Plant Physiol. (1985) 79, 489–493
0032-0889/85/79/0489/05/$01.00/0

Purification and Properties of Glyoxysomal Lipase from Castor Bean[1]

Received for publication April 8, 1985 and in revised form June 6, 1985

MASAYOSHI MAESHIMA[2] AND HARRY BEEVERS*
Biology Department, University of California, Santa Cruz, California 95064

ABSTRACT

The alkaline lipase in the glyoxysomes from the endosperm of young castor bean seedlings, an integral membrane component, was solubilized in deoxycholate:KCl and purified to apparent homogeneity. The molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 62,000 daltons. The enzyme reaction was markedly stimulated by salts and inhibited by detergents. Triricinolein, the endogenous storage lipid, was hydrolyzed by the purified enzyme which is therefore a true lipase. Treatment of intact glyoxysomes with trypsin strongly diminished the lipase activity but did not affect matrix enzymes. An antibody preparation raised in a rabbit against the purified enzyme inhibited the purified enzyme and that in glyoxysomal membranes.

During seed ripening, storage, and early growth of the castor bean, the acid lipase of the spherosome membrane is present in high activity (13). This enzyme described by Ory (16) has been intensively studied (2, 7, 13, 14) and is generally thought to be the enzyme that initiates the breakdown of the stored lipid (principally triricinolein) during its conversion to sucrose in the endosperm of the young growing seedling. A second acyl hydrolase, the alkaline lipase, appears in the endosperm after germination has begun and is the only known enzymically active integral membrane protein in the glyoxysomes. Muto and Beevers (14) described some properties of this enzyme in isolated glyoxysomal membranes and concluded from a limited investigation with palmitoyl glycerol esters that it hydrolyzed only monoacylglycerols. More recently, Lin *et al.* (10) have shown that alkaline lipases in seeds frequently show higher hydrolytic activity on their native triacylglycerol substrates with unsaturated acyl constituents than on those with saturated acyl substituents. In this investigation, we have identified the alkaline lipase as a major constituent of glyoxysomal membrane proteins on gels, purified it, investigated its properties, and prepared an antibody against it. As suggested by Lin *et al.* (10), the purified enzyme does hydrolyze the native triricinolein and thus is a true lipase (5).

MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* L. cv Hale) weighing 375 mg ± 5% were selected and soaked in running tap water (20°C) for 16 h. The end of this imbibition period was designated d 0 of germination. Germination was carried out in moist vermiculite in darkness at 30°C. Endosperm tissue was carefully removed and the rest of the seedling was discarded.

Preparation of Glyoxysomal Membrane. Two hundred g of endosperm from 4-d seedlings (*Ricinus communis* L. cv Hale) were chopped with a razor blade and then ground in a mortar in a total of 400 ml of grinding medium containing 0.4 M sucrose, 10 mM KCl, 1 mM EDTA, 10 mM DTT, 1 mM $MgCl_2$, and 165 mM Tricine-NaOH buffer (pH 7.5). The homogenate was filtered through a layer of nylon cloth, and centrifuged at 270*g* for 10 min. The supernatant was fractionated into the fat layer, supernatant, and particulate fractions by centrifugation at 10,800*g* for 30 min. The crude particulate pellet was suspended in 40 ml of grinding medium, and layered on linear gradients of 30 to 60% (w/w) sucrose. All sucrose solutions contained 1 mM EDTA (pH 7.5). After centrifugation for 2 h at 25,000 rpm in a Beckman SW 27 rotor, the glyoxysomal fraction was collected in 51% sucrose and diluted with 2 volumes of 1 M KCl containing 1 mM EDTA to extract the soluble glyoxysomal proteins. After standing for 30 min with stirring, the diluted glyoxysomal fraction was centrifuged for 1 h at 100,000*g*. The pellet was suspended in 10 mM Tricine-NaOH buffer (pH 7.5) containing 10% (w/v) sucrose and 5 mM DTT and then used as the crude glyoxysomal membrane fraction (33 ml). The supernatant was the KCl extract.

Purification of Alkaline Lipase from Glyoxysomal Membrane. All steps were performed at 0 to 4°C.

