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Isolation of Serine:Glyoxylate Aminotransferase from Cucumber Cotyledons[1]

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ABSTRACT

Serine:glyoxylate aminotransferase, a marker enzyme for leaf peroxisomes, has been purified to homogeneity from cucumber cotyledons (*Cucumis sativus* cv Improved Long Green). The isolation procedure involved precipitation with polyethyleneimine, a two-step ammonium sulfate fractionation (35 to 45%), gel filtration on Ultrogel AcA 34, and ion exchange chromatography on diethylaminoethyl-cellulose, first in the presence of pyridoxal-5-phosphate, and then in its absence. The enzyme was purified approximately 690-fold to a final specific activity of 34.4 units per milligram. Electrophoresis of the purified enzyme on sodium dodecyl sulfate-polyacrylamide gels revealed two polypeptide bands with apparent molecular weights of approximately 47,000 and 45,000. Both polypeptides coeluted with enzyme activity under all chromatographic conditions investigated, both were localized to the peroxisome, and both accumulated in cotyledons as enzyme activity increased during development. The two polypeptides appear not to be structurally related, since they showed little immunological cross-reactivity and gave rise to different peptide fragments when subjected to partial proteolytic digestion. Antiserum raised against either the denatured enzyme or the 45,000-dalton polypeptide did not react with any other polypeptides present in a crude cotyledonary homogenate. The purified enzyme also had alanine:glyoxylate aminotransferase activity, but was about twice as active with serine as the amino donor.

In cucurbits and related plant species with fat-storing cotyledons, the cotyledons serve as the site of lipid mobilization during early germination, then emerge above ground and become photosynthetic (2). The microbodies present at early stages (glyoxysomes) play a central role in fat mobilization, whereas those present after the onset of photosynthesis (peroxisomes) are involved in the glycolate pathway of photorespiration (2, 28, 29). The decrease in glyoxysomal enzyme activities usually occurs concomitantly with the increase in peroxisomal activities in the greening cotyledon (1, 12, 19, 29). Much interest has focused on this changeover in microbody function (2, 4, 7, 23, 29), but the mechanism is still unresolved.

Investigation of the transition from glyoxysomal to peroxisomal function and of the regulation of gene expression during this transition would be greatly aided by the availability of antibodies to one or more of these enzymes. We have already isolated HPR[3] from cucumber cotyledons and have prepared monospecific antibodies against the enzyme (26). SGAT is also a well-characterized marker enzyme for leaf peroxisomes (20, 28). This enzyme is responsible for the serine-linked transamination of glyoxylate to glycine in the photorespiratory glycolate pathway (28). SGAT has been localized to peroxisomes in cucumber cotyledons (26) and is known to increase dramatically in activity in greening cotyledons (19), a characteristic of peroxisomal enzymes (12, 22). Here, we report the isolation of SGAT from cucumber cotyledons and the preparation of antisera against the denatured enzyme and one of its two subunits.

MATERIALS AND METHODS

Materials. Spinach HPR ('glyoxylate reductase'), phenylmethyl sulfonyl fluoride, iodoacetamide, PLP, DEAE-cellulose, Polymin-P, L-serine, glyoxylate, V8 protease, proteinase K, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate were obtained from Sigma. HPL-C supplies were purchased from Beckman Instruments. Goat anti-rabbit IgG antibody conjugated to alkaline phosphatase was obtained from Kirkegaard and Perry Laboratories. Acrylamide, bis-acrylamide, and Coomassie brilliant blue were products of Bio-Rad, Ultrogel AcA 34 was supplied by LKB, and hydroxylapatite was purchased from Calbiochem-Behring. All other materials were obtained as described previously (26).

Buffers and Solutions. Buffer pH values were determined at 25°C unless otherwise noted. Grinding buffer contained 50 mM Tricine (pH 7.5), 5 mM EDTA, 10 mM DTT, and 1 mM each of the protease inhibitors phenylmethyl sulfonyl fluoride and iodoacetamide. Gel filtration buffer contained 50 mM Tricine (pH 7.5), 1 mM EDTA, 1 mM DTT, and 0.1 mM PLP. DEAE-cellulose buffer (DB) contained 1 mM EDTA and 1 mM DTT in K-phosphate (pH 7.5) at concentrations as specified. For the first of the two DEAE columns, 0.1 mM PLP was also present in the buffer (DB+PLP). The buffer used for hydroxylapatite chromatography (HB) contained 1 mM EDTA and 0.1 mM PLP in K-phosphate (pH 7.5). Polymin-P was purchased as a 50% (v/v) solution, diluted to a 10% (v/v) stock solution and titrated to pH 7.9 with HCl.

Enzyme Assays. SGAT activity was assayed using a coupled assay procedure (20) as described previously (26). One unit of SGAT activity is defined as that quantity of enzyme necessary to catalyze the formation of 1.0 μmol of hydroxypyruvate per min, measured as NADH oxidation by exogenously added HPR in the coupled assay. Similar coupled assays (20) were used for

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[3] Abbreviations: HPR, hydroxypyruvate reductase; SGAT, serine:glyoxylate aminotransferase; PLP, pyridoxal-5-phosphate; Polymin-P, polyethyleneimine; DB, buffer used to equilibrate and elute DEAE-cellulose columns; HB, buffer used to equilibrate and elute hydroxylapatite columns; AGAT, alanine:glyoxylate aminotransferase; IgG, immunoglobulin G.

alanine:glyoxylate aminotransferase (AGAT) and aspartate:α-ketoglutarate aminotransferase activities, except that the final incubation mixtures contained 1 mM glyoxylate, 20 mM alanine, and 0.062 units/ml lactate dehydrogenase or 30 mM α-ketoglutarate, 20 mM aspartate, and 2 units/ml malate dehydrogenase, respectively. Glutamate:glyoxylate aminotransferase activity was assayed as described by Noguchi and Fujiwara (19).

Gel Electrophoresis. Electrophoresis under denaturing conditions was performed according to the method of Laemmli (14). Mol wt were determined on 12% SDS-polyacrylamide gels with an acrylamide:bis-acrylamide ratio of 30:0.8. Polypeptide bands were visualized by either Coomassie brilliant blue (1) or silver staining (15). For immunoblot analyses, 10% SDS-polyacrylamide gels with an acrylamide:bis-acrylamide ratio of 30:0.2 were used to improve resolution of the SGAT bands.

Other Assays. Protein was determined with a modified Lowry procedure (3). Column effluents were monitored spectrophotometrically for protein at 280 nm. The conductivity of column fractions was determined with a Radiometer Copenhagen conductivity meter.

