

Cottonseed Malate Synthase¹

PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION

Received for publication March 18, 1987 and in revised form May 14, 1987

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ABSTRACT

Malate synthase (EC 4.1.3.2), an enzyme unique to the glyoxylate cycle, was purified to homogeneity from cotyledons of 72-hours, dark-grown cotton (*Gossypium hirsutum* L.) seedlings. Homogeneity of the enzyme was assessed by silver staining SDS-PAGE gels. Purification was accomplished by using a single buffer medium through six steps involving one ammonium sulfate fractionation and chromatography on three columns (Sephacryl S-300, DEAE Sephacel, Phenyl Sepharose). Large-scale preparation of glyoxysomes, a main step in all other published procedures, was not involved. The purified enzyme and that extracted from glyoxysomes appears to be a dodecamer with a native molecular weight of 750,000 (sedimentation coefficient of >20 Svedberg units [S] on sucrose gradients) composed of identical subunits (molecular weight approximately 63,000). The monomer (5S) occurs in the cytosol. Polyclonal antibodies raised in rabbits were judged to be monospecific for malate synthase by immunotitration, double immunodiffusion, and western blotting. Double immunodiffusion experiments revealed only partial immunological identity between the 5S (cytosolic) and 20S (glyoxysomal forms, although complete identity was observed between the 5S form in immature and germinated seeds, and the 20S form in immature and germinated seeds. Cross-reactivity of the cotton antimalate synthase serum was observed with extracts from five other oilseeds. Western blot analyses showed that malate synthase protein was not present in immature seeds prior to appearance of enzyme activity, but when present, subunit molecular weight was indistinguishable in immature, desiccated, and germinated seeds.

Malate synthase (EC 4.1.3.2), an enzyme specifically involved in glyoxylate cycle metabolism, catalyzes the aldol condensation of acetyl-CoA with glyoxylate to form malate and CoA. In higher plants, MS² is localized in glyoxysomes (a type of peroxisome) which occur almost exclusively in storage tissues of oilseeds where they are directly involved in reserve oil mobilization following germination (8, 27). Enzyme activity is not restricted to germinated seeds, but first appears during seed maturation (3, 6, 9).

Recent research on glyoxysomes and their enzymes has focused on their ontogeny and biosynthesis, mostly following seed germination (26). This work has not only been important for learning more about oilseed metabolism, but has contributed significantly toward the overall understanding of intracellular protein trafficking within eukaryotic cells (1, 16, 23). An essential

element for studying certain aspects of protein trafficking is the availability of monospecific antibodies to specific organellar proteins. As part of our ongoing efforts to elucidate mechanisms of glyoxysomal enzyme acquisition in cotton seeds, we have purified and raised antibodies to isocitrate lyase (4) and catalase (13) and now report results for MS.

MS has been partially purified from cottonseeds (20), purified to apparent homogeneity from only three oilseed species (cucumber 10, 22; castor bean 2, 5; corn 24), and purified to varying degrees in bacteria, yeast, and *Euglena* (Ref. 20 for references). In all procedures described for oilseeds, the enzyme was purified from glyoxysome fractions. In the procedure presented here the enzyme was extracted from homogenized cotyledons and purified using the same buffer medium for all steps. The final product was free of contaminating proteins as judged from silver-stained gels; this criterion for purity had not been applied previously to other purified MS preparations. New information is also presented on the immunological characteristics and cross-reactivity of the polyclonal antibodies raised in rabbits relative to different forms of MS in germinated and maturing seeds.

MATERIALS AND METHODS

Chemicals. PMSF, DTT, aprotinin, benzamidine-HCl, iodoacetamide, Sephacryl S-300, DEAE Sephacel, Phenyl Sepharose CL-4B, Protein-A Sepharose CL-4B, bovine thyroglobulin, BSA (98–99%), bovine heart L-lactic dehydrogenase, bovine liver catalase (C-10), glyoxylic acid (free acid and sodium salt), alkaline phosphatase conjugated goat anti-rabbit IgG, fast red violet LB salt, naphthol AS-BI phosphate (sodium salt), MOPS, Triton X-100, and PVP-10 were purchased from Sigma Chemical Co. Coenzyme A was from P-L Biochemicals, Inc. Acetic anhydride, sucrose (RNase free), hydrogen peroxide, ethylene glycol, K-phosphate, EDTA, and magnesium chloride were obtained from J. T. Baker Chemical Co. Acrylamide, bis-acrylamide, bovine serum gamma globulin, mol wt protein standards, and agarose immunodiffusion tablets were from Bio-Rad Laboratories. Serva Fine Biochemicals supplied SDS (research grade) and Serva Blue R (Coomassie blue R-250), and ammonium sulfate (special enzyme grade) came from Schwartz/Mann. Silver nitrate was purchased from Accurate Chemical and Scientific Corp. Deionized water (Barnstead Co.) was used to prepare all aqueous solutions.

Plant Material. Cotton plants, *Gossypium hirsutum* L. cv Deltapine 62 were grown under glasshouse conditions as described (15) for obtaining maturing seeds at varying ages. Commercial seeds were soaked in water with aeration for 4 to 6 h, then scrolled in moistened filter paper for germination and growth in the dark at 30°C (14). Cucumber seeds (*Cucumis sativus* L. cv Improved Long Green) were provided by W. M. Becker. D. Randall provided castor beans (*Ricinus communis* L.), J. Yamaguchi provided pumpkin seeds (*Cucurbita* sp. Amakuri Nankin), and sunflower seeds (*Helianthus annuus* L. Giant

¹ Supported by National Science Foundation grant DMB-8414857.