Step 1—Deoxycholate Fractionation. To the suspension of crude glyoxysomal membrane (3.0 mg/ml) were added solid KCl and 1% sodium deoxycholate to a final concentration of 0.1 M and 0.2 mg/mg of protein (0.06%), respectively, and the suspension was centrifuged at 100,000*g* for 1 h. The resulting yellow green supernatant was designated as supernatant I. The pellet was suspended in 10 mM Tricine-NaOH buffer (pH 7.5) containing 10% sucrose, 5 mM DTT to give a volume of 16.5 ml. Solid KCl and 1% sodium deoxycholate were added to give final concentrations of 0.5 M and 0.12%, respectively. After standing for 20 min at 4°C with stirring, the suspension was centrifuged at 100,000*g* for 1 h. The resulting supernatant (slightly yellow) was the DOC[3] extract.

Step 2—Polyethyleneglycol Fractionation. Polyethyleneglycol 6000 was added to the DOC extract to give a final concentration of 3% w/v. The precipitate was removed by centrifugation at 20,000*g* for 10 min, and to the supernatant was added PEG 6000 to give a final concentration of 15%. The mixture was centrifuged at 20,000*g* for 10 min and the pellet was suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 1 mM DTT, and 0.05% Triton X-100 (Tris/GDT buffer). The suspension was recentrifuged to remove undissolved proteins.

Step 3—CM-Cellulose Column Chromatography. Ten ml of

[1] Supported by grant PCM 84 03542 from the National Science Foundation.

[2] Present address: Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan.

[3] Abbreviations: DOC, deoxycholate; PB, phosphate buffer; IgG, immunoglobulin G; CM-cellulose, carboxymethyl cellulose.

the supernatant from step 2 was applied to a column (1.2 × 9 cm) of CM-cellulose (CM-52, Whatman) preequilibrated with Tris/GDT buffer. The lipase was eluted with the same buffer (pass-through fraction).

Step 4—Hydroxylapatite Column Chromatography. The eluate from step 3 was applied to a hydroxylapatite column (1.0 × 8 cm) preequilibrated with 50 mM K-phosphate (pH 8.0) containing 10% glycerol, 1 mM DTT, and 0.05% Triton X-100 (50 mM PB/GDT) and washed with 8 ml of 50 mM PB/GDT and 8 ml of 100 mM PB/GDT. Alkaline lipase was eluted from the column by 200 mM PB/GDT, and the peak fractions were pooled as purified glyoxysomal lipase.

Preparation of Antiglyoxysomal Lipase IgG. Antibody against the purified lipase was raised in a rabbit. The antigen solution containing 200 to 300 μg of purified enzyme was homogenized in an equal volume of Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the later injections. The homogenate was injected into a rabbit once a week for 5 consecutive weeks. The IgG fraction was purified from antiserum (105 ml) by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose (DE-52, Whatman) column chromatography. For the ion exchange step, 70 mM sodium phosphate buffer (pH 6.3) was used, and the early flow-through fraction was used for immunochemical analysis.

Immunochemical Analysis. Double immunodiffusion tests were carried out as described by Ouchterlony and Nilsson (17). The gel plate was prepared with 1% (w/v) agarose in PBS containing 0.5% Triton X-100 and 0.02% NaN_3. Each well (5 mm in diameter, 2 mm in depth) was filled with 30 μl of sample. After incubation at room temperature for 2 d, the plate was washed with PBS containing 0.5% Triton X-100 and 0.02% NaN_3 and stained with Coomassie brilliant blue R.

For immunotitration, glyoxysomal membrane (240 μg) and deoxycholate extract (79 μg) were mixed with antiglyoxysomal lipase IgG (2.6 mg/ml). After incubation at 25°C for 30 min, the lipase activity of the mixtures was measured. Nonimmune serum had no effect on lipase activity.

Enzyme Assay. Two different assays for lipase were used. The fluorometric method of Guilbault and Hieserman (6) was used with minor modifications in the greater part of these studies. Fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer set at 430 nm for excitation and 500 nm for emission. The measurements were carried out at 25°C. The reaction mixture used routinely contained 80 mM glycine-NaOH buffer (pH 9.0), 5 mM DTT, 0.83 mM *N*-methylindoxylmyristate in ethylene glycol monomethyl ether and enzyme, in 1.0 ml. For assays of the solubilized enzyme K-acetate was added to the reaction mixture to give a final concentration of 20 to 30 mM.

