Effect of Moisture, Microwave Heating, and Live Steam Treatment on Phospholipase D Activity in Soybeans and Soy Flakes[1]

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The impact of enzyme activity on the nonhydratable phospholipid content of crude soybean oil has been evaluated. A radiochemical method was used to assay phospholipase D activity in whole and flaked soybeans stored under a variety of storage and enzyme inactivating conditions. The crude enzyme was isolated and incubated with a mixture of ^{14}C-labeled and unlabeled phosphatidylcholine. The amount of liberated radioactive choline was used as a measure of enzyme activity. Whole soybeans with moisture contents of 8–18% were stored at 40°C and sampled weekly for up to four weeks. Although the enzyme was active in all samples, the optimum moisture content for enzyme activity was about 14%. Flaking and flake thickness were shown to increase phospholipas D activity. At moisture levels above 10%, flakes at .012″ showed about twice the activity of whole beans. As flake thickness was increased, enzyme activity decreased.

Whole soybeans with moisture contents of 12–18% were treated by microwave heating under controlled conditions. During the early stages of heating, the enzyme was activated, and then was gradually destroyed by the time the temperature of the beans reached 115-120°C. Approximately 8–10 min of microwave heating was required to completely destroy enzymatic activity. The inactivation of phospholipase D in soyflakes treated with live steam was also evaluated. The enzyme is rapidly destroyed at temperatures of about 110°C. Evaluations of flakes subjected to live steam and whole beans treated by microwave heating to inactivate phospholipase D suggest that heat, moisture and enzyme activity are important factors contributing to the formation of nonhydratable phospholipid in extracted crude oils.

KEY WORDS: Degumming, microwave treatment, nonhydratable phosphatides, phospholipase D, phospholipids, phosphorus, soybean oil, steam.

Crude solvent extracted soybean oil contains about 2–3% phospholipids whose affinity for water forms the basis for lecithin manufacture and production of degummed oil for further processing (1–3). Commercial degumming removes about 80–95% of the phosphorus-containing lipids from crude soybean oils (4–6). However, field, storage, and transportation damage to soybeans, and, to a lesser extent, normal bean processing, yield crude oil containing the so-called nonhydratable phosphatides (NHP) (7–9). By definition, the NHP are those phosphatides that, upon treatment of crude soybean oil with water, will not swell, form gels, and precipitate from the oil after agitation at elevated temperatures. Although the mechanism of NHP formation is not well understood, it has been suggested that NHP are formed either during storage of the whole bean or during the extraction process itself (8,10,11). In either case, NHP are thought to be the products of action of phospholipase D on the base group of phosphatidylcholine and/or ethanolamine (12,13).

Neilsen has reported the composition of the NHP to be phosphatidic and lysophosphatidic acids (8). Pretreatment of soybean flakes before solvent extraction using moist heat in a stirred bed (10,11) or infrared heating (14), give extracted crude oils which showed improved phospholipid removal by water degumming. While a direct correlation between phospholipase D inactivation and reduction in NHP content was suggested, enzyme activity in the treated flakes was not determined. We report here the effects of moisture, microwave heating, and live steam on the activity of phospholipase D in whole and flaked soybeans and the NHP content of extracted crude oil.

EXPERIMENTAL PROCEDURES

Williams certified seed grade soybeans were used. Over the course of this investigation, three different lots from a single source were used, but little difference in enzyme activity, either initially or after accelerated storage, was noted. Beans were tempered to the desired moisture level by placing them in plastic bags with the required amount of water and allowing them to equilibrate until all moisture had been adsorbed. Whole beans were cracked, flaked, and dehulled as described previously (9). Soyflakes, .012″ thick, were extracted with hexane for 5 hr in a modified Soxhlet extraction apparatus. After extraction, the miscella was filtered through paper, and the solvent was removed on a rotating evaporator. Degumming of the crude oil was carried out as described previously (5). American Oil Chemists' Society methods were used to determine phosphorus, free fatty acids, and peroxide values (15).