² Abbreviations: MS, malate synthase; PMSF, phenylmethylsulfonyl fluoride; DPA, days postanthesis; PBS, 10 mM Na phosphate, 0.9% w/v NaCl (pH 7.2); MOPS, morpholinopropane sulfonic acid.

Greystripe) were purchased locally.

Enzyme Purification. Cotton seedlings grown in the dark for 72 h were used as starting material. Cotyledon pairs (250) were removed from the scroled seedlings and added to 125 ml of homogenizing medium in a prechilled glass blender. All steps up through $(\text{NH}_4)_2\text{SO}_4$ fractionation were done at 4°C. The medium consisted of 100 mM K-phosphate, 8 mM MgCl_2 , 2 mM EDTA (pH 7.2), plus 1 mM PMSF (final concentration, added from a 100 mM stock solution in isopropanol just before blending). Cotyledons were homogenized at low speed until slurried, then at high speed for approximately 30 s more. This mixture was centrifuged at 27,000g for 60 min in a Beckman JA-20 rotor. Surface lipid layers were removed and the supernatant (approximately 140 ml) poured through one Whatman No. 1 filter. Sodium glyoxylate was added to a final concentration of 2 mM to prevent possible aggregation of MS (10), then membrane fragments and other debris were removed by centrifugation at approximately 200,000g for 1 h in a Beckman 50.2 Ti rotor. The supernatant was brought to 30% $(\text{NH}_4)_2\text{SO}_4$ (16.4 g/100 ml sample) slowly over a 2-h period with mixing by adding powdered $(\text{NH}_4)_2\text{SO}_4$. This was mixed for an additional hour, then centrifuged at 27,000g for 60 min (JA-20 rotor). The pellets were resuspended to approximately 5 ml with buffer A (100 mM K-phosphate, 8 mM MgCl_2 , 2 mM glyoxylate [pH 7.2], fresh 1 mM PMSF) which also was used for all subsequent steps unless indicated otherwise. These resuspended fractions were held at 4°C overnight without any appreciable loss of MS activity. All subsequent steps were done at room temperature.

Resuspended $(\text{NH}_4)_2\text{SO}_4$ fractions were desalted by passage over Sephadex G-25M (Columns PD-10, Pharmacia Fine Chemicals). The protein was applied to a 2.5×70 cm column of Sephacryl S-300 (superfine) equilibrated with degassed buffer A, and 5.0 ml fractions were collected by gravity flow. MS activity began to appear after collecting 25 fractions (void volume). Two peaks of MS activity were consistently recovered from this column. Typically 4 to 5 fractions exhibiting the highest specific activity in the second peak, were pooled for chromatography on a 2.5×28 cm DEAE Sephacel column equilibrated in buffer A, carefully checked at pH 7.2. Fractions (3.4 ml) were collected by pumping buffer A through the column at 50 ml/h. Cottonseed MS does not bind to this matrix under these conditions. To prepare antigen for antibody production, five peak activity fractions were pooled and applied to a 1.5×9 cm column of Phenyl Sepharose equilibrated with buffer A. The column with applied sample was washed thoroughly overnight with buffer A by pumping at 4 ml/h. MS activity was not in any of the wash fractions. The enzyme was eluted in 2.3-ml fractions at the same pump rate with a 100-ml gradient formed by equal volumes of buffer A and ethylene glycol. All fractions with MS activity, usually 5 to 6 in approximately 40% ethylene glycol, were pooled and used for antigen.

Protein was determined using the Coomassie brilliant blue dye-binding method (Bio-Rad Lab) with bovine serum gamma globulin as the standard. MS activity was assayed as described in detail by Miernyk *et al.* (21), except 70 mM MOPS (pH 8.2) and 0.04% Triton X-100 were substituted for 70 mM Tris-HCl (pH 8.0).

Antibody Production. The pooled fractions from Phenyl Sepharose chromatography were vacuum dialyzed and concentrated (-p Micro-ProDiCon, BIO-Molecular Dynamics) to 1 ml in 50 mM Tris-HCl, 8 mM MgCl_2 , 2 mM glyoxylate, 20% ethylene glycol (pH 7.5). Concentration of MS in dialysis membranes often resulted in loss of protein, presumably due to binding to the membrane. Enzyme (55 μg in 0.25 ml) was mixed with 0.75 ml PBS and 2 ml complete Freund's adjuvant (Difco, Lab), emulsified in a coupler, and injected subcutaneously in the lymph node area of all four legs of New Zealand White rabbits.

The same procedures and amounts were repeated on d 13. On d 36, 66 μg antigen (in 0.3 ml) was mixed with 1 ml PBS and injected intravenously into the marginal ear vein. Blood was collected via heart punctures on d 41 through 43, and antisera was pooled and stored at -20°C or -80°C.

IgGs were purified from 1 ml antiserum or preimmune serum using a Protein A-sepharose CL-4B column (1.4×1.0 cm) equilibrated with PBS. Column effluent after antisera addition was reapplied three times before extensive washing with PBS. Bound IgGs were eluted with 4 ml 0.1 M citrate-Na phosphate (pH 3.2), and immediately vacuum dialyzed to 1.0 ml against PBS. Preparations typically contained 4 to 5 mg/ml IgG.

