An Antibody to the Castor Bean Glyoxysomal Lipase (62 kD) also Binds to a 62 kD Protein in Extracts from Many Young Oilseed Plants

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ABSTRACT

An antibody raised against purified glyoxysomal lipase (triacylglycerol hydrolase EC 3.1.1.3) from castor bean (relative molecular weight of 62,000) also binds to a protein with a relative molecular weight of 62,000 in extracts of food reserve tissues from many young oilseed plants. These plants include Brassica napus L., Zea mays L., Arachis hypogaea L., Glycine max L., Gossypium hirsutum L., Cucurbita pepo L., Helianthus annuus L., Pisum sativum L., and Cicer arietinum L. The antibody caused inhibition of triacylglycerol hydrolisis by the lipases in extracts from seedlings of corn, oilseed rape, castor bean, soybean, and peanut. The pattern of antilipase binding to the 62 kilodalton protein in subcellular fractions from these other seedlings was consistent with the patterns of lipase activity reported in the literature and it is suggested that lipases from these oil seeds all have a subunit with a molecular weight of 62,000. The protein was only found in the food reserve tissues and was not present in extracts of roots and leaves of mature plants. In addition, the immunoreactive 62 kilodalton polypeptide was not detectable in lima beans and only at very low levels in kidney beans. Both these seeds are known to contain very little storage lipid and would not be expected to contain lipase. With the exception of the acid lipase of castor bean, ungerminated seeds do not generally contain active lipases. The immunoreactive 62 kilodalton protein could not be detected in the ungerminated seeds of most plants and only at very low levels in others.

Lipases (Triacylglycerol acyl hydrolases EC 3.1.1.3.) have been detected in the food reserve tissues of growing seedlings of many plants and especially in those which contain large amounts of triacylglycerols. The literature on plant lipases has been reviewed recently (4, 17) and, compared with lipases from animals, little is known about this class of hydrolytic enzymes. In general, lipase activity is not present in dry seeds but appears rapidly after germination of the seed. The lipase hydrolyzes triacylglycerols to glycerol and fatty acids which are converted to sugars and support growth of the young plant. The lipase activity declines as the storage lipids are exhausted. The intracellular location of lipase activity has been determined for a few species and is found to vary considerably. It is found with the lipid bodies of castor bean, cotton, corn and oilseed rape, the glycolyosomes of soybean, peanut and castor bean, and the light microsomal membranes of oilseed rape. Only the plant lipases to have been purified to date are the lipid body lipase of corn scutellum (8) and the glyoxysomal lipase of castor bean (13). The mol wt of these lipases measured by SDS-PAGE were reported to be 65,000 and 62,000 respectively. Antibodies were raised in a rabbit against the purified castor bean glyoxysomal lipase (13). The antilipase was used in these experiments to establish whether there was cross-reactivity with lipases in other young oilseed plants.

MATERIALS AND METHODS

Plant Material. Castor bean (Ricinus communis L. cv Hale) seeds were soaked for 1 d and germinated in moist vermiculite at 30°C for 4 d. Pea (Pisum sativum L. cv alaskan) 4 d, kidney bean (Phaseolus vulgaris L.) 4 d, lima bean (Phaseolus lunatus L.) 4 d, and three corn varieties (Zea mays L. cv Golden Bantam T-51 6 d, cv Trojan 6 d, and cv Hopie white 6 d) seeds were germinated in moist vermiculite for the days indicated. Erucic acid-free rape (Brassica napus L. cv Andor) 3 d, cotton (Gossypium hirsutum) 4 d, peanut (Arachis hypogaea L.) 4 d, sunflower (Helianthus annuus L.) 7 d, garbanzo bean (Cicer arietinum) 4 d, zucchini (Cucurbita pepo cv Burpee hybrid zucchini) 3 d, and soybean (Glycine max L.) 5 d were germinated on moist filter paper in deep Petri dishes. All seedlings except castor bean were grown in the dark at 25°C. In addition, seedlings of corn, rape, sunflower, cotton, and castor bean were grown up to plants for 6 weeks in a greenhouse.