Phospholipase D was isolated from soybeans by a modification of the method described by Nakayama (12), in which whole soybeans were soaked overnight in 100 mL of acetate buffer (pH 5.6) at 0°C. After the buffer was decanted, the beans were ground with a mortar and pestle. The macerated beans, along with the buffer, were then homogenized for 30 seconds in a Waring blender. The homogenate was filtered through cheesecloth under vacuum, and the crude enzyme-containing solution was isolated by centrifugation at 13,000 rpm for 30 min at 4°C and filtration prior to assay.

The use of radioactive substrates for determining phospholipase D activity has been shown to be both rapid and accurate (16). A variation of this method was used to determine the phospholipase D activity in the crude extract using L-α-phosphatidylcholine (choline-methyl-^{14}C; specific activity 10 μCi/mmol) as substrate. The substrate was suspended in (17) buffer (pH 5.6) and, after equilibration to 30°C, was shaken with crude enzyme solution for 10 min at 30°C. The enzyme reaction

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was stopped by addition of chloroform/methanol with vigrous mixing. Enzyme action liberates ^{14}C-methyl-labeled choline, which is isolated in the aqueous portion of the extraction solvent, while unreacted phosphatidylcholine remains in the organic layer. Following centrifugation, aliquots of the aqueous layer were removed for quantitation using a scintillation counter, and total liberated choline was calculated. Phospholipase D activity is reported here as micromoles of choline liberated per min per gram of whole bean.

Soybean samples were treated by microwave heating using a Despatch unit (Minneapolis, MN) equipped with a revolving carousel. Tempered beans (500 gm) were placed in a 21 × 21 cm Pyrex baking dish to a depth of about 1.2 cm and treated for 1-10 min at 0.6 milliamps current at 2450 megahertz. Bean temperatures achieved during microwave heating were determined immediately after treatment.

Soy flakes were treated with steam by placing 1,000 gm of flakes in a 23″ × 30″ × 1″ pan inside a steam-heated autoclave. Live steam was admitted, and the temperature rapidly elevated to 112-113°C (235°F). Exposure times were determined after treatment temperature was attained. Steamflow was discontinued, and the contents of the autoclave were vented. The samples were then removed for further processing.

RESULTS AND DISCUSSION

The changes in activity of phospholipase D during accelerated storage at 40°C were determined by tempering whole beans to moisture levels ranging from 8–18%, storing the tempered beans in a forced draft oven held at 40°C ± 1°C and sampling them weekly for up to four weeks. The effect of moisture on the activity of phospholipase D is shown in Figure 1. Upon receipt, this particular lot of soybeans had an enzyme activity of about 1.5 × 10^{-3} μmoles choline liberated/min/gm. During 40°C storage, all samples showed an increase in phospholipase D activity, most of which came in the initial part of the storage, i.e., one week. The optimum moisture level for storage of beans is 12%, i.e., minimum enzyme activity. Storage at moisture levels above or below 12% results in increased phospholipase D activity. It is interesting to note that the optimum moisture content for maximum enzyme activity during storage appears to be 14%.

When beans tempered to various moisture levels were microwave treated, the rise in bean temperature with time showed only slight differences over the range of 12-18% moisture. The activity of phospholipase D as a function of microwave treatment is shown in Figure 2, where enzyme activity is plotted against heating time. This lot of beans had an initial activity of about 2 × 10^{-3} μmole choline lib/min/gm, and activity increased to about 5.5 × 10^{-3} μmoles/min/gm during the initial stages of heating. This agrees with the observation that the enzyme is activated during the early stages of 40°C storage. As observed previously, 14% moisture appears to be the optimum for maximum enzyme activity.

