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Purification and Preliminary Characterization of Sucrose-Phosphate Synthase Using Monoclonal Antibodies[1]

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ABSTRACT

Monoclonal antibodies specific for sucrose phosphate synthase (SPS; EC 2.4.1.14) have been obtained for the first time. Three independent clones have been isolated which inhibited spinach (*Spinacia oleracea* L.) leaf SPS activity and facilitated the enzyme purification by immunoprecipitation. All three clones were specific for the spinach enzyme but neither inhibited nor precipitated the SPS present in tissue extracts of maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), soybean (*Glycine max* L.), and sugar beet (*Beta vulgaris* L.). The inhibition of SPS activity by all three clones was reversible in the presence of UDPG, suggesting the presence of an epitope at the substrate-binding site. Immunoprecipitates of active enzyme preparations consistently revealed the presence of a 120 kilodalton polypeptide, indicating that the enzyme may be a homotetramer with a native molecular weight of about 480 kilodaltons. The occasional appearance of a 52 kilodalton polypeptide in the immunoprecipitates of some enzyme preparations was not the result of proteolysis, was not necessary for enzyme activity, and did not contain an antigenic site as revealed by Western blotting experiments. All three antibodies bind weakly to the SDS denatured 120 kilodalton subunit bound to nitrocellulose. The specific activity of the purified spinach enzyme was determined for the first time to be approximately 150 units per milligram SPS protein (pH 7.5 and 25°C) based on quantitative immunoprecipitation of the enzyme.

Sucrose phosphate synthase (SPS)[3] is a pivotal enzyme in the sucrose synthesis pathway and plays a major role in the control of the leaf's capacity to synthesize sucrose and thus translocate photoassimilates to growing plant parts. Previous studies utilized the partially purified enzyme to establish the existence of intricate control strategies for SPS activities (for review see 6). Further understanding of the biochemical basis for these mechanisms as well as description of physical properties for SPS have awaited purification of the enzyme. Efforts to purify and characterize the physical properties of the enzyme using conventional chromatographic techniques have been frustrated by the lability of SPS activity and the low concentrations of the protein in photosynthetic tissues. In the successful purification efforts reported here, hybridoma cell fusion techniques (4) were utilized to produce monoclonal antibodies directed against partially purified spinach leaf SPS. Three independent clones have been isolated which sythesize monoclonal antibodies specific for the enzyme. The antibodies have facilitated the purification of spinach SPS, the identification of subunit composition, and the estimation of mol wt and enzyme specific activity.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma Chemical Co.[4] The DEAE-Fast Flow, Mono Q, and Polyanion SI anion exchange columns were obtained from Pharmacia. All SDS-PAGE and electroblotting reagents were of electrophoresis purity and purchased from Bio-Rad Laboratories. The affinity purified goat anti-mouse IgG horseradish peroxidase conjugate and 3,3′-diaminobenzedene color reagent used in Western blotting procedures were obtained from Bio-Rad Laboratories.

Plant Material

Spinach (*Spinacia oleracea* L., cv Dark Green Bloomsdale), maize (*Zea mays* L., cv Pioneer 3184), soybean (*Glycine max* [L.] Merr., cv Ransom), barley (*Hordeum vulgare* [L.] cv Boone), and sugar beet (*Beta vulgaris* L.) were grown in a soil mixture in pots under greenhouse conditions.

Preparation of Spinach SPS for Antigen

Spinach plants were harvested at 4 weeks postplanting by freezing with liquid N_2. Approximately 200 g of the powdered frozen tissue was homogenized using 1 L of a medium consisting of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, and 2.5 mM DTT using a chilled Waring blender. The homogenate was squeezed through 8 layers of cheesecloth and centrifuged at 20,000*g* for 10 min. The supernatant was fractionated with 6 to 11% PEG prepared in 50 mM Mops-NaOH (pH 7.5) and 2.5 mM DTT. The SPS-containing precipitate was collected at 30,000*g* and resuspended in a mini-

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[3] Abbreviations: SPS, sucrose-phosphate synthase; F6P, fructose 6-phosphate; UDPG, UDP-glucose; HRP, horseradish peroxidase.

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mal amount of a resuspension buffer (buffer A) consisting of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 2.5 mM DTT, 10% (v/v) ethylene glycol, and 0.1% (v/v) Tween 80. The precipitate was collected and resuspended in buffer A twice more. The supernatants, containing the SPS activity, were pooled and retained on ice.