A colorimetric method (9, 15) was used when the various glycerol esters were substrates. The free fatty acids released were measured as copper soaps, using 1,5-diphenylcarbazide. Stearic acid was used as the standard. The *A* at 550 nm was read with a Gilford 250 spectrophotometer. The 1-ml reaction mixture contained 80 mM glycine-NaOH (pH 9.0), 5 mM DTT, 1 (triacylglycerol), or 2 mM (monoacylglycerol) substrate, and enzyme preparation. The reaction, at 30°C, was started by addition of enzyme and stopped by addition of 6 ml of chloroform-*n*-heptane-methanol (4:3:2, v/v) solution. Emulsions of the substrate (4 or 8 mM) were prepared daily. The substrates were emulsified in 5% gum acacia and 0.025% β-D-octylglucoside for 1 min at 20 W in an Ultra Tip Model WW20 ultrasonic generator.

N-Methylindoxylmyristate was obtained from ICN Pharmaceuticals, and triolein and tripalmitin from Nu-Chek Laboratories. Triricinolein was isolated from castor oil on thin-layer plates (Silica Gel 60) obtained from E. Merk.

PAGE. Gel electrophoresis on 11% polyacrylamide gel containing 0.1% SDS was carried out by the method of Laemmli (8). Samples were dissociated in 2% SDS and 2% 2-mercaptoethanol at 70°C for 20 min. After electrophoresis the gel was stained with Coomassie brilliant blue R. BSA (67,000), beef liver catalase (60,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and Cyt *c* (12,500) were used as mol wt markers.

Estimation of Protein. Protein was determined after precipitation with trichloroacetic acid by the method of Lowry *et al.* (12) with BSA as the standard. SDS was added at a final concentration of 0.5% to prevent interference by Triton X-100 (4).

RESULTS

Purification of the Alkaline Lipase. The procedure outlined in "Materials and Methods" for the purification from glyoxysomes (Table I) was developed from preliminary trials in which the chromatographic properties and conditions for stability of the enzyme were established. Glyoxysomes isolated on sucrose density gradients were diluted in 1 M KCl and pelleted to give a crude membrane fraction. Treatment with 0.06% deoxycholate in 0.1 M KCl removed much of the protein but the lipase was retained. On adding 0.5 M KCl in 0.12% deoxycholate the lipase was solubilized. In the subsequent steps of PEG fractionation, CM-cellulose and hydroxylapatite chromatography, glycerol (10%) and Triton X-100 were present throughout to maintain solubility and activity. As shown in Table I and Figure 1, the procedure yielded a highly purified preparation in which only one band was visible after SDS-PAGE. The mol wt, by comparison with standards (Fig. 1) was 62,000 D, and this band can be recognized as a major component (No. 5) of the crude membrane preparation (Fig. 1A). Under nondenaturing conditions the lipase migrated very slowly as a broad band in PAGE and we were unable to assign a precise mol wt. The lipase (monomer) band at 62,000 D on SDS-PAGE gave no staining for glycoprotein using the thymol sulfuric acid method of Racusen (18).

Properties of the Purified Lipase. The pH optimum for the enzyme reaction was pH 9.0 and all measurements were made at this pH. After solubilization, as shown in Table II the reaction was markedly stimulated by Na^+ and K^+ salts and 30 mM

Table I. *Purification of Glyoxysomal Lipase*

Purification Step	Protein	Activity	Yield	Specific Activity	Purification
	mg	*nmol/min·mg*	*%*	*nmol/min·mg*	*–fold*
Glyoxysomal membrane[a]	99.0	3020	100	30.5	1
Deoxycholate extract	21.5	1980	66	92.1	3.0
PEG 6000 3–15% fraction	7.45	2000	66	268	8.8
CM-cellulose	6.27	1780	58	284	9.3
Hydroxylapatite	0.739	244	8.1	330	10.8

[a] The crude membrane fraction was prepared from glyoxysomes isolated from 200 g of endosperm from 4-d seedlings and contained about 44% of the total glyoxysome protein.

