Pergamon

Phytochemistry, Vol. 46, No. 3, pp. 441–447, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

PII: S0031-9422(97)00335-X

PURIFICATION AND CHARACTERIZATION OF SUCROSE-PHOSPHATE SYNTHASE FROM *PROSOPIS JULIFLORA*

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(*Received in revised form* 14 *March* 1997)

Key Word Index—*Prosopis juliflora*; sucrose-phosphate synthase; purification.

Abstract—Sucrose-phosphate synthase (SPS) was purified 4200-fold from the leaves of *Prosopis juliflora* and resulted in a final specific activity of 467 nkat mg^{-1} protein. The M_r of the native enzyme was 443 by gel filtration. The activity was optimum at pH 7.5 in MOPS buffer. Kinetic data for the forward reaction and both products and dead end inhibition indicated that the enzyme reaction follows an ordered bi-bi mechanism. The purified preparation of SPS was activated by glucose-6-phosphate and inhibited by inorganic phosphate. Both had a large effect only on the K_m of UDPG with inorganic phosphate acting antagonistically to glucose-6-phosphate. The enzyme was inhibited by anions and activated by 25 mM $MgCl_2$. The enzyme showed marginal sensitivity to -SH reagents, and the activity could be restored with DTT. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Sucrose-phosphate synthase (UDP glucose:D-fructose-6-phosphate-2-glucosyltransferase; EC 2.4.1.14) is one of the key enzymes of the sucrose biosynthesis pathway [1]. The enzyme catalyses the formation of uridine-diphosphate (UDP) and sucrose-6-phosphate from uridine-diphosphate glucose (UDPG) and fructose-6-phosphate (F6P). SPS has been isolated and characterized from both photosynthetic tissues, as in case of spinach [2], maize [3], soybean [4], and non-photosynthetic tissues as in case of wheat germ [5], potato tuber [6] and rice grains [7]. The activity of this enzyme is regulated by a variety of mechanisms including allosteric regulation by G6P and Pi [8], covalent modification of the enzyme [9] and by protein synthesis [10] in response to photosynthetic light conditions. The nature of regulation differs among different species as well as in response to the development stage and environmental conditions for a given species. To date, studies on SPS have been restricted to annuals, except for a report from our laboratory in which we demonstrated the requirement of protein synthesis for the activation of SPS in *Prosopis juliflora*. During investigation it was observed that the regulation of SPS in *Prosopis* is quite different from that of spinach. The objective of our work, was therefore to study the properties of the enzyme in detail and compare it with that of annual species. In this communication we report the purification and characterization of SPS from the tree species, *P. juliflora*. Besides other differences with the enzyme from annuals, the *P. juliflora* enzyme shows distinct characteristics in respect of modulation by G6P and Pi.

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RESULTS

Purification

SPS has been purified from spinach [11] using several steps including affinity column chromatography. These methods, applied as such to the *Prosopis* enzyme, do not work, since the amount of SPS present in *Prosopis* leaves is extremely small (sp. act. 0.11 nkat mg^{-1} protein) in the crude extract while in a plant like spinach it is (1.5 nkat mg^{-1} protein) in the crude extract. In view of the very small quantity of the enzyme in *Prosopis*, we avoided harsh and time consuming procedures during purification. Besides the small amount of enzyme to start with, there were other constraints also that influenced the final purification scheme. It was for example observed that more than 50% activity was lost when PEG was used for precipitation, (unlike spinach). Therefore, the protein in the crude homogenate was first adsorbed on to DEAE Sepharose CL 6B and eluted using linear gradient of KCl. Besides concentrating the enzyme, the fractionation by ion exchange had the added advantage

Table 1. Purification of sucrose-phosphate synthase from leaves of *P. juliflora*

Fraction	Protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1} protein)	Purification (fold)	Yield
Crude extract	675	4500	0.11	1	100
DEAE Sepharose	25.5	2400	1.6	14	53
Sepharose CL 6B	0.12	470	65	587	10
Mono Q (I) HR 5/5	0.011	57.5	90	811	1.3
Mono Q (II) HR 5/5	0.001	28.0	466	4200	0.62

of removing more than 90% of the protein, providing 14-fold purification in a single step. Subsequent gel filtration on Sepharose CL 6B yielded considerable purification but with very low recovery. It appeared that the enzyme became increasingly unstable as it approached purification. Final purification of 4200-fold was obtained on Mono Q column. The results of a typical purification scheme are summarized in Table 1. The final preparation had a sp. act. of 467 nkat mg^{-1} protein. Analysis of the SPS active fractions from each step in the purification by SDS-PAGE revealed a significant reduction in the number of silver-staining protein bands (data not shown). The final preparation however, still contained at least three additional proteins, none of which was *ca* the size 120 kDa, a size known to be that of the subunit size of the spinach enzyme [11]. The other proteins still co-purifying with SPS are thus unlikely to be sub-units of the enzyme. Purification steps to remove these co-purified proteins were not successful, resulting in the loss of enzyme activity.

