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Spherosomes of Castor Bean Endosperm

MEMBRANE COMPONENTS, FORMATION, AND DEGRADATION[1]

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ABSTRACT

The membrane components of the castor bean spherosomes were characterized. The storage triacylglycerols of isolated spherosomes were extracted with diethyl ether, and the membrane was isolated by sucrose gradient centrifugation. It had an apparent equilibrium density of 1.12 grams per cubic centimeter, and possessed an antimycin A-insensitive NADH cytochrome *c* reductase and an acid lipase. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in roughly equal amounts were the major phospholipids. The membrane proteins were resolved into several major and minor protein bands of molecular weights ranging from 10,000 to 70,000 by acrylamide gel electrophoresis, and the protein pattern in the gel was different from those of the endoplasmic reticulum, mitochondrial, and glyoxysomal membranes.

The varying amounts of spherosomal components in the seed were followed throughout seed maturation and germination. A striking similarity existed in the developmental pattern of each of the spherosomal components. This finding suggests that the spherosome is synthesized and degraded as one individual unit. The spherosomes isolated from maturing seeds exhibited rapid hydrolysis of the storage lipid *in vitro*, thus raising the problem of cellular control in preventing *in vivo* autolysis of the spherosomes during seed maturation.

In oil seeds, a substantial portion of the dry weight is comprised of storage triacylglycerols. These storage lipids are localized in organelles called spherosomes. Spherosomes are surrounded by a distinct membrane which has been proposed to represent a "half-unit" membrane (21). The chemical components other than the storage lipid of spherosomes have been studied only to a limited extent. The phospholipid composition of castor bean (6) and wheat aleurone layer (11) spherosomes, the protein composition, and equilibrium density of mustard spherosomal membrane (1), and the acid lipase of castor bean spherosomal membrane (15) have been reported. Such information is scattered, and a complete characterization of the spherosomal components of one seed species is lacking. During seed maturation, spherosomes may be formed by the budding off and enlargement of terminal ER[5] vesicles (18), or by the encasement of naked cytoplasmic lipid droplets with protein synthesized by adjacent ER (1). During germination, the spherosomal membrane actively participates in the initial enzymic hydrolysis of the storage triacylglycerol (14, 15, 19).

Among the various oilseeds, the process of lipid hydrolysis has been most thoroughly studied in castor bean (14–16, 18). In castor bean, an acid lipase is associated with the spherosomal membrane, and is responsible for the initial lipid hydrolysis (14–16). Unlike the activities of all other known enzymes involved in the subsequent lipid metabolic steps which increase concomitantly during germination (14), the lipase can be extracted in high activity from the dry seeds. The spherosomes isolated from the dry seeds, having both the lipase and storage triacylglycerol, can go through autolysis to give rise to fatty acids (16). However, the relationship of this lipase to the storage triacylglycerol is still unclear since it was reported that the activity of the enzyme and the amount of storage lipid did not match well during the course of germination (14). The lipase activity, although high in the dry seed, doubled during the 1st day of soaking and declined more rapidly than the total lipid during germination (14). It is not known whether or not there is an activation of the enzyme during soaking, and at what stage during seed maturation the active enzyme is formed which can hydrolyze the accumulating lipid.

In an attempt to resolve the above unknown steps, we followed the formation and degradation of the spherosomes and lipase during seed maturation and germination of castor bean. The detailed chemical components of the spherosomes isolated from the dry seeds were also studied. In this paper, we report our findings.

MATERIALS AND METHODS

Plant Materials. Castor bean (*Ricinus communis* L. var. Hale) was obtained from McNair Seed Co., Plainview, Texas. The seed was soaked in running tap water for 1 day and allowed to germinate in moist Vermiculite at 30 C. In the study of seed maturation, developing castor beans of an unknown variety were obtained from a local garden. Since the seeds did not mature at the same rate, even within the same inflorescence, they were selected and divided into four stages of maturation according to their morphology. The youngest seeds, stage 1, were surrounded by testae green in color, and their endosperms comprised only about 10% of the volume of the seeds. The testae of stage 2 seeds were 50% green and 50% brown. The endosperms of these seeds were about twice as large as those in stage 1. Stage 3 seeds had light brown seed coats and their endosperms comprised about 75% of the volume of the seeds. The most mature developing seeds, stage 4, had black seed coats and their endosperms filled all but the outer 1 mm of the volume of the seed.

