# Decomposition of Phospholipids in Soybeans During Storage

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ABSTRACT

Changes in lipid composition of soybeans and extracted oil during storage were analyzed. After soybeans with a moisture level of 13% had been stored for six months at 35°C, 45% of the total phospholipids had decomposed, and 72% of the total phospholipids originally extracted with the oil was lost. Phosphatidylcholine and phosphatidylethanolamine decreased significantly, and phosphatidic acid and lysophosphatidylcholine increased in soybeans during storage. Considerable amounts of phospholipids were removed by the degumming procedure. However, only phosphatidic acid remained constantly at a high level in the degummed oil. No difference was found between soybeans and extracted oil in fatty acid composition of phosphatidic acid. Phospholipase D activity was found in an acetate buffer extract of homogenized soybeans. Inhibition of initially strong enzyme activity became weaker the longer the soybeans were stored.

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Soybeans are a most valuable source for edible oils and vegetable proteins. Deterioration of components of field-damaged and storage-damaged soybeans has been investigated (List et al 1977, Robertson et al 1973, Saio et al 1980). Soybean oils and proteins are held in sacks of biological membranes, which are called spherosomes (or oleosomes) and protein bodies, respectively. When these sacks are broken during storage, the oils and proteins inside are exposed to cytoplasm and are then assumed to undergo deterioration and hydrolysis (Saio and Baba 1980). Prevention against degradation of spherosome and protein body membranes is necessary in order to increase the yields of oils and proteins from soybeans after storage.

This article describes changes that occur during storage of soybean phospholipids, which are the main lipid components of the membranes, and the relationship between phospholipids in soybeans and those in extracted and degummed oils.

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## MATERIALS AND METHODS

### Chemicals

Choline oxidase and peroxidase were purchased from Toyobo. CM-cellulose (CM-52) and 1PS filter paper were from Whatman, thin-layer chromatography (TLC) plates from Merck (Art. 5721), and Alumina Woelm N-Super I from Woelm Pharma GmbH and Co. Other chemicals were obtained from Nakarai Chemicals. The phosphatidylcholine emulsion was prepared from commercial soybean lecithin purified with an alumina column (Singleton et al 1965). Purified phosphatidylcholine in a test tube was dried under $N_2$ gas, placed in a desiccator for 30 min, and sonicated for 5 min in distilled water. Soybeans from the 1978 October crop from Indiana, Ohio, and Michigan were imported in May 1979 and immediately stored in a refrigerator. Some of these soybeans were used as a control. The soybeans had a moisture level of 13% and were stored at 35°C according to the method of Saio et al (1980).

### Lipid Extraction

Fifty grams of soybeans were ground by a mill for 1 min. The resulting powder was incubated in 250 ml of *n*-hexane or chloroform/methanol (2:1 v/v) for 45 min at 55°C. The extract was filtered through 1PS filter paper.

### Purification and Analysis of Phospholipid

Phospholipids were purified with CM-cellulose according to the method of Comfurius and Zwaal (1977). To analyze the phospholipids, two-dimensional TLC was conducted with solvents of chloroform/methanol/28% $NH_4OH$ (200:120:15 v/v) in the first dimension and chloroform/methanol/acetone/less acetic acid/water (200:150:100:15:10 v/v) in the second (Nishihara and Kito 1978). Phospholipids that had been separated on TLC were analyzed by determining phosphorus according to the method of Bartlett (1959) and Keenan et al (1968).

### Determination of Triglycerides

Triglycerides were determined colorimetrically by the method of Homer et al (1975).

### Analysis of Fatty Acids

The methylated fatty acids produced by treating phospholipids with anhydrous HCl-methanol were determined by gas liquid chromatography, using a column packed with 10% Silar 10 C, on 100–120 mesh Gas Chrom Q, $N_2$ flow rate 45 ml/min, under a temperature range from 160 to 240°C.

### Degumming

Crude oil (10 g) in a 50-ml glass centrifuge tube with a stopper was heated to 60°C under $N_2$ gas. After 0.025 ml of acetic anhydride had been added and agitated for 3 min, 0.2 ml of water was added, and agitation was continued for 3 min. Degummed oil was obtained by centrifugation and decantation.

### Crude Phospholipase D Preparation

Soybeans (3 g) were immersed overnight in 20 ml of 10 m*M* acetate buffer, pH 5.0, at 0°C. The swollen beans were homogenized in a mortar with a pestle and filtered through two layers of gauze. The residue was washed twice with 5 ml of the buffer. The filtered homogenate was centrifuged at 13,000 × *g* for 30 min at 0°C, and the supernatant was used as the crude enzyme extract.