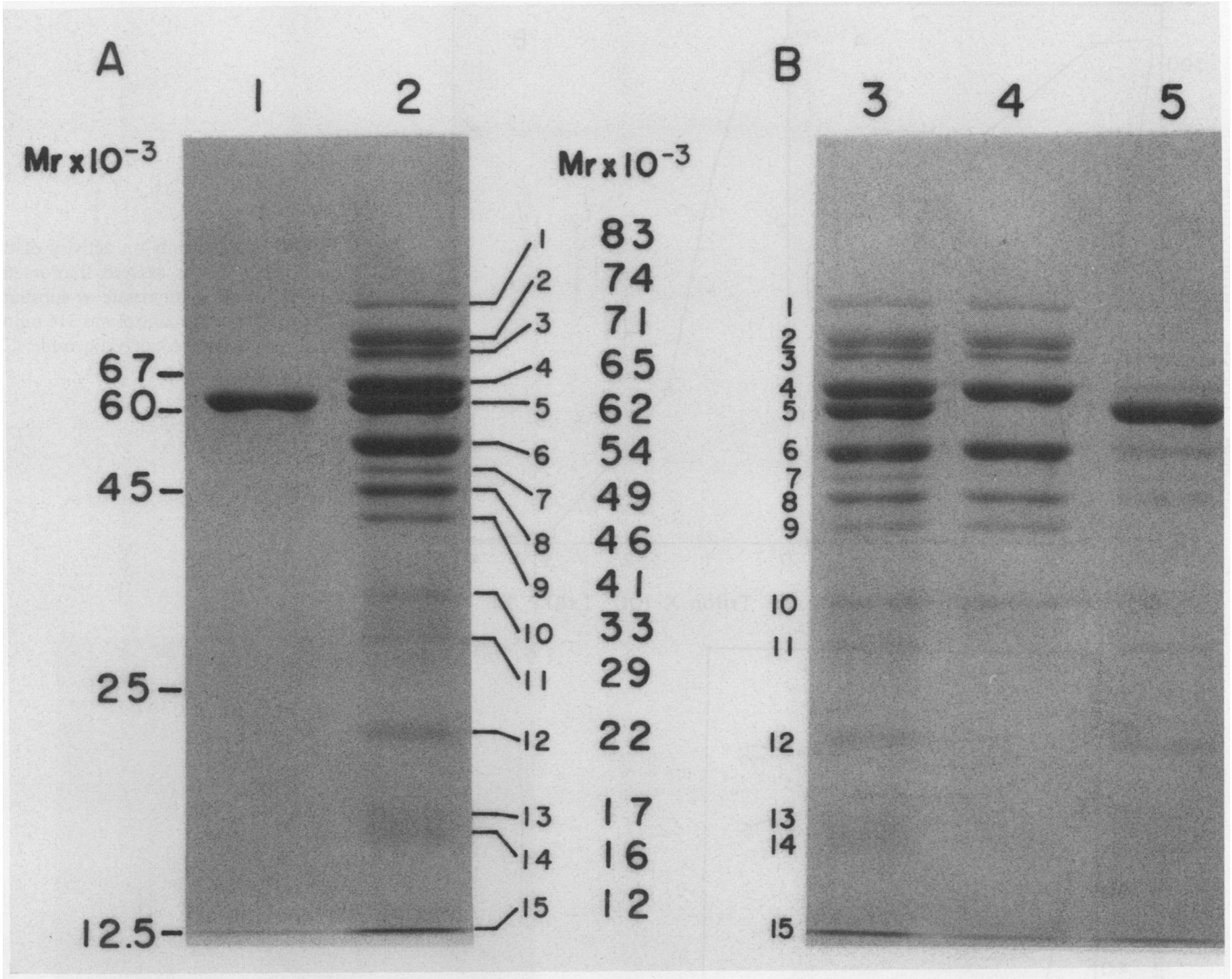

FIG. 1. SDS-PAGE of purified glyoxysomal lipase (A) and fractions of glyoxysomal membrane solubilized by deoxycholate and KCl (B). Lane 1, purified lipase (9 μg); lanes 2 and 3, crude glyoxysomal membrane fraction (60 μg); lane 4, supernatant I (36 μg); lane 5, deoxycholate extract (20 μg).