Isolation of Spherosomes and Other Organelles. For the preparation of spherosomes, 40 dehulled seeds were ground in 40 ml grinding medium (13) with a mortar and pestle. The pulp was filtered through a 44 μm^2 Nitex cloth and the final volume adjusted to 60 ml. The homogenate was then centrifuged at 10,000*g* for 30 min. The spherosome pad was removed from the surface with a

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[5] Abbreviations: ER: endoplasmic reticulum.

spatula. It was washed by resuspending in 30 ml of fresh medium, and the resuspension was recentrifuged at 10,000*g* for 30 min. The washed spherosome pad was removed and resuspended in grinding medium to give a final volume of 11 ml.

ER, mitochondria, and glyoxysomes were isolated from the endosperm of 4-day old castor bean seedlings by sucrose gradient centrifugation as described previously (13). The fractions were collected, diluted 2-fold with 50 mM Tricine (pH 7.5) in 0.3 M KCl, and centrifuged at 100,000*g* for 1 h to recover the membrane.

In the preparation of spherosomal membranes, the isolated spherosomes were resuspended in grinding medium and extracted three times with diethyl ether to remove the triacylglycerols. The trace amount of diethyl ether remaining was evaporated under a stream of N_2. The membrane fraction was centrifuged in a sucrose gradient composed of 10 ml 20% (w/w) sucrose on top of 24 ml of a 30–60% linear sucrose gradient. The gradient was centrifuged at 21,000 rpm for 4 h in a Beckman L2-65B ultracentrifuge with Spinco rotor 27, and fractionated. The sucrose concentration of each fraction was measured by refractometry.

Gel Electrophoresis. Slab gel electrophoresis was performed in 10% polyacrylamide gels with a stacking gel and a discontinuous buffer system (20). The organelle membranes were incubated for 1 h at room temperature in an equal volume of 10 mM K-phosphate (pH 7.4), 1% SDS, and 1% β-mercaptoethanol. Mol wt standards (all from Sigma) were β-galactosidase (*Escherichia coli*), phosphorylase *a* (rabbit muscle), BSA, catalase, glyceraldehyde-3-P dehydrogenase, chymotrypsinogen, myoglobin, and Cyt *c*.

Lipid Analysis. For the analysis of spherosomal lipids, spherosomes were isolated from dry seeds and washed as previously described. The lipids in the isolated spherosomes were extracted by the Bligh and Dyer method (3). The mass of the total lipid was determined by drying an aliquot of chloroform extract in a vacuum oven overnight and weighing the resulting lipid residue. Nonpolar lipids were separated by TLC on 250-μm Silica Gel G plates. The plates were developed with benzene-diethyl ether-ethanol (100:30:2, v/v/v). The various types of triacylglycerols were identified by comparison with castor oil standards and with their reported R_F values (7). The amount of each lipid class was determined by scraping and extracting the silica gel spots with chloroform-methanol (1:1, v/v), and measuring the number of ester bonds by the ferric hydroxamate assay (5).

Polar lipids were separated from the large excess of nonpolar lipids by column chromatography with silica gel. A sample of 100 mg total lipid was applied to the column (0.8 × 4.0 cm) in chloroform. Nonpolar lipids were eluted with 15 ml chloroform. Polar lipids were then eluted with 15 ml methanol. The polar lipids were then separated by TLC on 250-μm Silica Gel G plates. The plates were developed with chloroform-methanol-acetic acid-water (65:50:5:3, v/v/v/v). The various phospholipids were identified by comparison with known standards. The silica gel containing each identified phospholipid was scraped into a test tube and digested with 70% $HClO_4$. The resulting Pi was measured by a modification of the Bartlet method (5).