The partially purified SPS preparation was fractionated on a 30 mL DEAE-Fast Flow column (Pharmacia) equilibrated with buffer A. Weakly bound protein was eluted with 75 mM NaCl in the same buffer and the column developed with a 75 to 500 mM NaCl linear gradient. The SPS-containing fractions were pooled and diluted to 50 mM NaCl with buffer A before applying 15 to 20 mg amounts of protein to a Pharmacia FPLC Mono Q anion exchange column equilibrated in buffer A. SPS activity was eluted via a 0 to 300 mM linear NaCl gradient in the same buffer. Mono Q purified SPS fractions were diluted with buffer A to a NaCl concentration of <100 mM and applied to a Pharmacia FPLC Polyanion SI column equilibrated with the same buffer. The column was subsequently developed with buffer A containing a linear 0.1 to 1.0 M NaCl gradient.

Production and Screening on SPS-Specific Hybridomas

The Polyanion SI column fractions containing SPS activity were pooled and emulsified in an equal volume of Freund's complete adjuvant. Aliquots containing 50 μg of protein were injected intramuscularily into female Balb/c mice. Five weeks later each mouse was boosted with an additional 50 μg of protein emulsified in incomplete Freund's adjuvant and the sera tested for the capacity to inhibit SPS activity relative to a preimmune serum control. The sera obtained from two mice neutralized spinach SPS activity and, in ELISAs, bound to epitopes present in the partially purified SPS preparation bound to microtitre plates. A final intraperitoneal boost was given and the fusion performed 3 to 4 d later by the Hybridoma Service Laboratory, School of Veterinary Medicine, NC State University. The spleen cells were fused with a P3X-derived mouse myeloma cell line according to the procedure of Galfre and Milstein (2).

Approximately 900 viable hybrid cells were obtained and were first screened for their capacity to produce antibodies. Fifty to 100 μL of each clone was applied to 96-well microtiter plates and assayed for the presence of antibodies bound directly to the wells by the ELISA procedure described below. Those clones identified as antibody producers were further analyzed for spinach SPS specificity. The antibodies were tested for the capacity to bind to epitopes present in the Polyanion SI fractions via the ELISA assay. The positive results obtained in these assays were not conclusive proof of SPS recognition by the antibodies but provided a line of evidence to support the subsequent studies to test the capacity of the clones to specifically inhibit and/or precipitate SPS activity in solution. Ultimately, three stabilized hybrid cell lines were obtained that were judged to be specific for SPS. The single cell origin of the SPS-positive clones was verified by extensive recloning efforts via limiting dilution techniques. The stabilized clones were expanded into 500 mL cultures and the exhausted supernatants were aliquoted and stored at −80°C.

Antibody/Antigen Assays

ELISA Test

Five to 100 ng of the Polyanion SI purified SPS was applied to polystyrene microtiter wells previously treated for 30 min with a 1 mg/mL solution of poly-L-lysine. The enzyme solution was allowed to evaporate to dryness in the wells at 37°C overnight. The potential protein binding sites remaining were blocked by incubating the wells with 200 μL of 2% BSA (w/v) in 10 mM phosphate buffer (pH 7.5) and 150 mM NaCl (PBS) for 2 h at 37°C. The hybridoma culture supernatants were applied in 50 to 100 μL aliquots and incubated for 2h at RT. The wells were washed extensively with PBS containing 0.05% Tween 20 (v/v). The goat-anti-mouse alkaline phosphatase conjugate was diluted 1:3000 in PBS and applied to the wells in 100 μL aliquots and allowed to incubate at RT for 1 h. The wells were washed as above before applying 200 μL of a 5 mg/mL *p*-nitrophenol phosphate solution in a 10% diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$. Color development proceeded for up to 2 h at 37°C and was terminated by the addition of 100 μL of a 3 M NaOH. The production of *p*-nitrophenol was measured at 405 nm.

Immunoprecipitation of SPS Activity

In standard assays, approximately 0.003 units of SPS activity were incubated for 1 h on ice with untreated culture supernatant in amounts indicated in figure legends. Pansorbin (Calbiochem) cells were pelleted and resuspended to original volume in 50 mM Mops-NaOH (pH 7.5) and 2.5 mM DTT. Aliquots of this cell suspension were added to the immune complexes based on a 1:3 (v/v) ratio of Pansorbin and culture supernatant. The mixture was allowed to incubate an additional hour on ice before the cells were collected at 5000*g* for 1 min. In control incubation mixtures, the SPS-specific culture supernatants were substituted with exhausted culture media containing either no antibodies or antibodies shown to be specific for another protein. The supernatants were aspirated and assayed for SPS-dependent sucrose synthesis via the anthrone-based sucrose detection assay described below.

Antibody-Mediated Inhibition of SPS Activity

In standard assays, approximately 0.003 units of SPS activity were incubated with untreated culture supernatants in the amounts indicated in "Results." Immune complexes were allowed to form on ice for 1 to 2 h. SPS activity in the mixture was assayed via the anthrone color development as described below.