Immunochemical Analyses. Immature seeds and cotyledons of germinated seeds were homogenized with a motorized Teflon pestle at 4°C typically at a ratio of 1.5 to 2.0 vol medium per g fresh weight. The homogenates were centrifuged at 27,000g, 60 min; supernatants with lipid layer removed were used as extracts for immunotitration, Ouchterlony double immunodiffusion, and Western blotting experiments. Media used for homogenization were either 100 mM K-phosphate, 8 mM MgCl_2 , 2 mM EDTA (pH 7.2) ($\pm 0.05\%$ Triton X-100) or 100 mM Tris-HCl, 8 mM MgCl_2 , 2 mM EDTA (pH 7.5) ($\pm 0.05\%$ Triton X-100). PMSF was always added just before use to 1 mM. In some experiments, combinations of other protease inhibitors were added (see Figure legends).

For immunotitration experiments, 1 ml portions of extracts prepared in the K phosphate medium (minus Triton X-100) were incubated in 1.5 ml microfuge tubes for 1 h at room temperature, then overnight at 4°C following addition of varying amounts of anti-MS or preimmune serum. The samples were centrifuged for 15 min at 13,000 rpm (4°C) in a microfuge (model 235B, Fisher Scientific). Supernatants were assayed for catalase (14), isocitrate lyase (21), and MS activities which were compared to activities in samples incubated with PBS or preimmune serum. These control samples did not exhibit an appreciable loss in activity overnight.

Double-diffusion immunoprecipitation was performed in 1% (w/v) agarose incubated for 48 h in a humid atmosphere. Visualization of precipitation bands was enhanced by washing 1 to 2 d in PBS, then staining for 30 to 45 min in 0.125% (w/v) Coomassie blue R, 50% (v/v) methanol 10% (v/v) acetic acid. Slabs were destained first in 50% methanol plus 10% acetic acid for 1 h, then in 7% acetic acid, 5% methanol until bands were clearly resolved. Formation of precipitin bands was optimized for the various samples tested by placing 9 μl of a 1:8 dilution of anti-MS serum, or 1:6 dilution (in PBS) of IgGs prepared from this serum, in the center wells and 25 to 35 nmol/min MS activity in outer wells. For samples having low activity per ml, larger diameter outer wells were made in the agarose, and sample was repeatedly added until sufficient activity had been applied. Extracts of other oilseed cotyledons or endosperm (3 d) also were made in K phosphate medium containing 0.05% Triton X-100. Cottonseed extracts referred to as 'transition' are from seeds germinated and grown in the dark for 48 h, then put in the glasshouse under natural light for 24 h.

Electrophoretic (Western) blotting from SDS-PAGE gels was done as described in detail by Kuncce and Trelease (14), except with anti-MS serum. The extracts used for the blots shown in the Figures were prepared with K phosphate buffer (- Triton X-100). Extracts prepared in Tris-HCl yielded no distinguishable differences after blotting. Extracts boiled in SDS after freezing with liquid nitrogen, and glyoxysome isolation were accomplished as in Kuncce and Trelease (14). Solubilized glyoxysomal samples were prepared by slowly diluting glyoxysomes in 51% sucrose to 40% sucrose with Hepes gradient buffer, sedimenting them at 37,000g for 30 min, then resuspending the pellet in buffer A plus 1% Triton X-100. Routinely 95% of the gradient

MS was recovered in the glyoxysomal pellets following the sucrose dilution.

Gel Electrophoresis. SDS-PAGE was performed as described (14), except that proteins reduced with DTT were also alkylated with iodoacetamide for 30 to 60 min. Proteins on the gels were stained with silver according to the procedure of Wray *et al.* (29).

Rate-Zonal Centrifugation. Linear sucrose gradients (14 ml, 5–25% w/w) constructed on 1 ml 50% sucrose and having 0.8 to 1.0 ml applied sample were centrifuged for 19 h at 24,000 rpm ($4.32 \times 10^{11} \omega^2t$) at 5°C in a Sorvall AH-627 rotor. Sucrose solutions were made in 100 mM K-phosphate, 8 mM MgCl₂, 2 mM glyoxylate (pH 7.2). Approximate sedimentation coefficients were calculated according to the method of McEwen (18). Protein standards (lactate dehydrogenase-10 µg, bovine catalase-10 µg, and bovine thyroglobulin-2 µg) were run on separate gradients, and in some cases with 94 µg purified MS. All samples were prepared in the same solution as were the sucrose solutions, except extracts of germinated seeds included 1 mM PMSF and 1% (w/v) PVP-10.

RESULTS AND DISCUSSION

Purification of MS from other oilseeds for antibody production has involved isolation of large quantities of glyoxysomes requiring the use of zonal rotors (2, 10, 12), which are not commonly found in laboratories, or numerous tube gradients on swing-out rotors (5, 22). In these procedures, the enzyme was extracted from pelleted glyoxysomes or membrane pellets of osmotically shocked organelles using relatively high salt (0.6 M MgCl₂, 10, 12, 22; 0.2 M KCl, 2, 5) concentrations. The exception was the work by Servettaz *et al.* (24) who solubilized the maize enzyme from pelleted glyoxysomes in 10 mM phosphate buffer. Later studies on maize glyoxysomes indicated that MS was not membrane bound, therefore it was nearly quantitatively released without salt treatments (17). We had difficulty in consistently solubilizing more than 50% of the MS from cottonseed glyoxysomes isolated on sucrose gradients. Therefore, we chose to develop a relatively simple procedure for purifying MS from excised cotyledons without the need to purify glyoxysomes on a large scale.