Autolysis. Stage 4 developing castor bean spherosomes were incubated under the following conditions. The reaction mixture contained 100 mM citrate-NaOH buffer (pH 5), 5 mM DTT, and 0.5 ml isolated spherosomes (about 50 mg total lipid), in a total volume of 10 ml. At designated times 0.8-ml aliquots were removed and the lipids were extracted by the Bligh and Dyer method. The amounts of lipid ester bonds were determined by the ferric hydroxamate assay (5).

Assays. Acid lipase was measured by a fluorometric assay with *N*-methyl indoxylmyristate as the substrate (14). The lipase was measured in a reaction mixture of 4 ml, containing 100 mM citrate-NaOH (pH 5), 2 mM DTT, 250 μg/ml BSA, and 0.83 mM *N*-methyl indoxylmyristate (I.C.N. Pharmaceuticals) in ethylene glycol monomethyl ether (0.1 ml). The inclusion of BSA improved the kinetics of the acid lipase reaction.

Alkaline lipase was measured by the same procedure except citrate buffer was replaced by 0.1 M Tris-HCl (pH 9) and BSA was deleted.

NADH Cyt *c* reductase was assayed by initiating the reaction with NADH and measuring the reduction of Cyt *c* at 550 nm (8).

To measure the amount of protein in the isolated spherosome fractions, it was necessary to remove the lipids first. Nonpolar lipids were removed by several extractions with cold diethyl ether. Traces of ether were evaporated by a stream of N_2. The proteins were then precipitated with 10% trichloroacetic acid, and measured according to the method of Lowry *et al* (12).

Total lipids were measured in isolated spherosomes of each stage by extracting the spherosomes by the Bligh and Dyer method (3), and drying and weighing an aliquot of the chloroform extract.

Phospholipids were determined in the total lipid extract of the various isolated spherosome samples by digestion with 70% $HClO_4$, and measuring the resulting Pi by a modified Bartlet method (5).

RESULTS AND DISCUSSION

Biochemical Components of Spherosomes and Spherosomal Membrane. Spherosomes were isolated from dry castor beans by repeated washing and centrifugation. The chemical composition of the isolated organelles was analyzed (Table I). Most of the mass was represented by lipid and some 5% by protein. As expected, the overwhelming class of the lipid was triacylglycerols. Triacylglycerols with 3-ricinoleic acid and with 2-ricinoleic acid represented 66 and 23%, respectively, of the total triacylglycerols. The current finding of the triacylglycerol composition is in accord with that of a previous report (7). The polar lipids amounted to less than 1% of the total lipid. They contained roughly equal proportions of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Such a phospholipid composition is different from those of spherosomes of 2-day-old castor bean (6) and of wheat aleurone layer (11).

The membrane of isolated spherosomes was prepared using a method established previously (13) by extraction of the triacylglycerols with ether followed by sucrose gradient centrifugation

Table I. *Chemical Composition of Isolated Castor Bean Spherosomes*

The total lipid was measured as the mass of the chloroform-methanol extractable material, the nonpolar lipid by ester bond assay, and the polar lipids by measuring lipid-associated phosphate.

	Amount	Mol % of Each Lipid Class
	mg	
Total Protein	3.6	
Total Lipid	65.7	
Nonpolar Lipid	71.0	
Triacylglycerols (TR_0[a])		3
Triacylglycerols (TR_3)		66
Triacylglycerols (TR_2)		23
Triacylglycerols (TR_1)		5
Others		4
Polar Lipid	0.25	
Phosphatidylcholine		30
Phosphatidylethanolamine		26
Phosphatidylinositol		25
Phosphatidylserine		5
Phosphatidylglycerol		5
Others unidentified		8

[a] TR_0 to TR_3: triacylglycerols with 0, 1, 2, or 3 ricinoleins.