Detection of Antigenic Determinants by Western Blotting Techniques

SPS was immunoprecipitated, and nonspecific proteins removed by washing the Pansorbin cells successively 3 to 5 times in 50 mM Mops-NaOH (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 (v/v) followed by a final wash of 50 mM Tris-HCl (pH 7.0). The pellets were adjusted to 2% SDS and 2% 2-mercaptoethanol and boiled for 3 min to dissociate immune complexes before the Pansorbin cells were pelleted at 20,000*g* for 2 min. The supernatant containing the disso-

ciated SPS subunits and the heavy and light chains of the monoclonal antibodies was applied to 12.5% SDS polyacrylamide gels and fractionated using the system of Laemmli (5). The proteins were transferred to nitrocellulose for 3 h at 60V using the Tris/glycine/methanol buffer system of Towbin *et al.* (7).

Amido Black (0.1% [w/v] in 25% isopropanol [v/v]/10% acetic acid [v/v]) was incubated with control strips of the nitrocellulose blots for 1 min to directly stain the protein bands (3). The strips were destained in a solution of 25% isopropanol/10% acetic acid for 15 min and dried under pressure. The filter portions utilized in immunodetection of antigens were incubated with 2% BSA in PBS for 2 h at 37°C to block remaining protein binding sites. The blots were incubated separately with the three culture supernatants and the nonspecific antibody control in a 1:20 dilution in PBS for 3 h at RT. The blots were washed extensively with PBS containing 0.05% Tween 20 before goat anti-mouse HRP-conjugated antibodies diluted in PBS were added. The indicating antibodies were allowed to incubate for 2 h at RT before the filters were washed as above. Bound antibody was detected by the addition of 120 mL of PBS (at RT) containing 60 mg 3,3'-diaminobenzedene (solubilized in 20 mL cold methanol) and 60 μL of 30% H_2O_2 (added fresh). The reactions were allowed to proceed at RT until background color began to develop, then the nitrocellulose blots were washed extensively with distilled H_2O and dried between absorbent sheets away from direct light.

Sucrose Phosphate Synthase Assay

SPS was assayed by measurement of F6P-dependent formation of sucrose (+sucrose-P) from UDPG. The assay mixture (70 μL) contained 25 mM UDPG, 10 mM F6P, 10 mM $MgCl_2$, 50 mM Mops-NaOH (pH 7.5), and variable amounts of the enzyme. The mixtures were incubated at 25°C and reactions were terminated after 15 min by addition of 70 μL of 30% KOH. Unreacted F6P or fructose was destroyed by placing the tubes in boiling water for 10 min. After cooling, 1 mL of 0.14% anthrone in 13.8 M H_2SO_4 was added to the tubes and incubated at 40°C for 20 min. The tubes were allowed to cool, and the A_{620} was measured.

Determination of SPS Specific Activity

In order to measure the specific activity of spinach SPS, the activity in an enzyme preparation was established and the SPS protein was then precipitated immunochemically and purified by SDS-PAGE. The amount of SPS protein was quantitated from densitometric scanning of the Coomassie blue-stained gel bands. The SPS was rapidly extracted from fresh leaf tissue and desalted, and the extract was incubated with an excess of monoclonal antibodies and protein A for 3 h on ice. The complete removal of SPS protein from solution was verified by assaying for any remaining enzyme activity in the supernatants subsequent to the immune complex precipitation. The pelleted complexes were dissociated with 2% SDS and 2-mercaptoethanol and fractionated on 12.5% SDS gels along with 2, 4, and 8 μg amounts of BSA and phosphorylase b. The proteins were stained in 0.01% (w/v) Coomassie Blue in 40% methanol/10% acetic acid and destained in a solution of 40% methanol and 5% acetic acid. The gel bands were scanned using an EC910 densitometer (E-C Apparatus Corp) and Bascom-Turner recorder. A protein standard curve was constructed using peak area measurements for the two standard proteins. The total protein in the 120 kD subunit of SPS was determined by comparison with the empirically-derived standard curve.

RESULTS

Three stable independent hybridoma clones, designated 7C1, 9C9, and 3D4, were isolated which produce antibodies directed against spinach leaf sucrose phosphate synthase. Several lines of evidence have established specific recognition by all three antibodies of an epitope present on the SPS protein. Figure 1 confirms that incubation of the three culture supernatants with spinach leaf SPS resulted in concentration-dependent removal of enzyme activity from solution. While these results were obtained with Mono Q-purified SPS, the antibodies exhibited the capacity to indistinguishably bind and completely precipitate the SPS activity in crude extracts, and PEG and DEAE fractions as well. Because monoclonal antibodies recognize only one epitope, they often do not form the precipitating aggregates characteristic of polyclonal antibody immune complexes. Thus, precipitation of the immune complexes in these studies required an agent which specifically bound to the antibodies and was easily removed from solution. For our purposes, a formalin-fixed preparation of *S. aureus* cells (Pansorbin, Calbiochem) provided reproducible binding to the monoclonal antibodies via protein A and was utilized in all studies requiring precipitation of SPS from solution. Other precipitation studies performed with partially