Enzyme Isolation and Antibody Production. Cucumber seedlings (*Cucumis sativus* L. cv Improved Long Green) were grown in continuous light at 25°C on vermiculite saturated initially with modified Hoagland solution (11). The cotyledons from 7-d light-grown seedlings were harvested into liquid N_2, crushed to a powder, and homogenized in grinding buffer (2 ml/g tissue) using a Polytron homogenizer (Brinkman Instruments) with a PT 20 probe. All subsequent operations were carried out at 4°C. The homogenate was squeezed through three layers of cheesecloth, filtered through Miracloth, and centrifuged at 20,000*g* for 20 min.

Polymin-P was added to the resulting supernatant (S-1) to a final concentration of 0.1% (v/v). After stirring for 15 min, the solution was centrifuged at 10,000*g* for 10 min. To this second supernatant (S-2), solid $(NH_4)_2SO_4$ was added slowly with stirring to a final concentration of 19.4 g/100 ml (approximately 35% saturation). This solution was stirred for a further 30 min, then centrifuged at 20,000*g* for 10 min. Additional $(NH_4)_2SO_4$ (5.7 g/100 ml) was then added to the supernatant (S-3) to a final concentration of about 45% saturation. Stirring was continued for 30 min, followed by centrifugation at 20,000*g* for 10 min. The pellet (P-4) was resuspended in 20 ml gel filtration buffer, and the suspension was concentrated to a final volume of 11 ml by centrifugation through Amicon Centriflo cones (type CF25) at 1,000*g* for approximately 30 min.

The concentrated sample (S-5) was layered onto a 2.5 × 85-cm column of Ultrogel AcA 34 that had been equilibrated with gel filtration buffer. SGAT activity was eluted from the column at a flow rate of 27 ml/h, and 5-ml fractions were collected. The six fractions with peak SGAT activity were pooled and loaded onto a DEAE-cellulose column (2 × 17.5 cm) equilibrated with DB+PLP containing 5 mM K-phosphate (DEAE-I). The loaded column was washed with 2.4 column volumes of the same buffer, followed by 6.4 column volumes of DB+PLP containing 30 mM K-phosphate.

The column was washed with 55 ml DB+PLP containing 5 mM K-phosphate, and SGAT activity was eluted with a linear gradient of 5 to 250 mM K-phosphate in DB+PLP. Five-ml fractions were collected at a flow rate of 37 ml/h. Seventeen SGAT-containing fractions were pooled and concentrated to 4 ml with an Amicon ultrafiltration apparatus (PM-10 membrane). Repeated dilution and ultrafiltration (4 times with DB) removed the PLP and reduced the K-phosphate concentration to 5 mM. The SGAT preparation (final volume, 3.8 ml) was then applied to a second DEAE-cellulose column (1 × 6.4 cm) that had been equilibrated with DB containing 5 mM K-phosphate but no PLP (DEAE-II). In the absence of PLP, SGAT activity was recovered in the three void volume fractions (2.5 ml each).

SGAT was further purified by SDS-PAGE. The two predominant polypeptides, designated as SGAT-A (upper band) and SGAT-B (lower band), were eluted from the polyacrylamide gel, either together or separately, by the method of Hager and Burgess (10). Rabbit antisera to gel-eluted polypeptides were produced as previously described (26).

Hydroxylapatite Chromatography. SGAT was partially purified from homogenates of 7-d light-grown cotyledons (20 g) by Polymin-P precipitation and $(NH_4)_2SO_4$ fractionation as described above. The 35 to 45% $(NH_4)_2SO_4$-precipitable protein was resuspended in HB containing 5 mM K-phosphate. Ammonium sulfate was removed by repeated dilution and centrifugation through Amicon centriflo cones (type CF25). The sample (final volume, 3.5 ml) was layered onto a hydroxylapatite column (1.2 × 2.2 cm) that had been equilibrated with HB containing 5 mM K-phosphate. The column was washed with 5 ml of the same buffer and enzyme activity was eluted with a linear gradient of K-phosphate (5 to 250 mM) in HB. One-ml fractions were collected at a flow rate of 30 ml/h.

Mol Wt Determinations. The apparent mol wt of SGAT-A and SGAT-B were determined by comparison with known mol wt standards on SDS-PAGE.

The apparent native mol wt of both purified and partially purified SGAT were estimated by HPLC using a Spherogel TSK 3000 SW column (300 × 7.5 mm) protected by a Spherogel TSK SW precolumn. For mol wt calibration, proteins with known mol wt in the range 32 to 200 kD were used as standards on a column equilibrated with 100 mM Na-phosphate (pH 7.5), 150 mM NaCl, and 10 mM EDTA. The column was equilibrated in an ice bath with the above buffer (pH 7.3 at 0°C) and purified SGAT (35 μg) was applied. Fractions (250 μl) were collected on ice at a flow rate of 0.5 ml/min. SGAT elution volume was determined from both A_{280} and SGAT activity assays.

Partially purified SGAT was prepared as for hydroxylapatite chromatography except that the 35 to 45% $(NH_4)SO_4$ precipitable protein was resuspended in gel filtration buffer. Approximately 3 units of SGAT activity of this resuspended pellet were applied to the HPLC column after equilibrium with gel filtration buffer (pH 7.3 at 0°C) and eluted as described above. In this case, SGAT elution volume was determined from activity assays.

Subcellular Localization. To determine the subcellular location of SGAT-A and SGAT-B, cotyledonary homogenates from 4-d light-grown seedlings were subjected to sucrose gradient centrifugation as previously described (26). A 500*g* supernatant was layered onto a 27 to 60% (w/w) sucrose gradient, which was then centrifuged for 80 min at 20,000 rpm (40,300*g*) in a duPont/Sorvall SV-288 vertical rotor. Fractions (1.05 ml) were collected and assayed for sucrose density (calculated from refractive index), protein (A_{280}), cytochrome *c* oxidase activity (24), and SGAT activity. Selected fractions were analyzed for SGAT-A and SGAT-B by immunoblotting.

Immunoblotting. SGAT-A and SGAT-B were detected by the immunoblotting ('Western blotting') technique of Burnette (8) as modified by Tokuhisa *et al.* (27). Samples were electrophoresed through 10% SDS-polyacrylamide gels and the proteins were transferred by electroblotting to nitrocellulose paper (Millipore HAHY 304 FO). The blots were incubated with rabbit antiserum raised against either SGAT-B or a mixture of SGAT-A and SGAT-B, followed by goat anti-rabbit IgG antibody conjugated with alkaline phosphatase. Bound antibodies were detected by incubation of blots with the substrate 5-bromo-4-chloro-3-indoyl phosphate in the presence of nitroblue tetrazolium.

