Phospholipase D Activity in Hexane

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Phospholipase D converts phosphatidylcholine (PC) to phosphatidic acid (PA) at 65°C in water-saturated hexane. Presumably, the active site of the enzyme remains hydrated in the interior of a lipid micelle. Enzyme activity at elevated temperatures in a nonaqueous medium contrasts sharply with inactivation at high temperature in aqueous solution. Results demonstrate that nonhydratable phospholipids can be produced enzymatically under conditions comparable to those during oil extraction in commercial soybean processing.

KEY WORDS: Organic solvent activity, phospholipase D, soybeans.

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4, PLD) catalyzes hydrolysis of phosphatidylcholine (PC) and lyso-PC to phosphatidic acid (PA) and lyso-PA. Also, the enzyme catalyzes phosphatidyl transfer reactions whereby choline is replaced by alcohols rather than water. It is the hydrolysis of PC and other phospholipids (PL) that is responsible for the fouling of degummed oil during soybean oil refining (1). Partial hydrolysis products, PA and lyso-PA, are nonhydratable and are not removed with other PLs when solvent-extracted soybean oil is treated with water. Nonhydratable phospholipids (NHPL) contribute undesirable taste, color, odor and instability to processed oil. Should NHPL levels be too high, additional steps, such as phosphoric acid pretreatment and higher excesses of caustic soda (1), must be taken to improve quality but at higher costs.

The point(s) where PLD activity produces deleterious effects during soybean processing is uncertain. Blame has been placed upon poor seed quality. Studies have shown that beans with breaks, disease, and insect damage exhibit substantial enzymatic damage (2). Improper storage of beans is also known to increase NHPL levels (3). Soybean processing itself provides opportunity for unwanted PLD activity following cracking and during flaking of the beans (4). To remedy the latter, various treatments have been introduced into the processing sequence to inactivate the enzyme (4–7). Nevertheless, the problem of nonhydratable phospholipid persists and, in particular, is detrimental to foreign commerce in soybeans. Results presented here address a heretofore unsuspected cause of NHPL.

EXPERIMENTAL PROCEDURES

Crude PLD was isolated from 1989 soybeans (*Glycine max.* L., var. Century) by a method similar to that of Kouzeh Kanani *et al.* (8). Testae and hilum were removed initially, and the beans soaked overnight in 5 vol (v/w) of Tris-HCl buffer, pH 7.2. The entire isolation was carried out at 4°C. Soaked beans were blended for 60 s and allowed to stand one hour. Four suspensions and 30-min centrifugations at 14,000 × *g* removed cell debris and lipoidal material. The supernatant was treated with $(NH_4)_2SO_4$ to 20% (w/v), and the mixture was stirred for one hour, left overnight at 4°C, and then centrifuged at 14,000 × *g*. The sediment was dissolved in water and dialyzed against water containing 0.03% sodium azide for a minimum of 24 hr. The resultant solution (ca. 5μg protein/μL) was the PLD aqueous isolate.

Activity assays were conducted in 125 × 16 mm screw cap test tubes. Ten μL of 0.085 M $CaCl_2$ solution was added to each vial, and water was removed at 105°C. An equivalent of 800 μg of egg yolk PC in chloroform (Sigma Chemical Co., St. Louis, MO) was added, and the solvent was removed under dry nitrogen. One mL of water-saturated hexane was added, and the system was vortexed. For activity, either 4 μL of a PLD solution or blank was added. The ratio of PC to enzyme was thus approximately 800 μg PC to 10–12.5 μg protein, or 72 to 1 by weight. The tubes were flushed with nitrogen. Both samples and blank were buffered with 1.0 M sodium acetate, pH 6.0 on a 1:1 basis (v:v). The vials were sonicated in a bath sonicator for 5 min to form micelles, which were then placed in a temperature-controlled shaker bath (130 cycles/min and at either 26, 35, 45, 55, 65 or 75°C). Incubation was terminated by transfer of vials to an ice bath, addition of 0.8 mL of 1 N HCl and vortexing for 60 s.

