Biochem. J. (1987) 241, 71–74 (Printed in Great Britain)

Isolation and purification of chloroplastic spinach (*Spinacia oleracea*) sedoheptulose-1,7-bisphosphatase

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Higher-plant sedoheptulose-1,7-bisphosphatase was isolated and purified over 200-fold from spinach (*Spinacia oleracea*) chloroplast stromal extracts to apparent electrophoretic homogeneity by DEAE-Fractogel, molecular sieving on Sephadex G-200 and Blue B dye–matrix affinity chromatography. It is a protein of M_r 66000, made up of two apparently identical subunits (M_r 35000). The enzyme is activated by reduced thioredoxin f_b in the presence of dithiothreitol. Its specificity towards sedoheptulose 1,7-bisphosphate versus fructose 1,6-bisphosphate is high, but not absolute.

INTRODUCTION

In the reductive pentose phosphate cycle, the so-called 'Calvin cycle', of C_3 chloroplasts, hydrolysis of FBP and SBP plays an important role. However, there has been some doubt as to the existence of an SBPase distinct from FBPase [1–3]. Breazeale *et al.* [4] described an enzyme from spinach (*Spinacia oleracea*) chloroplasts that they called 'SBPase', but it was not purified to homogeneity. Recently we purified a chloroplastic phosphatase distinct from FBPase which we called the 'alternate' phosphatase [3,5], but it appeared that this latter enzyme was not a 'true' specific SBPase, since it hydrolysed FBP more readily than it did SBP. We describe here, for the first time, the isolation and purification to electrophoretic homogeneity of a phosphatase from C_3 chloroplasts that is highly specific for SBP. Some properties of this enzyme are given. This protein resembles the enzyme that Breazeale *et al.* [4] previously isolated. Several authors have described the isolation and partial purification of SBPase from other plants, including wheat (*Triticum aestivum*) [6] and pea (*Pisum sativum*) [7]. An SBPase from corn (*Zea mays*) has been purified to homogeneity [8].

MATERIALS AND METHODS

All enzymes, substrates and nucleotides were purchased from Boehringer, except SBP and bovine serum albumin (Sigma). Dye–matrix gels (Procion Red HE 3-B, Cibacron Blue 3GA, Orange A and Blue B) were obtained from Amicon. DEAE-cellulose (23 S) and Fractogel TSK-DEAE 650 (S) were purchased from Serva and Merck respectively.

FBPase was purified from spinach leaves as described by Zimmerman *et al.* [9] and chloroplastic thioredoxin f_b by the method of Buc *et al.* [10]. Intact spinach chloroplasts were isolated by the method of Mills & Joy [11]. Chloroplast intactness was assessed by the method of Lilley *et al.* [12]. The percentage intactness was 80%. Purity was evaluated by the use of marker enzymes: cytochrome oxidase for mitochondria and catalase for peroxisomes. Cytoplasmic contamination was estimated by monitoring the activity of NAD:malate dehydrogenase, which, unlike NADP:malate dehydrogenase, is soluble in cytoplasm [13,14], and of FBPase at pH 7.5 without DTT. Under these conditions, cytoplasmic FBPase alone is active [9]. All the tests proved that the contamination of chloroplasts was slight.

The native and monomeric M_r values for SBPase were estimated by the method of Whitaker [15] on Sephadex G-100 and G-200 columns calibrated with proteins of known M_r and by the method of Laemmli [16] on polyacrylamide slab gels under non-denaturing or denaturing conditions respectively. Proteins were stained with Coomassie Blue dye.

Hydrolysis of FBP by FBPase and the 'alternate' phosphatase was monitored by the method of Zimmerman *et al.* [9]. Hydrolysis of SBP was monitored as described by Woodrow & Walker (6).

To detect the different enzymic activities in the extracts, assays were run at 30 °C; FBPase activity was measured at pH 8.8 with 2.5 mM-FBP as substrate, in the absence of DTT; SBPase activity was revealed at pH 8.2, with 0.6 mM-SBP and 10 mM-DTT. To detect the 'alternate' phosphatase, assays at pH 8.8 and pH 7.5 were performed. At pH 8.8 this enzyme is not active [3], whereas the FBPase is. At pH 7.5 in the presence of 10 mM-DTT, both FBPase and the 'alternate' phosphatase are active [3]. A positive assay at pH 7.5 with 2.5 mM-FBP (plus 10 mM-DTT) and a negative assay at pH 8.8 (minus DTT) were evidence for 'alternate' phosphatase activity.

