Changes in Phospholipid Levels During High Moisture Storage of Soybeans

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ABSTRACT AND SUMMARY

Fungicide-treated and untreated commercial soybeans were stored at high temperature and humidity (35 C and 85% RH) in separate environmental chambers. Crude oil was extracted from the beans periodically during a 23-day storage period and its phospholipids separated by three-solvent system, two-dimensional thin layer chromatography. The amount of phospholipids was determined by spectrophotometric assay of inorganic phosphate liberated after perchloric acid digestion. The level of phospholipids increased substantially during storage in treated and untreated soybean samples. Phosphatidic acid, phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl choline were the major phospholipids. Phosphatidyl choline was the principal phospholipid in extracted crude oil from treated and untreated beans during storage. The major phospholipid levels increased more rapidly during the first 6 days of storage than during the remainder of the period in both samples. The data indicated that increased phospholipid levels occurred independently of mold growth and could have been the result of water imbibition and germination of the beans during storage.

INTRODUCTION

High humidity is a major problem for soybean growers throughout the United States, especially in the Southeast. With high temperatures, these conditions are conducive to mold growth on soybeans which contribute to bean deterioration during storage. Considerable production losses occur as a result (1). Previous studies have shown that high moisture is an absolute requirement for mold growth on soybeans (2). Oils extracted from storage-damaged soybeans (moldy) are characterized by high levels of free fatty acids and changes in inorganic phosphate and phospholipids (1-3). Such oils are very difficult to refine (1,4,5). Increased levels of these compounds are thought to be produced by mold growth (2). Since high humidity and temperature also support water imbibition and seed germination in soybeans, we decided to determine if these processes were also operative during bean storage and contributed to changes in the major phospholipid content of the oil.

EXPERIMENTAL PROCEDURES

Two samples of clean, whole soybeans (2.3 kg, mixed varieties) were spread in aluminum wire-screen containers (5 x 40 x 60 cm) and equilibrated at room temperature for 30 min. One sample was dusted with 50% Captan [N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboxiamide], a potent fungicide. Treated and untreated beans were then exposed to 35 C constant temperature and 85% RH in separate Climate-labs. Samples were taken periodically from treated and untreated beans during 23 days of storage and the extracted crude oil analyzed for major phospholipid content and total lipid phosphate. The 17- and 21-day samples from untreated beans included adhering seed-coat mold. Unexposed and mold-free beans were also analyzed and served as 0-day samples for both fungicide-treated and untreated beans.

Crude Oil Extraction

Treated and untreated beans were extracted similarly. Samples (5.0-5.5 g) were immersed in liquid nitrogen for 5 min and transferred to a large mortar which contained liquid nitrogen to a depth of about 5 cm. The beans were crushed to a fine powder by application of a downward pressure and rocking motion with the pestle. After liquid nitrogen had evaporated, the powder was quantitatively transferred to a homogenizer flask and allowed to stand for 10 min with 150 ml of chloroform-methanol (2:1). This solvent system has been used to extract phospholipids from soybeans and flaxseed (6-8). The mixture was blended at 35-40% of maximum speed for 4 min in a Virtis "45" homogenizer with ice packed around the flask to prevent heat build-up. The mixture was filtered with fine-sintered glass under vacuum, and solvent removed from the filtrate