Subcellular Fractionation. Plant material (1–3 g) was homogenized by first slicing with a razor blade and then grinding in an ice-cold mortar in 4 ml of grinding medium. The grinding medium contained 100 mM Hapes-NaOH (pH 7.5), 0.4 mM succrose, 2 mM DTT, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. All operations were carried out at 4°C. For cotyledons, leaves, and roots, Polyclar AT was added to the grinding medium at 5% (w/v). The brei was filtered through one layer of Miracloth and the residue rinsed with a further 3 ml of grinding medium and bulked with the rest. The homogenate was fractionated by differential centrifugation at 100g for 10 min, 10,000g for 30 min, and 100,000g for 60 min. The 100g pellet was discarded and, when present, the fat pad (collected after the 10,000g step) was resuspended in 1 ml of grinding medium and washed by flotation by centrifugation at 10,000g for 10 min. The triacylglycerols were removed by extracting the fat pad three times with diethyl ether. The remaining traces of ether were removed with a stream of nitrogen. The 10,000g and 100,000g pellets were each resuspended in 1 ml of grinding medium.

Sucrose Density Gradient Centrifugation. Organelles and membranes of 4 d castor bean endosperm were separated by isopycnic centrifugation as previously described (10) on a 36 ml 15 to 60% sucrose gradient. The gradient was fractionated with

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an ISCO 640 fractionator into 1.2 ml fractions. The sucrose density of each fraction was estimated with a Bausch and Lomb refractometer.

**Gel Electrophoresis.** SDS-PAGE was carried out on 1.5 mm thick slab gels consisting of 12% acrylamide, 0.27% bisacrylamide, 25% urea, and 0.37 M Tris-HCl (pH 8.8). The stacking gel consisted of 3% acrylamide, 0.08% bisacrylamide, 0.25 M Tris-HCl (pH 6.8). Aliquots of the fractions obtained from the differential centrifugation containing 7 μg of protein were incubated with 1.7% SDS, 1% β-mercaptoethanol, 8% glycerol, and 42 mM Tris-HCl (pH 6.8) at 70°C for 20 min before loading onto the gel. The gels were run at 90 V in the stacking phase and 150 V in the running phase. Mol wt standards lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde 3-P dehydrogenase (36,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase b (97,400), β-galactosidase (116,000), myosin (205,000), were purchased from Sigma. Gels which were not electroblotted were then stained with Coomassie brilliant blue.

**Western Blotting and Treatment with Antilipase.** The proteins were then electroblotted from the polyacrylamide gel onto nitrocellulose sheets with a Hoeffer Transblot apparatus at 1.8 amp for 2 h. The mol wt standard lanes were sliced off and stained with 0.1% amido black in methanol/H₂O/acetic acid (45:45:10). The slight shrinkage (1–2%) of the nitrocellulose due to the alcohol in the stain mixture was accounted for when measuring the distance from the origin for the mol wt standards. The nitrocellulose membrane was then blocked by incubation for 1

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**Fig. 1.** Subcellular fractions of castor bean (a), zucchini (b), and peanut (c) were separated by SDS-PAGE, Western blotted, and probed with antibodies, (i) to the castor bean glyoxysomal lipase and (ii) alkaline phosphatase conjugated goat anti-rabbit IgG before staining for the alkaline phosphatase. The subcellular fractions were FP (fat pad), 10 (10,000g x 30 min pellet), 100 (100,000g x 1 h pellet), and S (100,000g x 1 h supernatant). Figure 1d is a 12% gel showing the total protein for each of the fractions from castor bean and peanut.
The following fractions and conditions were used. Castor bean lipid body membranes, pH 7.0; oilseed rape 100,000 g pellet, pH 7.5; maize lipid body membranes, pH 7.0; peanut 10,000 g pellet, pH 9.0; soybean 10,000 g pellet, pH 9.0. Triolein (5 mm) was used as the substrate in all cases except for soybean where trilinolen was used.

Table I. Inhibition of Lipase by the Antilipase

<table>
<thead>
<tr>
<th>Plant</th>
<th>Lipase Activity (control)</th>
<th>Lipase Activity (with 5 μg antilipase)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol fatty acid released</td>
<td>mg⁻¹ protein min⁻¹</td>
<td></td>
</tr>
<tr>
<td>Castor bean</td>
<td>36.4</td>
<td>21.8</td>
<td>60</td>
</tr>
<tr>
<td>Oilseed rape</td>
<td>31.3</td>
<td>16.6</td>
<td>53</td>
</tr>
<tr>
<td>Maize</td>
<td>57.1</td>
<td>34.8</td>
<td>61</td>
</tr>
<tr>
<td>Peanut</td>
<td>3.3</td>
<td>1.8</td>
<td>55</td>
</tr>
<tr>
<td>Soybean</td>
<td>2.0</td>
<td>1.3</td>
<td>65</td>
</tr>
</tbody>
</table>

Table II. Effect of Antilipase and Other Proteins on Neutral Lipase Activity in the Lipid Body Membrane of Castor Bean