Phospholipase D appears to be fairly stable to heat. Irrespective of moisture levels, destruction of enzyme activity does not begin until temperatures have reached about 80–90°C. Heating times of 8–9 min are required to completely inactivate (97.5–99.0%) the enzyme with temperatures of 115–120°C. Relationships between temperature and phospholipase D activity during microwave heating are shown in Figure 3. The correlation coefficients between these two variables ranged from −0.94 to −0.98 and are significant at the 1% confidence level.

The effect of live steam on phospholipase D activity in soyflakes is shown Table 1. After 2 min at 112°C, about 75% of the activity remained, and after 3 min, about 5% remained. Complete destruction of phospholipase D was achieved in 4 min. Some variability in enzyme destruction was noted from different lots of beans. Another lot of beans yielded flake (14% moisture) in which the enzyme was essentially inactivated after 2 min treatment with live steam at 112°C.

The effect of cracking and flaking on phospholipase D activity was evaluated using whole beans tempered to known moisture levels. One portion was cracked, dehulled, and flaked, while the other was kept as intact beans. The phospholipase D activities of the whole and flaked beans are shown in Table 2. Also shown in Table 2 are the

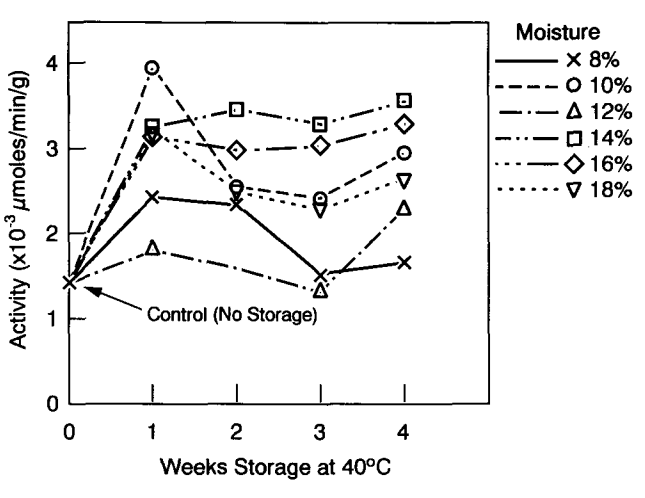

FIG. 1. Effect of storage at 40°C on phospholipase D activity in whole soybeans.

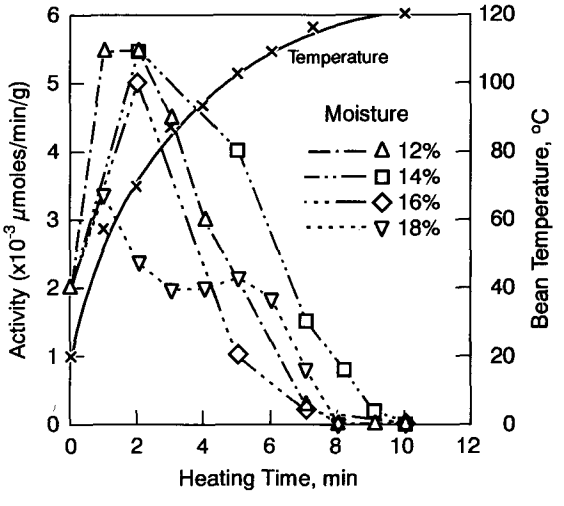

FIG. 2. Effect of microwave treatment on phospholipase D activity in whole soybeans.

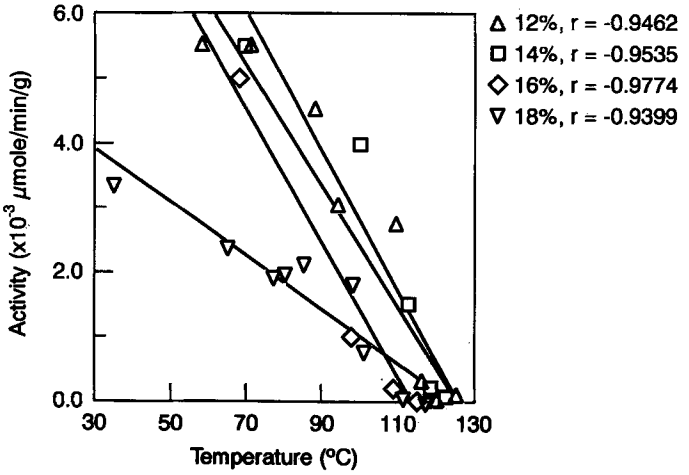

FIG. 3. Correlation of phospholipase D activity and bean temperature during microwave treatment.