Table II. *Effects of Salts on the Activity of Glyoxysomal Lipase (Purified through the CM-Cellulose Step)*

Salt	Concentration	Activity
	mM	*nmol/min·ml*
None		104
CH_3COOK	10	177
	20	250
	30	316
KCl	10	158
	30	300
CH_3COONa	10	131
	30	295
NaCl	10	150
	30	302

potassium acetate was included in the assays when purified enzyme was used. A similar stimulation by salts was observed for a rapeseed lipase by Rosnitschek and Theimer (19) but the lipase purified from lipid bodies of corn scutellum apparently did not require salts (11). In our initial work with the enzyme in crude glyoxysomal membrane preparations we reported that

Table III. *Hydrolysis of Various Substrates by the Glyoxysomal Lipase*

Experiment	Substrate	pH of Reaction Mixture	Fatty Acid Produced	
			Glyoxysomal membrane	Purified lipase
			nmol/min·mg protein	
1	Tripalmitin	9.0	4.5	5.2
	Triolein	9.0	5.2	15.5
	Triricinolein	9.0	5.1	18.6
2	Triricinolein	9.0	7.2	29.0
	Triricinolein	7.0	2.3	15.0

triacylglycerols were not hydrolyzed (14). Prompted by the observation of Huang (10) that lipases from other sources showed high activities on triacylglycerols containing unsaturated fatty acids, their natural substrates *in vivo*, we examined the substrate specificity of the membrane bound and purified lipase using the sensitive method of Nixon and Chan (15) to measure release of fatty acids. As shown in Table III triricinolein, the endogenous reserve lipid in the castor bean, was hydrolyzed. In the purified lipase preparation, this rate was considerably higher than that for two triglycerides containing oleic and palmitic acids as substitu-

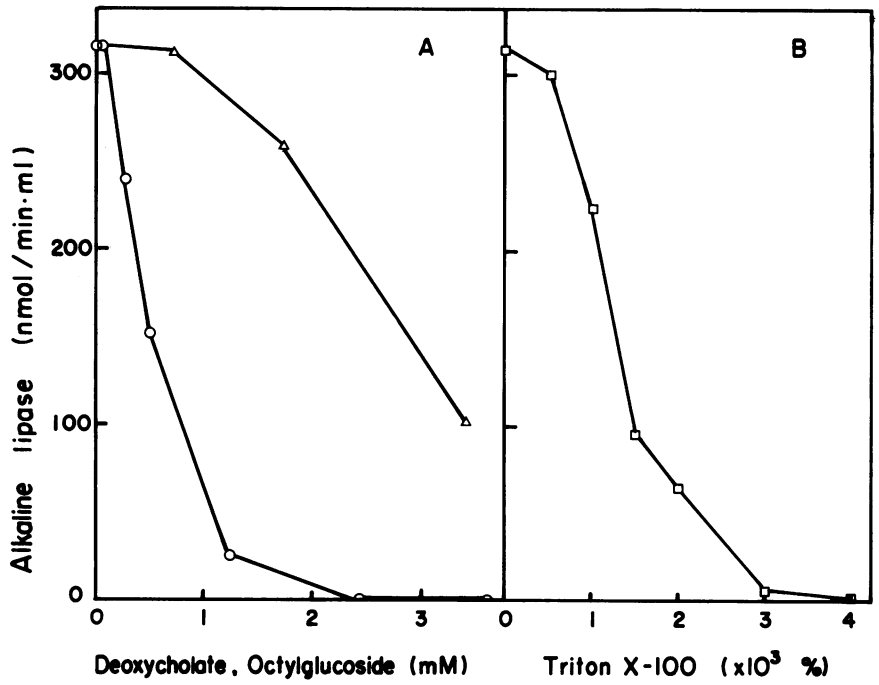

FIG. 2. Effects of detergents on activity of the purified lipase. Activity was assayed fluorometrically, with *N*-methylindoxylmyristate as substrate, at pH 9.0. The activity of the control was 316 nmol/min·ml. (○), deoxycholate; (△), octylglucoside; (□), Triton X-100.