A summary of the purification scheme and data for the steps are given in Table I. Purification to apparent homogeneity involved six main steps, including chromatography on three columns. Features of the procedure that make it relatively simple are (a) the same buffer medium (with minor modification) is used throughout, (b) only one ammonium sulfate fractionation is required, (c) all column chromatography is done at room temperature, (d) the ion-exchange chromatographic step does

not require fractionation with salt-gradient elutions, and (e) the enzyme is nearly homogeneous after passage through the DEAE Sephacel, such that this preparation can be used for most purposes other than antibody production.

The enzyme was purified at least 10 times in our laboratory with modifications for improvements. We consistently recovered approximately 80% of the enzyme (3-fold purification) in the supernatant following the two centrifugations (Table I). This high recovery is important because MS aggregates relatively easily (2, 7, 12) and usually requires high-salt solubilization from isolated glyoxysomes (see references above). It appears that most of the cotton enzyme can be solubilized from homogenates in K-phosphate media buffer with low salt. When other buffers were used for homogenization, the activity was reduced (20); we believe this was due to differential extractions of the enzyme and not enzyme inactivation.

Ammonium sulfate fractionation was specifically avoided in preparing castor bean MS because it caused aggregation (2, 5). Under our conditions, *i.e.* with MS in K-phosphate, fractionation between 0 to 30% saturation effectively concentrated the enzyme from large-volume supernatants and provided 4-fold more purification (Table I). However, we also experienced a 40% decrease in yield. Attempts to improve the yield by fractionation at higher concentrations were possible (up to about 45% saturation), but the specific activity was significantly decreased and resulted in our inability in later steps to produce a homogeneous MS preparation. Desalting on G-25 prior to sieving on Sephacryl S-300 proved necessary for partially clarifying the sample and giving consistent resolution on the S-300 column. Rate-zonal centrifugation of the desalted sample in the same buffer indicated that the enzyme was not aggregated, but was mostly in the 20S form (not shown).

Molecular sieving always yielded two MS peaks (Fig. 1), the first one eluting with most of the protein and shown to be highly aggregated MS (Fig. 2, panel B), whereas the enzyme in the second peak had a sedimentation coefficient of approximately 20S (data not shown). Attempts at varying chromatography conditions to eliminate this aggregation, which apparently occurs during the molecular sieving, were not successful. Servettaz *et al.* (24) showed only one MS peak (with a sedimentation coefficient of 20S) after (NH₄)₂SO₄ fractionation and filtration on Sepharose 6B. Köller and Kindl (10) resolved one MS peak from Sepharose 6B columns equilibrated with Tris buffer containing magnesium and glyoxylate (referred to as an 'oligomer') and obtained one peak from another Sepharose column lacking magnesium and glyoxylate which yielded an active 70 kD monomer. Thus, conditions and species source can influence the degree of aggre-

Table I. Summary of Malate Synthase Purification from a Homogenate of Cotyledons (250 pairs) Excised from Etiolated (3-D) Cotton Seedlings

Procedure	Enzyme Units	Protein	Specific Activity	Yield	Purification
	µmol/min	mg	units/mg	%	-fold
Blended homogenate ^a	250	4600	0.05	100	1
Supernatant ^b (180,000g, 60 min)	200	1180	0.17	80	3
Ammonium sulfate (0–30% pellet)	95.3	147	0.65	38	13
Sephacryl S-300	31.9	11	2.90	13	58
DEAE sephacel ^c (flow through)	15.3	3.0	5.10	6.1	102
Phenyl sepharose ^d	6.4	0.8	8.00	2.6	160

^a Medium—100 mM K-phosphate, 8 mM MgCl₂, 2 mM EDTA, 1 mM PMSF (pH 7.2). ^b Added 2 mM glyoxylate to above medium; used this medium without EDTA in all subsequent steps. ^c Enzyme (15 µg protein) appears homogeneous on Coomassie-stained SDS gels. ^d Enzyme (4 µg protein) appears homogeneous on silver-stained SDS gels.

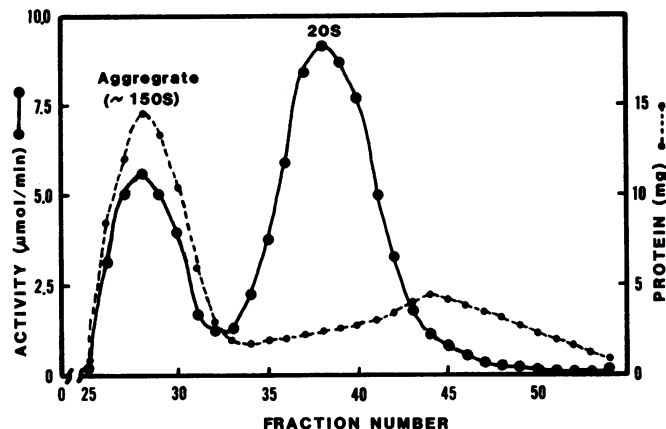


FIG. 1. Fractionation of malate synthase activity by gel filtration on a Sephacryl S-300 column. The column void volume was collected in the first 24 fractions. Enzyme in the first peak was shown to be an aggregate (Fig. 2). Fractions 35 through 40 were used for further purification.