### Assay of Phospholipase D Activity

Phospholipase D was assayed according to a modified method of Imamura and Horiuti (1978). The reaction was initiated by adding 0.05 ml of enzyme solution to 0.485 ml of reaction mixture composed of 1 μmole of phosphatidylcholine emulsion, 5 μmoles of $CaCl_2$, 20 μmoles of acetate buffer pH 4.8, and 0.035 ml of ethyl ether. The reaction was carried out for 20 min at 35°C and terminated by adding 0.2 ml of 50 m*M* ethylenediamine tetracetic acid in 1*M* Tris-HCl buffer, pH 8.0. The mixture was then heated in a boiling waterbath for 5 min. After cooling, the liberated choline was incubated with a mixture of choline oxidase, peroxidase, phenol 4-aminoantipyrine and was measured by the method of Imamura and Horiuti (1978).

### Determination of Protein

Protein was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard.

## RESULTS

### Degradation of Phospholipids During Storage

Soybean phospholipids and oils gradually decomposed during

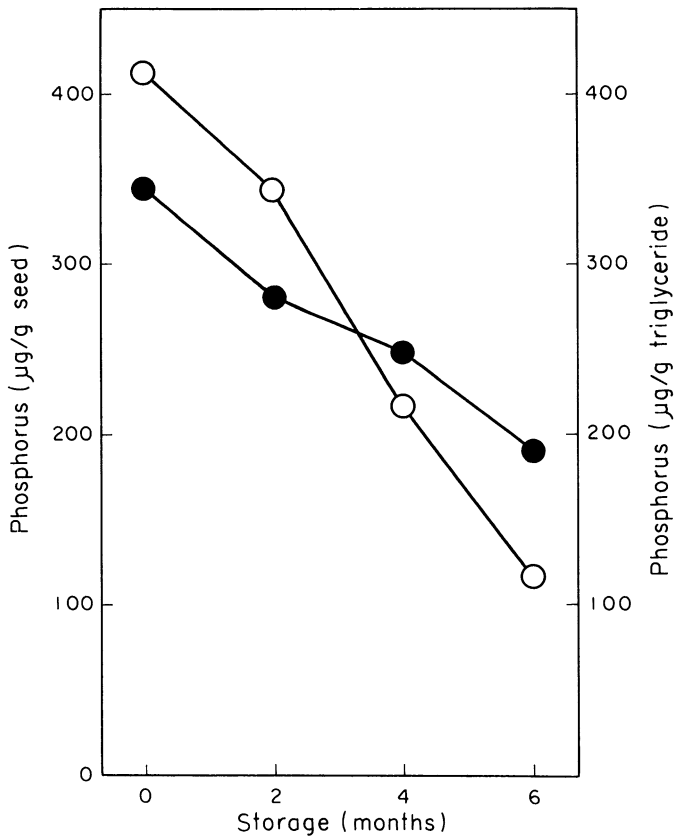


**Fig. 1.** Changes in the amounts of phospholipids during storage. • = phospholipid extracted with chloroform/methanol from whole soybean seeds, o = phospholipid in the *n*-hexane-extracted oil.

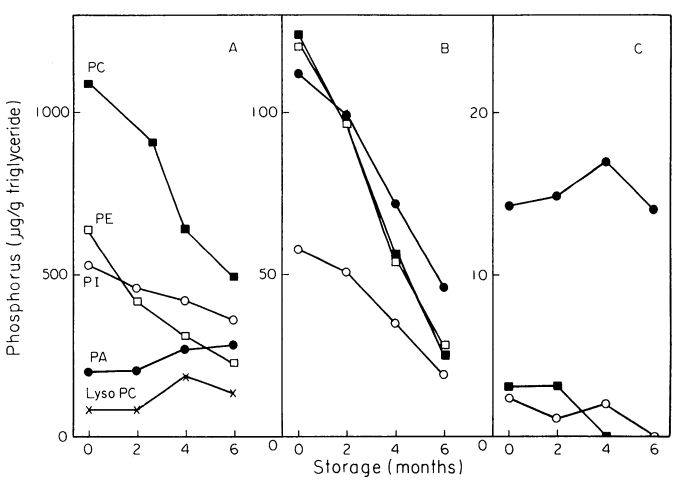


**Fig. 2.** Changes in the amounts of phospholipid classes during storage. **A,** phospholipid extracted with chloroform/methanol from whole soybean seeds; **B,** phospholipid in the *n*-hexane-extracted oil; **C,** phospholipid in degummed oil. ■, **PC** = phosphatidylcholine; □, **PE** = phosphatidylethanolamine; o, **PI** = phosphatidylinositol; •, **PA** = phosphatidic acid; ×, **Lyso PC** = lysophosphatidylcholine.