Peptide Mapping. SGAT-A and SGAT-B were subjected to partial proteolytic digestion by the method of Cleveland *et al.* (9) as modified by Riezman *et al.* (21). Purified SGAT was

electrophoresed into 10% SDS-polyacrylamide gels, and polypeptides were visualized by staining with Coomassie brilliant blue. SGAT-A and SGAT-B were excised from the gel separately. Approximately one-half of each band (about 7 μg protein) was loaded into 1-cm lanes of a 15% polyacrylamide gel and overlaid with about 5 μl of 20% sucrose, followed by either *Staphylococcus aureus* V8 protease (128 ng/lane) or proteinase K (32 ng/lane) in 20 μl of 10% sucrose. Samples were then electrophoresed two-thirds of the way through the stacking gel, at which time the current was turned off for 15 min to allow for partial proteolytic digestion. Thereafter, electrophoresis was resumed until the bromophenol blue tracking dye reached the bottom of the gel. The digestion products were visualized by silver staining.

RESULTS

SGAT Purification. The isolation of SGAT from 600 g (fresh weight) of cucumber cotyledons is summarized in Table I and Figures 1 to 3. The Polymin-P step precipitated about 65% of the S-1 protein, leaving 96% of the initial SGAT activity in the supernatant. A two-step $(NH_4)_2SO_4$ fractionation (35 to 45%) resulted in recovery of 50% of the initial activity, with an overall purification of 27-fold to this point (Table I). A further 5-fold enrichment was achieved by gel filtration on Ultrogel AcA (Fig. 1). The pooled SGAT-containing fractions from this column (Fig. 1, shaded region) were analyzed by SDS-PAGE (Fig. 3, lane c).

SGAT was further purified on DEAE-cellulose, based on the observation (R. Keith, unpublished observations) that the binding of SGAT to DEAE-cellulose is markedly affected by the presence or absence of PLP. In the presence of 0.1 mM PLP (DEAE-I, Fig. 2), SGAT binds to the DEAE-cellulose until the K-phosphate concentration reaches about 115 mM. Elevation of the K-phosphate concentration of the eluting buffer to 30 mM (Fig. 2, 'step') resulted in the removal of substantial protein without any loss of SGAT. Subsequent elution of the column with a linear gradient of K-phosphate (Fig. 2, 'gradient') resulted in the recovery of SGAT-enriched fractions (Fig. 2, shaded region). The gel profile of the fraction that contained maximum enzyme activity is shown in Figure 3, lane e.

After removal of PLP from the DEAE-cellulose eluate and return of the K-phosphate concentration to 5 mM, SGAT did not bind to DEAE-cellulose but was recovered in the void volume (DEAE-II, elution profile not shown). Gel profiles of the three void volume fractions with SGAT activity are shown in Figure 3, lanes f, g, and h. These three fractions accounted for 18% of the original enzyme activity (Table I). The first void volume

Table I. *Summary of Serine:Glyoxylate Aminotransferase Purification*

Fraction	Enzyme Activity	Specific Activity	Purification	Yield
	units[a]	*units/mg*	*-fold*	*%*
Filtrate	601	0.050	1.0	100
S-1[b]	583	0.088	1.8	97
S-2[c]	580	0.255	5.1	96
S-3[d]	332	0.221	4.4	55
S-5[e]	301	1.35	27	50
Ultrogel pool[f]	319	6.37	127	53
DEAE-I pool[g]	168	13.4	267	28
DEAE-II pool[h]	108	28.4	568	18
DEAE-II fraction 1[i]	21.7	34.4	687	

[a] One unit of SGAT catalyzes NADH oxidation at the rate of 1.0 μmol/min in the coupled assay described in the text. [b] Initial 20,000g supernatant. [c] Polymin-P supernatant. [d] 35% $(NH_4)_2SO_4$ supernatant. [e] Resuspended 35 to 45% $(NH_4)_2SO_4$ pellet. [f] Pooled fractions with peak SGAT activities from the Ultrogel column. [g] Pooled fractions with peak SGAT activities from the DEAE-I column. [h] Values represent a summation of the data for the three void volume fractions from the DEAE-II column. [i] Data for single fraction from DEAE-II column with the highest specific activity.

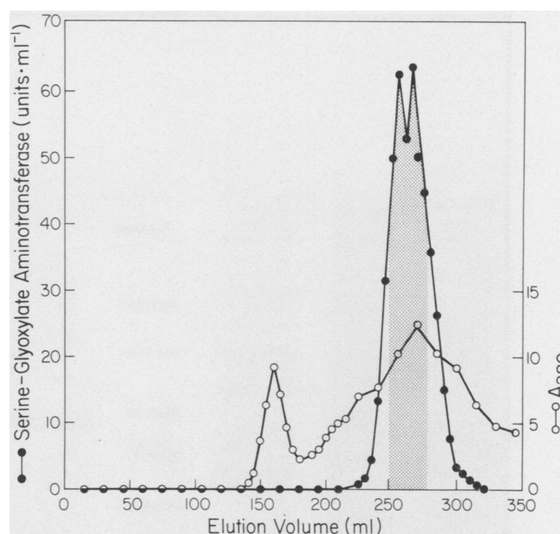

FIG. 1. Gel filtration of SGAT on Ultrogel AcA 34. The pellet from the second $(NH_4)_2SO_4$ precipitation (P-4) was resuspended in gel filtration buffer and the suspension was applied in a volume of 9.5 ml to a 2.5 × 85-cm column of Ultrogel AcA 34 equilibrated and eluted with the same buffer. Five-ml fractions were collected at a flow rate of 27 ml/h. The effluent was assayed for SGAT activity (●) and for protein (A_{280}; ○). The peak fractions of SGAT activity (shaded region; elution volume of 250 to 275 ml) were pooled, concentrated, and applied to the DEAE-cellulose column (Fig. 2).

