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# Involvement of Glyoxysomal Lipase in the Hydrolysis of Storage Triacylglycerols in the Cotyledons of Soybean Seedlings[1]

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## ABSTRACT

**The total cotyledon extract of soybean (*Glycine max* [L.] Merr. var. Coker 136) seedlings underwent lipolysis as measured by the release of fatty acids. The highest lipolytic activity occurred at pH 9. This lipolytic activity was absent in the dry seeds and increased after germination concomitant with the decrease in total lipids. Using spherosomes (lipid bodies) isolated from the cotyledons during the peak stage of lipolysis (5–7 days) as substrates, about 40% of the lipase activity was found in the glyoxysomes after organelle breakage had been accounted for; the remaining activity was distributed among other subcellular fractions but none was found in the spherosomal fraction. The glyoxysomal lipase had maximal activity at pH 9, and catalyzed the hydrolysis of tri-, di-, and monoacylglycerols of linoleic acid, the most abundant fatty acid in soybean. The spherosomes contained a neutral lipase that could hydrolyze monolinolein and *N*-methylindoxylmyristate, but not trilinolein. This spherosomal lipase activity dropped off rapidly during early seedling growth, preceding lipolysis. Spherosomes isolated from either dry or germinated seeds did not possess lipolytic activity, and spherosomes from germinated seeds but not from dry seeds could serve as substrates for the glyoxysomal lipase. It is concluded that the glyoxysomal lipase is the enzyme catalyzing the initial hydrolysis of storage triacylglycerols.**

During seedling growth of oil seeds, the reserve triacylglycerols in the storage tissues are rapidly mobilized. Triacylglycerols are hydrolyzed to fatty acids which are channeled through $\beta$-oxidation, and the resulting acetate is processed by the glyoxylate cycle (1, 9). Although $\beta$-oxidation and the glyoxylate cycle are known to occur exclusively in the glyoxysomes, the subcellular location of the enzyme(s) that catalyzes the initial triacylglycerol hydrolysis has not been well-documented. In castor bean, the spherosomes[3] containing the reserve triacylglycerols possess an acid lipase,[4] which is already active in the dry seed before germination (12, 14, 15). In jojoba, an alkaline lipase (wax ester hydrolase) is present in the spherosomes (wax bodies) of germinated but not dry seeds (8). In other oilseed species, lipase activities in the spherosomes of dry or germinated seeds are absent or have not been reported (7). It has recently been postulated that in some oilseed species the spherosomes possess active lipases in postgermination, but the enzymes are readily detached from the spherosomes during organelle preparation (16, 17). In addition to metabolizing the resulting fatty acids after lipolysis, the glyoxysomes also possess lipase activity; however, the enzyme from castor bean (12) and peanut (7) can hydrolyze only monopalmitin but not di- or tripalmitin. It was suggested that the mechanism of initial triacylglycerol hydrolysis may be different in various oilseed species (7).

We have analyzed the pattern of triacylglycerol hydrolysis in soybean seeds during germination and seedling growth. Among other findings, we observed the appearance of triacylglycerol-hydrolyzing lipase activity in the total cotyledon extract in postgermination and the association of this enzyme with the glyoxysomes. In the present paper, we report our findings and discuss the possible existence of this enzyme in glyoxysomes of other oilseed species.

## MATERIALS AND METHODS

**Plant Materials.** Soybean seeds (*Glycine max* [L.] Merr. var. Coker 136), generously supplied by J. Stanton of the Coker Pedigreed Seed Co., were selected for uniformity. Only those seeds having weights between 0.19 to 0.21 g were used. The seeds were soaked in water for 12 h at 26°C and allowed to germinate in moist paper towels at 26°C in darkness. In the postgermination study, seedlings were selected by both chronological age and hypocotyl length.

**Preparation of Homogenates for Lipolysis Studies.** The whole dry seeds and the cotyledons of seedlings were used. The dry seeds and cotyledons were washed 3 times with distilled $H_2O$. Twenty dry seeds or cotyledon pairs were ground in 25 ml grinding medium with a mortar and pestle. The grinding medium contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 0.15 M Tricine buffer adjusted to pH 7.5 with KOH. The homogenate was filtered through a piece of Nitex cloth (Tetko, Elmsford, NY) of pore size 20 × 20 $\mu$m, and the filtrate was used directly for lipolysis studies.