Molecular properties

The purified SPS preparation was essentially free of phosphoglucoisomerase and SPS kinase activity which were reported to co-purify with SPS on ion exchange column [12, 13]. Incubation of aliquots with assay buffer containing either F6P or G6P in the absence of UDPG did not lead to the production of the other sugar phosphate. The optimal pH and temperature for the *Prosopis* SPS were found to be 7.5 and 25° respectively (data not shown). In fact, the pH dependence curve was typically bell-shaped with optima at 7.5. The native M_r of the purified SPS was found to be 443 k on Superose 6 gel filtration column.

The effect of salts on the enzyme was typical of the enzyme from the C_3 plants like spinach [13]. As shown in Table 2, 20 mM $MgCl_2$ or $MnCl_2$ showed considerable activation of SPS. *Prosopis* SPS was found to be inhibited to varying extents by several anions, namely, SO_4^{-2}, PO_4^{-3} and fluoride, while chloride and acetate were non-inhibitory. The inhibition was highest (>70%) with fluoride (Table 2). The influence of various nucleosides mono-, di- and triphosphates on SPS showed that ADP and AMP had no effect, ITP provoked very slight inhibition, and ATP, GTP and CTP inhibited the reaction by 30–50% in the absence of any $MgCl_2$. The inhibition produced by the latter three nucleotides was however, totally reversed by 20 mM $MgCl_2$.

Table 2. Effect of metal ions, anions and nucleotides on sucrose-phosphate synthase activity. The concentrations used were 20 mM for metal ions, 50 mM for anions and 2 mM for nucleotides. For the effect of metal ions and nucleotides, assays for test and controls were carried out without $MgCl_2$ and expressed as % activity. All the anions were used as sodium salts and assays for test controls were carried out in presence of 20 mM $MgCl_2$. The activity values are means ± s.d. from three separate samples

Reagents tested	% activity
$MgCl_2$	152±1.0
$MnCl_2$	148±0.4
$CaCl_2$	99±1.3
NaCl	99±1.6
KCl	99±0.3
Chloride	95±1.4
Fluoride	25±0.6
Acetate	77±1.0
Sulphate	36±0.5
Phosphate	32±0.5
Bicarbonate	77±2.1
Citrate	61±0.1
ATP	53±0.9
ITP	83±0.8
GTP	48±0.2
CTP	60±0.6
ADP	97±0.03
AMP	99±1.3

The requirement of -SH groups for the enzyme activity was also tested using different -SH protecting and modifying reagents. Table 3 shows the protection offered by various -SH protecting groups. 2-ME and DTT gave around 40% protection at 1 mM while

Table 3. Effect of sulphydryl compounds on sucrose–phosphate synthase activity. The purified preparation of SPS was desalted on Sephadex G-25 column using 50 mM MOPS buffer containing 10 mM $MgCl_2$ and assayed in the presence and absence of sulfhydryl reagents as described in the Experimental. The activity was taken as 100% in the absence of effector. Values are means ± s.d. from three separate samples.

Conc. (mM)	Percentage activity			
	DTT	2-ME	Cysteine	GSH
0.1	101 ± 0.3	109 ± 0.5	125 ± 0.6	115 ± 2.0
1.0	144 ± 2.4	141 ± 1.7	130 ± 0.3	125 ± 1.2
10.0	145 ± 0.4	149 ± 0.7	135 ± 0.9	130 ± 1.3
20.0	143 ± 0.5	150 ± 0.3	133 ± 0.9	131 ± 1.6

the protection offered by the other two reagents, viz. cysteine and glutathione was only 25%. The enzyme also showed marginal inhibition with -SH modifying reagent, DTNB (25% at 1 mM DTNB). Preincubation of the enzyme with substrates did not protect the enzyme from DTNB inhibition (data not shown).