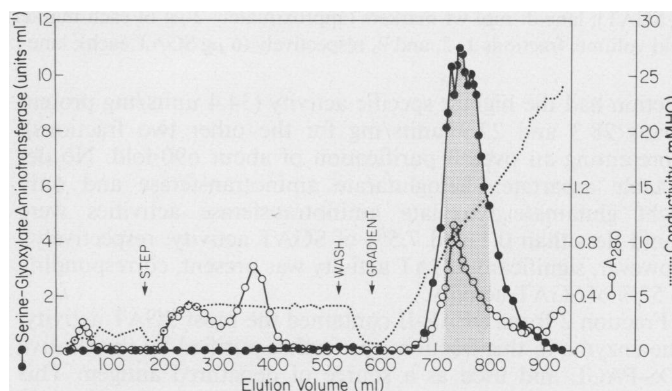

FIG. 2. Ion exchange chromatography of SGAT on DEAE-cellulose in the presence of PLP (DEAE-I). The pooled SGAT-enriched fractions (28 ml total) from the Ultrogel column (Fig. 1) were loaded onto a 2 × 17.5-cm column of DEAE-cellulose equilibrated with DB containing 0.1 mM PLP and 5 mM K-phosphate. Five-ml fractions were collected at a flow rate of 37 ml/h. In the presence of PLP, SGAT remained bound when the column was washed with this buffer and during the subsequent step elution with 30 mM K-phosphate. Upon elution with a linear gradient of K-phosphate (5 to 250 mM), SGAT activity was recovered from the column. The eluate was assayed for SGAT activity (●), protein (A_{280}, ○), and K-phosphate concentration (conductivity, · · · ·). The peak SGAT-containing fractions (shaded region; elution volume of 720 to 800 ml) were concentrated and dialyzed by ultrafiltration into DB containing 5 mM K-phosphate and no PLP, then applied to a second DEAE-cellulose column (DEAE-II) that was equilibrated and eluted in the absence of PLP.

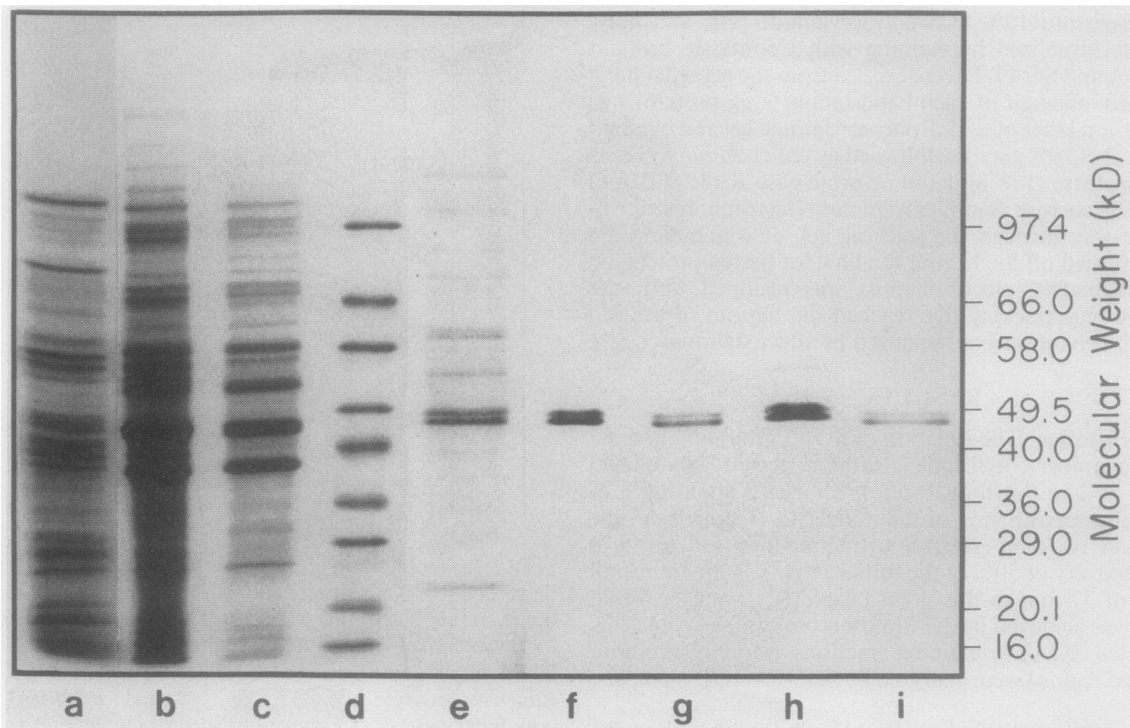

FIG. 3. Progress of SGAT purification as assayed by SDS-PAGE. Aliquots from each of the latter stages of the SGAT purification procedure were subjected to SDS-PAGE on 7.5 to 15% acrylamide gradient gels and polypeptides were visualized by Coomassie blue staining. Lane a: 35% $(NH_4)_2SO_4$ supernatant (2 μg SGAT); lane b: resuspended pellet from 35 to 45% $(NH_4)_2SO_4$ precipitation (12 μg SGAT); lane c: Ultrogel pool (11 μg SGAT); lane d: mol wt markers (approximately 2 μg of each marker); lane e: DEAE-I peak activity fraction (5 μg SGAT); lanes f–h: DEAE-II void volume, fractions 1, 2, and 3, respectively (6 μg SGAT each); lane i: SDS-PAGE eluate of DEAE-II void volume fraction 2 (6 μg SGAT).

fraction had the highest specific activity (34.4 units/mg protein *versus* 28.3 and 22.9 units/mg for the other two fractions), representing an overall purification of about 690-fold. No detectable aspartate:α-ketoglutarate aminotransferase and only slight glutamate:glyoxylate aminotransferase activities were found (less than 0.1 and 7.5% of SGAT activity, respectively). However, significant AGAT activity was present, corresponding to 52% of SGAT activity.

Fraction 2 from DEAE-II contained the most SGAT activity. The enzyme in this fraction was further purified by preparative SDS-PAGE and used as a source of denatured antigen. This electrophoretically purified preparation contained only two prominent bands, designated as SGAT-A and SGAT-B (Fig. 3, lane i). No other bands were detectable even when the gel was deliberately overloaded with 32 μg protein/lane (data not shown).

Hydroxylapatite Chromatography. To determine if the AGAT activity measured in the purified SGAT preparation represented a contaminating enzyme, a partially purified SGAT sample was subjected to hydroxylapatite chromatography. AGAT:SGAT activity ratios were 0.72:1 in the crude homogenate and 0.74:1 in the $(NH_4)_2SO_4$-purified fraction which was applied to the hydroxylapatite column. AGAT activity could be resolved into two peaks; 25% of the activity eluted at about 20 mM K-phosphate and the remainder of the activity coeluted with SGAT at about 70 mM K-phosphate. No SGAT activity or immunodetectable SGAT protein was found in the first AGAT peak. The second peak contained AGAT and SGAT activity in a ratio of 0.55:1, nearly the same as the ratio measured for purified SGAT (0.52:1).