One unit of activity is defined as the amount of enzyme required to hydrolyse 1 μmol of substrate/min at 30 °C and at pH 8.8 under conditions giving the maximum rate.

Protein concentration was determined by the method of Bradford [17], with bovine serum albumin (Sigma, highest grade) as standard.

RESULTS

All the operations were performed at 4 °C. Intact chloroplasts isolated from 1 kg of spinach leaves (see ref.

Abbreviations used: DTT, dithiothreitol; FBP, fructose 1,6-bisphosphate; SBP, sedoheptulose 1,7-bisphosphate; FBPase, fructose-1,6-bisphosphatase; SBPase, sedoheptulose-1,7-bisphosphatase.

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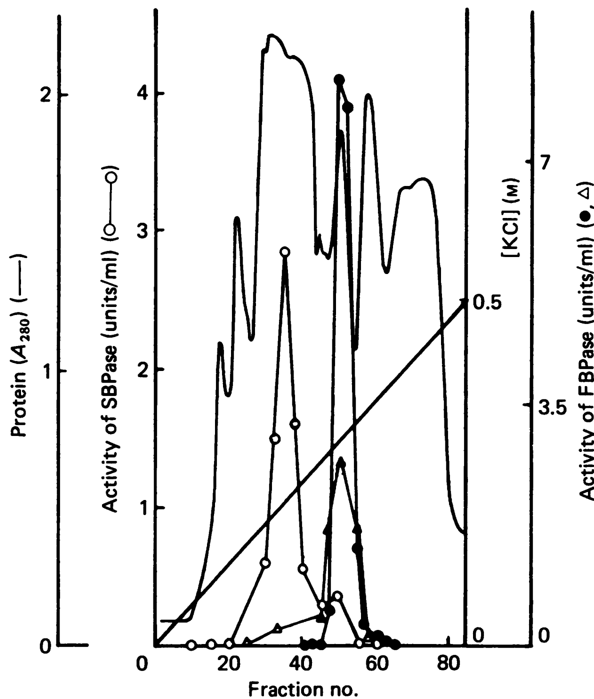

Fig. 1. Fractionation of SBPase by ion-exchange chromatography

The chloroplast stromal extract in 5 mM-phosphate buffer, pH 7.5, containing 1 mM-EDTA was subjected to DEAE-Fractogel chromatography. The column (1.6 cm × 15 cm) was equilibrated with the same buffer. Elution was effected with a linear 0–0.5 M-KCl gradient in the same buffer. The volume of fractions was 3.14 ml. Phosphatase activity was monitored at pH 8.8 minus DTT (●) and at pH 7.5 plus DTT (△) with FBP and with SBP at pH 8.2 plus DTT (○). ——, A_{280}.

[11]) were broken by osmotic shock in 5 mM-EDTA/ 0.1 M-phosphate buffer, pH 7.5 (138 ml), to release their stromal contents. After centrifugation (20000 *g*, 1 h), the supernatant (130 ml) was dialysed and passed through a DEAE-Fractogel column equilibrated with 5 mM-phosphate buffer, pH 7.5, containing 1 mM-EDTA and eluted with a linear gradient of 0–500 mM-KCl in equilibration buffer (Fig. 1). The first peak corresponds to SBPase and the second to FBPase activities respectively. Under different experimental conditions (on DEAE-cellulose column equilibrated with 30 mM-Tris/HCl buffer, pH 7.9), three peaks of phosphatase activities were revealed. The first one corresponded to SBPase, the second and the third to the 'alternate' phosphatase and FBPase activities respectively [3]. In phosphate buffer, the 'alternate' phosphatase was inactivated and therefore only the SBPase and FBPase peaks were detected on the DEAE-Fractogel column (Fig. 1). The active fractions of the first peak of Fig. 1 were pooled, concentrated and subjected to molecular sieving on Sephadex G-200. The elution pattern is shown in Fig. 2(*a*). Those fractions that were not active with FBP as substrate, at pH 8.8 without DTT or at pH 7.5 with DTT were pooled and concentrated and dialysed against 5 mM-phosphate buffer, pH 7.5, containing 0.1 mM-EDTA. The concentrate was applied to a Blue B dye–matrix column equilibrated with the same buffer (Fig. 2*b*). Although SBPase did not bind to this dye–matrix gel, many proteins did (Table 1). At this stage, SBPase appeared homogeneous by native and SDS/Gradipore electrophoresis (Fig. 3). Among the dye–matrix columns evaluated (Blue A, Blue B, Red and Orange), only the Blue B gave a homogeneous enzyme on Gradipore slab gels. In every case, SBPase did not bind to the dye. Table 1 summarizes the purification protocol. The inactivation of enzyme during dialysis after gel filtration was responsible for the low yield obtained between this step and affinity chromatography (23%).