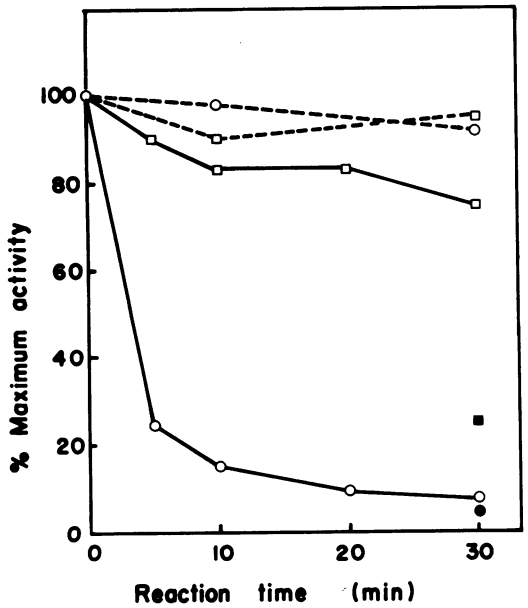

FIG. 3. Effect of trypsin on lipase and isocitrate lyase activities in intact glyoxysomes. Samples of glyoxysomes (230 μg protein) in 20 mM Tris-HCl (pH 8.0) containing 0.5 M sucrose and 1 mM $CaCl_2$ were incubated at 25°C. One-half of the samples received trypsin (1 μg) in addition. At the indicated intervals, trypsin inhibitor (10 μg) was added to the samples containing trypsin and isocitrate lyase (□) and lipase (○) measured in these (——) and in control samples (– – –). The closed symbols show the isocitrate lyase (■) and lipase (●) activities observed in a parallel reference experiment in which glyoxysomes broken osmotically (by dilution with 3 volumes of buffer not containing sucrose) were exposed to trypsin for 30 min. The maximum (0 time) activities were 128 nmol/min·ml for isocitrate lyase and 61.9 nmol/min ml for lipase.

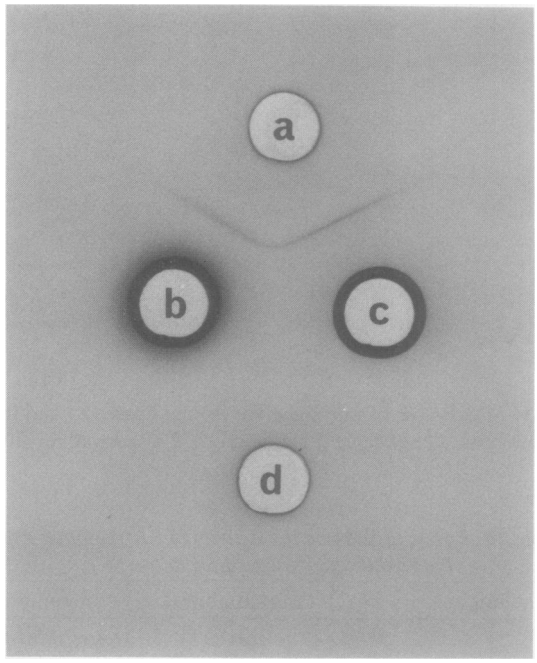

FIG. 4. Double immunodiffusion test. The gel plate was prepared with 1% agarose in PBS containing 0.5% Triton X-100 and 0.02% NaN_3. Well a, antiglyoxysomal lipase IgG (2 μg); well b, glyoxysomal membrane fraction treated with 1% Triton X-100 (33 μg); well c, purified lipase (20 μg); well d, nonimmune serum (60 μg).

ents.

The effects of detergents on enzyme activity of the purified lipase are shown in Figure 2. Deoxycholate, octylglucoside, and Triton X-100 all inhibited the reaction, the concentrations required for 50% inhibition were 0.019, 0.082, and 0.0012%, respectively. It should be noted that the inhibitions by deoxycholate and Triton X-100 are reversible, since both of these reagents were used during the purification procedure.