storage. After the soybeans had been stored for 6 months, 45% of the total phospholipids had decomposed (Fig. 1). On the other hand, the yield of the total extracted oil decreased to 85% (data not shown), and 72% of the total phospholipids originally extracted with the oil was lost (Fig. 1). The amount of triglyceride extracted from soybean by chloroform/methanol and *n*-hexane was approximately 14 and 11% of the weight of the soybeans, respectively. Among phospholipid classes, phosphatidylcholine and phosphatidylethanolamine decreased significantly from 43 and 25% to 33 and 15%, respectively, and phosphatidic acid and lysophosphatidylcholine increased in soybeans during storage (Fig. 2A). Total amounts of phospholipids dissolved in the extracted oil

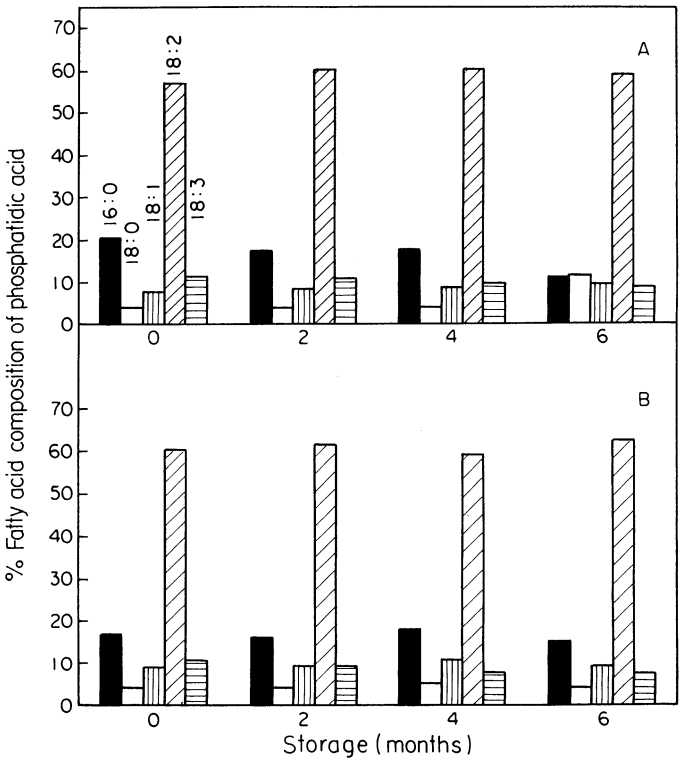


**Fig. 3.** Fatty acid composition of phosphatidic acid. **A**, whole soybean seeds; **B**, the *n*-hexane-extracted oil. ■ = palmitic acid, □ = stearic acid, ▥ = oleic acid, ▨ = linoleic acid, ▤ = linolenic acid.

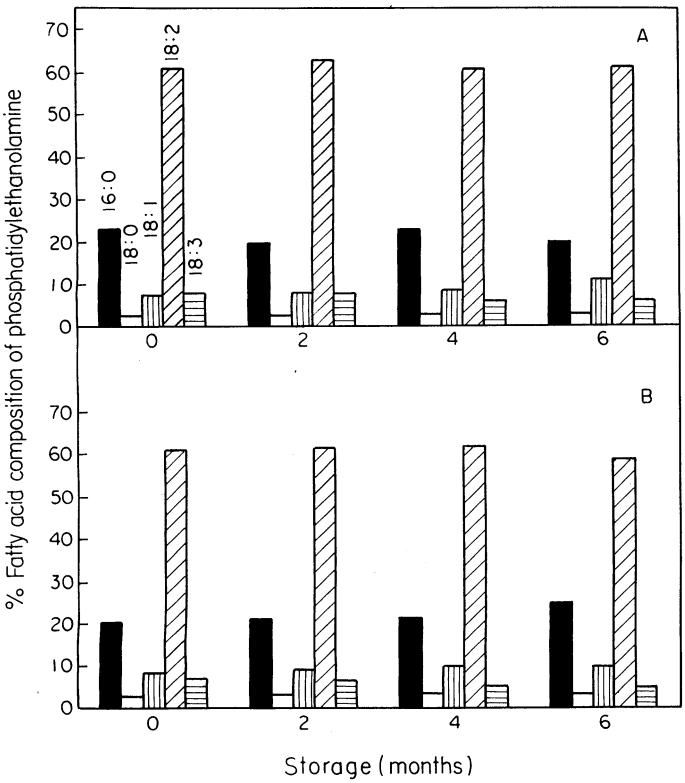


**Fig. 5.** Fatty acid composition of phosphatidylethanolamine. ■ = palmitic acid, □ = stearic acid, ▥ = oleic acid, ▨ = linolec acid, ▤ = linolenic acid.