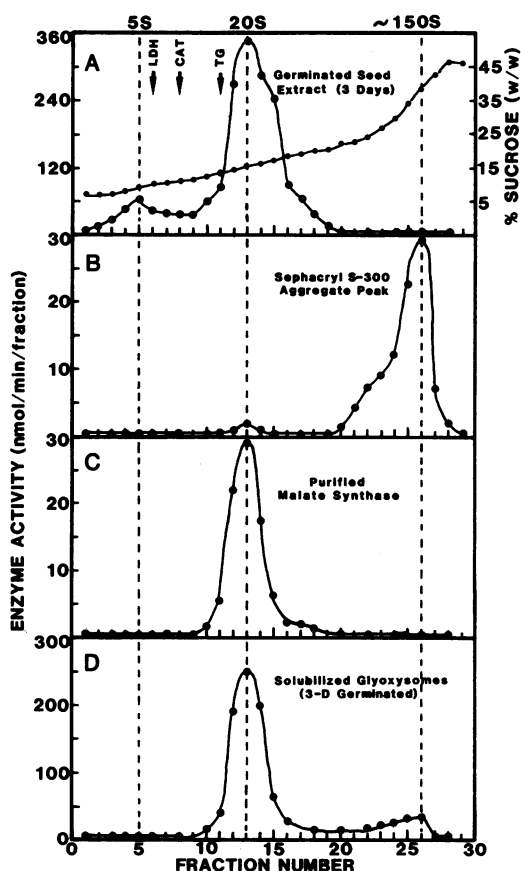


FIG. 2. Profiles of malate synthase activity following rate-zonal centrifugations on sucrose gradients (5–25% w/w) for 19 h (5°C) in a Sorvall AH-627 rotor at 24,000 rpm. Positions of standards used for verification of calculated S values and native mol wt determinations (in separate gradients or with purified malate synthase) are indicated by arrows. LDH-lactate dehydrogenase (136 kD); CAT-catalase (232 kD); TG-thyroglobulin (699 kD).

gation (or disaggregation) of MS, particularly during molecular sieving on columns. Selecting five to six of the highest specific activity fractions (Fig. 1) provided us with sufficient enzyme to proceed.

Ion-exchange chromatography was also employed by others. The maize enzyme was recovered as an elutant from a batch DEAE cellulose procedure (24), whereas the cucumber enzyme was chromatographed and eluted with salt gradients from the cation exchanger CM-Sephadex (10, 22). The flow-through fractions from the DEAE Sephacel column yielded a cotton enzyme preparation (about 3 mg) that was judged homogeneous on Coomassie-blue stained SDS-PAGE gels. The specific activity was about 5.1 μmol substrate/min·mg protein with about 100-fold purification from the original homogenate. This specific activity is comparable to those obtained for the purified castor bean enzyme, *i.e.* 2.6 (2) and 5.75 (5), yielding approximately 0.2 and 2.2 mg, respectively. Staining proteins on gels with silver, however, showed that our preparation was still contaminated, mostly with lower mol wt polypeptides. Although this enzyme preparation was acceptable for use as standards and so forth, it was not deemed suitable as antigen for production of potentially monospecific antibodies.

Phenyl sepharose chromatography was required to obtain our final enzyme preparation. Kruse and Kindl (12) employed this as a final step in the purification of 5S cytosolic MS from cucumber; the enzyme was eluted with an increasing gradient of ethylene glycol and decreasing gradient of KCl. We were able to elute the cotton enzyme with only an increasing ethylene glycol gradient. Elution occurred at approximately 40% ethylene glycol. The yield was reduced to about 0.8 mg with a specific activity of about 8 μmol substrate/min·mg protein. Values for the purified enzymes from corn and cucumber ranged from 15 to 25 μmol substrate/min·mg protein (10, 22, 24). Silver staining SDS-PAGE gels containing 3 or 4 μg protein per lane indicated the final preparation was not detectably contaminated with other proteins (Fig. 3). The purified enzyme preparations described by others were assessed for purity on Coomassie blue-stained gels.

Following rate-zonal centrifugation, the purified MS exhibited a sedimentation coefficient of about 20S (Fig. 2, panel C). This

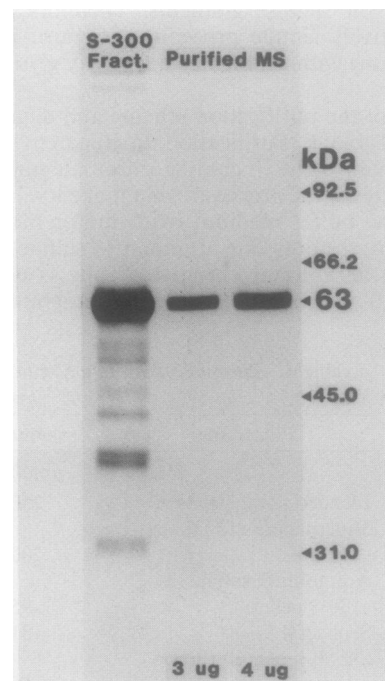


FIG. 3. Silver-stained gel (10%) after SDS-PAGE of pooled fractions (five) from the 20S malate synthase peak (Fig. 1) (15 μg) and the Phenyl Sepharose column (purified MS). With 4 μg protein applied, only a 63 kD subunit was observed in the final enzyme preparation used for raising antibodies in rabbits.

corresponded to the predominant form found in crude homogenates used as starting material (Fig. 2A) and to the MS released from glyoxysomes isolated previously on sucrose gradients (Fig. 2D). Notably, a small portion of 5S MS occurred in homogenates which did not appear in the purified preparation, and some aggregated MS occurred in solubilized glyoxysome samples that did not appear in the homogenate or purified MS. The medium for sample preparation and gradients was the same in all cases, allowing one to compare the occurrence of various forms of MS. The effects of varying media components on MS forms has been well documented by Kruse and Kindl (12).