<table>
<thead>
<tr>
<th>Addition</th>
<th>Lipase activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol fatty acid released mg⁻¹ protein min⁻¹</td>
<td></td>
</tr>
<tr>
<td>Control (H₂O)</td>
<td>49.0</td>
<td>100</td>
</tr>
<tr>
<td>Antilipase</td>
<td>7.9</td>
<td>16</td>
</tr>
<tr>
<td>BSA (delipidated)</td>
<td>44.2</td>
<td>90</td>
</tr>
<tr>
<td>Pure IgG</td>
<td>49.3</td>
<td>101</td>
</tr>
</tbody>
</table>

h with 3% BSA in saline buffer containing 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The blocking buffer was replaced with a 1:1500 dilution of the antilipase in blocking buffer (80 μg antilipase in 50 ml). The antilipase was prepared by Maishima and Breevers (13) against purified castor bean glyoxysomal lipase. After 2 h incubation with the antibody the membrane was washed twice for 20 min with saline buffer plus 0.5% Triton X-100 and once with saline buffer. The membrane was then incubated for 90 min with a 1:2000 dilution of alkaline phosphatase conjugated goat anti-rabbit from Boehringer Mannheim Biochemicals. The membrane was washed as after the antilipase incubation and then stained for alkaline phosphatase with 60 ml of 180 mM Tris-HCl (pH 8.0) containing 20 mg naphthol AS-MX phosphate and 300 mg fast red TR salt which were purchased from Sigma. Staining was stopped after about 30 s by rinsing the blot with distilled water.

Protein was determined by the method of Lowry et al. (11) after a TCA precipitation step.

Fumarase (16), catalase (12), and alkaline lipase (13) were assayed by establishing methods.

Lipase was also measured by assaying for released fatty acids as described by Huang (5).

RESULTS AND DISCUSSION

Figure 1, a, b, and c show some examples of nitrocellulose blots of the proteins of subcellular fractions from castor bean, peanut, and zucchini after separation by SDS-PAGE and incubation with antibodies to the castor bean glyoxysomal lipase. It can be seen that in some fractions there is only one strongly stained band which is located at a position equivalent to a mol wt of 62,000. In other fractions no staining was detectable at 62,000. The mol wt of the protein which bound the antilipase was about 62,000 in all the seedlings where staining occurred. The mol wt were never more than 63,000 or less than 61,000. For comparison, the total protein for each fraction from castor bean and peanut is shown in separate gels run under identical conditions and stained with Coomassie blue (Fig. 1d). There are many proteins in each fraction so it is clear that the antibody was very specific in binding strongly to the protein at 62,000. In some cases the blots showed faint traces of staining at other mol wt but the gels stained for total protein show that these proteins were particularly abundant and thus the specific staining in terms of antibody binding per unit protein was very much lower than the 62,000 band. There were only two significant exceptions to this where strong binding to a protein with a higher mol wt was detected. These were an 82,000 protein in the lipid body membranes of zucchini and maize and in the 100,000 g pellet of maize. Western blots of fractions which reacted with the antilipase were also probed with preimmune serum from the same rabbit which was used to make the antilipase. No binding was detected at 62,000 with preimmune serum.

It was shown in a previous paper (13) that the antilipase inhibited the glyoxysomal lipase activity of castor bean. If the antibody was binding to the same binding site in the lipases from seedlings of other plants then it might be expected that the activity of these would also be reduced in the presence of the antibody. The antibody was added at a titer estimated from the previous data (13) to give 50% inhibition. Table I shows that the lipase from all five plants tested was inhibited from 35 to 50% by antilipase. It is known that some proteins inhibit lipase activity by binding at the interface between the emulsified triacylglycerol droplets and the aqueous medium thus decreasing the substrate surface concentration for binding of the lipase and
causing inhibition (2, 15). To check this possibility of nonspecific inhibition, a similar experiment was performed on the neutral lipase from castor bean lipid body membranes using pure rabbit IgG from Sigma and pure delipidated BSA at the same protein concentration as the antilipase. A higher titer of antilipase was used in this experiment and as shown in Table II the antibody inhibited by the lipase by 84%. On the other hand, pure rabbit IgG did not inhibit lipase at all and delipidated BSA gave only 10% inhibition. It would therefore seem that the inhibition of lipase by the antibody was due to specific binding to the enzyme.