Lipid recovery began with hexane removal under nitrogen and extraction of the lipids according to the Bligh-Dyer method (9). Individual lipids were isolated by means of laboratory-prepared 0.25 mm thickness thin-layer chromatography (TLC) plates (Merck 60 G silica gel). The solvent system was chloroform:methanol:ammonia (65:35:4 v/v/v). Spots were located with 8-anilino-napthalene-1-sulfonate. Individual spots were transferred to 150 × 16 mm digestion tubes. Phosphorus analyses were as previously described (10).

Aqueous phase PLD activities were measured by a procedure generously provided by the Sigma Chemical Co. Protein was determined by the Coomassie binding assay of Bradford (11).

RESULTS AND DISCUSSION

In contrast to the work of others who have characterized pure PLD from non-seed sources (12–14), the intent of this investigation was to examine a crude preparation in which the PLD was more likely to be associated with other proteins/subunits present under processing conditions. Such a preparation should optimize retention of polypeptide subunits/cofactors that contribute to total native activity. Currently, no critical

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

peptide cofactors are ascribed to PLD (15), but the enzyme is especially difficult to purify in active form.

PLD is known to be inactivated at elevated temperatures in aqueous media (16). Table 1, however, shows that PLD is not affected at 65°C in a "nonaqueous" environment. PLD in hexane continued to produce PA throughout a 60-min treatment at 65°C. The PLD activity of enzyme previously heated in aqueous suspension at 65°C or 100°C for 10 min was reduced nearly eight-fold, yet some activity remained. Untreated PLD run at 65°C in an aqueous buffered medium (Sigma assay) yielded an enzyme activity of only 1 nM PA/hr.

Obviously, the term "nonaqueous" must be used cautiously. In these experiments, PLD was administered to the PC-hexane solution in a buffered aqueous solution. Buffering of the solution defined the enzyme's conformation at an assigned pH as prescribed for lipase reactions in non-aqueous media (17). Sonication was required to establish micelles, which consist of the aqueous phase surrounded by a PC-enzyme membrane. The micellar PC-protein membrane formed here should be essentially the reverse of that found in lipid bodies (18,19). The membrane itself possesses alkyl chain character, but the hydrophilic choline group resides now on the interior surface of the membrane and acyl chain terminal groups from the hydrophobic membrane exterior. The enzyme must possess partial hydrophobic character to facilitate hydrolysis of PC that is composed mostly of 18-carbon acyl chains (20).

PLD preheated in aqueous suspension did not exhibit significant activity when transferred to hexane. It is therefore hypothesized that upon sonication the enzyme that remains active is not in the aqueous interior of the micelle and that the PLD active site rests within the micelle membrane or is in the vicinity of the PL choline groups. The enzyme's "nonaqueous" conformation must be unique and essential to the hydrolysis activity, for were its conformation similar to that which exists in aqueous suspension, the enzyme would have been deactivated. Furthermore, the small but significant activity displayed in hexane by the preheated inactivated PLD suggests that some degree of active conformation is either retained or established, more likely the latter. As such, it represents a renaturation of the enzyme, albeit small, under the experimental conditions prevailing here.

Incubation beyond one hour at 65°C resulted in a decrease of PLD activity from ca. 40 nM PA/hr at one hour to ca. 20 nM PA/hr at five hours (data not shown). A gradual loss of activity should be expected even in enzymes stabilized by "nonaqueous" environments (17), but in this case the loss of activity would be consistent with characteristics of the system. PC conversion to PA should lead to destruction of the micellar structure and ultimately to a more highly hydrated enzyme that is readily deactivated at 65°C.

Table 2 shows that the "nonaqueous" reaction appears to be temperature-dependent. Each value is the result of five reactions. This work differed slightly in that the parent PLD isolate was not prebuffered with 1.0 M sodium acetate. As a result, the pH of the active site is not well monitored. The parent solution was measured at 6.2, but the presence of calcium chloride in the reaction tube may have lowered the pH further and thus altered the enzyme conformation prior to micelle formation. With the exception of the 26°C datum, the rate of reaction approximately doubles with each ten degree temperature rise, giving a very satisfactory Q_{10} value of 2. Figure 1, an Arrhenius plot, yields an activation energy of 12,840 cal mole^{-1} and a frequency factor of 7.36×10^9. At this time, there is no explanation offered for the seemingly anomalous 26°C value.