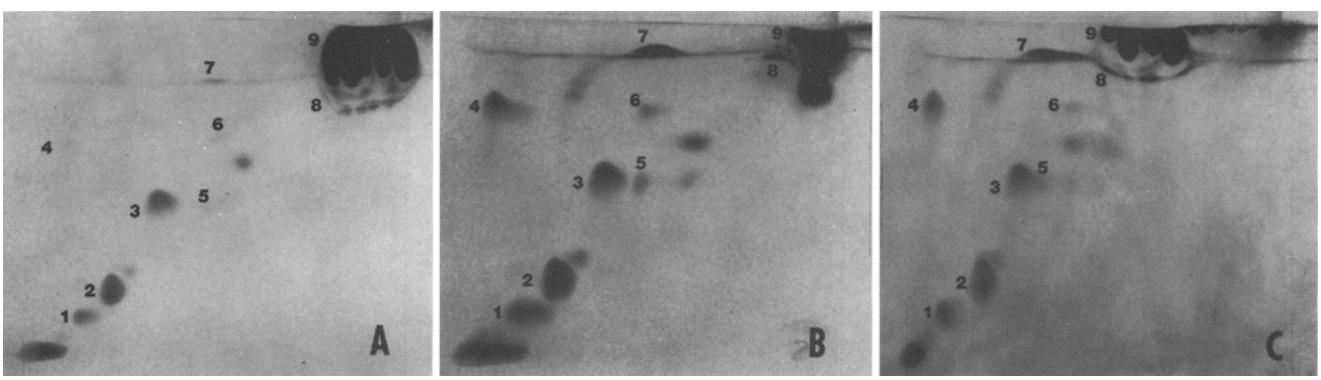

FIG. 1. Separation of soybean oil phospholipids by two-dimensional thin layer chromatography. (A) Three-solvent system, two-dimensional thin layer chromatography of soybean oil phospholipids from zero-day beans. 1. Pure chloroform, 2. chloroform-methanol-7N ammonium hydroxide, from left to right. 3. chloroform-methanol-acetic acid-water, from bottom to top. Origin is lower left-hand corner. (B) Same as A, except oil from soybeans stored for 21 days. (C) Same as B, except initial chromatography with chloroform omitted. Compound identification on all plates: 1. phosphatidyl inositol, 2. phosphatidyl choline, 3. phosphatidyl ethanolamine, 4. phosphatidic acid, 5. and 6. unidentified phospholipid, 7. free fatty acids, 8. unidentified phospholipid, 9. triglycerides.

by reduced pressure at 35 C with a rotary evaporator.

The extract was redissolved in chloroform (125 ml) and shaken with saturated sodium chloride (50 ml). The chloroform layer was removed, and the aqueous salt phase was re-extracted twice with 25 ml of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and filtered through Whatman #4 filter paper. The chloroform was removed under reduced pressure at 35 C. The pressure was further reduced to 60 mm Hg at 35 C for 15 min, and the oily extract flushed with dry nitrogen to remove residual chloroform. The extract was transferred to a 16 x 75 mm polyethylene centrifuge tube and stored at 2 C for 30 min for the complete precipitation of a fine suspension, thought to be proteolipid (9). The extract was centrifuged at 23,400 x g at 0 C for 10 min and the crude oil was carefully removed with a transfer pipet and stored at -10 C. No additional phospholipids could be obtained by this procedure, even when the powdered beans were extracted for 24 hr with the solvent. The yield was 3.0 g of crude oil per 15 g (wet wt) of soybeans from treated and untreated samples.

Phospholipid and Inorganic Phosphate Determinations

Phospholipids were separated by three-solvent system, two-dimensional thin layer chromatography (TLC) on precoated Silica Gel 60 plates (0.25 mm) (E. Merck, Darmstadt, Germany). All tanks were lined with Whatman #1 chromatography paper to saturate with developing solvent. Five microliters of crude oil were applied to each plate (20 x 20 cm) and developed in chloroform (1st dimension). The solvent front was allowed to run for 14 cm and the plate air-dried in the hood for 10 min before two-dimensional chromatography (2D-TLC) (10). The chromatogram was developed in the same direction as before with chloroform-methanol-7N ammonium hydroxide (65:30:4) (v/v) until the solvent front had run for 10 cm. The chromatogram was air-dried in the hood for 23 min and then developed in the second dimension with chloroform-methanol-acetic acid-water (170:25:25:4) (v/v) until the front had run for 9 cm. The phospholipids were identified with molybdate and specific spray reagents (11) and by comparison with R_f values of standard phospholipids (Supelco, Inc., Bellefonte, PA) similarly chromatographed. The plates shown in Figure 1 were sprayed with a modified anisaldehyde-sulfuric acid reagent (12).

After identification, plates to be quantitatively analyzed were visualized with I_2 vapor. Individual phospholipid spots were outlined with a sharp blade and quantitatively scraped into 15 x 150 mm culture tubes. A portion of the developed plate, devoid of compounds, was also scraped into a tube to serve as a blank. Three to four plates were required to provide sufficient sample for each analysis. In this manner, total lipid phosphate analyzed ranged from 0.3 to 0.7 μmoles which is well within the linear quantitation range of the procedure. The phospholipids and blank were digested with 0.8 ml 70% perchloric acid at 130 C for 30 min in a Temp-blok module heater. After the initial digestion, a small glass bead was added to each tube and digestion completed with a micro-burner. Inorganic phosphate was determined spectrophotometrically at 820 nm (13).

For the analysis of total lipid phosphate, triplicate 5 μl samples of oil were separated by TLC with chloroform (solvent front—14 cm), and all phospholipid spots combined and analyzed as a single sample. Two different oil samples (six spots) could thus be conveniently determined from a single plate. Total lipid phosphate of the oil was determined from polar lipid spots as described above, except 1.0 ml of perchloric acid was used for the digestion. This procedure was found to be less hazardous than direct digestion of crude oil with perchloric acid. Total weight of the oil on each chromatogram was computed from oil density measurements and standard volumes of applied oil. Individual phospholipids and total lipid phosphate were determined in duplicate for each sample.

