# Phospholipid Degradation in Membranes of Isolated Soybean Lipid Bodies

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**Membrane degradation was observed in lipid bodies isolated from soybean seeds and incubated at 30°C for 6- and 18-day periods. Three events occurred during degradation. Phosphatidylcholine and phosphatidylethanolamine (PE) were converted to phosphatidic acid presumably by phospholipase D, but PE decreased even in the absence of phospholipase D enzymic activity. A substance that cochromatographed with cardiolipin appeared as a product after 18 days incubation.[1]**

Concern about the quality of U.S. export soybeans has focused on increased levels of free fatty acids in processed, degummed oil (1,2). In addition, high oil losses are incurred in processing due to the emulsifying properties of phospholipids (3). Residual phospholipids also result in poor flavor stability and darkening of the finished oil products (4).

In oil refining, phospholipids are either hydratable and can be removed by degumming, or they are nonhydratable (5) and require alkali refining. The greater the amount of nonhydratable phospholipid, such as phosphatidic acid (PA), the greater the cost for the finished oil.

High levels of free fatty acids can result from lipase action on triglycerides that are held in lipid bodies (oleosomes). Presumably, for such lipolysis to occur, the membrane of the lipid body must be perturbed to expose the oil to cytoplasmic enzymes.

This report examines the action of constitutive enzymes on predominant phospholipids in membranes of isolated lipid bodies during their storage. Degradation of the membrane is largely caused by the action of phospholipase D, which converts phosphatidylcholine (PC) to PA (6). Previous studies have followed changes in stored, whole soybeans (7–9), but there is little information on biomechanisms by which degradation occurs.

## EXPERIMENTAL PROCEDURES

*Lipid body isolation.* The general procedure for lipid body isolation followed that of Yatsu and Jacks (10). Testae and hypocotyls of 70 g of soybeans (*Glycine max.* L.), Century variety, 1986 harvest, were removed and the beans were then surface sterilized with 10% Chlorox® solution for several minutes followed by twenty rinsings with distilled water. The beans were soaked overnight in 280 ml of 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 7.2, at 4°C. All procedures were conducted at 4°C. After the soak, an additional 70 ml of buffer was added, and the beans were homogenized in a Waring Blendor® for 60 sec. The homogenate was filtered through two layers of cheesecloth and centrifuged 20 min at 31,300 *g* and 4°C. The floating fat pad was removed and rehomogenized in five volumes of cold, fresh buffer. This centrifugation-homogenization procedure was performed five times followed by a wash in cold distilled water containing cycloheximide (50 mg/L) and sodium azide (0.02%) to control microbial contamination. Two subsequent washings were made with 0.5 N sodium acetate buffer, pH 5.6, with the two additives. The bulk of the acetate buffer was removed from the lipid bodies by repeated centrifugation at 31,300 *g* and withdrawal of solution from beneath the compressed lipid body pad. This lipid body isolate was stored at 4°C.

*Lipid body incubation.* Samples of 0.2 to 0.3 g of wet lipid body isolate were mixed thoroughly with 5 ml of 0.1 M sodium acetate buffer, pH 5.6. These and unagitated samples without buffer were incubated at 30°C. Incubation was terminated by immersing each sample tube in boiling water for 10 min to inactivate enzymes.

*Phospholipid extraction.* Lipids were extracted into chloroform by the Bligh-Dyer method (11). Phospholipids were isolated with a Waters SEP-PAK silica cartridge. Chloroform was used to remove nonpolar lipids, followed by acetone and then methanol to collect the polar lipids. After the removal of methanol, the residue, redissolved in chloroform, was filtered through sintered glass and the filtrate was concentrated and stored under $N_2$.

*Separation and quantitation of phospholipids.* Phospholipids were separated by two-dimensional silica gel TLC (Merck 60G) with chloroform-methanol-ammonia (65:30:4 by vol), adapted from Rouser *et al.* (12), followed by chloroform-acetone-methanol-acetic acid-water (50:20:10:15:5 by vol) (13). Spots were located with 8-anilino-naphthalene-1-sulfonate, and phospholipids were identified by comparison to standards from Sigma Chemical Company (St. Louis, MO) and Avanti Polar Lipids, Inc. (Birmingham, AL). Phospholipid silica spots and a plate background spot were analyzed for phosphorus by combined Bartlett (14) and Broeckhuyse (15) variations of the Fiske-SubbaRow method (16). Absorbance was measured at 820 nm.

Microbial count samples (0.1 to 0.2 g) were suspended in 2 ml of sterile 0.1% peptone water. After dispersal in the diluent, the sample were serially diluted and spread-plated on tryptone-glycose-yeast extract agar (tryptone, 5.0 g; glucose, 1.0 g; yeast extract, 5.0 g; dipotassium hydrogen phosphate, 1.0 g; agar, 20 g; distilled water 1 L). Duplicate plates were incubated aerobically for 14 days and anaerobically (BBL GasPak system) for 5 days at 30°C.