TABLE 1

Effect of Live Steam on Phospholipase D Activity in 14% Moisture Soyflakes

Exposure time (seconds)[a]	Enzyme activity[b]	% Destroyed
0	4×10^{-3}	0
30	4.05×10^{-3}	0
60	3.71×10^{-3}	7.3
120	3.06×10^{-3}	23.5
180	1.75×10^{-4}	95.8
240	1.0×10^{-5}	99.8

[a]112°C.

[b]μmoles choline liberated/min/gm beans.

TABLE 2

Effect of Flaking, Flake Thickness and Moisture on Phospholipase D Activity

	Phospholipase-D Activity[a]			
Moisture %	Flake thickness	Flakes	Whole beans	% Increase
10	0.012	4.1	4.4	0
12	0.012	8.5	5.0	170
14	0.012	8.6	4.9	175.5
16	0.012	8.2	4.5	182.2
14	0.006	8.45	4.9	172.2
14	0.012	7.65	4.9	156.1
14	0.018	7.45	4.9	152.0
14	0.029	7.20	4.9	146.9

[a]μmoles choline/min/gm $\times 10^{-3}$.

effects of flake thickness on phospholipase D activity. With the exception of the 10% moisture beans, flakes had from 1.5–1.8 times the enzyme activity of whole beans. Flake thickness appears to be inversely related to phospholipase D activity. Flakes ranging from .006 to .029" show enzyme activity ranging from about 8.5×10^{-3} to 7.2×10^{-3} activity units, respectively. A plot of the log enzyme activity vs flake thickness yields a straight line. Results shown in Table 2 provide evidence that rupture of the cell walls degrades the oleosomes or lipid sacs to such an extent that enzyme activity is enhanced.

The slow rate of NHP formation in whole beans may be accounted for by several factors, as shown in Table 2: i) During storage as whole beans, where no cell wall disruption has occurred, the enzyme only shows about half the activity of flakes; and ii) the flake thickness data shows that enzyme activity decreases as flake thickness is increased. Thus, in whole beans, the effects of phospholipase D would be minimized as compared to flaked beans.

A recent study (18) has shown that, at the molecular level, intensive membrane degradation occurs in lipid bodies isolated from soybeans. Phospholipase D was shown to convert phosphatidylcholine and phosphatidylethanolamine to phosphatidic acid fairly rapidly at 30°C.

Figure 4 shows results of degumming of oil extracted from 14% moisture beans after storage for up to 20 weeks at 40°C. Two phenomena are observed: i) The phosphorus content of the crude oil decreases with storage; and ii) the NHP content of the crude oil increases with storage, i.e., a greater amount of the crude oil phosphorus content is retained in the degummed oil. Both observations indicate phospholipid deterioration. To what extent phospholipase D is involved is open to speculation, but the enzyme is active over the course of the storage experiments.

The formation of nonhydratable phosphatide in flakes at 10 and 14% moisture levels was evaluated, with the former representing normal soyflake processing moisture and the latter where the enzyme phospholipase D is most active. The flakes were stored at 40°C for periods up to 36 hr, the crude oil extracted with hexane, and finally degummed. Results are shown in Figure 5. Oils from the 10% moisture flakes yielded crude oils having NHP contents in the ranges usually found in normal commercial processing streams, i.e., 50–80 ppm phosphorus, and the levels of NHP did not increase with flake storage. However, the same flakes tempered to 14% moisture after 4 hr storage at 40°C yielded oil with extremely high levels of NHP. Even the control flake (no storage) gave an oil that degummed poorly. The differences in the degumming of the oils with respect to flake moisture cannot be explained entirely by the phospholipase D activity, since