(Fig. 1). Similar to the jojoba spherosomal membrane, the castor bean spherosomal membrane, along with its phospholipid, protein, acid lipase, and NADH-Cyt reductase, migrated to an apparent equilibrium density of 1.12 g/cm^3. A substantial amount of spherosomal membrane components were solubilized and remained at the top of the gradient. The solubilization presumably occurred during the ether extraction of the triacylglycerols. The equilibrium density of 1.12 g/cm^3 is different from that reported for mustard spherosomal membrane (1.27 g/cm^3) (1). It is surprising for a structure with lipid and protein as the two major components to have an equilibrium density as high as 1.27 g/cm^3. An equilibrium density of 1.12 g/cm^3 in our study is a reasonable value since many cellular membrane components, including ER, Golgi, vacuole membrane, plasma membrane, and protein body membrane (17) have values in this range. The glyoxysomal membrane which is especially enriched in protein has an equilibrium density of 1.21 g/cm^3 (9).

The spherosomal membrane isolated by sucrose gradient centrifugation had a phospholipid to protein ratio of 0.77. The Cyt *c* reductase activity was insensitive to 1 μM antimycin A, and the activity toward NADH was 13.6 times higher than that toward NADPH. The protein components of the spherosomal membrane were analyzed by 10% slab gel electrophoresis (Fig. 2). Major and minor protein bands of diverse mol wt ranging from 10,000 to 70,000 were resolved, and the protein pattern was very different from those of KCl-washed membranes of mitochondria, ER, and glyoxysomes. In mustard, the proteins of the spherosomal membrane were also reported to be different from those of the ER in SDS-gel electrophoresis (1). In our experiments (Fig. 2), we were able to obtain a protein pattern from the glyoxysomal membrane similar to that reported (2, 4), having five to six major bands of mol wt ranging from 50,000 to 75,000 in the absence of deoxycholate treatment. However, our finding of the protein pattern of the ER membrane (from 4-day-old seedlings) is quite different from that (from 3-day-old seedlings) reported earlier (4).

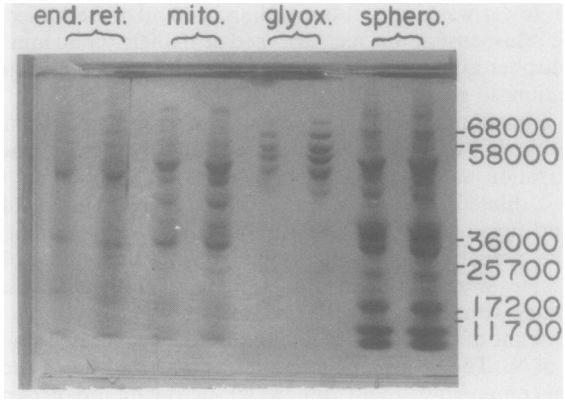

FIG. 2. Photograph of 10% SDS-acrylamide gel after electrophoresis of organelle membrane of castor bean. In each organelle preparation, the amount of protein applied to the right slot was twice that to the left slot. Mol wt shown on the right.

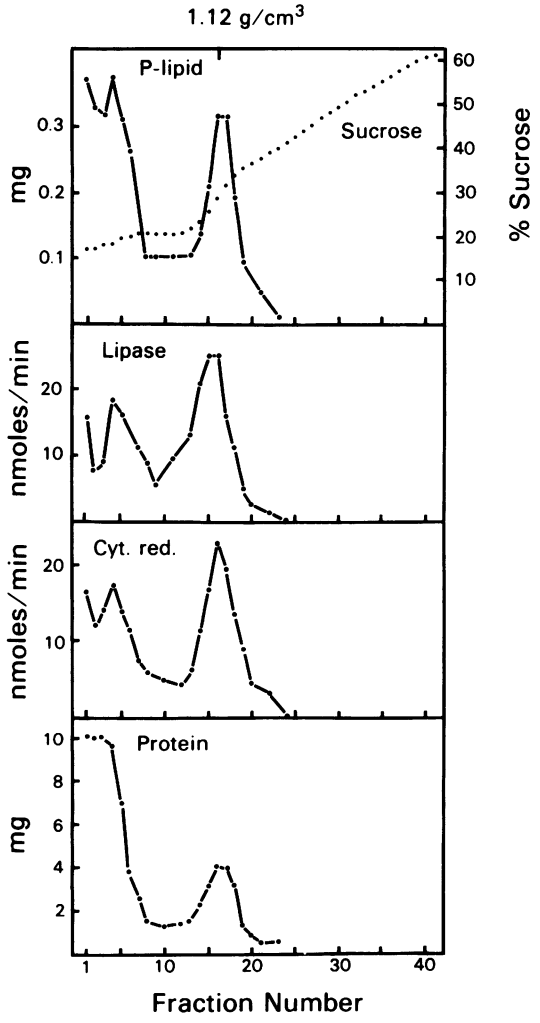

FIG. 1. Sucrose gradient centrifugation of spherosomes after the triacylglycerol had been extracted with diethyl ether. Contents are expressed as amount per gradient fraction.