## RESULTS

Table 1 presents the concentrations of phospholipid species in lipid bodies after 0, 6, and 18 days of incubation at 30°C without buffer. An additional 18-day sample examined for bacteria and fungi showed no microbial contamination. In this and subsequent experiments, no aerobic or anaerobic organisms were detected at the lowest dilution, i.e., 0.1 to 0.2 g of sample in 2 ml of diluent. If any microorganism had been present, its

TABLE 1

**Phospholipid Concentrations (mg PL/100 mg Lipid Bodies)[a]**

| Days of Incubation | Phospholipid | | | | | | Total PL |
|---|---|---|---|---|---|---|---|
| | PA | PC | PE | PI | PG | CL | |
| 0 | 0.10 ± 0.01 | 0.55 ± 0.02 | 0.13 ± 0.01 | 0.15 ± 0.01 | 0.033 ± 0.004 | ND[b] | 0.97 ± 0.05 |
| 16 | 0.18 ± 0.03 | 0.37 ± 0.03 | 0.10 ± 0.02 | 0.15 ± 0.01 | 0.033 ± 0.005 | ND[b] | 0.83 ± 0.10 |
| 18 | 0.14 ± 0.02 | 0.33 ± 0.03 | 0.06 ± 0.01 | 0.16 ± 0.02 | 0.027 ± 0.004 | 0.032 ± 0.006 | 0.74 ± 0.09 |

[a]Abbreviations are: PA = Phosphatidic acid; PC = Phosphatidylcholine; PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PG = Phosphatidylglycerol; CL = Cardiolipin/Diphosphatidylglycerol; PL = Phospholipid.
[b]None detected.

concentration would have been less than 10 per gram of sample.

Initial work (unpublished) provided results similar to those presented here, but the data were suspect due to the discovery of bacterial and fungal growth. As a consequence, sodium azide and cycloheximide were introduced to prevent contamination. These antibiotics neither inhibited the activity of phospholipase D nor altered the degradation observed in oleosomes.

Table 1 shows that PA increased during the first six days and changed little thereafter. PC declined rapidly at first and then its rate of disappearance slowed. Phosphatidylethanolamine (PE) also declined in quantity, but at a slower rate than PC — 23% compared to 33% in the first 6 days and more rapidly, 40% compared to 16%, in the next 12 days. Phosphatidylinositol (PI) appeared unaffected. Phosphatidylglycerol (PG) decreased 16–18% during incubation, but this result is well within standard deviation for the analysis. A substance tentatively identified as cardiolipin (CL) appeared after prolonged incubation. Consistent with these chemical changes there was a 24% overall decrease in recoverable phospholipid.

The initial oleosome material, when well-mixed after centrifugation and prior to incubation, was opaque and off-white, but during incubation it became transparent and yellow-brown. We view this change in appearance as evidence of a change in the organization of oleosome constituents due to concomitant chemical changes.

Table 1 also illustrates variable enzyme activity in the isolated oleosomes. Results at time zero reveal little variance in phospholipid levels. As the material aged, hydrolysis proceeded, and the analyses show a much larger variation between samples as if the lipolytic enzymes were distributed nonuniformly or their specific activities and stabilities were being affected differentially. Sample heterogeneity cannot be excluded, but it is equally probable that real differences in lipid body enzymes or their microenvironments caused such results. The variation in phospholipid composition is especially apparent in PE levels after 6 days. PE in samples 6 and 7 was not degraded to the extent that it was in the other three 6-day samples. Similarly, sample 15 shows less degradation of PC than expected in comparison to PE or other 18-day samples. Such nonuniformity suggests unrecognized variables. In this regard, the presence of calcium, a cofactor or potentiator of phospholipase D, was confirmed at a concentration of $1.3 \times 10^{-6}$ moles $Ca^{2+}$/g oleosome material.

The effect of incubation on dispersed lipid bodies is presented in Table 2. In this situation, the first group of samples was at pH 5.6, an optimum pH for phospholipase D activity (17). The second group was at pH 7.2, which was used in isolating the lipid bodies and which is less conducive to phospholipase action (18). The oleosome material was from a different preparation than that used in work reported in Table 1. The phospholipid composition of different preparations incurred only minor fluctuations even though the lipid body isolation procedure is lengthy. Samples (2–3 mg each) were incubated at 30°C without agitation to minimize abrasion and shear during lengthy incubation. Both groups were virtually free of bacterial contamination at the end of the incubation period.