Evidence that the lipase is on the exterior of the glyoxysomal membrane was obtained in the following experiment. When purified glyoxysomes were disrupted by osmotic shock, the activities of isocitrate lyase, a matrix component, and the lipase, an integral membrane protein, were both strongly reduced by exposure to trypsin (Fig. 3). However, when intact glyoxysomes in 0.5 M sucrose were incubated in trypsin, the lipase activity was rapidly inhibited, while the isocitrate lyase was scarcely affected. Thus, it appears that if the native substrate triricinolein had

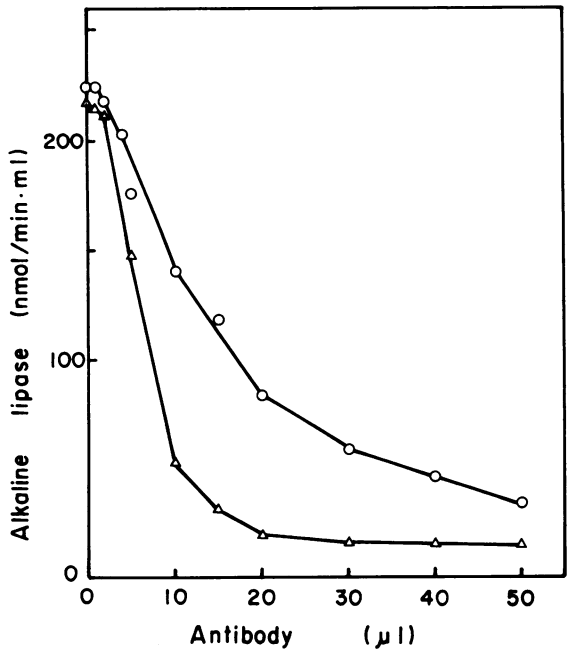

FIG. 5. Effect of antiglyoxysomal lipase IgG on lipase activity in glyoxysomal membrane fraction and deoxycholate extract. Various amounts of antiglyoxysomal lipase IgG (2.6 mg/ml) were added to glyoxysomal membrane fraction (240 μg) and deoxycholate extract (79 μg), and then the mixtures were incubated at 25°C for 30 min. The lipase activity was measured fluorometrically. (○), Glyoxysomal membrane; (△), deoxycholate extract.

access to the glyoxysome, it would be hydrolyzed on the exterior surface. The glyoxysomes contain the enzyme that converts fatty acids to their CoA derivatives (3) and all of the enzymes of oxidation and the glyoxylate cycle required to convert these to succinate (1, 2).

Antibody Experiments. Antibody to the purified glyoxysomal lipase was raised in a rabbit and partially purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography as described in "Materials and Methods." As shown by the double immunodiffusion test in Figure 4, a single precipitin line was produced when the antiglyoxysomal lipase IgG was incubated with either glyoxysomal membrane or the purified lipase and these lines fused completely. No precipitin was produced from nonimmune serum. Incubation of solubilized lipase (DOC extract) with increasing amounts of antibody led to almost total loss of enzyme activity (Fig. 5), and the lipase in the crude membrane fraction of glyoxysomes responded similarly, although roughly twice as much antibody was required for 50% inhibition.

DISCUSSION

Previous attempts to solubilize and purify the glyoxysomal lipase were unsuccessful (14). The purification described here depends on the selective solubilization of proteins of the crude glyoxysomal membrane preparation in deoxycholate KCl, and the inclusion of glycerol and Triton X-100 during the steps of polyethyleneglycol fractionation, CM-cellulose, and hydroxylapatite chromatography. In SDS-PAGE, a single band of mol wt 62,000 D was obtained which corresponded to a major constituent of the crude membrane fraction (Fig. 1). Tests for glycosylation were negative. Lipase activity in intact glyoxysomes was accessible to digestion by exogenous trypsin while isocitrate lyase, a known matrix component, was not. Antibody to the purified lipase gave a single band in double diffusion Ouchterlony tests with crude and purified enzyme and strongly inhibited enzyme activity.