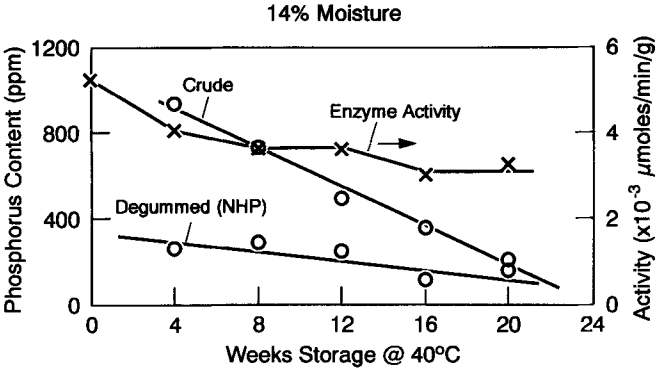

FIG. 4. Nonhydratable phosphatide formation in crude oil extracted from whole soybeans stored at 40°C, 14% moisture.

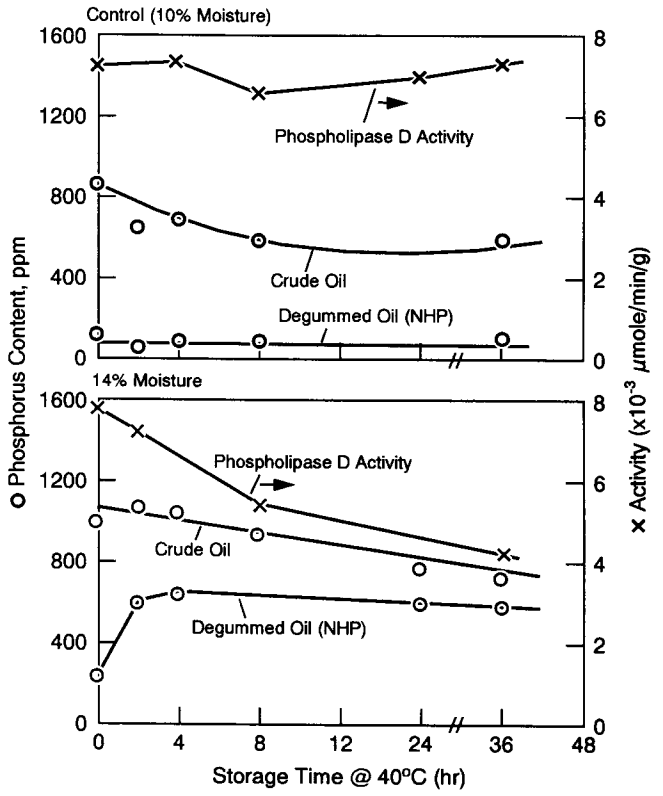

FIG. 5. Nonhydratable phosphatide formation in 10 and 14% moisture flakes stored at 40°C.

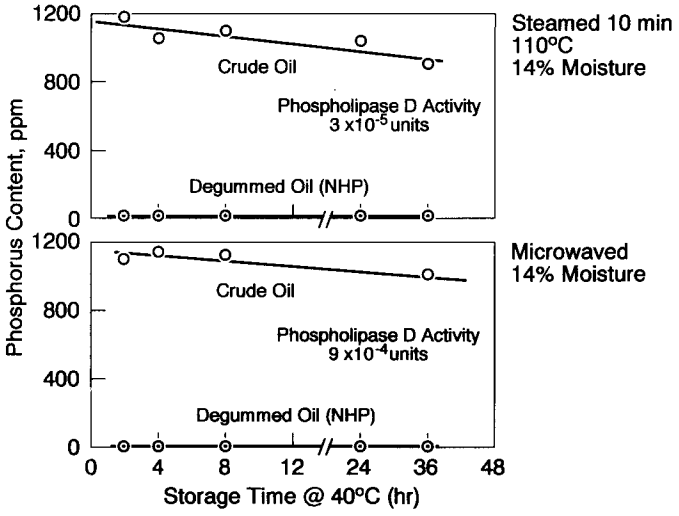

FIG. 6. Nonhydratable phosphatide formation in microwave and live-steam treated soyflakes (14% moisture).

