

# A product-regulated fructose 2,6-bisphosphatase occurs in green leaves

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An enzyme catalyzing the hydrolytic conversion of fructose 2,6-bisphosphate (Fru-2,6-P<sub>2</sub>) to fructose 6-phosphate (Fru-6-P) and P<sub>i</sub> has been identified and purified from plants, specifically the cytosolic fraction of spinach leaf parenchyma cells. Partially purified preparations of the enzyme, designated fructose 2,6-bisphosphatase (Fru-2,6-P<sub>2</sub>ase), were inhibited by products of the reaction (i.e., P<sub>i</sub> and Fru-6-P) but showed no response to a protein phosphorylation system known to inhibit the corresponding enzyme in mammalian cells. Fru-2,6-P<sub>2</sub>ase co-purified with fructose 6-phosphate,2-kinase, the enzyme catalyzing the synthesis of Fru-2,6-P<sub>2</sub>. The observed pattern of regulation of the enzymes functional in the synthesis and breakdown of Fru-2,6-P<sub>2</sub> reinforces the conclusion that chloroplasts play a role in controlling cytosolic carbon processing in leaves.

*Fructose 2,6-bisphosphate*

*Fructose 2,6-bisphosphatase*  
*Glycolysis*

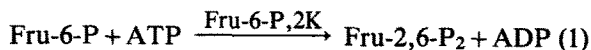
*Sucrose synthesis*

*Gluconeogenesis*

*Enzyme regulation*

## 1. INTRODUCTION

We recently reported evidence for the occurrence of an enzyme, designated fructose 6-phosphate,2-kinase, that catalyzes the synthesis of fructose 2,6-bisphosphate from fructose 6-phosphate and ATP in the cytosolic fraction of spinach leaf parenchyma cells [1]:



Fru-6-P,2K was regulated allosterically by

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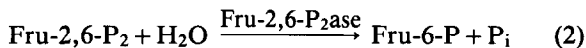
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**Abbreviations:** Fru-2,6-P<sub>2</sub>, fructose 2,6-bisphosphate; Fru-2,6-P<sub>2</sub>ase, fructose 2,6-bisphosphatase; Fru-6-P,2K, fructose 6 phosphate,2-kinase; PFP, pyrophosphate-linked phosphofructokinase (EC 2.7.190); PGA, 3-phosphoglycerate; Fru-1,6-P<sub>2</sub>, fructose 1,6-bisphosphate; Glu-1,6-P<sub>2</sub>, glucose 1,6-diphosphate

metabolite effectors that are formed and transported by chloroplasts (P<sub>i</sub>, an activator and 3-phosphoglycerate or PGA, an inhibitor). This finding supported earlier evidence that Fru-2,6-P<sub>2</sub> functions in the leaf cytosol to regulate carbon flow (i.e., sucrose synthesis and breakdown) through activation of a glycolytic enzyme (pyrophosphate-linked phosphofructokinase or PFP) and through inhibition of an enzyme of gluconeogenesis (fructose 1,6-bisphosphatase) [2,3]. Recent evidence showing changes in the concentration of Fru-2,6-P<sub>2</sub> in leaves as a result of illumination status is in accord with this conclusion [4].

If Fru-2,6-P<sub>2</sub> plays a dynamic role in carbon interconversions, an enzyme catalyzing its selective hydrolysis should be present in plant tissues. While such an enzyme has been described for animal systems [5–7], there is to date no evidence for its presence in plants. We have therefore investigated this problem and now report evidence for the occurrence of a product-inhibited enzyme catalyzing the hydrolysis of Fru-2,6-P<sub>2</sub> (Fru-2,6-P<sub>2</sub>ase) in the

cytosolic fraction of spinach leaf parenchyma cells:



The regulatory properties of the enzyme support the role for effector metabolites in controlling the level of Fru-2,6-P<sub>2</sub> in leaves suggested in [1].

## 2. MATERIALS AND METHODS

Spinach leaves (*Spinacea oleracea*) were obtained from a local market. All biochemicals including Fru-2,6-P<sub>2</sub> and coupling assay enzymes were obtained from Sigma Chemical Co. (St Louis MO).

### 2.1. Fru-2,6-P<sub>2</sub>ase 'bioassay'

Fru-2,6-P<sub>2</sub>ase was assayed at 20°C as described for Fru-6-P,2K except that the disappearance of Fru-2,6-P<sub>2</sub> was measured [1]. The reaction mixture contained: 0.1 M Tris-HCl (pH 7.4), and 0.1 μM Fru-2,6-P<sub>2</sub>. The reaction was started by adding enzyme; final vol. 0.1 ml. At zero time, 2 min, and 4 min, an aliquot of the reaction mixture was analyzed for Fru-2,6-P<sub>2</sub> by the PFP method in which NADPH oxidation is followed spectrophotometrically at 340 nm [1,2]. The amount of Fru-2,6-P<sub>2</sub> hydrolyzed was evaluated by comparing the extent of PFP activation in this assay to that effected by known amounts of Fru-2,6-P<sub>2</sub>.