The native M_r of SBPase, as estimated by non-denaturing Gradipore-slab-gel electrophoresis and Sephadex G-200 or G-100 molecular sieving, is about 66000 [64000, 68000 and 66000 respectively; Figs. 3(*a*), 4(*a*) and 4(*b*)]. Its monomeric M_r, as estimated by SDS/Gradipore-slab-gel electrophoresis, is about 35000 (Fig. 3*b*), suggesting that the SBPase holoenzyme is made up of two apparently identical subunits.

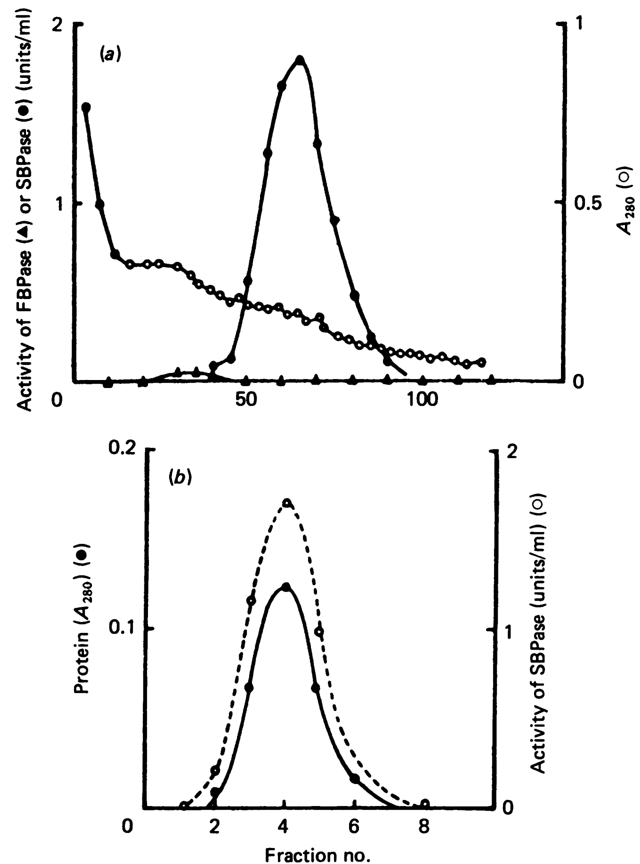

Fig. 2. Molecular sieving and affinity chromatography of SBPase

(*a*) Sephadex G-200 chromatography of SBPase. The column (2.5 cm × 110 cm) was equilibrated with a 0.1 M-phosphate buffer, pH 7.5, containing 1 mM-EDTA and 0.1 M-NaCl. The volume of fractions was 1.8 ml. The absorbance at 280 nm (○) was monitored and activity was estimated with FBP at pH 8.8 minus DTT (▲) and with SBP at pH 8.2 plus DTT (●). (*b*) Blue B affinity chromatography. The column (1 cm × 2 cm) was equilibrated with a 5 mM-phosphate buffer, pH 7.5, containing 0.1 mM-EDTA. The column was washed with the same buffer. Enzyme was eluted in the void volume of the column. The volume of fractions was 0.5 ml. ○, Activity measured at pH 8.2 (plus DTT) with SBP. ●, A_{280}.