Kinetic analysis

The dependence of the initial velocity of the enzyme reaction upon substrate concentration was studied in case of the substrates, UDPG and F6P. Concentrations of the substrates were varied systematically over a range, suitable to obtain kinetic constants. To illustrate the results, double reciprocal plots of reaction rates against varying concentrations of one substrate were obtained at constant concentrations of the other substrate (Fig. 1). The entire range of concentrations shown was analysed on two separate occasions and the data so obtained was plotted. The results fitted a pattern of linear non parallel lines intersecting to the left of the vertical axis. This pattern indicates that the enzyme follows an ordered bi-bi mechanism (both substrates must combine with the enzyme in a sequential manner before the first product is released). The K_m values obtained from the data were 20 mM for UDPG as one substrate and 2 mM for F6P as the other substrate. The bi-bi mechanism was studied further for inhibition by the product (UDP) and dead end inhibitor (inorganic phosphate). The product inhibition was studied using a series of UDP concentrations with different concentrations of UDPG and F6P (Fig. 2). The figure clearly indicates that UDP is a competitive inhibitor when UDPG is the variable substrate while with F6P as a substrate, the inhibition by UDP was non-competitive. The data indicated that substrate binding and product release from SPS follows an obligatory sequence. The dead end inhibition pattern (data not shown) indicated that the inhibitory effect of inorganic phosphate towards UDP-glucose was competitive. This shows that UDP glucose was the first substrate to be bound to the enzyme and was followed by F6P. The inhibitory pattern produced by UDP and Pi thus clearly support the ordered bi-bi mechanism for the enzyme action.

The enzyme purified from *P. juliflora* was activated with G6P and inhibited with Pi. From the data in Fig. 3 it is clear that there was a four-fold increase in activation of the enzyme, when G6P concentration was increased from 0 to 20 mM. In case of Pi, the inhibition was partial with only 25% of the activity that was observed with 20 mM G6P being observed in presence of 15 mM Pi. SPS showed hyperbolic substrate saturation kinetics with UDPG and F6P in presence of G6P and Pi. Both G6P and Pi had a large effect on the K_m for UDPG, while V_{max} changed only slightly. In presence of 15 mM Pi, the K_m for UDPG increased from 20 mM to 77 mM (*ca* 4-fold) and it decreased to 4.6 mM (*ca* 4-fold) in presence of 20 mM G6P. In contrast to the large effect on the K_m of UDPG, G6P and Pi had very little effect on other substrate, F6P (Fig. 4b). In presence of 15 mM Pi or 20 mM G6P, the K_m for F6P remained unchanged while the V_{max} was decreased slightly. In presence of both modulators an antagonism was observed between activation by G6P and inhibition by Pi of the enzyme. Thus K_m for UDPG increased from 4.6 mM to 20 mM in presence of both 20 mM G6P and 15 mM Pi, while the K_m for F6P remained unaffected (Fig. 4a,b).

DISCUSSION

These studies provide the first report on the characterization of SPS from a tree species. In previous work SPS has been purified to various extents. In spinach the purification of SPS ranged from 10 to 49-fold [8, 14, 15], 11-fold for the maize enzyme [3], 56-fold for the pea enzyme [12], 41-fold for the potato tuber enzyme [6] and 74-fold for the enzyme from soybean leaf [4]. There was a report of a near homogenous preparation from spinach [11], but the enzyme was used for raising polyclonal antibodies and not for characterization. The sp. act. of the 4200-fold purified *Prosopis* SPS was 467 nkat mg^{-1} protein, which is 30–40% of the pure preparation from spinach (1300 nkat mg^{-1} protein) and much higher than the