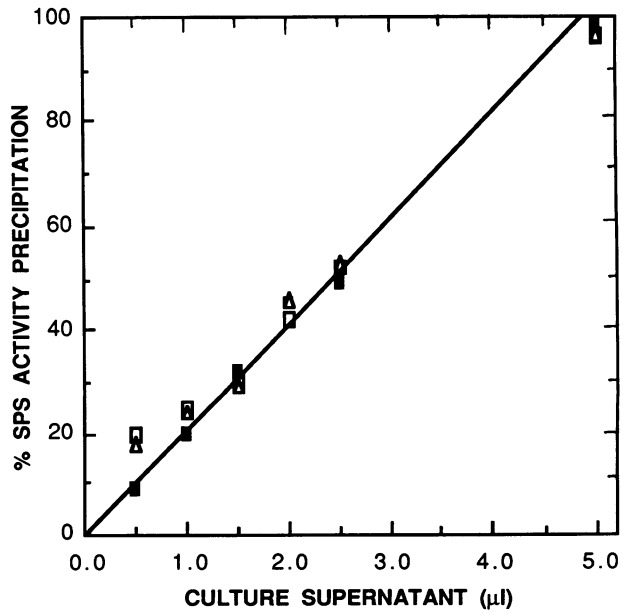

Figure 1. Antibody concentration dependence of SPS precipitation in solution. Approximately 0.003 units of Mono Q-purified spinach SPS were incubated for 1 h on ice with the indicated volumes of culture supernatant from clones 7C1 (□), 9C9 (■), and 3D4 (△). Immune complexes were precipitated with an excess of protein A and then the incubation supernatants assayed for remaining SPS activity as described in "Materials and Methods."

purified antibodies (fractionated on phenyl superose and estimated to be approximately 80% homogeneous by SDS-PAGE) confirmed that the apparent precipitation of SPS activity in these assays was antibody-dependent (data not shown). Based on these results and those in Figure 1, the precipitation of SPS activity was attributed to antibody binding. Therefore, untreated culture supernatants were routinely utilized in all subsequent antigen precipitation experiments.

In the absence of the protein A preparation, clones 7C1, 9C9, and 3D4 were found to partially inhibit SPS activity in solution. At concentrations of antibody sufficient to precipitate 100% of the SPS activity, all three monoclonals maximally inhibited the enzyme 80 to 85% (Fig. 2). Because direct inhibition of SPS activity suggested antibody binding near the active site, the substrates UDPG and F6P, and activator G6P were investigated for potential effects on antibody:antigen association. It was found that the apparent inhibition of SPS activity was readily reversible in the presence of increasing concentrations of UDPG (Fig. 2) but could not be mimicked by equivalent concentrations of salt, F6P, or G6P (data not shown). Significantly, treatment of immunoprecipitated SPS with UDPG did not result in recovery of enzyme activity nor release of SPS protein as revealed by SDS-PAGE (data not shown). These results suggest that the partial inhibition of SPS activity measured in the presence of excess antibody (80–85% maximum) may be explained by the dissociating effect of UDPG present in the reaction mixture. Evidence in support of this was obtained from a time course of SPS activity assayed in the presence of the antibodies and 25 mM UDPG which revealed a slow partial recovery of the enzyme activity during the assay (data not shown).

Spinach SPS was immunopurified in order to verify that all three clones precipitated identical proteins and to identify subunit composition for the enzyme. Initial characterizations of immune complexes by SDS-PAGE revealed the antibody-dependent precipitation of two polypeptides for all three monoclonal antibodies (Fig. 3). The proteins exhibited mol wt of approximately 120 and 52 kD and were immunoprecipitated from enzyme preparations partially purified by DEAE and Mono Q chromatography.

Transfer of the separated immunoprecipitates to nitrocellulose and subsequent immunochemical analysis revealed that antibody bound weakly to the denatured 120 kD polypeptide but did not recognize the precipitated 52 kD protein (Fig. 4). The 52 kD band appears as a clear area at the lower edge of the heavily stained antibody heavy chain. In addition, all three clones recognized low levels of an apparent degradation product exhibiting a mol wt of 30 kD. The presence of the 30 kD protein as well as a 90 kD polypeptide in the immunoprecipitates was only detectable in more highly purified preparations of SPS. The 90 and 30 kD proteins were present in very minor amounts relative to the 120 kD protein as esti-