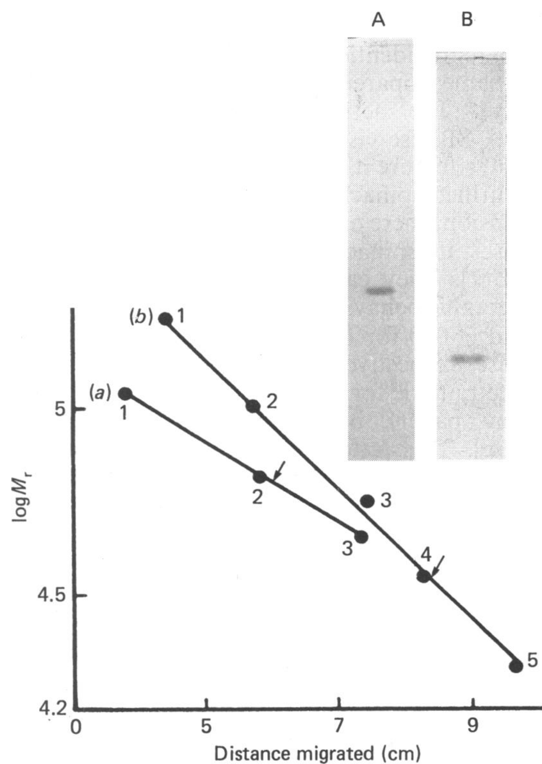

Fig. 3. M_r and apparent electrophoretic homogeneity of SBPase

(*a*) M_r of the native enzyme. Protein markers were: 1, glucose-6-phosphate dehydrogenase (M_r 110000); 2, bovine serum albumin (M_r 67000); 3, ovalbumin (M_r 45000). The arrow indicates the electrophoretic mobility of the phosphatase. Inset A: non-denaturing electrophoresis of the phosphatase in a polyacrylamide gradient (7.5–20%, w/v) gel. A 12 μg portion of protein from the pooled and concentrated active fractions of the Blue B dye–matrix column were applied. (*b*) M_r of the denatured and reduced enzyme. Protein markers were: 1, macroglobulin (M_r 170000); 2, phosphorylase (M_r 97400); 3, glutamate dehydrogenase (M_r 55400); 4, lactate dehydrogenase (M_r 36500); 5, trypsin inhibitor (20000). The arrow indicates the migration of the phosphatase. Inset B: denaturing electrophoresis of the phosphatase in a polyacrylamide gradient (5–25%, w/v) gel. A 10 μg portion of protein was applied.

Previous studies have shown activation by reduced thioredoxins of different enzymes, including the 'alternate' phosphatase [3] and FBPase [18]. We have studied the sensitivity of oxidized SBPase to reduced thioredoxin f_b and DTT, with SBP and FBP as substrate at pH 7.5 (Fig. 5). SBPase was activated by reduced thioredoxin f_b much more quickly and to a greater extent than by DTT alone. Enzyme activity was also higher with SBP as substrate than with FBP. The difference in the enzyme concentration was responsible for the apparent discrepancy between the results in Fig. 2(*a*) and Fig. 5, curve 1. Indeed, the enzyme concentration was much higher (0.5 μM) for the activation experiments than for a routine purification step (where the enzyme was diluted).

The SBPase activity was evident only when the chloroplast envelope membranes were disrupted; this was shown by monitoring the SBPase activity of chloroplasts in a reaction mixture that contained either 0.33 M- or no sorbitol. Enzyme activity was observed only in the absence of sorbitol.

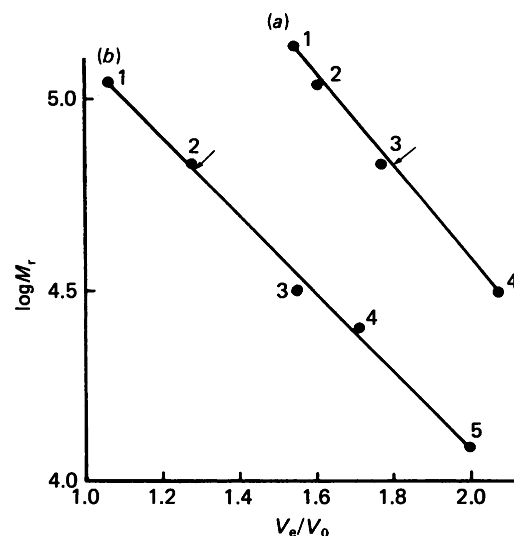

Fig. 4. Determination of the M_r of SBP by molecular sieving

(*a*) Sephadex G-200 column. Protein markers were: 1, fructose-1,6-bisphosphatase (M_r 140000); 2, glucose-6-phosphate dehydrogenase (M_r 110000); 3, bovine serum albumin (M_r 67000); 4, deoxyribonuclease (M_r 31000). (*b*) Sephadex G-100 column. Markers: 1, glucose-6-phosphate dehydrogenase (M_r 110000); 2, bovine serum albumin (M_r 67000); 3, deoxyribonuclease (M_r 31000); 4, α-chymotrypsinogen (M_r 25000); 5, cytochrome *c* (M_r 12300). The arrow in both panels indicates the elution volume of the phosphatase.

Table 1. Purification of SBPase from spinach leaf (1 kg) intact chloroplasts at 4 °C

Activation of the enzyme was performed with 10 mM-DTT for 15 min at 30 °C and pH 8.2. Activity was monitored as described in the Materials and methods section with SBP (2 mM) as substrate

Fraction*	Activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)
Chloroplast stromal extract	86.4	258	0.33	100
DEAE-Fractogel	69.6	112	0.62	81
Sephadex G-200	51.6	3.2	16.1	60
Matrex Blue B	11.9	0.17	70.0	14

* See also Figs. 1, 2(*a*) and 2(*b*).