TABLE 1

Phospholipase D Reactivities at 65°C in hexane[a]

Series	nM PA/hr[b]
1. (PLD unaltered)	45 ± 6
2. (PLD 100° heat-pretreated)[c]	6 ± 3
3. (PLD 65° heat-pretreated)[c]	6 ± 3

[a]Trials were performed for 60 min with shaking (130 motions/min). Protein concentration was 2.5 μg/μ.
[b]PA = phosphatidic acid.
[c]Enzyme isolate heat-pretreated for 10 min.

Significance. What relationships do these results bear with the production of soybean oil and lecithin? Racicot and Handel (21) observed a much varied phosphorus content on a day-to-day basis while examining the crude and degummed oil productions of four companies. Kouzeh Kanani *et al.* (4) pointed out that favorable conditions for enzymatic activity exist after the cracking of the soybean during processing. Obviously, the problem exists in more than one oil refinement step.

The reaction examined consisted of reverse micelles with a buffered interior, whereas intact lipid bodies are true micelles. The importance of the "nonaqueous" PLD activity in this model system is that it demonstrates that NHPLs can be formed during hexane extraction of steam-treated flakes if the enzyme exists in lipid-rich regions that preserve its activity in the same way that hexane preserved activity in these experiments. There is as of yet no information regarding the fate of the PLs during the hexane extraction phase. With an expectation that water is not thoroughly removed during the preparatory flaking, it is conceivable that reverse micelles form or a similar physical state results. At the elevated extraction temperature, PL hydrolysis conditions appear to be nearing optimum enzymatic activity. Lastly, the appearance of PA, when previously heat-treated inactivated PLD was utilized, is evidence of enzyme reactivation or that a more hydrophobic PLD exists, which is resistant to high-temperature aqueous denaturation. This enzyme may be present in lesser amounts and/or may be kinetically slower than the superficially observed enzyme. In either case, different extraction conditions may increase this restored activity. In summary, the requirement for inactivating the PLD enzyme is now even more necessary.

To achieve inactivation, two immediate routes might be explored. The first is the addition of enzyme-inactivating agents, perhaps metal chelators or chemical denaturants prior to oil extraction. The second is to further examine processing parameters (tempera-

TABLE 2

Production of Phosphatidic Acid with Increasing Temperature[a]

	Temperature (°C)					
	26°C	35°	45°	55°	65°	75°
nM PA in 5 hr	41 ± 1	32 ± 6	54 ± 18	103 ± 44	200 ± 10	304 ± 10
nM PA/hr	8	6	10	21	40	61

[a]Each run is the result of five simultaneous trials. The PLD isolate was unbuffered and had a protein concentration of 5.0 μg/μL.

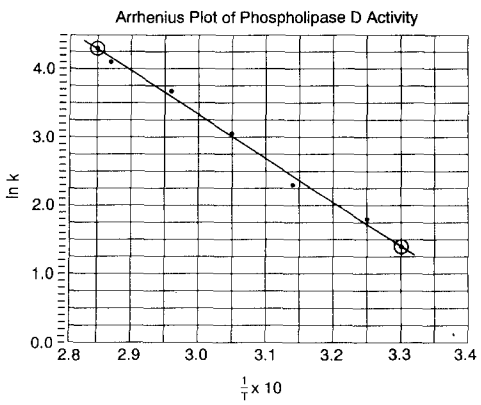

FIG. 1. Arrhenius plot of phospholipase D activity. PC concentration is 800 μg/mL. Temperatures are 35, 45, 55, 65 and 75°C.

ture, concentrations, solvent types) to find conditions that minimize PL hydrolysis. In either case, subsequent manufacturing steps must be surveyed as well for subsequent deleterious effects. A longer-term approach would isolate the enzyme or enzymes involved, identify them, and genetically alter them to accommodate processing.

The important oxidative stability of soybean oil is greatly influenced by minor components in oil, one of which is PL (22). The type and content of minor components in crude oils are dependent upon extraction solvents, extraction temperature, and oilseed pretreatment (23,24). Hexane at 65°C has been presented as a likely factor in NHPL formation by PLD. Further investigation of "nonaqueous" activities of other enzymes, their kinetics, temperature responses, and stabilities is warranted. Such investigations present opportunities for new insight analogous to that gained on lipases (17,25).

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