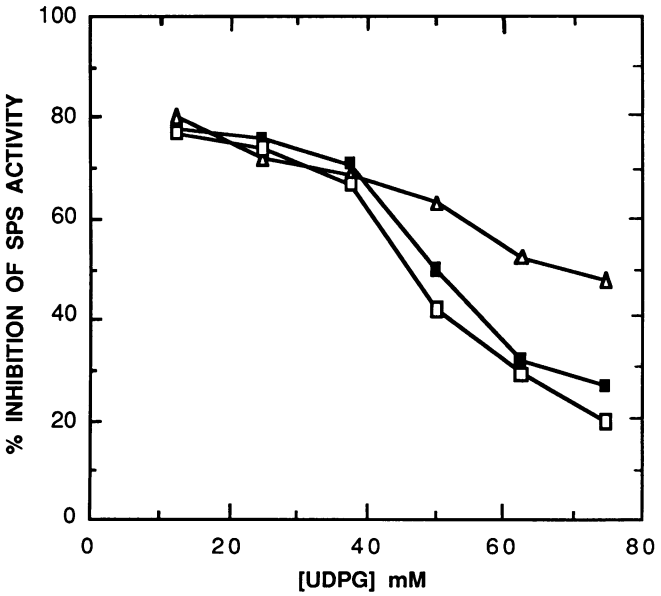

Figure 2. UDPG-mediated reversal of antibody-induced inhibition of SPS activity. Spinach SPS (0.004 units) was incubated with 25 μL of culture supernatant from clones 7C1 (□), 9C9 (■), and 3D4 (△) as described in Figure 1 (in the absence of protein A). The SPS activity was assayed for 10 min as described in "Materials and Methods" in the presence of 10 to 75 mM UDPG.

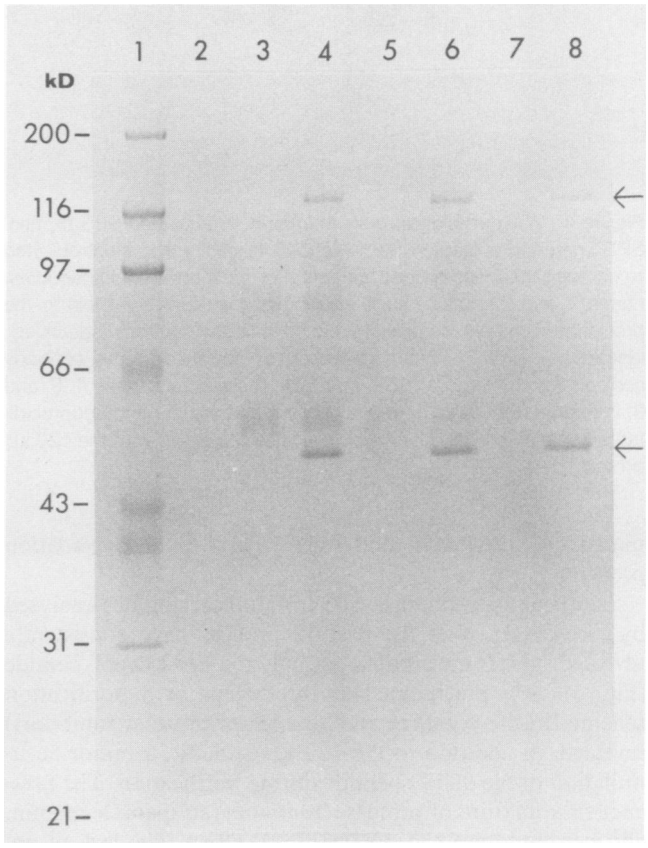

Figure 3. Analysis of immune complex composition by SDS-PAGE. Approximately 0.5 units of DEAE-purified SPS activity were incubated with 0.5 mL of each culture supernatant plus 0.2 mL of Pansorbin. SPS control precipitations contained Pansorbin but no culture supernatant, and antibody control precipitations were performed in the absence of enzyme. Lane 1, mol wt standards (Bio-Rad); lane 2, SPS control; lanes 3, 5, and 7, antibody control precipitations for clones 7C1, 9C9, and 3D4, respectively; lanes 4, 6, and 8, SPS precipitation in the presence of clones 7C1, 9C9, and 3D4, respectively. The arrows indicate the location of the immunoprecipitated 120 and 52 kD proteins.

