enzymes. Indeed, when compared to the activity of phosphatase III, phosphatase I was twice as active and phosphatases IIa and IIb were 10-fold less active in Jerusalem artichoke tuber than in spinach leaf (*cf.* Figs. 1 and 4).

The various phosphatases found in Jerusalem artichoke tuber shared many properties with their spinach leaf counter-

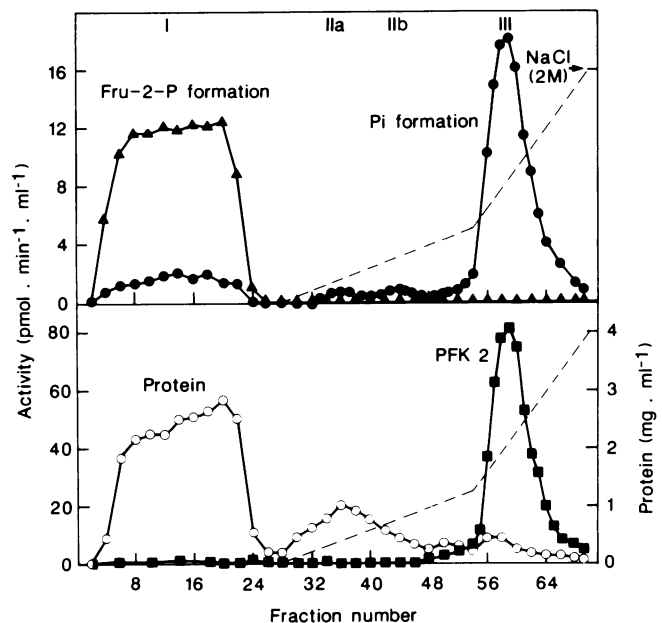


Figure 4. Elution profile of Jerusalem artichoke tuber phosphatases from the blue Sepharose column. A portion (55 mL) of a 6% PEG supernatant of Jerusalem artichoke tuber was loaded on the column and fractions of 3.5 mL were collected. The hydrolysis of Fru-2,6-P₂ was measured by the radiochemical assay at 0.1 μ M substrate (upper panel). The activity of PFK 2 was also measured (lower panel).

parts (not shown). Indeed, phosphatase I was not retained on blue Sepharose and hydrolyzed $[2\text{-}^{32}\text{P}]\text{Fru-2,6-P}_2$ mostly into $[^{32}\text{P}]\text{Fru-2,6-P}_2$, since more than 80% of the incubation product could not be precipitated as an ammonium-magnesium salt. Phosphatases IIa and IIb hydrolyzed Fru-2,6-P_2 into Fru-6-P and Pi with K_m values higher than $50\text{ }\mu\text{M}$ and were inhibited by divalent cations. Phosphatase III produced Fru-6-P and Pi with a K_m of 20 nM and copurified with PFK 2.

In Vivo Disappearance of Fru-2,6-P_2

To estimate the rate at which phosphatases acting on Fru-2,6-P_2 are expected to operate *in vivo*, we looked for conditions under which the rate of degradation of Fru-2,6-P_2 was maximal. Because Stitt *et al.* (20) reported that the concentration of Fru-2,6-P_2 is very low in spinach leaf discs incubated in the presence of mannose, we further investigated the effect of this sugar on Fru-2,6-P_2 concentration in Jerusalem artichoke tuber slices.

Slices of Jerusalem artichoke tuber prepared as described earlier (15) were incubated successively in water for 80 min and in 50 mM mannose for 125 min and were returned to water again for 120 min. Control slices were kept in water throughout the experiment. Figure 5 shows that Man-6-P accumulated in slices incubated in the presence of mannose. During the same time and as expected from Stitt *et al.* (20), the concentration of Fru-2,6-P_2 decreased at an average rate of 30 pmol/min/g of fresh tissue. These changes were associated with a decrease in ATP and, to a lesser extent, in Glc-6-P . When the mannose-treated slices were rinsed and further incubated in water, Man-6-P disappeared progressively, while ATP, Glc-6-P and Fru-2,6-P_2 increased to the control values, indicating that the viability of the cells was preserved during mannose treatment.

The effects of 2 mM Man-6-P on the activities of PFK-2 and of phosphatases IIa, IIb, and III from Jerusalem artichoke tuber were investigated. We found that 2 mM Man-6-P inhib-

ited by 35 and 85%, respectively, the activity of PFK 2 and that of phosphatase III measured at $0.1\text{ }\mu\text{M}$ Fru-2,6-P_2 but had no effect on the activity of phosphatases IIa and IIb.

DISCUSSION

Properties of the Phosphatases Acting on Fru-2,6-P_2 in Higher Plants

Three similar types of phosphatase acting on Fru-2,6-P_2 were found in such different tissues as spinach leaf and Jerusalem artichoke tuber. We will successively discuss the properties and potential role of each of these types of phosphatase.