The castor bean spherosomal membrane shares some similarities with the membrane of the ER, including the equilibrium density and the antimycin A-insensitive NADH-Cyt reductase. However, the protein and phospholipid components of the two membrane systems are different. Since the spherosomal membrane has been proposed to be derived directly (18) or indirectly (1) from the ER, it is reasonable that they should have some similarities. The differences in protein and lipid components may simply indicate that the spherosomal membrane originates only in specialized regions of the ER.

Formation and Degradation of Spherosomes. The formation and degradation of spherosomes and their membrane constituents during seed maturation and germination were followed. Spherosomes were isolated from the endosperm at various developmental stages, and the amount of lipid, phospholipid, acid lipase, protein, and Cyt reductase were measured. The levels of the various components increased and decreased in parallel patterns throughout the whole span of maturation and germination (Fig. 3). This striking similarity in developmental pattern of the spherosomal components indicates that the whole organelle is synthesized and degraded as one individual unit. No addition or removal of spherosomal components was detected throughout development.

Our finding negates the idea that during maturation, most of the triacylglycerol is formed massively as lipid droplets in the cytoplasm and the membranes are synthesized subsequently. However, the possibility still exists that a small percentage of the spherosomes are formed at time intervals, with the lipid droplet being synthesized before the membrane. During triacylglycerol accumulation, the triacylglycerol was synthesized together with an acid lipase in the same organelle (Fig. 3). This lipase was always in an active form, and spherosomes isolated from the maturing seed went through complete autolysis *in vitro* (Fig. 4). Our finding excludes the possibility that the acid lipase is formed only during the final period of seed dehydration so that the enzyme does not have the proper environment to hydrolyze the storage triacylglycerol. Whether or not the organelle does go through a partial