In previous discussions of the possible physiological role of the glyoxysomal lipase, a primary participation in lipid hydrolysis was thought to be unlikely because apparently only monoacylglycerols were hydrolyzed (2, 14). However, as now shown (Table III), the native triacylglycerol with the unsaturated ricinoleic acid as substituent is hydrolyzed, as suggested from work on other seeds by Huang's group (7, 9, 10). The enzyme is a true lipase and from the trypsin experiment (Fig. 3) is accessible to native substrate on the outer surface of the glyoxysome. Thus, one obstacle against the possible role of the glyoxysomal lipase as the primary one in lipid hydrolysis is removed. As in other seeds (7, 9, 10, 19), this capacity of lipase associated with the glyoxysomes develops during early growth and coincides with the onset of fat breakdown. As Huang (7) has emphasized, the lipase in the membrane of the spherosomes of castor bean is not a common feature among fatty seedlings and yet it seems inevitable from its location that it would be responsible for hydrolysis. Nevertheless, it is clear that this enzyme is introduced into the spherosomes in parallel with fat deposition during fruit development, and the activity of the extracted enzyme is highest during the earliest period of seedling growth before any fat breakdown occurs *in vivo* (13, 14). Thus, if the spherosomal lipase has a role in hydrolysis, it is necessary to explain why it is totally prevented from functioning during ripening and in the first days of seedling growth before the organelle and enzyme machinery, which are necessary for the complete conversion of fat to sucrose, are assembled (2).

LITERATURE CITED

1. BEEVERS H 1982 Glyoxysomes in higher plants. Ann NY Acad Sci 386: 243–253
2. BEEVERS H 1982 Fat metabolism in seeds: role of organelles. *In* JFGM Wintermans, PJC Kuiper, eds, Biochemistry and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 223–235
3. COOPER TG 1971 The activation of fatty acids in castor bean endosperm. J Biol Chem 246: 3451–3455
4. DULLEY JR, PA GRIEVE 1975 A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal Biochem 64: 136–141
5. GALLIARD T 1980 Degradation of acyl lipids: hydrolytic and oxidative enzymes. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 4. Academic Press, New York, pp 85–116
6. GUILBAULT GG, J HIESERMAN 1969 Fluorometric substrate for sulfatase and lipase. Anal Chem 41: 2006–2009
7. HUANG AHC 1984 Plant lipases. *In* Brockman HL, B Borgstrom, eds, Lipase. Elsevier Press, Amsterdam, pp 419–442
8. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 222: 680–685
9. LIN YH, AHC HUANG 1983 Lipase in lipid bodies of cotyledons of rape and mustard seedlings. Arch Biochem Biophys 255: 360–369
10. LIN YH, RA MOREAU, AHC HUANG 1982 Involvement of glyoxysomal lipase in the hydrolysis of storage triacylglycerols in the cotyledons of soybean seedlings. Plant Physiol 70: 108–112
11. LIN YH, AHC HUANG 1984 Purification and initial characterization of lipase from the scutella of corn seedlings. Plant Physiol 76: 719–722
12. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
13. MOREAU RA, KDF LIU, AHC HUANG 1980 Spherosomes of castor bean endosperm. Plant Physiol 65: 1176–1180
14. MUTO S, H BEEVERS 1974 Lipase activities in castor bean endosperm during germination. Plant Physiol 54: 23–28
15. NIXON M, SHP CHAN 1979 A simple and sensitive colorimetric method for the determination of long-chain free fatty acids in subcellular organelles. Anal Biochem 97: 403–409
16. ORY RL 1969 Acid lipase of castor bean. Lipids 4: 177–185
17. OUCHTERLONY G, LA NILSSON 1973 Immunodiffusion and immunoelectrophoresis. *In* DM Weir, ed, Handbook of Experimental Immunology. Blackwell Scientific Publications, Oxford, pp 19.1–19.39
18. RACUSEN D 1979 Glycoprotein detection in polyacrylamide gel with thymol and sulfuric acid. Anal Biochem 99: 474–476
19. ROSNITSCHEK I, RR THEIMER 1980 Properties of a membrane-bound triglyceride lipase of rapeseed (*Brassica napus* L.) cotyledons. Planta 148: 193–198