**Organelle Fractionation.** In the preparation of spherosomes, the homogenate obtained after filtration was centrifuged at 10,000 *g* for 10 min. The spherosome pad was removed with a spatula. It was washed by resuspending in 30 ml fresh grinding medium, and the resuspension was centrifuged at 10,000 *g* for 10 min. This washing procedure was repeated 2 more times. The spherosome pad after the final washing was removed and resuspended in grinding medium to give a final volume of 10 ml (from 20 seeds).

The method of preparing spherosomal membranes followed that described earlier (10, 11). The isolated spherosomes were resuspended in grinding medium and extracted three times with diethyl ether. The trace amount of diethyl ether remaining was evaporated under a stream of $N_2$.

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[3] The spherosomes in oil seeds should be termed lipid bodies, but for convenience we use the term spherosomes in this paper.

[4] The definition of a lipase (EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of triacylglycerols. Until the discovery reported in this paper, the glyoxysomal lipase, which was shown to hydrolyze monoacylglycerols but not tripalmitin, did not fit to the above definition. In this report, for convenience we will use the term lipase for any enzyme that hydrolyze mono-, di-, or tri-acylglycerols.

In the preparation of subcellular organelles by sucrose density gradient centrifugation, the cotyledons were chopped into a fine mince with a razor blade in a Petri dish containing grinding medium (10 g tissue in 12 ml medium). The tissue mince was ground gently with a mortar and pestle. The homogenate was filtered through one layer of Nitex cloth (pore size 20 × 20 μm) and layered directly onto a sucrose gradient. The gradient consisted of 6 ml 20% (w/w) sucrose on top of 22 ml of a 30% to 60% linear sucrose gradient. All the sucrose solutions contained 1 mM K-phosphate (pH 7.5). The gradient was centrifuged at 21,000 rpm for 4 h in a Beckman L2-65B ultracentrifuge (Beckman Instruments) with Spinco rotor 27, and fractionated.

**Assays.** The activities of catalase, Cyt *c* oxidase, and Cyt *c* reductase were assayed spectrophotometrically as described earlier (10). Lipase activity was assayed by either a fluorometric or a colorimetric method. In the fluorometric method (12), a reaction mixture of 4 ml containing 0.1 M cacodylate buffer (pH 6.5) (or other buffers as stated), 2 mM DTT, and 0.42 mM *N*-methylindoxylmyristate in ethylene glycol monomethyl ether (0.05 ml) was used at room temperature (24°C). In the colorimetric assay (13), the fatty acids released were converted to copper soaps and quantitated using 1,5-diphenylcarbazide as the color reagent. The reaction mixture contained, in a final volume of 1 ml, 0.1 M Tris-HCl buffer (pH 9.0) (or other buffers as stated), 5 mM DTT, 2.5 mM substrate, and enzyme. The substrates, except isolated spherosomes, were emulsified in 5% Gum Acacia (Specialty Chemicals, Morristown, NJ) for 30 s at high speed with a Bronwill Biosonik IV ultrasonic generator (VWR Scientific, San Francisco, CA) fitted with a microprobe. The reaction was carried out at 34°C in a shaker-water bath, and the release of fatty acids was measured at time intervals.

Tripalmitin, tristearin, triolein, 1,3-dilinolein and monolinolein were obtained from Sigma. Trilinolein was obtained from NU Chek, Prep, Inc. (Elysian, MN), and *N*-methy-indoxylmyristate from ICN Pharmaceuticals (Cleveland, OH). In some experiments, the trilinolein was further purified by TLC (plates coated with 250 μm Silica Gel G from Brinkman Instruments, Inc., Westbury, NJ) using a developing solvent of hexane:dimethyl ether:acetic acid (50:50:1, v/v/v).

The lipids were extracted by the Bligh and Dyer method (2). The total lipid was determined by drying an aliquot of the chloroform extract in a vacuum oven overnight and weighing the lipid residue. Protein was assayed by the Bradford method (3).

## RESULTS

**Lipolysis and Enzyme Activities During Germination and Seedling Growth.** When soybean seeds were allowed to germinate in darkness at 26°C, the amount of total lipid in the cotyledons remained unchanged for 3 d and then declined (Fig. 1). After 13 d of seedling growth, about 50% of the lipid had been consumed. Beyond 13 d, the seedlings became infected by microorganisms.