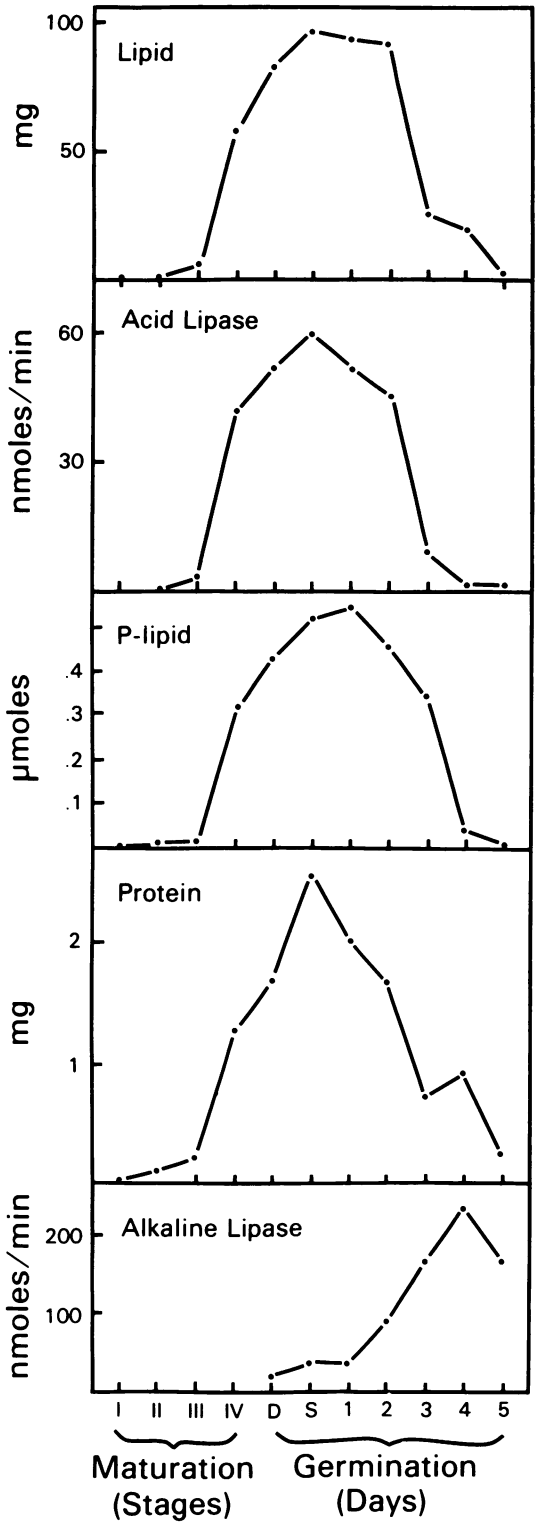

FIG. 3. Compositional changes in spherosomes isolated from castor bean during seed maturation and germination. Stages of seed maturation were arbitrarily designated according to morphological criteria. Whereas seeds of *R. communis* L. var. Hale were used in the germination study, the seeds used in the maturation study were of an unknown variety of *R. communis* L. obtained from a local garden. Dry seeds (D) were soaked for 24 h (S) and allowed to germinate for various days. Contents are expressed as amounts per seed.

autolysis *in vivo* is unknown. The maturing cell may have a control mechanism, such as an elevation of the pH, so that wasteful spherosome autolysis does not occur.

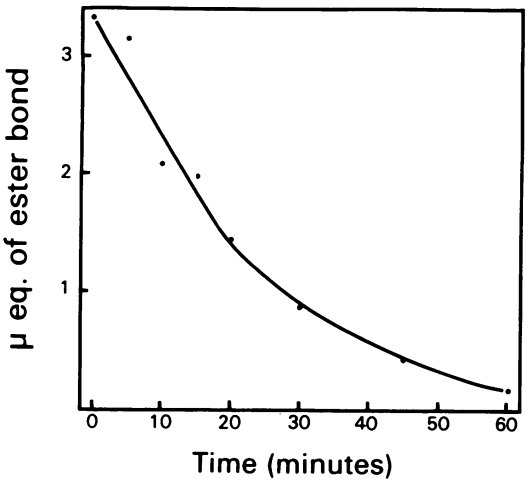

FIG. 4. Autolysis of spherosomes isolated from maturing castor bean (stage 4).

Since at all stages during germination, the spherosomes contain an acid lipase which can hydrolyze triacylglycerol to glycerol and fatty acid, the participation of a second enzyme in lipid hydrolysis appears to be unnecessary. It was shown that spherosomes isolated from the dry seed can go through autolysis to produce fatty acid (16). Furthermore, this autolysis is complete in less than 1 h, indicating that the *in vitro* acid lipase activity is high enough to account for the *in vivo* lipolysis. Alkaline lipase of the glyoxysomes, a possible candidate for the second hydrolytic enzyme (14), did not appear in full activity until most of the lipid was depleted (Fig. 3). The function of the glyoxysomal alkaline lipase which can hydrolyze monoacylglycerols but not di- or triacylglycerols (14) is still unknown.

At the beginning of our studies, we recognized that it was difficult to obtain a quantitative yield of spherosomes from the dry seeds by our aqueous extraction procedure due to the highly dehydrated nature of the seed. No problem was encountered with seed from other stages of maturation and germination. In order to quantitate the spherosomal components in the dry seeds, the following procedure was used. Dry seeds were ground in the aqueous grinding medium as usual, and the amount of lipid and other components in isolated spherosomes was measured. The dry seeds were also ground vigorously with a chloroform and methanol extraction (3) to recover the total lipid quantitatively. The amount of total lipid obtained by organic solvent extraction in comparison with that by aqueous solvent extraction was used to calculate the effectiveness of the aqueous solvent extraction. An average of 28% more lipid was extracted with organic solvent than with aqueous solvent. After correction for incomplete extraction of lipid and other spherosomal components by the aqueous medium (Fig. 3), the levels of acid lipase and total lipid followed each other closely before and immediately after imbibition. The incompleteness of aqueous solvent extraction as compared with organic solvent extraction appears to be the reason for the increase in the total lipase (by aqueous solvent extraction) but not the total lipid (by organic solvent extraction) during imbibition that has been reported earlier (14). In reality, no increase in acid lipase activity occurred.