Mol Wt. The two polypeptide bands present in the purified SGAT preparation were shown by SDS-PAGE (Fig. 3) to have apparent mol wt of approximately 47 kD (SGAT-A) and 45 kD (SGAT-B).

The mol wt of enzymically active SGAT was determined by HPLC (Fig. 4). With a partially purified preparation, SGAT activity was maximal at an elution volume of 8.65 ml, corresponding to an apparent mol wt of approximately 170 kD (Fig. 4a). However, with a purified enzyme preparation, SGAT activity peaked at an elution volume of 10.2 ml, corresponding to an apparent mol wt of approximately 62 kD (Fig. 4c). As the vertical lines on Figure 4 emphasize, neither elution profile showed a peak or shoulder of activity in the peak region of the other profile. The elution profiles of immunodetectable SGAT-A and SGAT-B closely paralleled the elution profiles of enzyme activity for both the partially purified enzyme (Fig. 4b) and the pure enzyme (Fig. 4d).

Developmental Appearance of SGAT. Activity of SGAT increased substantially between 3 and 7 d in cotyledons of light-grown seedlings, but remained at very low levels in dark-grown seedlings (Fig. 5a). Similar patterns were seen for both SGAT-A and SGAT-B when cotyledonary homogenates were analyzed by immunoblotting (Fig. 5b). Neither band was immunologically detectable before day 3, and both increased dramatically and synchronously thereafter in the light. Neither band was detectable in the dark at any point, presumably because our immunological assay was not sensitive enough to detect the small amount of protein responsible for the slight activity seen in dark-grown cotyledons.

Subcellular Localization of SGAT. Fractionation of cotyledonary homogenates from 4-d light-grown seedlings on a sucrose density gradient established the peroxisomal localization of both SGAT-A and SGAT-B (Fig. 6). SGAT activity banded at the characteristic peroxisomal density of 1.26 g/cm^3, clearly distinct from the mitochondrial (Cyt *c* oxidase) peak (Fig. 6a). Both polypeptides cobanded with enzyme activity, and neither was detectable anywhere else on the gradient (Fig. 6b).

Relationship of SGAT-A and SGAT-B. Since SGAT-A and

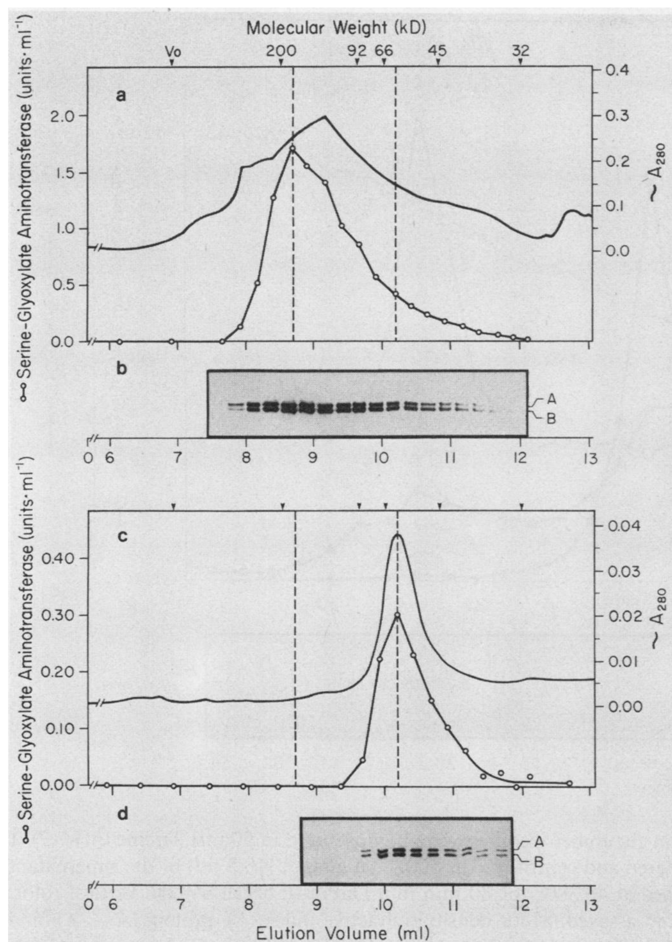

FIG. 4. Estimation of SGAT mol wt by HPLC. SGAT was applied to a Spherogel TSK 3000 SW column, the column was washed with the appropriate equilibration buffer and 250-μl fractions were collected at a flow rate of 0.5 ml/min. The effluent was monitored continuously for protein at 280 nm (——). Fractions were assayed for SGAT activity (a and c, O) and for immunodetectable SGAT (b and d). (a and b), Partially purified SGAT (3 units of activity in 280 μl of the resuspended 35 to 45% $(NH_4)_2SO_4$ pellet), column equilibrated with gel filtration buffer (pH 7.3 at 0°C). (c and d), Purified SGAT (35 μg from void volume fraction 1 of DEAE-II in 280 μl), column equilibrated with 100 mM sodium phosphate (pH 7.3 at 0°C), 150 mM NaCl, and 10 mM EDTA.

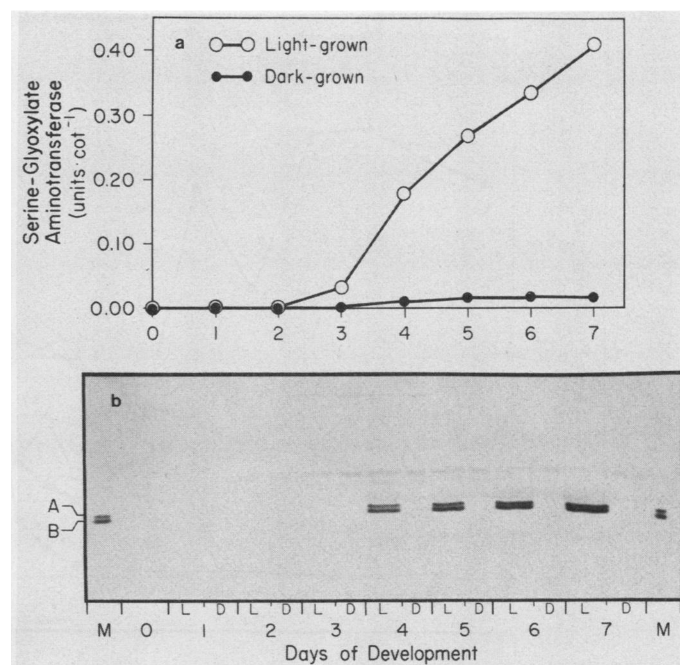

FIG. 5. Developmental appearance of SGAT in cucumber cotyledons. Cotyledons from cucumber seedlings grown at 25°C for the indicated lengths of time in either continuous white light (L, O) or darkness (D, ●) were homogenized in grinding buffer. Homogenates were centrifuged at 10,000g for 10 min and the supernatants were assayed (a) for SGAT activity and (b) for immunodetectable SGAT-A and SGAT-B polypeptides. Each lane in the immunodetection assay was loaded with an amount of supernatant that corresponded to 0.025 cotyledon, except the marker (M) lanes, which contained 0.1 μg of purified SGAT.