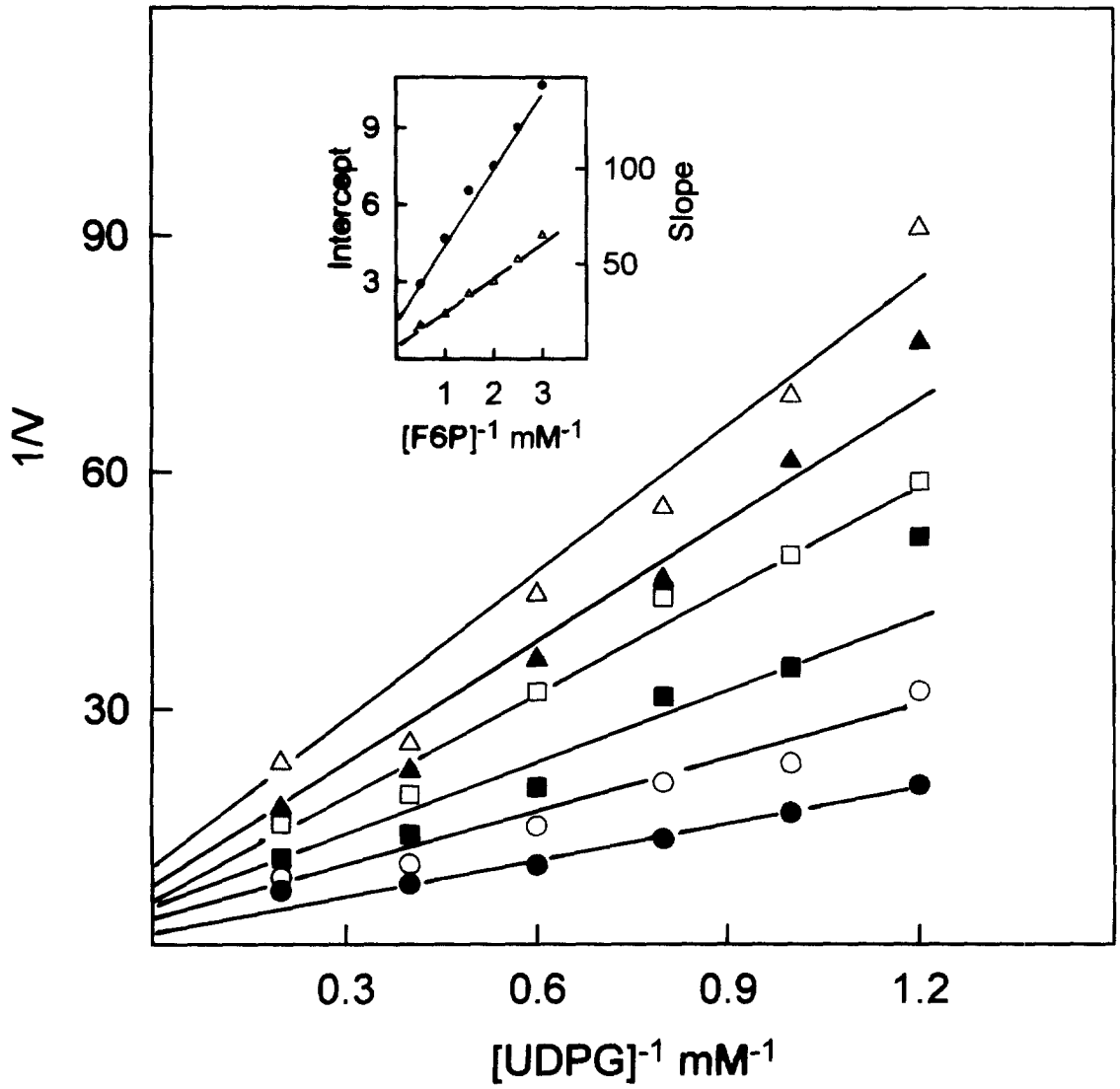

Fig. 1. Double-reciprocal plots of initial velocity of *Prosopis* SPS with UDP-glucose as the variable substrate. The assays of SPS were carried out in absence of G6P. The concentrations of F6P were : (△), 0.33 mM; (▲), 0.5 mM; (□), 0.66 mM; (■), 1.00 mM; (○), 1.67 mM and (●), 2.00 mM. The slopes (△) and intercepts (●) of the primary plot are replotted as a function of the concentration of F6P in the secondary plot.

earlier preparations from maize leaf (8 nkat), rice scutellum (34 nkat), pea seeds (70 nkat) and potato tuber (67 nkat) used for the characterization [6, 12]. The native M_r of *Prosopis* enzyme, 443 k is close to that reported (450 k to 480 k) for the enzyme from other crop species [11, 12].