Catalase is a glyoxysomal enzyme that has been used as a marker for the development of gluconeogenesis from storage lipids in various oil-seed species (1, 6). In the cotyledons of germinated soybean seeds, catalase activity increased many-fold to a peak at day 5 and then declined slowly (Fig. 1).

At the peak stage (5–7 d) of lipolysis, the total cotyledon extract was analyzed for its ability to release free fatty acids from the endogenous lipids at various pH values (Fig. 2). The highest activity was at pH 9 and a smaller peak of activity (about 40% as high as that at pH 9) was present at pH 5.

With this information, the endogenous lipolytic activity of the total cotyledon extract was followed at pH 9 and pH 5 during germination and seedling growth (Fig. 1). The pH 9 activity rose many-fold after germination and then declined; the pattern of activity followed closely that of catalase. In contrast, the pH 5 activity rose after the first day of germination and remained roughly the same during postgerminative growth. These data suggest that the pH 9 activity, rather than the pH 5 activity, belongs to the enzyme catalyzing the actual lipolysis of the storage triacylglycerols.

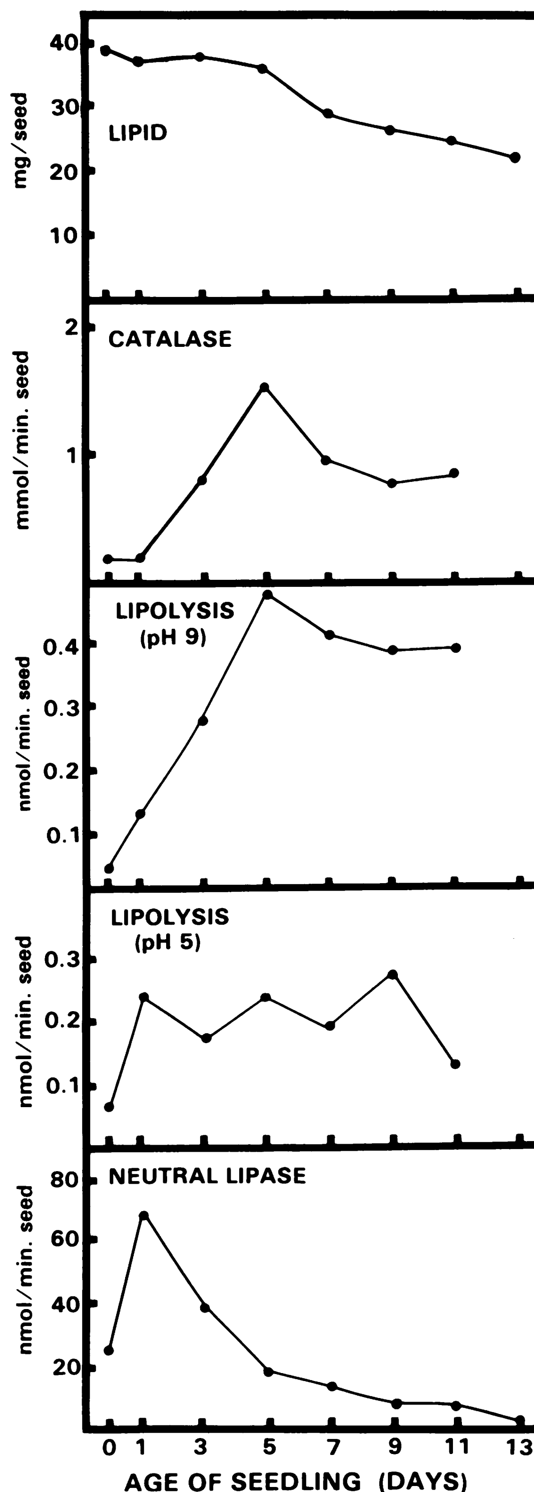


FIG. 1. Compositional changes in the cotyledons of soybean seedlings. Lipolysis was carried out with the total homogenates at pH 9 or 5, and the free fatty acids released were monitored. Neutral lipase activity was measured by a fluorometric assay at pH 6.5 using *N*-methylindoxylmyristate as an artificial substrate.