Phosphatase I

Phosphatase I is an acid phosphatase, active on a wide variety of substrates. Indeed, even after a 150-fold purification, this cationic protein was still able to hydrolyze all substrates tested, including $\alpha\text{-Glc-1,6-P}_2$, Fru-2,6-P_2 , phosphoenolpyruvate and *p*-nitrophenylphosphate. Moreover, the activity on phosphoenolpyruvate was competitively inhibited by Fru-2,6-P_2 and Fru-1,6-P_2 , with K_i values corresponding to the respective K_m values. Phosphatase I breaks both the osyl- and the ester-phosphate links of $\alpha\text{-Glc-1,6-P}_2$ or of Fru-2,6-P_2 . In the case of Fru-2,6-P_2 , however, it produces about 10-fold more Fru-2-P than Fru-6-P , indicating that it splits the furanosyl phosphate linkage less easily than the ester phosphate one. The occurrence of an enzyme forming Fru-2-P from Fru-2,6-P_2 has not been previously reported in plants, but is well established in yeast (8, 17); in the latter case, the enzyme formed similar amounts of Fru-2-P and of Fru-6-P .

Phosphatase I has a higher activity on the β - than on the α -anomeric form of Glc-1-P or Glc-1,6-P_2 . Such a relative specificity for the β -anomeric form of sugar phosphates has already been observed for an acid phosphatase of mung bean

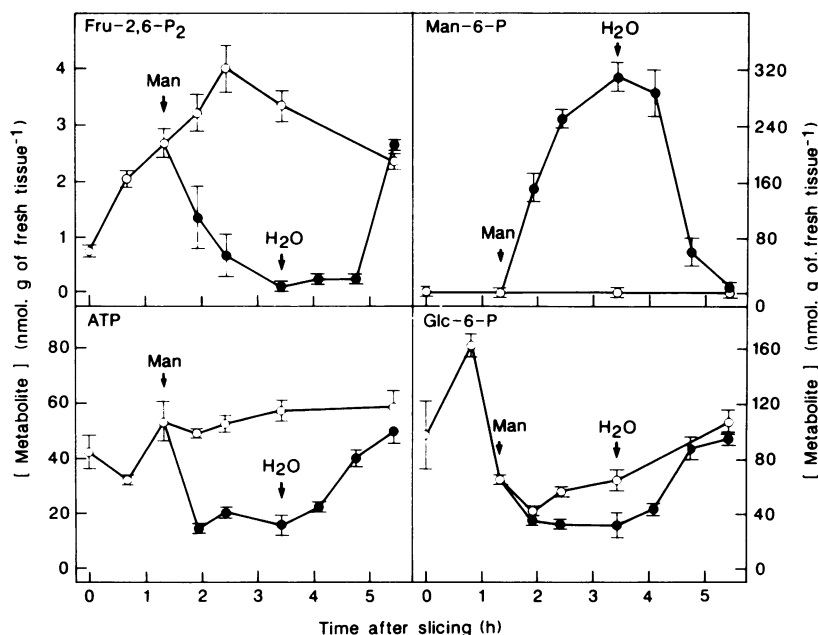


Figure 5. Effect of mannose on the concentration of several metabolites in slices of Jerusalem artichoke tuber. The slices were incubated successively in water (80 min), in 50 mM mannose (125 min), and in water (120 min). Controls were kept in water throughout the experiment (open circles). Values shown are means $\pm$ SEM for three different slices.

(7). The acid phosphatase of spinach leaf has been previously shown to be localized in the vacuole (1).

Phosphatase II

This enzyme specifically cleaves the osyl-phosphate linkage of several phosphate sugars. The specificity studies indicate that it has more affinity on one hand, for bisphosphoric esters and, on the other, for their β -anomeric form. Fru-2,6-P₂ is the only phosphate ester in plants that fulfills these two conditions. Despite the fact that, in our hands, the apparent M_r of phosphatase II was 50,000 rather than 97,000, the enzyme most likely corresponds to the specific FBPase 2 described by Macdonald *et al.* (13, 14). Indeed, as described by these authors, it is inhibited by divalent cations, by Fru-1,6-P₂ and by Pi, and displays a relatively high (30 μ M) K_m for Fru-2,6-P₂. The occurrence of a phosphatase hydrolyzing Fru-2,6-P₂ into Fru-6-P and Pi with a low affinity has also been reported in mung bean seedlings (2), in corn seedlings (18), in the endosperm of castor bean (11) and in the leaves of other C3 (lettuce, pea) and C4 (sorghum, amaranthus) plants (13, 14).

Phosphatase III

This enzyme is the low- K_m FBPase 2 known to be associated with PFK 2 in a bifunctional protein and previously purified and characterized by Larondelle *et al.* (12). The activity of this enzyme can easily be measured by the release of [³²P]Pi from [2-³²P]Fru-2,6-P₂, as well as by the formation of a [³²P] phosphoenzyme. In spinach leaf, as well as in Jerusalem artichoke tuber, its maximal activity is about one-tenth of that of PFK 2. The lower PFK 2/FBPase 2 ratio shown in Figure 1 or 4, may be due to a partial proteolysis that affects the PFK 2 much more than the FBPase 2 activity (12). The failure of Macdonald *et al.* (13, 14) to detect the presence of this well-defined enzyme is most likely explained by the lack of sensitivity of their assay procedure.