The 5S form in homogenates likely was lost during $(\text{NH}_4)_2\text{SO}_4$ fractionation. In cucumber, the 5S monomeric form does not readily aggregate (12), hence this may be evidence that the cotton 5S form was not lost during the molecular sieving step. The occurrence of some aggregated MS in the glyoxysome fraction appeared to be a consequence of isolating glyoxysomes in sucrose gradients. Aggregates of MS were shown to be associated with ER fractions isolated from castor bean (7) or cucumber (12) when they were isolated in Tricine buffer. Cottonseed homogenates prepared and centrifuged in K phosphate buffer did not reveal MS associated with ER (see Ref. 28 for further discussion).

Values for the sedimentation coefficient and mol wt of purified MS determined by rate-zonal centrifugation are as variable as the number of studies reporting them. For cucumber MS (10), estimations of 18.6S and 540 kD were presented. Sedimentation and gel filtration behavior of the maize enzyme (24) yielded values of 20S and 500 kD. Using the same two criteria, Bowden and Lord (2) reported values of 21.6S and 575 kD for castor bean MS. Variations also appeared for determinations of subunit mol wt on SDS-PAGE gels; values for castor bean MS were 59 kD (5) and 64 kD (2), for cucumber MS they were 57 kD (22) and 63 kD (10). No data were given for the corn enzyme (24). Others (2, 24) concluded that MS was a multimeric enzyme of identical subunits, whereas Köller and Kindl (10) indicated that cucumber MS was an octamer of identical subunits. Our contribution to these data on oilseed MS are in agreement in terms of subunits, *i.e.* we observe a single subunit with an estimated mol wt of 63 kD (Figs. 2, 6, 7; Ref. 28), but believe the cotton enzyme exists in glyoxysome as a dodecamer. Previous work with the partially purified enzyme (20) indicated a native mol wt of 750 ± 8 kD from rate-zonal centrifugations and calculations of Martin and Ames (20). In those experiments, cotton MS migrated further than β -galactosidase (540 kD). Gel filtration on Bio-Gel A-15 indicated a mol wt of 730 kD (20) wherein MS also eluted ahead of β -galactosidase. Bowden and Lord (2) stated that the castor bean MS eluted ahead of ribulose-1,5-bisphosphate carboxylase (550 kD) on Sepharose 6B. In the present study, we included thyroglobulin (699 kD) as a standard in separate rate-zonal gradients and in gradients with purified MS. The position of thyroglobulin is depicted in Figure 2A, illustrating that cotton MS migrates further down the gradient under identical conditions. Our S values from numerous experiments (calculated from Tables in McEwen, 18) averaged near 21S, but because of variability in our data and those in the literature (see above), we rounded the value to indicate approximately 20S in the Figures. At the present time we believe that native cottonseed MS is a dodecamer with a mol wt of approximately 750 kD, consisting of 12 identical subunits, each with mol wt of approximately 63 kD.

Antibody Characterization. The fidelity and activity of the polyclonal antibodies raised to purified cottonseed MS in rabbits were first tested in immunotitration experiments (Fig. 4). Fifty microliters of antiserum precipitated essentially all of the MS in a ml of immature seed extract containing $0.4 \mu\text{mol}/\text{min}$ activity, whereas nearly 200 μl of antiserum was needed to remove the MS in 1 ml of germinated seed extract containing about 3-fold

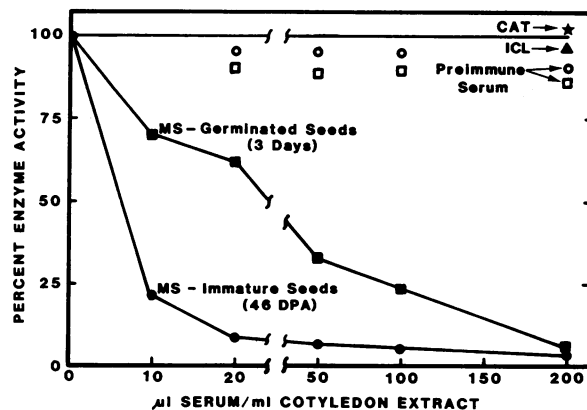


FIG. 4. Immunotitration of malate synthase in K-phosphate buffered ($-$ Triton X-100) cotyledon extracts of immature and germinated seeds containing 0.4 and $1.2 \mu\text{mol}/\text{min}$ activity per ml, respectively. Antimalate synthase serum proportionately precipitated malate synthase in the two extracts, but did not complex any catalase (CAT) or isocitrate lyase (ICL) during 16 h incubation at 4°C .