**Subcellular Localization of the Alkaline Lipase Activity.** The

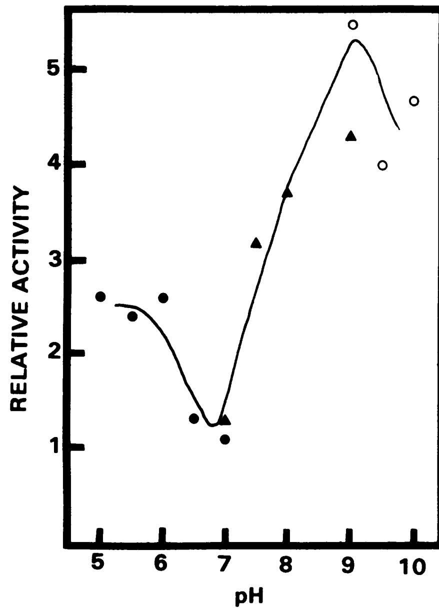


FIG. 2. Effect of pH on the lipolytic activity of the total homogenate from the cotyledons of 5- to 7-d-old soybean seedlings. (●), Succinate-NaOH: (▲), Tris-HCl; and (O), glycine-HCl.

Table I. *Subcellular Localization of Alkaline Lipase in the Cotyledons of 5 to 7-day-old Soybean Seedlings*

The various organelles were separated from the total cotyledon extract by sucrose density gradient centrifugation. Spherosomes isolated from 5- to 7-d-old seedlings were used as substrates. Enzyme activities are expressed on per 100 seed basis.

| | Alkaline Lipase | Catalase | Cytochrome Oxidase | Cytochrome Reductase |
|---|---|---|---|---|
| | *nmol/min (% total)* | | *μmol/min (% total)* | |
| Glyoxysomes | 18.7 (21.0) | 1.09 (49.9) | 0.04 (3.4) | 0.03 (1.3) |
| Mitochondria | 13.0 (14.8) | 0.64 (2.9) | 0.87 (77.9) | 0.34 (14.1) |
| Membrane fraction | 17.8 (19.1) | 0.30 (1.4) | 0.12 (10.7) | 0.85 (35.4) |
| Supernatant | 43.9 (45.1) | 1.00 (45.8) | 0.09 (8.0) | 1.18 (49.2) |
| Spherosomes | 0 (0) | —[a]— | — — | — — |

[a] —, not tested.

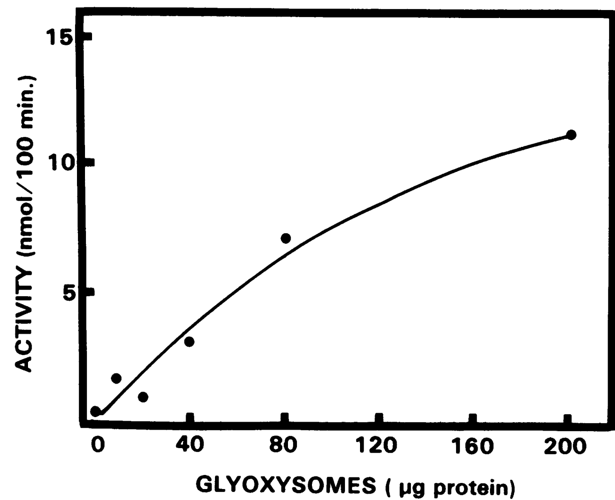


FIG. 3. Glyoxysomal lipase activity as a function of glyoxysomal proteins. Spherosomes (3.5 mg/ml) isolated from the cotyledons of 5- to 7-d-old seedlings were used as substrates.

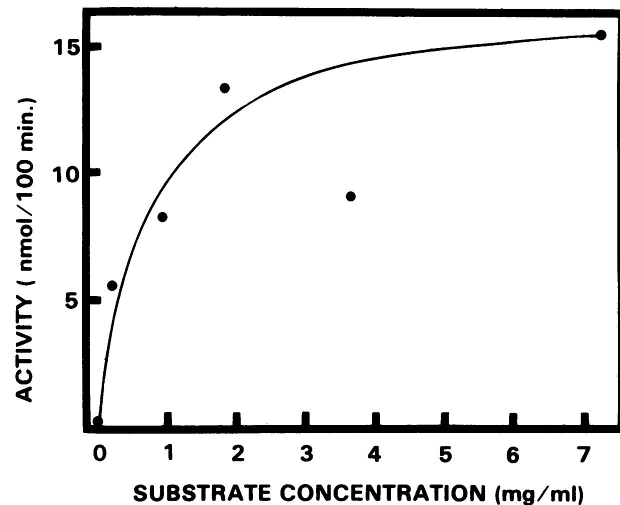


FIG. 4. Lipase activity of the glyoxysomes as a function of substrate concentration. Spherosomes isolated from the cotyledons of 5- to 7-d-old seedlings were used as substrates. Glyoxysomes containing 80 μg protein were used in each assay.