the 10% moisture flakes had enzyme activities that were high and relatively constant throughout the entire storage period, whereas some loss of activity was observed in the 14% moisture flakes.

Moisture, along with phospholipase D activity, appears to be a key factor in NHP formation. At low-moisture levels (< 12%) the enzyme is potentially active; however, the water activity is below that required for phospholipase D reaction. At higher moisture levels (i.e., 14%) water activity may be sufficient for reaction to form NHP (19).

This effect is more clearly shown in studies in which phospholipase D was inactivated by live steam and microwave heating (Fig. 6). Whole soybeans (14% moisture) were treated by microwave heating for 10 min to inactivate the enzyme. The beans were cracked, dehulled, and flaked. Another portion of the same beans was cracked, dehulled, and flaked, and then the flakes were treated with live steam for 10 min. The treated flakes were stored for up to 36 hr at 40°C prior to extraction. Crude oils from both microwave treated soybeans and steam-treated flakes show a gradual decrease in extractable phosphorus upon storage at 40°C. The degummed oils, however, are extremely low in phosphorus, i.e., 10–20 ppm. Thus, NHP formation is effectively inhibited in whole beans by microwave treatment or in flakes by live-steam treatment. This effect may result from both destruction of phospholipase D and moisture removal. A decrease in extractable phosphorus in the crude oils in the absence of enzyme activity may result from morphological changes in the flake itself. Heat treatment or cracking of oilseeds and oil-bearing materials in general is known to affect the yield of neutral oil (20). Moisture present in the seed or flake may well affect the yield of phosphatide as well.

Results obtained with live-steam treatment of flakes are in accord with Kock (10), who reported that yields of lecithin were about twice that encountered in conventional processing. Similarly, microwave heating increases the phosphorus levels of crude oil to values between 1000–1500 ppm. Hexane normally extracts only about 50% of the total phosphatides from soybeans. Heat treatment with live steam or microwave treatment, apparently through moisture removal, enzyme inactivation, or morphological changes in the lipid bodies present in the flakes, results in more complete extraction of phosphatides.

NHP formation is complex. Certainly, phospholipase D involvement may be inferred from the fact that destruction of enzymatic activity results in virtual elimination of the problem. However, based on studies reported here, other factors may be involved. These include flake thickness, moisture content, and possibly morphological changes in the flake brought about by heat. It has been suggested that phospholipase D is activated by organic solvents like hexane. An increase of phospholipase D activity by diethyl ether has been reported (12). However, our studies show that full fat and hexane-defatted flakes show little difference in enzyme activity. Also, hexane added to the enzyme assay sample did not increase phospholipase D activity. For example, crude enzyme isolated from 14% moisture flakes, assayed at 55°C in the presence of up to 500 μL hexane, showed an activity of 6.9×10^{-3} μmoles choline/min/gm compared to 6.6×10^{-3} for a control. These results suggest that hot hexane does not activate phospholipase D to any great extent during solvent extraction.

Moisture, heat, and enzymatic activity, in combination, are important factors which lead to NHP formation. Evidence presented here indicates that although heat is normally used in commercial processing of beans into flakes, temperatures used may not be sufficient to inactivate phospholipase D (D.C. Tandy, private communication). Temperatures of 150–170°F (66–77°C) are normally

used in conditioning of cracked beans prior to flaking. Even with the residence time of 20 min commonly used in commercial practice, these conditions would probably not inactivate phospholipase D. The mechanism of NHP formation is currently being investigated and will be reported later.

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