SGAT-B copurified with enzyme activity and behaved identically under all conditions we have examined (chromatographic elution, developmental appearance, and subcellular localization), the structural similarity of the two polypeptides was examined. Antiserum raised against either isolated SGAT-B or the purified SGAT-A/B mixture was used to assess the immunological cross-reactivity of the two polypeptides (Fig. 7). (Antiserum was not raised against SGAT-A because it was not possible to isolate SGAT-A in a form that was uncontaminated by SGAT-B.) Antiserum against SGAT-B reacted strongly against a single band (Fig. 7, lane b). Only a slight amount of reactivity was observed against a band with a mol wt comparable to that of SGAT-A. The two bands recognized by antiserum raised against SGAT-B seem to be the same as those recognized by antiserum raised against SGAT-A/B, since a mixture of the two antisera visualized only two bands (Fig. 7, lane c). The preimmune control showed no reactivity with either band (lane d).

The structural similarity of SGAT-A and SGAT-B was also examined by comparing the polypeptide fragments generated from each by partial proteolytic digestion (Fig. 8). Major differences were observed in the digestion patterns when either *S. aureus* V8 protease (lanes and b) or proteinase K (lanes c and d) were used. These data suggest that SGAT-A and SGAT-B differ significantly in their amino acid sequences.

DISCUSSION

In most previous investigations of SGAT from plant sources, the enzyme has been partially purified to resolve it from other aminotransferases, and its kinetic properties have been studied (30). Ion exchange chromatography was used to obtain partially purified SGAT from wheat leaves (13) and from isolated spinach leaf peroxisomes (20). Hydroxylapatite chromatography has also been used either as a single purification step for spinach leaf SGAT (16) or following $(NH_4)_2SO_4$ fractionation and ion exchange chromatography for SGAT from oat (6) and kidney bean (25) leaves. Recently, Noguchi and Hayashi (17) purified to homogeneity a peroxisomal aminotransferase from spinach leaves which they identified as tryptophan:glyoxylate aminotransferase. However, they found their enzyme to be 15 times more active with serine than with tryptophan, and suggested that it might be identical to SGAT. The enzyme was purified approximately 1000-fold to a final specific activity of about 14 units SGAT activity/mg protein, with a yield of 5.5%.

The purification reported here for SGAT from cotyledons of 7-d light-grown cucumber seedlings is more rapid, has higher yields, and results in an enzyme preparation with higher specific activity than any procedure yet reported. SGAT was purified approximately 690-fold to a specific activity of 34.4 units/mg protein with a final yield of 18% of the initial homogenate activity.

Our isolation procedure takes advantage of the finding that SGAT binds to DEAE-cellulose in the presence, but not in the