total cotyledon homogenate was examined by sucrose density gradient centrifugation. Similar to the results obtained from other oilseed species (6), the total homogenate was resolved into the following distinct subcellular fractions: spherosomes floating on top, soluble fraction immediately below the spherosome layer, a membrane fraction (Cyt reductase as marker) at the interface between densities 1.11 and 1.13 g/cm$^3$, mitochondria (Cyt oxidase as marker) at density 1.17 g/cm$^3$, and glyoxysomes (catalase as marker) at density 1.25 g/cm$^3$.

Spherosomes isolated from 5- to 7-d-old seedlings were used as substrates for the assay of lipase activity. The spherosomes were isolated from the total homogenate by repeated centrifugation and resuspension of the lipid layer. When the alkaline (pH 9) lipase activity was assayed in the various subcellular fractions (Table I), 21% (average of four experiments) of the activity was recovered in the glyoxysomes, 14% in the mitochondria, 19% in the membrane fraction at density 1.12 g/cm$^3$, 45% in the soluble fraction, and 0% in the spherosomes (*i.e.* there was no lipolytic activity associated with the spherosomes). In the current experiment, about 50% of the glyoxysomes were broken during organelle preparation. This figure is based on the recovery of catalase in isolated glyoxysomes (Table I), assuming that *in vivo* all the catalase activity is exclusively localized in the glyoxysomes. This recovery estimation is valid even if catalase is localized in the glyoxysomal matrix and the lipase is restricted to the glyoxysomal membrane, since the calculation is based on the recovery of the two enzymes in the glyoxysomal band which contains only intact glyoxysomes and not glyoxysomal ghosts. After accounting for organelle breakage, we estimated that the glyoxysomes contain about 40% of the total lipase activity. The remaining lipase activity may represent the same enzyme in other subcellular sites or belong to other enzymes unrelated to the gluconeogenesis from triacylglycerols.

**Properties of the Glyoxysomal Lipase.** Isolated glyoxysomes were used to study the properties of the lipase. The lipase activity in the current assay system was proportional to the amount of glyoxysomes added, up to 100 μg/ml of glyoxysomal protein (Fig. 3). The activity was roughly proportional to the amount of spherosomes (0–2 mg lipid/ml) added (Fig. 4). When tested between pH 7 to 10, the activity was highest at pH 9 (Fig. 5).

Of the substrates tested (Table II), the enzyme was most active toward monolinolein and *N*-methylindoxymyristate. It catalyzed the hydrolysis of dilinolein and trilinolein at a relatively lower

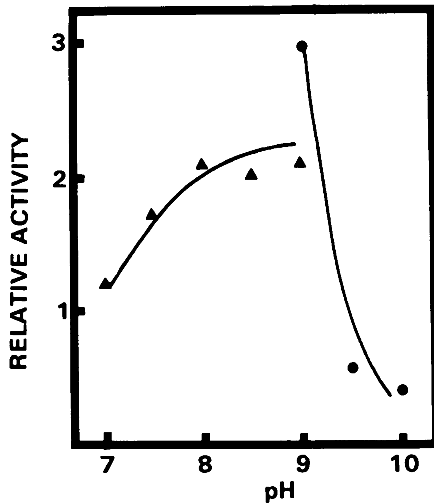


FIG. 5. Effect of pH on the lipase activity in isolated glyoxysomes. Trilinolein was used as the substrate. (▲), Tris-HCl; (O), glycine-HCl.