The purified preparation of the enzyme from the tree species showed properties similar to those of the crop species with a few differences. Thus *Prosopis* SPS had an optimum pH at 7.5, like the enzyme from pea, while pH values ranging from 6.5 to 7 have also been reported for other species [12, 15, 16]. It was found that while monovalent cations have no effect in the reaction, divalent ions like Mg^{2+} and Mn^{2+} produced stimulation in agreement with the results reported for wheat germ [5] and spinach [13]. On the contrary, various anions seem to have an inhibitory action, with the effect being more marked for fluoride, sulphate and phosphate as has been shown for wheat germ [5] and rice [7]. However, the SPS from wheat germ was not inhibited by fluoride [5]. Also in the case of SPS from wheat germ, the high concentration of Mg^{2+} modifies the anion inhibition. Like SPS from spinach [15] and wheat germ [5] the activity of *Prosopis* SPS is also affected by nucleoside triphosphates. The addition of Mg^{2+} resulted in cessation of inhibition with recovery of activity to control values. The *Prosopis* leaf enzyme did not show a requirement for -SH groups for stability but if 2-ME was not included in the buffer during purification 20–30% activity was irreversibly lost. The effect of -SH protecting and modifying reagents showed that -SH groups may not

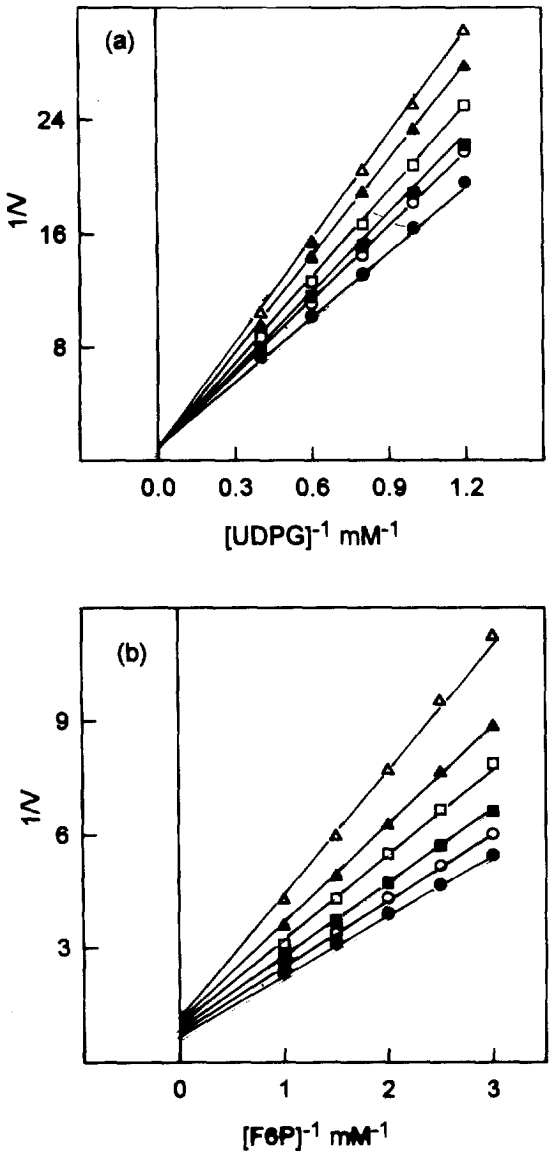

Fig. 2. Inhibition of *Prosopis* SPS by UDP with (a) UDPG as the variable substrate. The assays of SPS were carried out in absence of G6P. The concentration of F6P was 4 mM. The concentrations of UDP were (△), 10 mM; (▲), 5 mM; (□), 3 mM; (■), 1 mM; (○), 0.5 mM; (●), 0 mM. (b) Inhibition with F6P as the variable substrate. The concentration of UDPG was 10 mM.

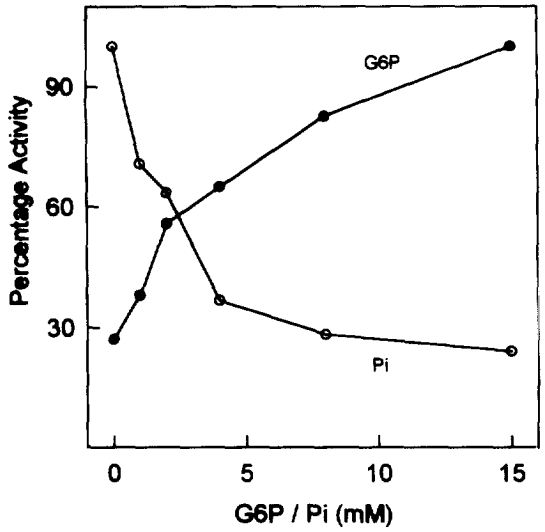

Fig. 3. Effect of various concentrations of G6P (●) and Pi (○) on *Prosopis* SPS. The activity of SPS was assayed in presence of given concentration of G6P or F6P as mentioned in Experiments. For activation by G6P the activity in presence of 15 mM G6P was taken as 100% and for Pi inhibition, the activity in absence of Pi was taken as 100%.