Role of Each of the Phosphatases in the Process of Fru-2,6-P₂ Hydrolysis *In Vivo*

Phosphatase I corresponds most likely to an unspecific acid phosphatase. Since all the acid phosphatase activity of spinach leaf has been reported to be located in the vacuole (1), phosphatase I is not expected to affect the cytosolic concentration of Fru-2,6-P₂, *in vivo*. In contrast, because of its association with PFK 2, the enzyme that forms Fru-2,6-P₂ in the cytosol (20), phosphatase III is supposed to be cytosolic. The localization of phosphatase II is less clear. Although they did not use a method that preserves the integrity of the vacuoles, Macdonald *et al.* (13) assumed that this enzyme is cytosolic, because they did not recover it in a particulate fraction. Nevertheless, we will assume here that phosphatase II and III are cytosolic enzymes.

If we do not consider the numerous inhibitory effects of some metabolites on their kinetic properties, we can calculate that, in spinach leaf, at a physiological concentration of 10 μ M Fru-2,6-P₂, phosphatase II is 200-fold more active than phosphatase III (Table IV). Due to the difference in the affinity

of these enzymes for Fru-2,6-P₂, this ratio diminishes when the concentration of Fru-2,6-P₂ is lowered. At 50 nM Fru-2,6-P₂ and less, phosphatase III becomes more active than phosphatase II. In Jerusalem artichoke tuber, phosphatase II (a + b) is proportionally less active (Table IV). At a concentration of Fru-1,6-P₂ lower than 1 μ M, it is less active than phosphatase III.

Transposition of these calculations to *in vivo* situations are rendered difficult by the marked changes in affinity and maximal activity provoked by the presence of a number of metabolites. It is in fact obvious that phosphatase II, if it is cytosolic, has to be strongly inhibited to allow the accumulation of Fru-2,6-P₂, especially in slices of Jerusalem artichoke tuber in which the Fru-2,6-P₂ cytosolic concentration could reach 100 μ M or more (15), but also in spinach leaf where, at 10 μ M Fru-2,6-P₂, its activity, if not inhibited, is about 20-fold higher than the maximal activity of PFK 2. Pi (K_i = 0.25 mM), Fru-1,6-P₂ (K_i = 0.1 mM) and, to a lesser extent, divalent cations are potential physiological inhibitors.

To verify the assertion that the hydrolysis of Fru-2,6-P₂ *in vivo* is driven by phosphatase II in addition to phosphatase III, one would need to find a condition *in vivo* where the disappearance of the phosphoric ester is faster than what can be accounted for by phosphatase III. To the best of our knowledge, the most dramatic drop of the Fru-2,6-P₂ concentration has been described in spinach leaf disks, following a dark-light transition (21). From these results, and assuming a Chl content of 2.5 mg/g of fresh tissue, we calculated a maximal rate of Fru-2,6-P₂ breakdown of about 120 pmol/min/g of fresh tissue. Nevertheless, the maximal activity of phosphatase III is quite sufficient for this rate of hydrolysis (Table IV).

We, then, turned to the model of slices of Jerusalem artichoke tuber. This model has, in fact, the main advantage of showing dramatic changes in the Fru-2,6-P₂ concentration, while having a low activity of the bifunctional PFK 2/FBPase 2 (fivefold less active than in spinach leaf, when expressed per g of fresh tissue). The most important drop in the concentration of Fru-2,6-P₂ that we could observe occurred when slices, incubated in water to allow the accumulation of Fru-2,6-P₂, were then transferred to a 50 mM mannose solution. Fru-2,6-P₂ was degraded at a rate of 30 pmol/min/g of fresh tissue,

Table IV. Comparison of the Maximal Rate of Fru-2,6-P₂ Degradation Observed *In Vivo* and the Calculated Activities of Phosphatases II and III at 10 μ M Fru-2,6-P₂

Values are expressed in picomols hydrolyzed per min and per g of fresh tissue.

	Spinach Leaf	Jerusalem Artichoke Tuber
Maximal rate of degradation <i>in vivo</i>	120 ^a	30
Phosphatase II or IIa + IIb activity	50,000	500
Phosphatase III activity	250	40

^a Calculated from the results reported in Stitt *et al.* (21), with the assumption that the Chl content of spinach leaf is 2.5 mg per g of fresh tissue.

which can, in this case also, be accounted for by the maximal extractable activity of phosphatase III (Table IV). The progressive accumulation of Man-6-P is, however, expected to reduce this activity, which is also partially inhibited by Fru-6-P ($K_i = 0.2$ mM for phosphatase III of spinach leaf [12]). Our *in vivo* observations do, therefore, not rule out the involvement of phosphatase II in the degradation of Fru-2,6-P₂.

The mechanism by which mannose causes a decrease in the concentration of Fru-2,6-P₂ has not been investigated. It could involve a lowering of ATP, Fru-2,6-P₂, or Pi, which are, respectively, two substrates and an essential activator of PFK 2 (12).

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