### 2.2. Fru-2,6-P<sub>2</sub>ase spectrophotometric assay

Fru-2,6-P<sub>2</sub>ase could also be conveniently assayed (but with less sensitivity) by measuring the appearance of Fru-6-P in an Aminco DW-2 dual wavelength spectrophotometer. The spectrophotometric assay provided proof of the formation of Fru-6-P as a product of the Fru-2,6-P<sub>2</sub>ase reaction. The reaction mixture contained enzyme as indicated, 0.1 M Tris-HCl (pH 7.4), 10 units glucose 6-phosphate dehydrogenase, 10 units phosphoglucose isomerase; 1 mM NADP; and 0.1 μM (or the indicated amount) of Fru-2,6-P<sub>2</sub>; final vol. 1.0 ml. The reaction was started by adding enzyme. The NADP reduced was measured at 340 nm. This assay was about 1/5th as sensitive as the bioassay described above.

### 2.3. Purification of Fru-2,6-P<sub>2</sub>ase

Fru-2,6-P<sub>2</sub>ase was purified at 4°C from spinach

leaves, as described for Fru-6-P,2K [1] [homogenization of leaves, polyethylene glycol fractionation, DEAE (DE-52)-cellulose and hydroxyapatite chromatography] except that the polyethylene glycol fraction was 8–14% and the Tris-HCl buffer used after polyethylene glycol precipitation was pH 7.4. The enzyme, which was free of non-specific phosphatase activity, was concentrated by dialysis against 50 mM Tris-HCl buffer (pH 7.4), containing 50% glycerol and 0.1% 2-mercaptoethanol. The dialyzed Fru-2,6-P<sub>2</sub>ase preparation could be stored at –20°C for several days without appreciable loss of activity.

Protein was determined by the method of Bradford [8].

## 3. RESULTS AND DISCUSSION

As found for Fru-2,6-P<sub>2</sub> [1] and Fru-6-P,2K [2], Fru-2,6-P<sub>2</sub>ase was localized in the cytosolic fraction of leaf parenchyma cells. Partially purified preparations of Fru-2,6-P<sub>2</sub>ase showed a broad optimum at pH 7.4 (fig.1) and were preferential for Fru-2,6-P<sub>2</sub> as substrate (fig.2). The substrate

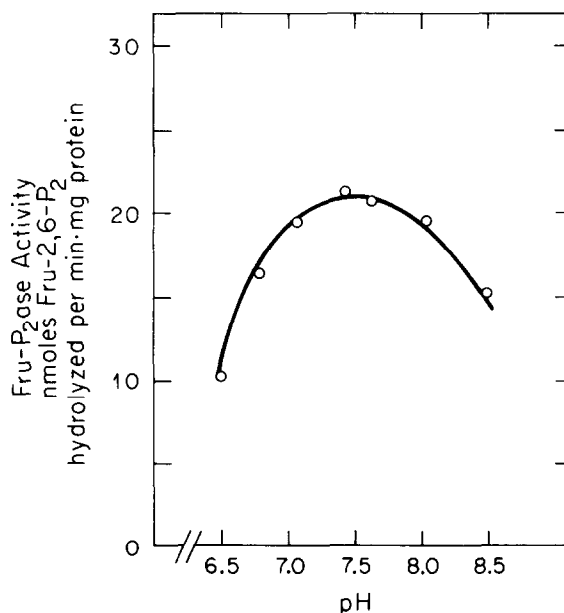


Fig.1. Effect of pH on spinach leaf Fru-2,6-P<sub>2</sub>ase; Fru-2,6-P<sub>2</sub>ase (25 μg) was assayed by following Fru-2,6-P<sub>2</sub> disappearance in the bioassay procedure.

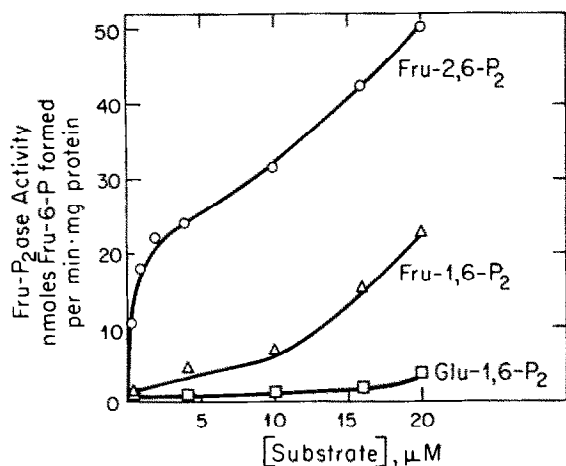


Fig. 2. Substrate specificity of spinach leaf Fru-2,6-P<sub>2</sub>ase; Fru-2,6-P<sub>2</sub>ase (45  $\mu$ g) was assayed by following Fru-6-P formation in the spectrophotometric assay.