Table II. *Hydrolysis of Various Acylglycerols and N-Methylindoxylmyristate by Isolated Soybean Glyoxysomes*

The assays were carried out with Tris-HCl buffer (ph 9.0).

| Substrates | Hydrolysis Activity | |
|---|---|---|
| | Fatty acids released | Relative activity |
| | *nmol/min* | *%* |
| Trilinolein | 0.017 | 10 |
| Triolein | 0 | 0 |
| Tristearin | 0 | 0 |
| Tripalmitin | 0 | 0 |
| Dilinolein | 0.024 | 14 |
| Monolinolein | 0.17 | 100 |
| *N*-Methylindoxylmyristate | 0.19 | 111 |

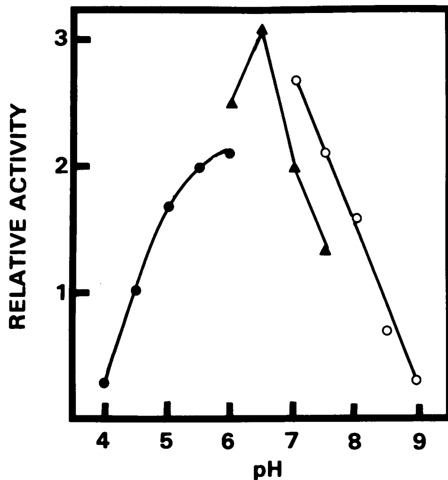


FIG. 6. Effect of pH on the lipase activity in isolated spherosomes. *N*-methyindoxylmyristate was used as a fluorometric substrate. (●), Succinate-NaOH; (▲), imidazole-HCl; and (O), Tris-HCl.

rate, but could not hydrolyze triolein, tripalmitin, or tristearin. The major fatty acids of soybean are linoleic acid (49%) and oleic acid (22%), and most of the triacylglycerols contain one or two linoleic acids (5).

**Spherosomes and Neutral Lipase Activity.** When *N*-methylindoxylmyristate was used as a substrate in a fluorometric assay for lipase activity, activity was found in the spherosomes. This spherosomal lipase activity had optimal activity at pH 6.5 (Fig. 6). When the spherosomal membrane was extracted from isolated spherosomes by a method established for other oilseed species (10, 11), a part of the activity was recovered in the membrane fraction.

The activity of this spherosomal lipase, as assayed in the total homogenate (Fig. 1) or in the isolated spherosomal membrane (Fig. 7), was present in the dry seeds and declined rapidly following germination. The decline preceded the disappearance of storage triacylglycerols (Fig. 1). Presumably, the spherosomal enzyme is not the enzyme catalyzing the initial triacylglycerol hydrolysis. Supporting evidence comes from the inability of the isolated spherosomes, from either dry seeds or 5- to 7-d-old seedlings, to undergo autolysis. Such an autolysis has been observed with castor bean spherosomes (14, 15) which were also used in control experiments in the current study. Furthermore, the membranes of the spherosomes isolated from dry seeds were able to hydrolyze monolinolein but not trilinolein.

In our study of the glyoxysomal lipase, we used spherosomes isolated from 5- to 7-d-old seedlings as the native substrates. The spherosomes from dry seeds could not serve as substrates (Fig. 8). Apparently, following germination, there is a change in the spherosomes such that the storage triacylglycerols in the spherosomes somehow become available for lipolysis by the glyoxysomal lipase.

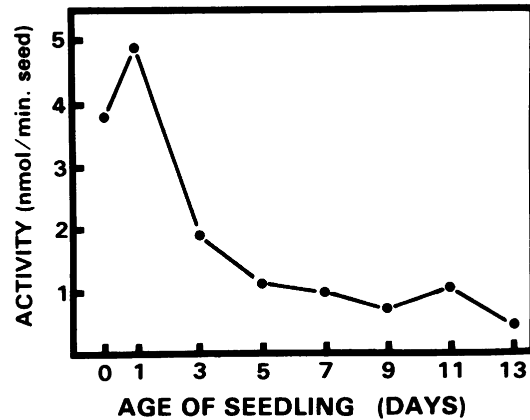


FIG. 7. Changes in the lipase activity in the membrane of isolated spherosomes in post-germination. *N*-methylindoxylmyristrate was used as a fluorometric substrate, and the activity was assayed in imidazole-HCl buffer at pH 6.5.

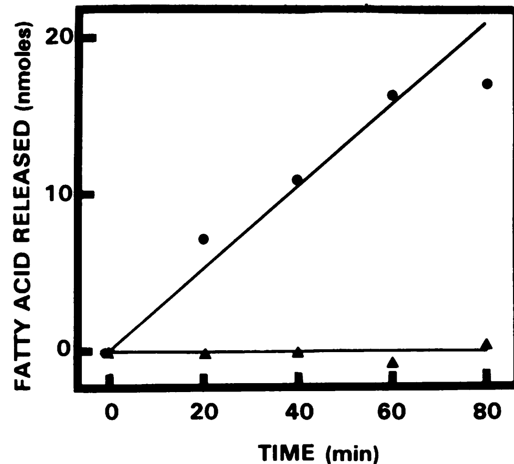


FIG. 8. Ability of spherosomes isolated from either dry seeds (▲) or cotyledons of 5- to 7-d-old seedlings (●) to serve as substrates (3.5 mg/ml) of the glyoxysomal lipase.