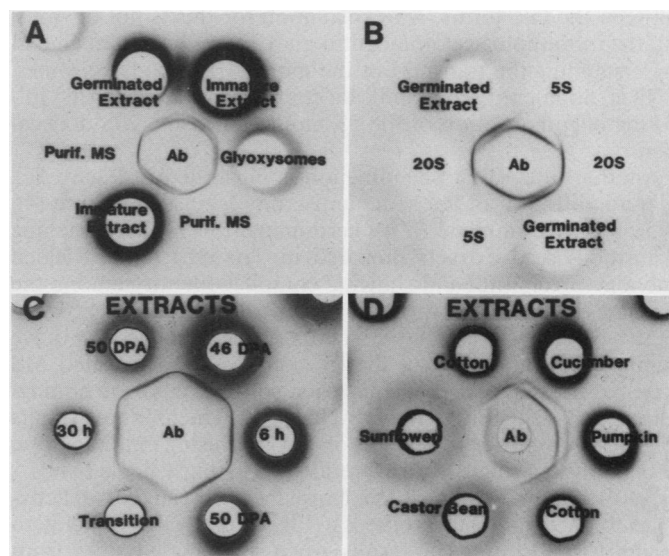


FIG. 5. Precipitin band formations in Ouchterlony double immunodiffusion plates with antimalate synthase serum (Ab) and various samples. Optimal band formations were achieved with $10 \mu\text{l}$ of 1:8 dilutions of antiserum (center wells) and varying amounts of each sample (outer wells) having 25 to 35 nmol/min activity. A, Extracts prepared with 100 mM K-phosphate ($+$ Triton X-100) and glyoxysomes isolated on sucrose gradients. B, Extracts as in A, 5S and 20S enzyme from rate-zonal gradients such as Figure 2A. C, Extracts prepared with 100 mM Tris-HCl buffer ($+$ Triton X-100). D, Extracts prepared with 100 mM K-phosphate ($+$ Triton X-100).

more units. Catalase and isocitrate lyase activities were unaffected by addition of MS antibodies. The results with catalase were especially significant because it was difficult to purify MS free of catalase contaminants in other studies (10, 24).

Another common test for evaluating the quality of antibodies raised to a particular protein is Ouchterlony double immunodiffusion. If the antibodies were monospecific, one would expect single precipitin bands and the absence of spurs between the purified enzyme and extracts from which it was derived. As illustrated in Figure 5A, two precipitin bands were clearly evident between the antiserum (or IgG preparations from this serum) and extracts prepared in K phosphate. In contrast, a single band was apparent with glyoxysome extracts and purified MS in the

outer wells. An obvious interpretation is that a non-MS contaminant is being recognized in the extracts that is absent or perhaps has a low titer in glyoxysomes and/or the purified preparation. The absence of spurs, however, indicated complete serological identity. Close inspection of Figure 5A shows that the purified and glyoxysomal bands are completely fused with the outer bands in the extracts. This leads one to suspect that different forms of MS, rather than a contaminant, are responsible for the bands in extracts. The results shown in Figure 5B confirmed this suspicion. Germinated-seed extracts again revealed two bands, the outer one being contiguous and fused with 20S MS, and the inner one contiguous and fused with 5S MS. The data in Figure 5, A and B, therefore, demonstrate that the antibodies can recognize the 5S and 20S forms as separate entities in the immunodiffusion plates when the samples are prepared in K-phosphate. This confirmed results presented in Figure 2; 5S and 20S forms occurred in seed extracts whereas only the 20S form occurred in solubilized glyoxysomes and the purified preparation. The data in Figure 5A indicated serological identity between the 5S form in immature and germinated seeds, and the 20S form in immature and germinated seeds. However, Figure 5B reveals spurs between the 5S and 20S forms, indicating incomplete identity between the two forms. An explanation for this is not obvious; but the immunological nonidentity may be an important clue in understanding the transport of the monomer into glyoxysomes. That is, nonidentity may indicate conformational change(s) following oligomerization of the 5S monomer to the 20S dodecamer.

Another interesting phenomenon is illustrated in Figure 5C. Immunodiffusion of the same antiserum against immature (46 and 50 DPA), imbibed (6 h), germinated (30 h), and greening (transition) seed extracts prepared in Tris-HCl buffer yielded only one precipitin band showing complete identity among the samples. We are uncertain of the reason for the disparity between Figure 5, A and C, but the results are repeatable. We have unpublished data showing that 5S and highly aggregated MS occurred in rate-zonal gradients when cotton seeds were homogenized and centrifuged in Tris-HCl. The Ouchterlony results (Fig. 5C) may be due to only the 5S form diffusing in the agarose, or perhaps to a single large aggregate producing a single band.

Antibodies to cottonseed MS showed widespread cross-reactivity with MS from other oilseeds (Fig. 5D). It was difficult to produce uniformly clear results when applying samples from different species with varying enzyme units per ml, but precipitin bands were observed with all species shown in Figure 5D and with watermelon (not shown) and soybean (25). Soybean and cottonseed extracts were made in Tris-HCl containing 0.5 M NaCl (25); under these conditions, a single precipitin band occurred between cotton anti-MS and the two extracts. In addition, no spurs were observed between the soybean and the cotton extracts. The results in Figure 5D were complicated by the appearance of multiple bands and spurs. Complete identity was observed between the two MS forms in the cucurbits (cucumber and pumpkin). Partial identity was apparent with both these forms and cotton (see spurs). The sunflower and castor bean extracts exhibited only one inner band. This suggested that only a 5S form of MS was extracted with the K phosphate. This interpretation was confirmed when rate-zonal centrifugation of the castor bean extract (similar to that used for Fig. 5D) revealed only a 5S form on the gradient (data not shown). However, when castor beans were extracted with K phosphate containing 0.2 M KCl, two bands showing partial identities with both bands from cotton extracts were observed (data not shown). Similar studies with the sunflower were not done.