We should emphasize that the two-lipase system appears to be unique to castor bean. In many other plant species, the spherosomes of dry seeds possess little or no lipase activity at acidic, neutral, or alkaline pH values (10). However, the glyoxysomes obtained from their germinating seedlings contain an alkaline lipase similar to that of the castor bean (8, 10). On the other hand, the cotyledons of germinating jojoba contain an alkaline lipase associated with the spherosomes and have no glyoxysomal lipase

(13). These studies suggest that there are diverse mechanisms of storage lipid hydrolysis among the oil seeds of various plant species.

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LITERATURE CITED

1. BERGFELD B, YN HONG, T KÜHNL, P SCHOPFER 1978 Formation of oleosomes (storage lipid bodies) during embryogenesis and their breakdown during seedling development in cotyledons of *Sinapis alba* L. Planta 143: 297–307
2. BIEGLMAYER C, H RUIS 1974 Protein composition of the glyoxysomal membrane. FEBS Lett 47: 53–55
3. BLIGH EG, WJ DYER 1959 A rapid method of total lipid extraction and purification. Can J Biochem 37: 911–915
4. BOWDEN L, JM LORD 1976 Similarities in the polypeptide composition of glyoxysomal and endoplasmic reticulum membrane from castor-bean endosperm. Biochem J 154: 491–499
5. DITTMER JC, AW WELLS 1969 Quantitative and qualitative analysis of lipids and lipid components. Methods Enzymol 14: 482–531
6. DONALDSON RP 1976 Membrane lipid metabolism in germinating castor bean endosperm. Plant Physiol 57: 510–515
7. DONALDSON RP 1977 Accumulation of free ricinoleic acid in germinating castor bean endosperm. Plant Physiol 59: 1064–1066
8. HUANG AHC 1975 Comparative studies on glyoxysomes from different fatty seedlings. Plant Physiol 55: 870–874
9. HUANG AHC, H BEEVERS 1973 Localization of enzymes within microbodies. J Cell Biol 58: 379–389
10. HUANG AHC, RA MOREAU 1978 Lipases in the storage tissues of peanut and other oil seeds during germination. Planta 141: 111–116
11. JELSEMA CL, DJ MORRÉ, M RUDDAT, C TURNER 1977 Isolation and characterization of the lipid reserve bodies, spherosomes, from aleurone layers of wheat. Bot Gaz 138: 138–149
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, AHC HUANG 1977 Gluconeogenesis from storage wax in the cotyledons of jojoba seedlings. Plant Physiol 60: 329–333
14. MUTO S, H BEEVERS 1974 Lipase activities in castor bean endosperm during germination. Plant Physiol 54: 23–28
15. ORY RL, LY YATSU, HW KIRCHER 1968 Association of lipase activity with the spherosomes of *Ricinus communis*. Arch Biochem Biophys 264: 255–264
16. ORY RL, AJ ST ANGELO, AM ALTSCHUL 1960 Castor bean lipase: action on its endogenous substrate. J Lipid Res 1: 208–213
17. QUAIL PH 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
18. SCHWARZENBACH AM 1971 Observations on spherosomal membranes. Cytobiologie 4: 145–147
19. WANNER G, RR THEIMER 1978 Membranous appendices of spherosomes (oleosomes). Possible role in fat utilization in germinating oil seeds. Planta 140: 163–169
20. WEBER K, M OSBORNE 1975 Proteins and sodium dodecyl sulfate: molecular weight determination on polyacrylamide gels and related procedures. *In* H Neurath, RL Hill, eds, The Proteins. Academic Press, New York, pp 180–225
21. YATSU LY, TJ JACKS 1972 Spherosome membranes. Half unit-membranes. Plant Physiol 49: 937–943