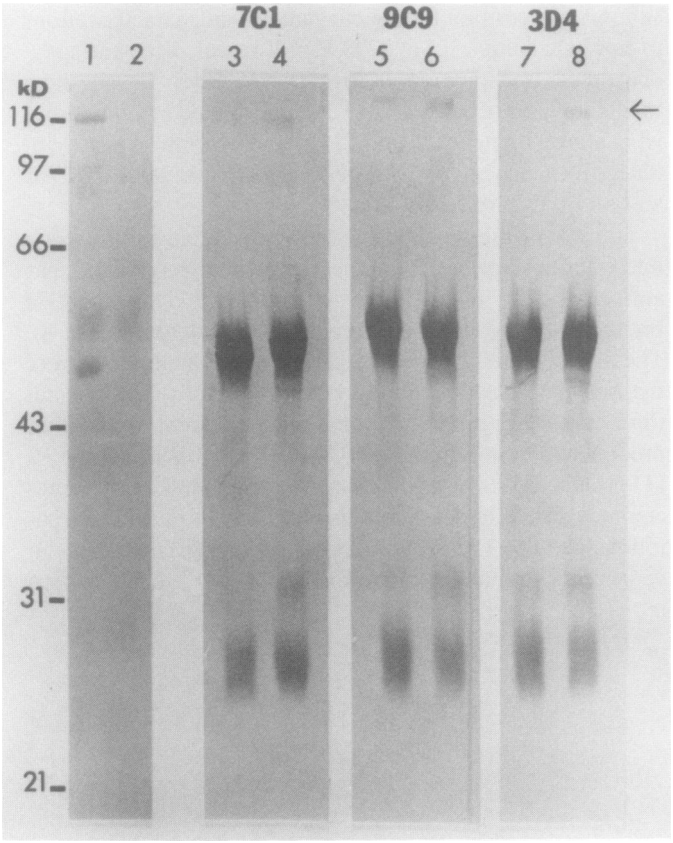

Figure 4. Western blot analysis of immunoprecipitated SPS protein. SPS immunoprecipitated from Mono Q fractions and electroblotted to nitrocellulose was probed with each of the monoclonal antibodies. Lanes 1 and 2, Amido black staining of transferred protein in the precipitated immune complexes and an antibody control, respectively; lanes 3, 5, and 7, immunodetection of epitopes in the antibody controls for clones 7C1, 9C9, and 3D4, respectively; lanes 4, 6, and 8, immunodetection of epitopes present in complete immunoprecipitates containing SPS. The arrow indicates the position of the 120 kD protein band.

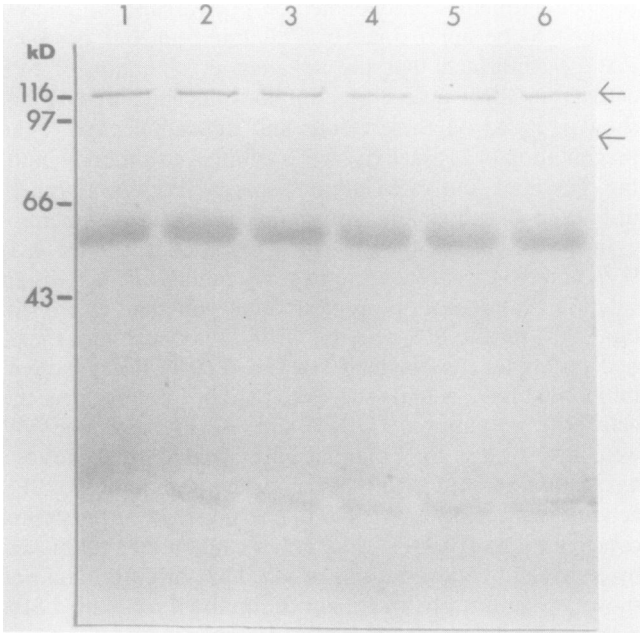

Figure 5. SDS-PAGE analysis of SPS composition at several stages of purification in the presence and absence of protease inhibitors. Lanes 1, 2, and 3, SPS immunoprecipitated from a crude extract, 6 to 11% PEG fraction and DEAE anion exchange chromatography, respectively, where purification was carried out sequentially in the absence of protease inhibitors; lanes 4, 5, and 6 correspond to SPS immunoprecipitated from the analogous stages of purification performed in the presence of 1 mg/L leupeptin, 1 mg/L antipain, and 1 mM benzamidine. The arrows indicate the 120 kD subunit and the 90 kD degradation product. The 52 kD protein was not observed.

mated by SDS-PAGE and were judged to be degradation products.

Significantly, numerous SPS immunoprecipitates analyzed by SDS-PAGE over the past 12 months have consistently revealed substantial amounts of only the 120 kD polypeptide (Fig. 5). SPS precipitated at three steps of a purification scheme (in the presence and absence of protease inhibitors) revealed, in addition to the 120 kD subunit, a minor accumulation of the 90 kD peptide during purification. The presence of a mixture of protease inhibitors (antipain, leupeptin, and benzamidine) had no significant effect. The lack of appearance of the 52 kD polypeptide suggests that, when observed, the 52 kD protein was not generated by protease activity during enzyme purification. The negative Western blot results from this protein suggest that the 52 kD polypeptide does not contain the antigenic site (Fig. 4). Recent analysis of SPS activities in crude extracts and DEAE fractions were indistinguishable from those obtained earlier with similar preparations exhibiting both the 52 and 120 kD polypeptides in the immunoprecipitates.