Immunological variations described above for MS have not been reported by any others. Dommies and Northcote (5) showed one immunodiffusion band between their antiserum and purified

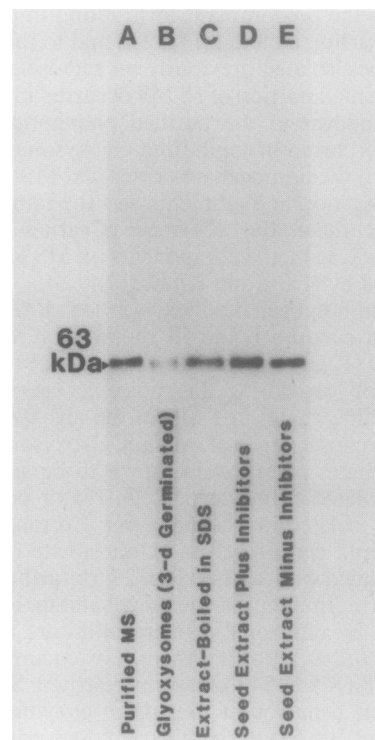


FIG. 6. Western blot stained for malate synthase following transfer from a SDS-PAGE gel (4% stacking, 8% separating, 17 cm long), demonstrating monospecificity of the antiserum and comparing subunit mol wt of purified malate synthase with enzyme extracted under varied conditions. Each lane had 3 to 5 nmol/min MS activity except C which had 100 μ g protein. Inhibitors included in lane D sample were 1 mM benzamidine-HCl and PMSF, 5 mM EDTA and iodoacetamide, and 2 μ g/ml aprotinin.

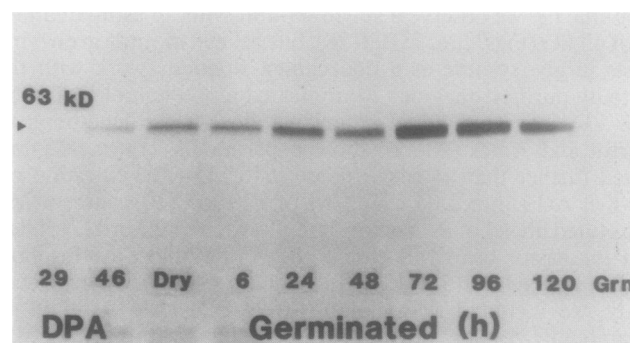


FIG. 7. Western blot prepared as in Figure 6 comparing subunit mol wt and the relative amount of malate synthase in maturing (DPA), dry, germinated and green seeds. Each lane contained 25 μ g protein of extracts prepared with 100 mM K-phosphate, 8 mM $MgCl_2$, 2 mM EDTA (pH 7.2), and 1 mM PMSF and benzamidine-HCl.

MS. Köller and Kindl (11) stated in their "Methods" section that Ouchterlony plates were used to show that they were dealing with monovalent serum, but conditions or results were not presented. Riezman *et al* (22) showed an immunoelectrophoretogram with two joining arcs which indicated complete identity between a basic and acidic form. Bowden and Lord (2) stated that single precipitin bands formed between their antiserum and KCl extracts of glyoxysomes and purified MS; both these sources were shown to contain only a 30S form of MS. Thus, it is not that the interesting phenomena reported here are inconsistent with previous results, but that the same experiments under these

conditions have not been performed, *i.e.* data are not presented for double immunodiffusion between anti-MS serum and crude extracts except for soybean (25).

Another procedure for assessment of antiserum monospecificity is western blot analyses (Fig. 6). Peptides from cotyledon and glyoxysome extracts and purified MS were electrotransferred from SDS-PAGE gels to nitrocellulose, then probed with anti-MS antiserum followed by anti-rabbit IgGs conjugated to alkaline phosphatase. Cotyledon extracts were treated in several manners to curtail or prevent potential proteolysis prior to gel application. In all cases, only a single band in the 63 kD region appeared on the blots (Fig. 6), demonstrating monospecificity of the antiserum to solubilized glyoxysomes and cotyledon extracts. These data also indicated that the subunit mol wt of the purified enzyme was not detectably altered during the purification procedures. Verification of this is given in the accompanying paper where *in vivo* and *in vitro* translated products are compared with the purified enzyme (28).

Results of our immunotitration experiments (Fig. 4) demonstrated that the antiserum was specific for MS in both immature and germinated seeds. We used the Western blot procedure to test for the occurrence of possible subunit mol wt variations, and for the relative amount of MS at various stages of seed maturation (29–48 DPA), desiccation (>54 DPA), germination (0–12 h), heterotrophic seedling growth (12–120 h in the dark), and phototrophic growth (10 d in the glasshouse) (Fig. 7). The blot, with the same amount of protein per lane, showed that subunit mol wt of MS was indistinguishable at the various stages, and that MS increased in relative amount to about 72 h postimbibition before declining. These latter results paralleled previous results where the relative activity of MS was measured at different stages in cotyledon extracts (19). Activity was not detectable until about 42 DPA; the blot indicated that inactive enzyme was not present in immature seeds prior to that time (at 29 DPA). Of considerable interest, however, was the finding that MS can be translated *in vitro* from poly(A)+RNA extracted from immature seeds at 28 DPA (28). Peak activity of MS in dark-grown seeds occurred at about 3 d, then declined slowly. MS activity was not detectable in photoautotrophic glasshouse-grown seeds, nor was the protein detectable on Western blots. This may indicate that the enzyme is somehow selectively removed from glyoxysomes as they are converted to leaf-type peroxisomes in the light. This hypothesis is tenable if one adheres to the concept of the 'one-population' model proposed nearly 15 years ago (see Ref. 8 for review and discussion).

The relationships shown in Figure 7 between MS in immature and germinated seeds had not been demonstrated before with any oilseeds, but can be done relatively easily on western blots with monospecific antibodies. With such information, questions arise relative to the biogenesis of glyoxysomes and acquisition of their enzymes during seed maturation and postgerminative growth. As part of our continuing efforts to understand and fully appreciate this aspect of plant physiology, two of us (28) have examined the biogenesis of MS in cottonseeds, exploiting some of the potential uses of the antibodies prepared and reported on in this paper.

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