Table 2 shows in relative terms that the phospholipids were degraded faster at lower pH, as would be expected of hydrolysis by phospholipase. Degradation of PC was similar to that in non-dispersed lipid bodies, but there was no accompanying large increase in PA. There was a large decrease in PE, but PI concentration increased as if PI was either completely inert or was being augmented perhaps by phosphatidyl transfer. If it is assumed that PI remains constant as it did in non-dispersed lipid bodies (Table 1), the values in Table 2 indicate that the concentrations of other phospholipids declined. If such were true, and sample 6 (Table 2, pH 5.6) had a PI concentration of 0.16 mg/100 mg lipid bodies, other phospholipid levels would be as follows: 0.06 mg PA, 0.26 mg PC and 0.02 mg PE. These values are all lower than those found for non-dispersed lipid bodies (Table 1). Alternatively, if PC and PE were converted to PI or substances assayed as PI, the apparent concentration of PI might easily reach 30–40% with less change in PA, PC, and PE.

## DISCUSSION

Previous studies on the degradation of phospholipids in soybeans have been conducted on whole beans subjected to moisture, heat and freezing (3,8,19–22). The premise of this work is that subtle perturbation of seed substructures, either physically or biochemically, can lead to a change in otherwise undamaged beans.

The evidence of enzymic activities, that produce changes in isolated lipid bodies which mimic those in intact seed, offers prospects of simpler systems for investigating the roles of subcellular components in seed destabilization.

In as much as phospholipids make up most of the oleosome membrane, their cumulative loss (Table 1) means the membrane itself broke down. While the to-

**TABLE 2**

**Phospholipid Concentrations (% by weight) from Lipid Bodies[a]**

| Days Incubated | pH 5.6[b] | | | | pH 7.2[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | PA | PC | PE | PI | PA | PC | PE | PI |
| 0 | 12 | 60 | 15 | 14 | | | | |
| 1 | 15 | 53 | 11 | 20 | 13 | 58 | 15 | 14 |
| 2 | 12 | 50 | 9 | 29 | ND[d] | ND | ND | ND |
| 4 | 13 | 50 | 6 | 31 | 10 | 54 | 12 | 24 |
| 8 | 14 | 51 | 2 | 33 | 12 | 59 | 4 | 25 |
| 12/15[e] | 12 | 52 | 4 | 32 | 12 | 54 | 4 | 30 |
| 29 | 5 | 50 | 2 | 43 | 11 | 54 | 4 | 31 |

[a]Abbreviations are as in Table 1.
[b]In 0.1 M Na acetate buffer.
[c]Without buffer.
[d]Not determined.
[e]Incubated for 12 d at pH 5.6 or 15 d at pH 7.2.

tal oleosome phospholipid content was decreasing, the total lipid extracted increased slightly, which would be consistent with the destruction of substructure binding or occluding additional lipid. The 24% loss of phospholipids on incubation is not unprecedented. Priestly and Leopold have examined the degradative effects of natural and accelerated aging on germinating soybean seeds (23,24). They also observed losses of extractable phospholipids, but only in acceleratedly aged beans. In contrast, Pearce and Abdul Samad found reductions in phospholipids in both aging environments with peanuts (25). Neither group was able to give reasons for the observed losses. Nakayama *et al.* observed a 40% loss of phospholipids in soybeans stored for six months at 35°C and 13% moisture level (8). Saio *et al.* examined the phosphorus level in soakwater and witnessed phosphorus losses up to 25% on soybeans stored at 35°C and 80% humidity (20). Apparently, phospholipids are hydrolyzed and resultant phosphate is carried away in the aqueous portion of the initial Bligh-Dyer extraction.

PC hydrolysis likely results from phospholipase D activity. This enzyme could also be credited with the pronounced reduction of PE except for the results of the second series (Table 2). When oleosomes were immersed in sodium acetate buffer, phospholipase D hydrolysis of PC was negligible while PE was still acted upon at a rate equal to or greater than that of the series without buffer solution.

This evidence suggests that phospholipase D may be bound to the lipid bodies in a way that leaves it easily inactivated or carried away into solution either freely or with initial disruption of the membrane. Pertinent to this question, Yoshida found phospholipase D to bind quite tightly to microsome membranes in the bark tissue of black locust trees (26), and Galliard cited the existence of both soluble and particulate-bound phospholipase D (18).

A different form of phospholipase D must account for the reduction of PE in the second series (Table 2). PE hydrolysis was vigorous even in suspended lipid bodies, which indicates that if the PE hydrolysis is due to enzymatic activity then such an enzyme is membrane-bound. From the data obtained, PE reduction is not dependent upon phospholipase D, but phospholipase D activity may be dependent or related to the disappearance of membrane PE. Possibly more than one phospholipase is associated with lipid bodies.