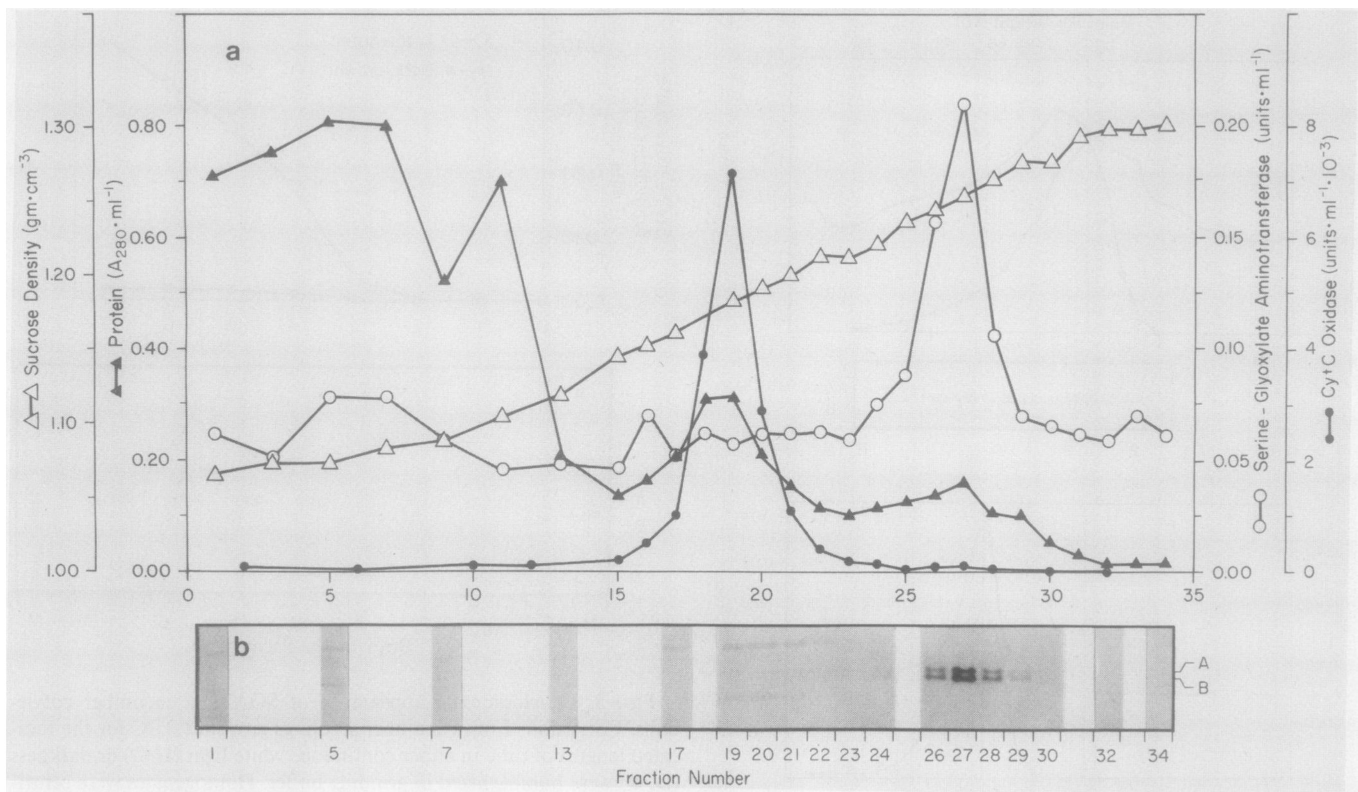

FIG. 6. Subcellular localization of SGAT. Cotyledons from 4-d light-grown cucumber seedlings were homogenized in 50 mM Tricine (pH 7.7), 1 mM EDTA, 10 mM DTT, and 20% (w/w) sucrose. The homogenate was filtered and centrifuged at 500*g*. An aliquot (10.5 ml) of the supernatant was layered onto a 20 to 60% (w/w) sucrose gradient (25 ml) and centrifuged at 40,300*g* for 80 min in a DuPont/Sorvall SV-288 vertical rotor. Fractions (1.05 ml) were collected dropwise from the bottom of the tube and assayed (a) for density (refractive index, △), protein (A_{280}, ▲), and activities of SGAT (○) and Cyt *c* oxidase (●), and (b) for immunodetectable SGAT-A and SGAT-B polypeptides. Each lane in the immunodetection assay was loaded with 25 µl of the appropriate fraction, so that 0.1 µg of enzyme was present in the lane from the fraction with the maximum SGAT activity (fraction 27).

absence, of PLP. Chromatography in the presence of PLP (DEAE-I) resolves SGAT from the bulk of the proteins, which bind only weakly or not at all. Subsequent passage of the enzyme over DEAE-cellulose in the absence of PLP (DEAE-II) separates it from most of the remaining proteins. The reason for this effect of PLP is not known. One suggestion is that because of its nucleophilic phosphate and carbonyl groups, PLP may bind directly to DEAE-cellulose and SGAT might then bind to the PLP. This explanation would be consistent with our observation that large amounts of PLP bound to DEAE-cellulose when the column was equilibrated with PLP-containing buffers. Moreover, both SGAT and PLP were released from the column at about the same K-phosphate concentration.

Analysis of the flow-through fractions from the second DEAE-cellulose column by SDS-PAGE revealed the presence of two major polypeptides, SGAT-A and SGAT-B, with mol wt of 47 and 45 kD, respectively. These two polypeptides accounted for more than 90% of the protein by densitometric scanning (data not shown). Further purification by SDS-PAGE yielded a preparation in which these two polypeptides were the only proteins detectable (greater than 98% pure) even when subsequent SDS-polyacrylamide gels were intentionally overloaded. Additional evidence of enzyme purity was provided by immunoblotting. Antisera raised against the SDS-PAGE purified enzyme detected only the 47 and 45 kD bands in immunoblots of total soluble cotyledonary protein.

The presence of two bands in the purified SGAT preparation does not appear to be an artifact of proteolysis during tissue extraction. Both bands were still detected on an immunoblot of 7-d light-grown cotyledons when proteolysis was minimized by quick-freezing of the cotyledons (in liquid N_2) and lyophilization, followed by rapid extraction into a boiling SDS gel buffer containing 1% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 5% (w/v) sucrose, and 60 mM Tris (pH 6.8; D. E. Titus, unpublished observations).

The antiserum raised against gel-eluted SGAT is specific for the denatured form of the enzyme. Addition of this antiserum and formalin-fixed *S. aureus* cells to homogenates of 7-d light-grown cotyledons failed to immunoprecipitate SGAT activity, and neither polypeptide was detected in the *S. aureus* pellet by immunoblotting. However, if the homogenate was made 2% (w/v) with respect to SDS and boiled for 5 min, both SGAT-A and SGAT-B could be readily immunoprecipitated (D. Hondred, unpublished observations).

SGAT could be recovered in two enzymically active forms, differing in mol wt as determined by HPLC. Partially purified SGAT, presumably similar to the native form of the enzyme, has an apparent mol wt of 170 kD, whereas purified SGAT has a mol wt of 62 kD. This difference may reflect the dissociation of SGAT from an oligomer to a monomer during the purification procedure, perhaps as a result of removal of PLP prior to the second DEAE-cellulose column.

Most plant and animal aminotransferases have mol wt in the range of 75 to 115 kD, although values as high as 132 and 248 kD have been reported for the ornithine:α-ketoglutarate aminotransferases of rat liver and pig kidney, respectively (5, 30). The peroxisomal aminotransferase described by Noguchi and Hayashi (17) is more comparable in size to cucumber SGAT, since

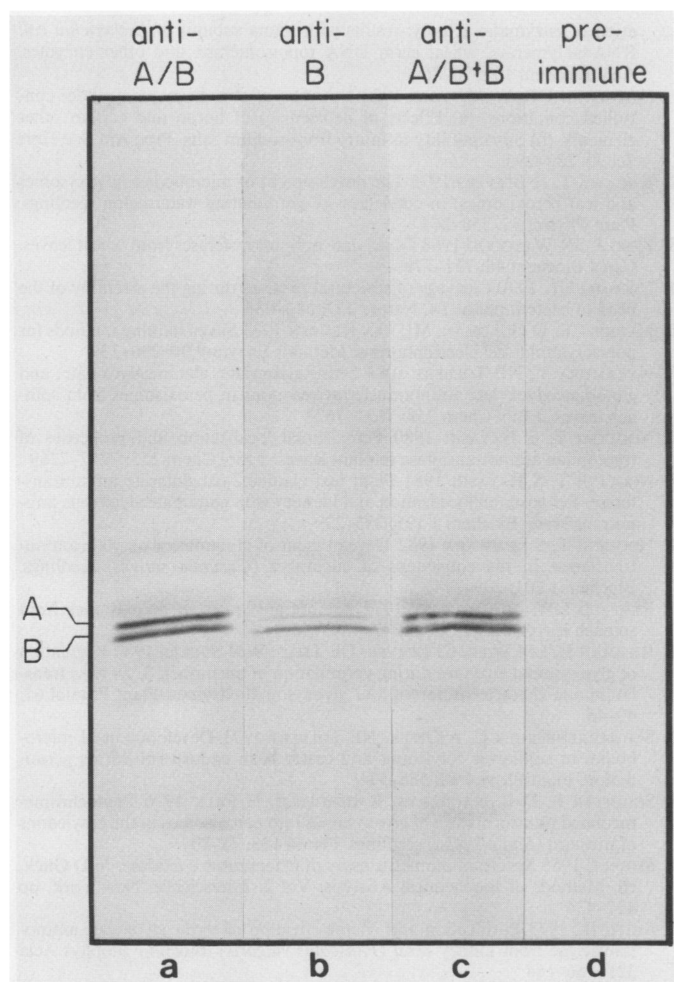

FIG. 7. Immunoblot analysis of SGAT-A and SGAT-B. S-1 extracts of 7-d light-grown cotyledons were subjected to SDS-PAGE on 10% acrylamide gels and electroblotted onto nitrocellulose paper. The nitrocellulose blot was incubated with rabbit anti-SGAT antisera as indicated, followed by goat anti-rabbit IgG antibody conjugated with alkaline phosphatase. Lane a: incubated with antiserum against a mixture of SGAT-A and SGAT-B (anti-A/B); lane b: incubated with antiserum against SGAT-B (anti-B); lane c: incubated with a mixture of anti-A/B and anti-B antisera; lane d: incubated with preimmune serum.