The monoclonal antibodies were used to purify spinach SPS and facilitate the first determination of enzyme specific activity. SPS activity was rapidly measured in desalted crude leaf extracts which were subsequently divided and incubated with an excess of monoclonal antibodies. The immune complexes were precipitated by the addition of excess Pansorbin. The complete removal of SPS protein from solution was verified by activity assays performed on the resulting supernatant. Any soluble immune complexes remaining in the supernatant would be detected as a result of the dissociating effect of UDPG and the slow recovery of SPS activity observed during extended assays (data not shown). The precipitated immune complexes were dissociated and fractionated along with known amounts of two standards, BSA and phosphorylase b, by SDS-PAGE. An empirical standard curve was obtained that related amount of protein in a gel lane with staining intensity (by densitometric scanning) (Fig. 6A). Thus, the amount of SPS 120 kD protein could be quantitated from the standard curve (Fig. 6B). The specific activity was determined to be approximately 150 units/mg SPS protein at pH 7.5 and 25°C.

The antibodies produced by the 7C1, 9C9, and 3D4 clones were tested for cross-reactivity with SPS isolated from other plant species. The species investigated included those unrelated to spinach such as maize, barley, and soybean, as well as sugar beet which is more closely related. The antibodies were tested for their capacity to bind to the SPS in these extracts using both direct enzyme inhibition and activity precipitation assays. The results of Table I reveal the absence

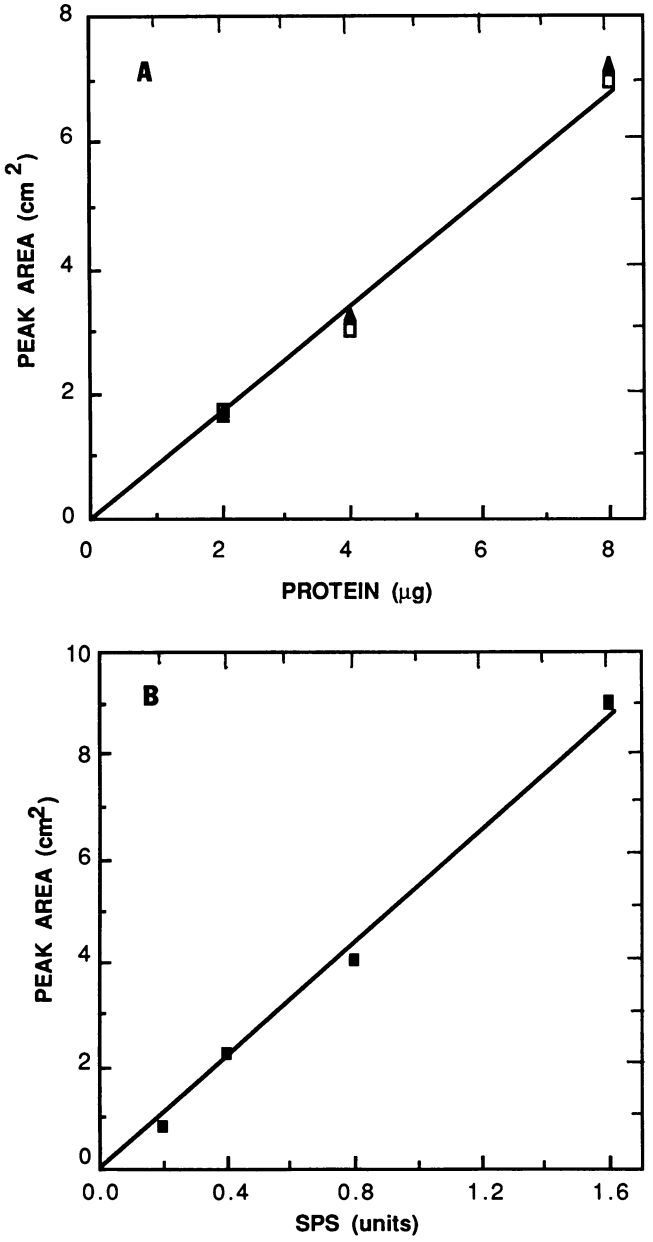

Figure 6. Specific activity determination for spinach SPS. A, A protein standard curve was established using BSA (□) and phosphorylase b (▲) by scanning the Coomassie blue-stained SDS-PAGE fractionated proteins as described in "Materials and Methods;" B, spinach SPS was immunoprecipitated in the amounts indicated, fractionated by SDS-PAGE, and the stained 120 kD subunit quantitated by densitometric scanning.

of both SPS activity inhibition and precipitation for all plant species investigated as a result of enzyme incubation with each of the three monoclonal antibodies. The antibodies do not bind to the active SPS enzyme in solution nor bound to a nitrocellulose membrane (data not shown). The results suggest potential differences in SPS protein sequence of conformation at the UDPG binding site that confer antigenic properties specific for one species but not others.