RESULTS AND DISCUSSION

The separation of phospholipids in crude soybean oil by thin layer chromatography is shown in Figure 1. Phosphatidic acid, phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl choline were the major phospholipids from treated and untreated beans during storage. Unidentified phospholipids were observed but were not determined since their levels did not appear to change as much as did the four major phospholipids. Figures 1A and 1B show the increase in phospholipid content of oil from untreated soybeans stored for 0 and 21 days. Chromatograms from treated beans gave similar results. Figures 1B and 1C compare the separation of lipids by the three-solvent system, 2D-TLC and by the standard two-solvent system, 2D-TLC. Separation of phosphatidyl ethanolamine (spot #3) from an unidentified phospholipid (spot #5) is better in Figure 1B than in Figure 1C and permitted a more precise quantitation of phosphatidyl ethanolamine.

During storage, the content of major phospholipids increased substantially in crude oil from treated and untreated beans (Table I). The substantial increase in phosphatidic acid in treated and untreated beans may be of interest to refiners since it is the principle nonhydratable phospholipid responsible for refining losses (5). Phosphatidyl inositol levels increased fivefold in 21 days of storage in untreated beans and almost threefold in treated beans after 23 days (Table I). Initially, phosphatidyl inositol ranked third in abundance among the four phospholipids but became the second most abundant after 6 days in untreated beans. In treated beans, this lipid did not become second in abundance until 23 days (Table I). Phosphatidyl choline was the principal phospholipid in extracted oil in both treated and untreated beans and increased twofold after 21 and 23 days of storage. Phosphatidyl ethanolamine also increased about twofold in content during the same period from both samples (Table I).

Major phospholipid levels in extracted oil from untreated beans ranged from 1.1% (0-day) to 3.1% (21 day) on a weight basis and 1.6% (0-day) to 3.2% (23 day) from treated beans (Table II). Values of 9-10% have been reported for total phospholipids in soybean oil (7,14). The soybeans used in the present study were smaller (5.0 x 7.0 mm) and much lighter (151.4 mg/bean) than average, but were judged mature by germination tests (90%). Mature soybean size and weight are reported to be 7.0 x 7.0 mm and 220-260 mg/bean, respectively (7,14). Also, the beans we used were produced locally, and growth environment has been found to affect the chemical composition of soybeans (15-17). Possibly the phospholipid levels could also be affected. The lower percentage of phosphatidyl choline in 0-day beans, 33-35% as compared to 45-46% (7,18), may have been the result of these effects (Table II).

Initially, the composition of the phospholipid fraction of crude soybean oil was similar to previously reported values, except for phosphatidyl choline. Only phospholipids from treated beans at the 4th and 10th day of storage showed similar composition of the major phospholipids with those of reported values (Table II) (7,14,18). Individual phospholipid levels and composition changed the most during the first 4 and 6 days of storage in treated and untreated beans (Table I and II). These data indicate that the rate of increase in phospholipid levels was greatest between 0 and 4-6 days of storage.

Mold growth was detected by visual observation of

TABLE I

Major Phospholipids in Extracted Oil from Stored Soybeans[a]

Days stored	Without fungicide				With fungicide			
	Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl ethanolamine	Phosphatidyl choline	Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl ethanolamine	Phosphatidyl choline
0	tr	1.95 ± 0.15	3.81 ± 0.01	5.10 ± 0.10	0.46 ± 0.23	3.00 ± 0.25	4.61 ± 0.23	6.23 ± 0.04
4	-	-	-	-	1.29 ± 0.01	6.19 ± 0.48	7.97 ± 0.09	14.29 ± 0.66
6	1.83 ± 0.21	6.59 ± 0.10	5.80 ± 0.40	8.80 ± 1.12	0.49 ± 0.0	7.07 ± 0.0	6.02 ± 0.05	10.76 ± 0.005
10	-	-	-	-	1.54 ± 0.22	6.17 ± 0.30	7.67 ± 0.22	13.21 ± 0.41
14	-	-	-	-	2.33 ± 0.06	6.73 ± 0.20	7.23 ± 0.52	12.72 ± 0.59
16	-	-	-	-	2.35 ± 0.27	7.22 ± 0.61	8.26 ± 0.39	12.67 ± 0.44
17	2.99 ± 0.43	9.10 ± 0.02	6.65 ± 0.15	12.15 ± 0.45	-	-	-	-
21	3.31 ± 0.32	9.83 ± 0.58	6.87 ± 0.13	11.29 ± 1.01	-	-	-	-
23	-	-	-	-	2.41 ± 0.23	8.31 ± 0.15	7.66 ± 0.21	13.73 ± 0.31

[a]Mean values (mg phospholipid/g oil) with standard error of the mean.