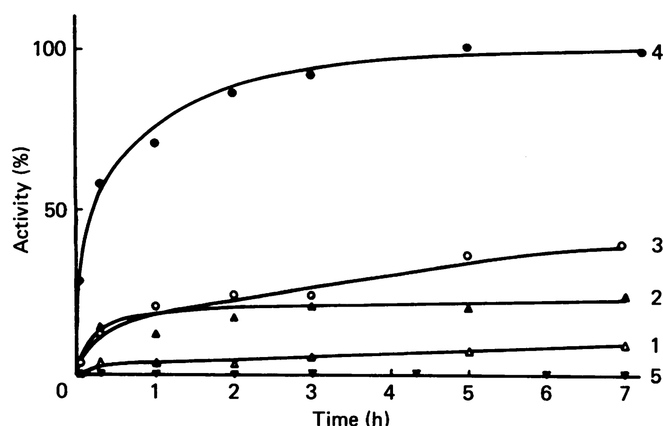

Fig. 5. Activation of SBPase by thioredoxin f_b

The enzyme (0.5 μM) was preincubated at pH 7.5 and 30 °C in 0.1 M-phosphate buffer containing 2 mM-EDTA with either 2 mM-DTT alone (1 and 3) or 2 mM-DTT plus 1.35 μM-thioredoxin f_b (2 and 4). At various times, enzyme activity was determined by using an aliquot of the preincubation mixture with either FBP (1 and 2) or SBP (3 and 4) as substrate. 5, Oxidized (inactive) enzyme activity towards both substrates. The reaction mixture contained 10 mM-$MgCl_2$ and either 2.5 mM-FBP or 0.6 mM-SBP; the pH of the mixture was 7.5 and 8.2 respectively.

DISCUSSION

For the first time SBPase has been purified to apparent electrophoretic homogeneity from intact C_3 chloroplasts. The spinach chloroplast enzyme is different from both FBPase and the 'alternate' phosphatase previously described [1,3]. Its M_r is 66000 and the protein is made up of two apparently identical 35000-M_r subunits.

This enzyme displays a high specificity towards SBP, but yet displays slight activity with FBP. Its activity depends on its redox status: oxidized, it is totally inactive; reduced by a dithiol (DTT or thioredoxin f_b), it is active, and much more so with the latter (thioredoxin f_b) than the former (DTT). This sensitivity to reduced thioredoxin f_b agrees with the light-activation of the enzyme *in vivo*, as described by Breazeale *et al.* [4].

The low overall purification yield (14%) of this enzyme is due to its instability at 4 °C (see [4]), like the 'alternate' phosphatase but unlike FBPase. However, the enzyme can be stored at −80 °C in 10% (v/v) glycerol for several months without any loss of activity.

The purification protocol given here has two supplementary steps compared with the one described by Breazeale *et al.* [4] for the spinach chloroplast enzyme (a DEAE-Fractogel step and affinity chromatography). The purification protocol of the corn enzyme [8] displays one more step (pH 4.6 precipitation), and affinity chromatography is replaced by a hydroxyapatite step. The spinach enzyme, like that from corn [8], is made up of two apparently identical subunits and the dimers have about the same apparent native M_r (66000 and 76000 respectively). The activity of the purified spinach chloroplast SBPase is dependent on a reductant, thioredoxin, a feature it shares with the corn leaf and the partially purified spinach chloroplast enzymes.

In conclusion, there are at least three dithiol-activated phosphatases in spinach leaf chloroplasts [3,4,9; the present work]. They can be distinguished by (i) their chromatographic behaviour, (ii) their native M_r (140000, 100000 and 66000 for FBPase, 'alternate' phosphatase and SBPase respectively) and (iii) their quaternary structure [tetrameric for FBPase [19], dimeric for SBPase (the present paper), but unknown for the alternate phosphatase]. This latter enzyme and the SBPase are inactive at pH 8.8 in the absence of DTT, in contrast with FBPase. The 'alternate' phosphatase is the only one to be inhibited by P_i under our experimental conditions. All three phosphatases are activated (reduced) by reduced thioredoxin and, at least for FBPase and SBPase, are light-controlled *in vivo*.

Received 24 June 1986/31 July 1986; accepted 9 September 1986

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