DISCUSSION

Spinach SPS-specific monoclonal antibodies have been isolated and used to purify SPS, yielding important information

Table I. *Cross-Reactivity of Antibodies Produced by Clones 7C1, 9C9, and 3D4 with Maize, Soybean, Barley, and Sugar Beet Leaf SPS*

Species	Inhibition of SPS[a]			Precipitation of SPS[b]		
	7C1	9C9	3D4	7C1	9C9	3D4
		%			%	
Spinach	55	46	37	100	82	80
Maize	9	3	7	5	10	7
Soybean	2	5	5	0	5	0
Barley				15	0	8
Sugarbeet				3	0	0

[a] Incubation mixtures contained 0.002 units of SPS activity and 15 μL of culture supernatant from each of the clones. Reactions were terminated and developed after 20 min as described in "Materials and Methods." Data are expressed as percent inhibition of SPS activity based on control assays containing antibody-free culture media. [b] Incubation mixtures contained 0.002 units of SPS activity, 15 μL of the hybridoma culture supernatants and an excess of protein A (Pansorbin). Activity in the supernatant after precipitation of immunecomplexes was assayed as described above. The data are expressed as the percent of control SPS activity measured in the presence of antibody-free cultures.

about enzyme subunit composition, specific activity, and active site characteristics. The spinach enzyme, which has a native mol wt of 480 kD (1), appears to be a homotetramer composed of four subunits of 120 kD each. Specific activity estimates of 150 units/mg enzyme protein have been obtained for spinach SPS using quantitative immunoprecipitation of enzyme activity from leaf extracts.

In addition to the 120 kD polypeptide, a 52 kD protein was detected in early immune complexes. Subsequently, the 52 kD component was not precipitated during numerous experiments, making its validity as a component of the SPS holoenzyme doubtful. Its early isolation with the 120 kD protein in the precipitates was antibody-dependent but the 52 kD protein did not contain the antigenic site for the monoclonal antibodies as indicated by negative Western blotting results. This suggests that its presence in the immune complexes was due to association with the 120 kD polypeptide in the tissue extracts. The loss of the 52 kD component during the mild conditions of SPS immunopurification, however, implies an extremely weak association with the 120 kD subunit that is uncharacteristic of subunit-subunit interactions. Further, SPS preparations apparently lacking the 52 kD protein (as suggested by its absence from immunoprecipitates) exhibited activities similar to earlier preparations which contained the 52 kD component. The 52 kD protein could not be generated at any stage of SPS chromatographic purification protocols in the absence of protease inhibitors suggesting that it is not a product of SPS proteolysis. At the present time, we can definitely conclude that the 52 kD polypeptide is not an obligatory subunit of the enzyme. However, the possibility remains that the polypeptide is a protein which interacts weakly with the SPS holoenzyme; the significance of the interaction (if any) remains to be established.

The 120 kD polypeptide is believed to be the sole subunit of SPS based on enzyme activity dependence and analysis of immunoprecipitates by SDS-PAGE. Specific removal of the 120 kD protein from tissue extracts by antibody precipitation

resulted in complete elimination of enzyme activity. The antigenic site for all three antibodies was found to reside on the 120 kD protein as revealed by Western blotting analysis. To date, efforts to dissociate the immune complexes and recover an active 480 kD enzyme composed solely of 120 kD subunits have not been successful. Elution of SPS from immunoaffinity columns containing the monoclonal antibodies have required harsh, denaturing conditions (data not shown).

The binding of all three antibodies to spinach SPS resulted in inhibition of the enzyme activity and suggested the presence of an epitope near the active site. The addition of UDPG specifically reversed the antibody-induced inhibition of SPS activity presumably through dissociation of antibody binding at the enzyme active site. The addition of F6P or G6P to the antibody:antigen incubations, however, had no effect on the extent of antibody-dependent SPS inhibition. These results suggest that distinct sites exist on the enzyme for the substrates and activator. Once the immune complexes were bound to protein A, however, UDPG-dependent dissociation of antibody and antigen was prevented, possibly suggesting that steric hinderance or a conformational change in the immune complex prevented access of UDPG to the antigenic site. The similar behavior observed for all three independent clones implies recognition of a common epitope on the spinach SPS protein.

To date, all three antibodies exclusively recognize the spinach SPS. The antigenic site, or possibly antibody access to it, appears to be lacking in the enzyme from maize, barley, soybean, and sugar beet. This would suggest active site differences between spinach SPS and the enzyme extracted from both the closely related and relatively unrelated species. Previous studies have established the existence of kinetic and regulatory differences in SPS extracted from some of these species (for review see Ref. 6). It is significant to note that physical differences at the SPS active site as detected by the monoclonal antibodies apparently exist as well. Additional comparisons of physical characteristics in these species, which may provide clues to regulatory differences, could potentially be obtained through further immunochemical applications. Sufficient quantities of the spinach SPS 120 kD subunit have been immunopurified for production of polyclonal antibodies which, if cross-reactive with other plant species, will facilitate these studies.

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