specificity of the enzyme was also apparent from its great affinity for Fru-2,6-P<sub>2</sub> ( $s_{0.5} = 0.6 \mu\text{M}$ ) and its relatively high turnover rate in the presence of this substrate ( $V_{\text{max}} = 25.5$  munits/mg) (table 1). Thus, like its counterpart from animal cells, leaf Fru-2,6-P<sub>2</sub>ase seems to be an enzyme that functions mainly if not exclusively in the hydrolysis of Fru-2,6-P<sub>2</sub> [5–7]. However, unlike the mammalian enzyme, leaf Fru-2,6-P<sub>2</sub>ase was not affected by a protein phosphorylation system (beef heart protein kinase catalytic subunit). Leaf Fru-2,6-P<sub>2</sub>ase was also unaffected by reduced thioredoxins or

Table 1

Substrate affinity and maximal velocity of cytosolic Fru-2,6-P<sub>2</sub>ase from spinach leaves

Substrate	Substrate required for half maximal velocity ( $s_{0.5} \mu\text{M}$ )	Maximal velocity ( $V_{\text{max}}$ , munits/mg) <sup>a</sup>
Fru-2,6-P <sub>2</sub>	0.6	25.5
Fru-1,6-P <sub>2</sub>	15.0	6.7
Glu-2,6-P <sub>2</sub>	18.2	3.2

<sup>a</sup> One unit = The amount of enzyme which catalyzes 1  $\mu\text{mol}$  Fru-6-P formation/min in the spectrophotometric assay

disulfide compounds such as oxidized glutathione, by divalent cations such as  $\text{Mg}^{2+}$  (5–30 mM), or EDTA (10 mM).  $\text{MnCl}_2$  strongly inhibited the enzyme ( $I_{0.5} = 1 \text{ mM}$ ) (not shown).

Fru-6-P<sub>2</sub> from leaves was found [1] to respond strikingly to key metabolite effectors ( $\text{P}_i$ , an activator; and PGA, an inhibitor). With this perspective, we tested the effect of a variety of cytosolic metabolites on Fru-2,6-P<sub>2</sub>ase and observed strong inhibition by the products of its reaction; i.e.,  $\text{P}_i$  and Fru-6-P (fig. 3). No effect was observed with the other metabolites tested (mM): PGA, 3; glucose 6-phosphate, 10; glucose 1-phosphate, 2; fructose 1,6-bisphosphate, 1; sucrose, 100; dihydroxyacetone phosphate, 3; UDP-glucose, 3; cAMP, 10; and AMP, 10. We found no evidence for an activation of Fru-2,6-P<sub>2</sub>ase with any of the agents tested. Mammalian Fru-2,6-P<sub>2</sub>ase is also strongly inhibited by Fru-6-P [5,6].

A final point worth noting concerns the relationship between Fru-2,6-P<sub>2</sub>ase and Fru-6-P<sub>2</sub>K. As is the case for mammalian cells [5,6], the activities responsible for the synthesis and hydrolysis of Fru-2,6-P<sub>2</sub> in leaves co-purified throughout the above procedure. In view of their similarities, it will therefore be of interest to determine whether the two activities also reside on a single protein in leaves.

The present results provide evidence for an enzyme that selectively degrades Fru-2,6-P<sub>2</sub> in the cytosolic fraction of leaf parenchyma cells. The enzyme, Fru-2,6-P<sub>2</sub>ase is under regulation by Fru-6-P and  $\text{P}_i$ , two key leaf metabolites that also serve as

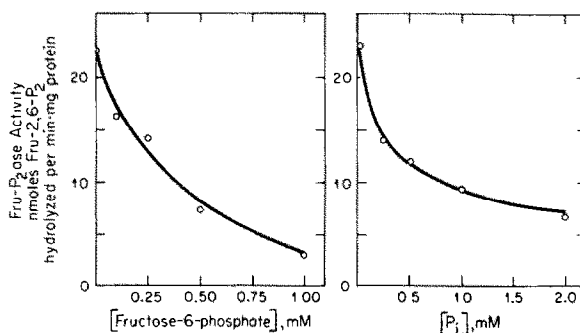


Fig. 3. Inhibition of spinach leaf Fru-2,6-P<sub>2</sub>ase by its products. Fru-2,6-P<sub>2</sub>ase (25  $\mu$ g) was assayed by following Fru-2,6-P<sub>2</sub> disappearance in the bioassay procedure.

reaction products. It is significant to note that the concentration of both of these compounds in the cytosol changes in accordance with the metabolic state of chloroplasts [9]. The pattern of regulation of Fru-2,6-P<sub>2</sub>ase by these metabolites thus supports the view [1] that Fru-2,6-P<sub>2</sub> represents a regulatory link between chloroplasts and the cytosol of photosynthetic leaf cells.

#### ACKNOWLEDGEMENTS

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