In TLC, there was no appreciable difference in the lipid pattern (mostly triacylglycerols) between the spherosomes from dry seeds and those from 5- to 7-d-old seedlings. Also, lipids extracted from either dry seeds or 5- to 7-d-old seedlings could be hydrolyzed by the glyoxysomal lipase. Presumably, there is a change in the spherosomal membrane following germination. This assumption is indirectly supported by the finding that the spherosomal membrane lipase activity was drastically reduced 5 d after germination (Figs. 1 and 7).

## DISCUSSION

The glyoxysomal lipase of soybean has a developmental pattern similar to that of catalase in postgermination. The activity is at its highest during the period of rapid lipolysis reaching, at days 3 to 7 when the lipolysis is most active, 3 nmol fatty acid released/min · seed at 34°C (after the incomplete tissue homogenization has been accounted for). This amount of lipase activity is close to the rate of *in vivo* lipolysis of approximately 2.5 $\mu$mol triacylglycerol disappeared/seed · day at 26°C (Fig. 1). The activity is associated with the glyoxysomes. Thus, besides the $\beta$-oxidation and glyoxylate cycle activities, the glyoxysomes also possess lipase activity for the hydrolysis of the triacylglycerols to fatty acids.

The glyoxysomal lipase is active toward trilinolein, dilinolein, and monolinolein, but cannot hydrolyze triolein, tristearin, and tripalmitin. This substrate specificity reflects the ability of the enzyme to catalyze the hydrolysis of the native soybean triacylglycerols which contain a high percentage of linoleic acid (5). The enzyme also hydrolyzes the native substrates of isolated spherosomes.

When *N*-methyindoxylmyristate was used as an artificial substrate for the assay of lipase activity, the glyoxysomes isolated from many oilseed species were found to possess an alkaline lipase (6). In castor bean (12) and peanut (7) where the substrate specificity of the glyoxysomal lipase had been tested, the enzyme was active toward monopalmitin but could not hydrolyze dipalmitin and tripalmitin. Based on this substrate specificity, it was suggested that the glyoxysomal lipase is not the enzyme responsible for the initial triacylglycerol hydrolysis. Our current finding on the substrate specificity of the soybean glyoxysomal lipase opens the possibility that the lipase in the glyoxysomes of other oilseeds are active toward unsaturated triacylglycerols. If so, the glyoxysomal lipase in various oilseed species may play a significant role in the triacylglycerol hydrolysis *in vivo*, inasmuch as the fatty acids in a great variety of species are predominantly unsaturated (5). Indeed, we have found that glyoxysomes isolated from some other oilseed species, including castor bean and peanut, contain lipase that can hydrolyze trilinolein but not tripalmitin (work in progress).

The substrate specificity of the soybean glyoxysomal lipase and its optimal activity at alkaline pH are similar to those of an enzyme described in rapeseed (16). The rapeseed lipase was found not in the glyoxysomes but in a membrane fraction isolated at a density of 1.1 g/cm$^3$ on sucrose gradients. The membrane was thought to have been derived from the spherosomal membrane. It remains to be seen whether or not the difference in the subcellular location of the soybean and rapeseed lipase is due to a difference in the species or in methodology.

Electron microscopy has established that in various oilseed species, the glyoxysomes are in direct contact with the spherosomes (4). The soybean glyoxysomal lipase can act on spherosomes isolated from seedlings but cannot act on those from dry seeds. This finding indicates that the spherosomes have to go through modification in postgermination before their triacylglycerols can be metabolized by the glyoxysomes. Already, we have shown a modification of the spherosomal lipase during the early stage of postgermination. Modification of the spherosomes in postgermination was also noted in jojoba where the spherosomal lipase, fatty alcohol dehydrogenase, and fatty aldehyde dehydrogenase appear only after germination (8, 10). It remains to be seen how the soybean spherosomes are modified such that the triacylglycerols become available to the glyoxysomal lipase.

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