Presence of cardiolipin in soybean seed has been observed previously (27). Still, the emergence of cariolipin here at an advanced stage of incubation is surprising and its purpose is without explanation. Most likely it is synthesized by phosphatidyl transfer (28). Similarly, it is possible that PI levels could remain unchanged if inositol were available for phosphatidyl transfer.

Lynch and Thompson noted that a membrane's physical state (29) is controlled by composition and environmental conditions. In this regard, Qu *et al.* have shown the proteins of lipid bodies vary greatly among plant species (30). And, as the phospholipids, Witt and Gercken commented that a certain composition appears to be necessary to maintain membrane structure and function (31). They added that changes in phospholipid composition lead to structure alterations that are not well understood. Furthermore, Priestly and Leopold cited that lipid peroxidation is evident in the polar lipids of the membrane fraction of soybeans aged naturally, but not in acceleratedly aged beans (25). In either case, they commented that deteriorative changes, which are capable of causing aging in seeds, are probably manifold and may vary according to storage conditions (24).

## REFERENCES

1. Anonymous, In Brief, *Agricultural Outlook 125*:9 (1986).
2. Anonymous, ASA Topics, *J. Am. Oil Chem. Soc. 63*:1224 (1986).
3. Kouzeh Kanani, M., D.J. van Zuilichem, J.P. Roozen and W. Pilnik, *Lebensm.-Wiss. u. Technol. 17*:39 (1984).
4. Evans, C.D., P.M. Cooney, C.R. Scholfield and H.J. Dutton, *J. Am. Oil Chem. Soc. 31*:295 (1954).
5. Nielsen, K., *Studies on the Non-Hydratable Soybean Phosphatides*, Maxson and Co. Ltd., London, 1956.
6. Nakayama, Y., and M. Kito, *Agric. Biol. Chem. 45*:2155, 1981.
7. Mounts, T.L., J.M. Snyder, T.T. Hinsch, A.J. Bongers and A.R. Class, *J. Am. Oil Chem. Soc. 64*:682 (1987).
8. Nakayama, Y., K. Saio and M. Kito, *Cereal Chem. 58*:260 (1981).
9. Urbanski, G.E., L.S. Wei and A.I. Nelson, *J. Food Sci. 45*:208 (1981).
10. Yatsu, L.Y., and T.J. Jacks, *Plant Physiol. 49*:937 (1972).
11. Bligh, E.G., and W.J. Dyer, *Can. J. Biochem. 37*:911 (1959).

12. Rouser, G., S. Fleischer and A. Yamamoto, *Lipids 5*:494 (1970).
13. Yavin, E., and A. Zutra, *Anal. Biochem. 80*:430 (1977).
14. Bartlett, G.R., *J. Biol. Chem. 234*:466 (1959).
15. Broekhuyse, R.M., *Biochem. Biophys. Acta 152*:307 (1968).
16. Fiske, C.H., and Y. SubbaRow, *J. Biol. Chem. 66*:375 (1925).
17. Heller, M., in *Advances in Lipid Research*, Vol. 16, edited by R. Paoletti and D. Kritchevsky, Academic Press, New York, 1974, p. 267.
18. Galliard, T., in *Methods in Enzymology*, Vol. 31, edited by S. Fleischer and L. Packer, Academic Press, New York, 1974, p. 520.
19. Wilson, R.F., and R.W. Rinne, *Plant Physiol. 57*:270 (1976).
20. Saio, K., I. Nikkuni, Y. Ando, M. Otsuru, Y. Terauchi and M. Kito, *Cereal Chem. 57*:77 (1980).
21. Kanamoto, R., Y. Wada, G. Miyajima and M. Kito, *J. Am. Oil Chem. Soc. 58*:1050 (1981).
22. Racicot, L.D., and A.P. Handel, *Ibid. 60*:1098 (1983).
23. Priestley, D.A., and C.A. Leopold, *Plant Physiol. 63*:726 (1979).
24. Priestley, D.A., and C.A. Leopold, *Physiol. Plant. 59*:467 (1983).
25. Pearce, R.S., and Abdul Samad, I.M., *J. Exp. Bot. 31*:1283 (1980).
26. Yoshida, S., *Plant Physiol. 64*:241 (1979).
27. Wagner, H., and P. Wolff, *Fette, Seifen, Anstrichm. 6*:125 (1964).
28. Galliard, T. in *The Biochemistry of Plants*, Vol. 4, edited by P.K. Stumpf and E.E. Conn, Academic Press, New York, 1980, p. 105.
29. Lynch, D.V., and G.A. Thompson, Jr., *Trends Biochem. Sci. 9*:442 (1984).
30. Qu, R., S.-m. Wang, Y.-h. Lin, V.B. Vance and A.H.C. Huang, *Biochem. J. 235*:57 (1986).
31. Witt, W., and G. Gercken, *Biochim. Biophys. Acta 862*:100 (1986).

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