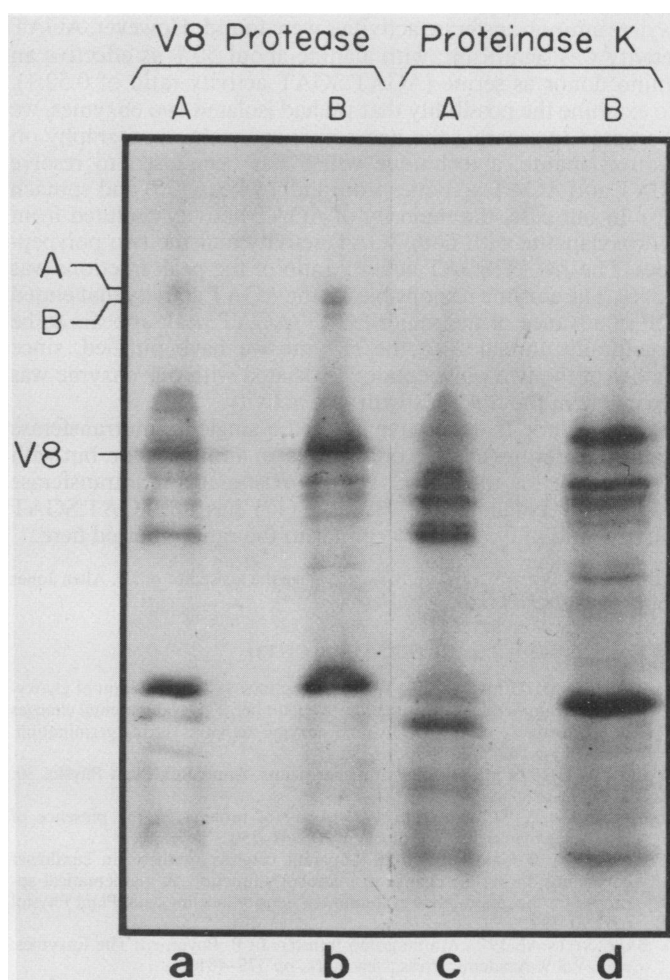

FIG. 8. Peptide mapping of SGAT-A and SGAT-B. Purified SGAT was subjected to SDS-PAGE to resolve bands A and B, which were excised from the gel separately and loaded into a second gel with either *S. aureus* V8 protease (lanes a and b) or proteinase K (lanes c and d). Samples were electrophoresed two-thirds of the way through the stacking gel and incubated for 15 min to allow partial proteolytic digestion. Electrophoresis was then continued until the dye front reached the bottom of the gel, and digestion products were visualized by silver staining. Lane a: SGAT-A, V8 protease; lane b: SGAT-B, V8 protease; lane c: SGAT-A, proteinase K; lane d: SGAT-B, proteinase K.

it has a mol wt of 185 kD and a subunit mol wt of 46 kD, suggesting that the enzyme exists as a homotetramer. These values are in good agreement with those for the enzyme we have isolated, except that we detect two subunits.

We are aware of no previous reports of aminotransferases with nonidentical subunits, but the evidence seems strong that both of the polypeptides we have isolated are subunits of the same enzyme complex. The light-modulated appearance of both polypeptides closely paralleled the appearance of SGAT activity in the cotyledons of developing seedlings, and both polypeptides banded with SGAT in a sucrose gradient at the density of peroxisomes. Moreover, both polypeptides coeluted with SGAT activity under all chromatographic conditions investigated, including HPLC, size exclusion chromatography, ion-exchange chromatography, and hydroxylapatite chromatography.

It is especially significant that both polypeptides bound to DEAE-cellulose in the presence but not in the absence of the aminotransferase coenzyme PLP just as enzyme activity did. In addition, the HPLC data showed that both polypeptides coeluted with enzyme activity for crude [$(NH_4)_2SO_4$-fractionated] and for purified enzyme preparations, even though the apparent mol wt of SGAT in the two preparations differed greatly (170 kD for the former, 62 kD for the latter). Though apparently part of the same enzyme complex, SGAT-A and SGAT-B do not seem to be structurally related, since they exhibited little immunological cross-reactivity and gave rise to different polypeptide fragments when subjected to partial proteolytic digestion.

From the mol wt of the native enzyme and the occurrence and sizes of two polypeptides in the purified preparation, we conclude that cucumber SGAT probably exists as an oligomer consisting of two nonidentical subunits, probably either as a heterotetramer or a highly elliptical heterodimer.

Plant peroxisomes have been reported to contain three additional aminotransferase activities: AGAT, aspartate:α-ketoglutarate aminotransferase, and glutamate:glyoxylate aminotransferase (16, 18, 20, 30). Since aminotransferases often exhibit broad substrate specificities (5, 17, 30), we assayed our purified SGAT preparation for these additional activities. No significant aspartate:α-ketoglutarate aminotransferase or glutamate:gly-

oxylate aminotransferase activities were found. However, AGAT activity was significant, with alanine about 50% as effective an amino donor as serine (AGAT:SGAT activity ratio of 0.52:1). To examine the possibility that we had isolated two enzymes, we attempted to separate the two activities by chromatography on hydroxylapatite, a technique which has been used to resolve SGAT and AGAT activities from kidney bean (25) and spinach (16). In our case, the majority of AGAT activity coeluted from hydroxylapatite with both SGAT activity and the two polypeptides. The AGAT:SGAT activity ratio of the peak fractions was 0.55:1. The enzyme responsible for the AGAT activity that eluted well in advance of the major SGAT/AGAT peak appears to be structurally unrelated to the enzyme we have purified, since neither of the two polypeptides associated with our enzyme was detectable in the fractions with that activity.

We conclude that we have isolated a single aminotransferase that can use either alanine or serine as an amino donor, but with a preference for the latter. The peroxisomal aminotransferase reported by Noguchi and Hayashi (17) has an AGAT:SGAT activity ratio of 0.52:1, very similar to the ratio reported here.

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