be directly involved in catalysis and further may also not be necessary to maintain an appropriate enzyme conformation. The complete inhibition of SPS by PCMB was reported in wheat germ [5] and maize [17]. In contrast, SPS activity in potato tuber [18] and spinach [17] was inhibited only to the extent of 25% by PCMB.

Kinetic analysis of the enzyme showed that the mechanism of catalysis could be ordered bi-bi as reported earlier for the wheat germ enzyme [19] and for the spinach enzyme [15]. The predicted inhibition by product UDP and dead end inhibitor Pi was also observed. The K_m for F6P (2 mM) of *Prosopis* was 10 times higher than the K_m of SPS from maize leaf but compares favourably with the K_m values of SPS from spinach, wheat germ, potato tuber and rice scutellum which ranged from 1.6 to 4.9 mM. The K_m for UDPG of spinach SPS was reported to be 1.9 mM which is much lower than that of the *Prosopis* enzyme while that of potato tuber, rice scutellum and maize leaf ranged from 15 to 34 mM. The higher values of K_m for UDPG in absence of G6P may not represent the affinity of UDPG for SPS under physiological conditions, because SPS has been shown to be strongly modulated by metabolites like G6P and Pi in several species [3, 6, 8, 20]. In *Prosopis* also the enzyme was activated by G6P and inhibited by Pi. These effectors acted antagonistically to each other and generated large changes in the affinity for one of the substrates (UDPG) only. For instance in the case of SPS from spinach, G6P and Pi have been reported to alter only the affinity for F6P while in maize, the affinity for UDPG was changed. In contrast, for the SPS from potato tuber the affinity of both substrates appeared to be altered by G6P and Pi. Apparently *Prosopis* enzyme resembles maize SPS with respect to the effect on UDPG and F6P. Though there are differences with respect to the effect of metabolites, the antagonistic interaction between G6P and Pi appears to be a common feature in all the species and may have some physiological implications.

A comparison of the *Prosopis* SPS with SPS from other crop species revealed that the tree enzyme had several properties, which are distinct from crop species, especially in the modulation by G6P and Pi. The fundamental differences observed in the properties of

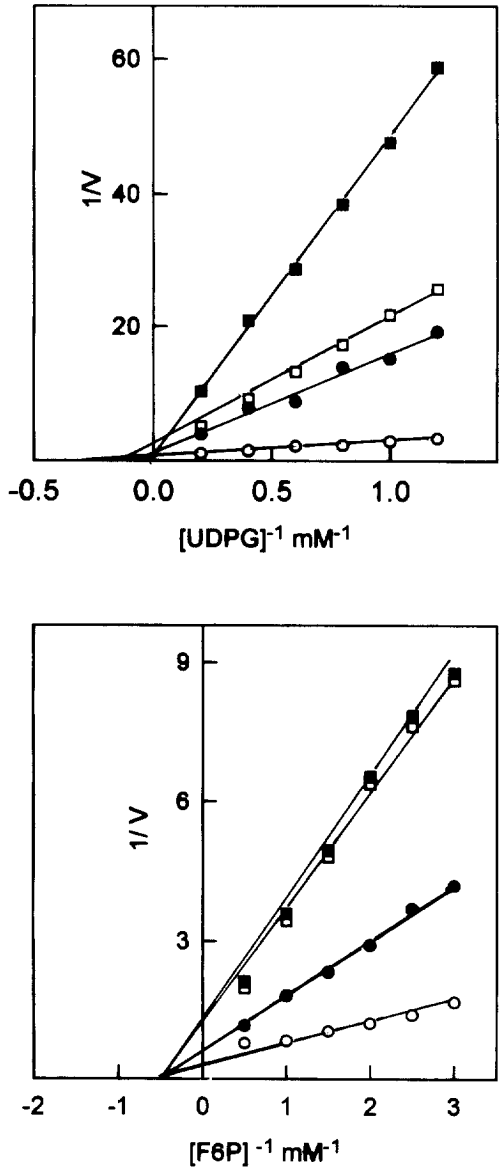

Fig. 4. Effect of various concentrations of Pi and G6P on the substrate saturation kinetics of *Prosopis* SPS. The activity of *Prosopis* enzyme was assayed in the presence of (a) 4 mM F6P and varying UDPG; (b) 10 mM UDPG and varying concentrations of F6P. The concentrations of metabolites used were (■) 15 mM Pi, (□) 15 mM Pi and 20 mM G6P, (●) no addition and (○) 20 mM G6P.