TABLE II

Changes in % Phospholipids (PL) in Oil, Composition of the Major Phospholipids, and Bean Wet Weight during Storage

Days stored	% Major PLs in oil	Phospholipid composition[b]								Bean wet wt[c], mg
		Without fungicide				With fungicide				
		Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl ethanolamine	Phosphatidyl choline	Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl ethanolamine	Phosphatidyl choline	
0	1.3[a]	0	13.47	26.31	35.22	2.41	15.74	24.18	32.68	151.4 ± 6.0
4	2.9	-	-	-	-	3.90	18.73	24.12	43.25	-
6	2.3[a]	7.15	25.77	22.68	34.41	1.80	26.14	22.26	39.79	165.8 ± 7.6
10	2.8	-	-	-	-	4.84	19.42	24.15	41.59	-
14	2.9	-	-	-	-	7.23	20.88	22.43	39.46	-
16	3.0	-	-	-	-	6.93	21.30	24.37	37.88	-
17	3.1	8.71	26.51	19.37	35.40	-	-	-	-	-
21	3.1	9.52	28.27	19.75	32.47	-	-	-	-	178.2 ± 8.2
23	3.2	-	-	-	-	6.76	20.24	21.47	38.49	-

[a]The % PL at 0-day in untreated beans was 1.1%, treated beans was 1.6%. At 6-day, untreated was 2.3%, and treated was 2.4%.

[b]As % P_i (from major phospholipids) of the total lipid P_i.

[c]Average weight of ten untreated beans with standard error of the mean.

untreated beans during storage. No growth was detected during the first 6 days of storage. The beans became slightly moldy by the 14th day, very moldy by the 17th day, and extremely moldy by the 21st day. Mold completely covered the outer seed coats for 21-day soybeans. Mold growth was never visually detected on beans treated with 50% Captan. During the first 6 days of storage, the level of the major phospholipids increased twofold (Table I and II), whereas mold growth was not detected on untreated beans. During the last 4 days of storage, however, the level of these phospholipids changed very little (Table I and II) while mold growth increased substantially. Phospholipid levels also increased more rapidly in the first 4 days of fungicide-treated beans, strongly suggesting that these changes can occur independently of mold growth.

Germination studies (48 hr) showed that, with water imbibition of soybeans, total phospholipid levels increased after an initial lag phase (14). We exposed soybeans to conditions which could promote water imbibition and germination as well as mold growth (35 C and 85% RH). The seed coats from both treated and untreated beans after 16 days were soft and easy to remove, and the endosperms soft and spongy. The increase in untreated bean weight was 9.5 and 17.7% after 6 and 21 days (Table II). The data show a higher rate of moisture uptake during the first 6 days than during the last 15 days. The moisture uptake during storage probably accounts for the observed increases in phospholipid levels.

The results of these experiments with soybeans stored under high temperature and humidity suggest that increased phospholipid levels in extracted oil occurred independently of mold growth on the beans and may have resulted from water imbibition and the onset of seed germination.

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REFERENCES

1. Evans, C.D., G.R. List, R.E. Beal, and L.T. Black, JAOCS 51:444 (1974).
2. Milner, M., and W.F. Geddes, Cereal Chem. 23:225 (1946).
3. Robertson, J.A., W.H. Morrison, and D. Burdick, JAOCS 50:443 (1973).
4. Guillaumin, R., and N. Drouin, Rev. Fr. Corps Gras 13:185 (1966).
5. Hvolby, A., JAOCS 48:503 (1971).
6. Singh, H., and O.S. Privett, Lipids 5:692 (1970).
7. Privett, O.S., K.A. Dougherty, W.L. Erdahl, and A. Stolyhwo, JAOCS 50:516 (1973).
8. Zimmerman, D.C., and H.J. Klosterman, Ibid. 42:58 (1965).
9. Allen, C.F., and P. Good, Ibid. 42:610 (1965).
10. Hitchcock, C., and B.W. Nichols in "Experimental Botany," Vol. IV, Edited by J.F. Sutcliff and P. Mahlberg, Academic Press, London and New York, 1971, p. 287.
11. Skipski, V.P., and M. Barclay in "Methods in Enzymology," Vol. XIV, Edited by J.M. Lowenstein, Academic Press, London and New York, 1969, pp. 542-548.
12. Krebs, K.G., D. Heusser, and H. Wimmer in "Thin-layer Chromatography," Second edition, Edited by Egon Stahl, Springer-Verlag, New York, 1969, p. 857.
13. Rouser, G., A.N. Siakotos, and S. Fleischer, Lipid 1:85 (1966).
14. Harwood, J.L., Phytochemistry 14:1985 (1975).
15. Chapman, G.W., J.A. Robertson, D. Burdick, and M.B. Parker, JAOCS 53:54 (1976).
16. Hammond, E.G., W.R. Fehr, and H.E. Snyder, Ibid. 49:33 (1972).
17. Howell, R.W., and F.I. Collins, Agron. J. 49:593 (1957).
18. Wilson, R.F., and R.W. Rinne, Plant Physiol. 54:744 (1974).

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