The results of binding of the antibody to the 62 kD protein in the seedlings that were tested are given in Table III. The intensity of staining of the 62 kD bands in all the fractions from each seedling was measured with a scanning densitometer, summed, and the proportion of staining in each fraction was calculated. The proportions are intended only to give a guide to the relative amounts of binding observed between fractions from each tissue. It has been shown that the lipid bodies isolated from soybean or peanut seedlings do not have any lipolytic activity over the physiological pH range (6, 9). It can be seen from Table III and Figure 1c that there was no binding of the antilipase to the 62 kD protein of the lipid body membranes from soybean and peanut. The most intensity stained bands for these seedlings were in the 10,000g pellet. The lipase in these seedlings is known to be located in the glyoxysomes which would be present in this fraction (1). The species which are known to have lipase activity in the lipid bodies of the seed food reserves namely, castor bean, corn, oilseed rape, and cotton all bound the antilipase to the 62 kD protein of the lipid body fraction (Table III). The lipid body membranes of sweet corn showed low binding compared to the wild corn and the variety Trojan, which contain much more storage triacylglycerol in the scutellum. Very little staining was detected in the 100,000g supernatant or soluble fractions for most of the seedlings, as expected from the finding that lipases are usually bound to membranes. However, the three corn varieties showed substantial amounts of immunoreactive 62 kD protein in the soluble fraction and soybean and peanut also showed some staining in the 100,000g supernatant (Table III). It was noted by Lin and Huang (8) that the lipid body lipase of corn tended to separate from the membrane relatively easily and that during seedling growth lipase activity in the soluble fraction increased. The lipase from corn which has been purified (8) was estimated to have a mol wt of 65,000. The protein from corn which bound the antilipase in our system had a very similar mol wt of 61,000-63,000. Since the antilipase also inhibited the corn lipase activity (Table I) it is likely that these proteins are the same.

Since lipase activity was absent or found at very low levels in ungerminated seeds of all species studied, except castor bean, it was of interest to see if the 62 kD protein could be detected with the antibody in extracts of ungerminated seeds. No binding was detected in extracts of the ungerminated seeds of peanut, soybean, cotton and corn. Very low levels of staining were detected in the ungerminated seeds of castor bean and oilseed rape where the majority was located in extracts of lipid bodies. The acid lipase of the castor bean lipid bodies is present at highest activity in the ungerminated seed. Since very little antilipase binding was detected in extracts of the ungerminated castor bean seeds it would appear that the acid lipase does not bind the antibody. This might not be unexpected since the acid lipase is unique among lipases of oil seeds in being present in ungerminated seeds.

Roots and leaves from mature plants of castor bean, sunflower, oilseed rape, cotton, and corn were homogenized and fractionated in the same way as the seedlings and tested for antilipase binding as before. However, in no case was binding detected at 62,000 or at any other mol wt (Table III). Thus, it would appear that the lipase in these plants is detectable by this method only in food reserve tissues of the growing seedling.

Isopycnic sucrose density gradient centrifugation was used to separate the membranes and organelles from 4 d castor bean endosperm. Aliquots of each fraction were separated by SDS-PAGE and western blotted. After probing with the antilipase and staining, the intensity of staining was measured with the densitometer. Figure 2 shows the distribution of alkaline lipase activity and antilipase binding between the organelles and membranes of 4 d castor bean endosperm. As found previously (14), the catalase and alkaline lipase, which are glyoxysomal markers, were found to peak at a density of 1.24 g ml⁻¹. There was also some alkaline lipase activity in slightly lighter fractions which were not associated with catalase and these were presumably glyoxysomal membranes from glyoxysomes broken during homogenization. It can be seen that the staining intensity for the antilipase on the western blot correlated very closely with the peaks of alkaline lipase activity. There was a small amount of activity associated with the endoplasmic reticulum and a small peak of staining coincided with this. The lipid bodies of the fat pad have not been included with this figure but it should be noted that the lipid body membranes contain a neutral lipase (3) and also bind the antilipase to a 62 kD protein (Table III). To date only two plant lipases have been purified to homogeneity. One is from the lipid bodies of corn scutellum (8) and the other from the glyoxysomes of castor bean (13). Lipase activities from several other plants have been detected and in some cases char-
acterized (5). The intracellular localization of lipases varies between species as do other properties of the lipases such as pH profile and substrate specificity. It might, therefore, be suspected that the proteins would be quite different. However, from the antilipase binding studies described in this paper it appears that seedlings of the oilseeds studied contain a lipase subunit with the same mol wt of approximately 62,000.

LITERATURE CITED
1. COOPER TG., H BEEVERS 1969 Mitochondria and glyoxysomes from castor bean endosperm. J Biol Chem 244: 3507-3513