Prosopis SPS may have a crucial role in regulation *in vivo*.

EXPERIMENTAL

Materials. The plants of *Prosopis juliflora* were raised in nursery and grown under natural conditions on the terrace garden of the institute. The leaves of two year old seedlings were used for the enzyme isolation. Substrates, buffers and other chemicals were from Sigma. Column materials, DEAE Sepharose CL 6B, Sepharose CL 6B were from Pharmacia. Prepacked columns Mono Q HR 5/5 and Superose 6 HR 10/30 were from Pharmacia Biotech; M_r markers for gel filtration were from Sigma.

Methods. For purification of SPS all steps including HPLC were carried out at 4°. 25 g of leaves of *P. juliflora* were quickly frozen in liquid N_2 and homogenized in a 75 ml of 50 mM MOPS–KOH buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 25 mM 2-ME and 0.02% Triton X-100. The homogenate was filtered through 4 layers of muslin cloth, centrifuged at 40000 *g*, 20 min. The supernatant was loaded onto a DEAE Sepharose CL 6B column (30 ml bed volume) equilibrated with extraction buffer without Triton X-100 (buffer A). The enzyme was eluted with a linear gradient of 50–400 mM KCl in 200 ml of buffer A. SPS activity was eluted in a single peak with *ca* 0.3 M KCl. Peak frs were pooled and applied to gel filtration column of Sepharose CL 6B (bed vol. 450 ml) equilibrated with buffer A and eluted with same buffer. The 2 most active frs were pooled and applied on Mono Q HR 5/5 equilibrated with buffer A and eluted with a 7.5 ml of linear gradient of 0–30 mM KCl followed by 30–35 mM KCl (2.5 ml). 4 active frs were pooled and diluted × 4 with buffer A and reloaded on Mono Q HR 5/5 and eluted using the same gradient system as used for Mono Q I. The active frs (0.5 ml each) were pooled and used as the final prepn of enzyme.

SPS activity was measured by a fixed point determination of sucrose-phosphate produced [13]. The reaction mixt. contained 50 mM MOPS buffer pH 7.5, 4 mM F6P, 10 mM UDPG, 25 mM $MgCl_2$ in a final vol. of 100 μl. All assays were run at 25°. For kinetic studies, substrates and effectors were varied as specified in figure legends. The assay was terminated with 30% KOH and unreacted F6P was destroyed by heating the tubes at 100° for 10 min. Blanks containing the complete assay mixt. were terminated at zero time with 30% KOH. After cooling 1 ml of 0.14% anthrone in 13.8 M H_2SO_4 was added and the tubes were incubated at 40° for 20 min prior to measuring *A* at 620 nm. For experiments with sulphydryl modifying reagents, the enzyme was first desalted to remove 2-ME and then pretreated with a specified concn of sulphydryl modifying reagent for 10 min at 25°. One unit of activity is defined as the activity necessary to produce 1 nmol of sucrose-phosphate in 1 sec. The activity of phosphoglucoisomerase was measured according to [4] and SPS kinase activity was measured as Mg ATP dependent inactivation of SPS by endogenous kinase [13].

Native M_r was estimated by gel filtration on a column of Superose 6 HR 10/30 attached to HPLC system. Samples containing blue dextran, protein M_r standards and sucrose–phosphate synthase were loaded, separately, on a Superose 6 column that had been equilibrated with 50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA and 25 mM 2-ME. The elution vol. of blue dextran was taken as the void

vol. Standards were: bovine thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa).

SDS-PAGE was carried out using 10% polyacrylamide gels in a continuous buffer system as described [21]. Proteins were detected by Ag staining by the method of ref. [22]. Proteins were estimated by the method of ref. [23].

Acknowledgement—We would like to thank S. A. Ranade for critical reading and helpful suggestions on the manuscript.

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