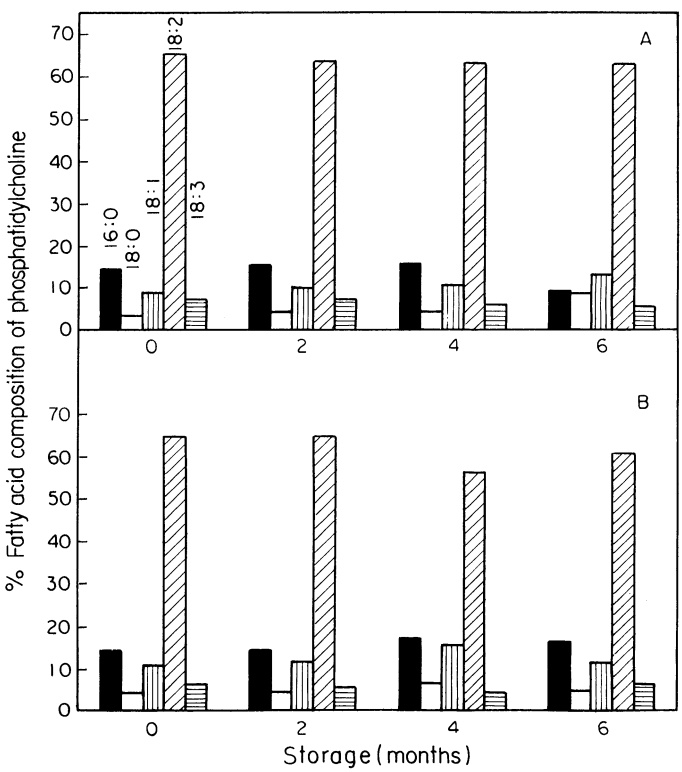


**Fig. 4.** Fatty acid composition of phosphatidylcholine. ■ = palmitic acid, □ = stearic acid, ▥ = oleic acid, ▨ = linoleic acid, ■ = linolenic acid.

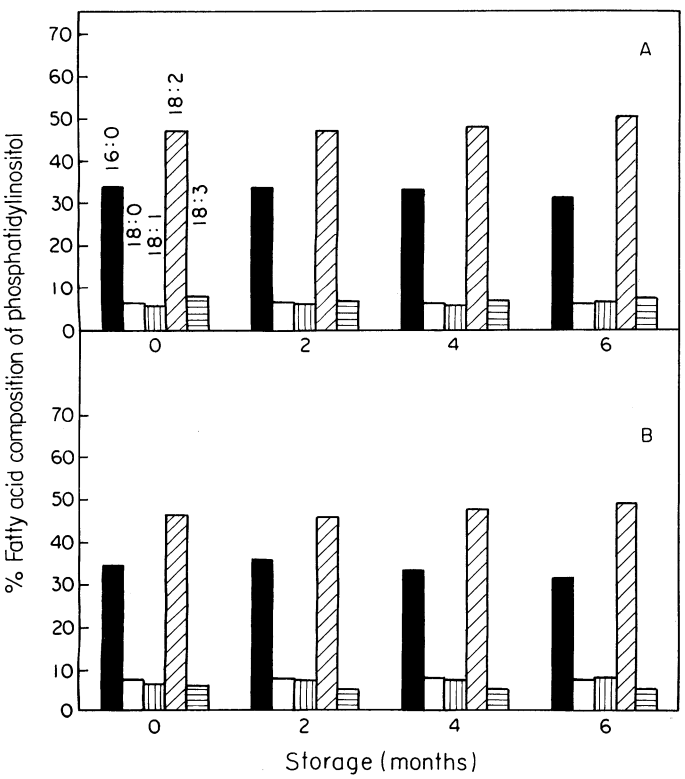


**Fig. 6.** Fatty acid composition of phosphatidylinositol. ■ = palmitic acid, □ = stearic acid, ▥ = oleic acid, ▨ = linoleic acid, ▤ = linolenic acid.

decreased with storage (Fig. 2B). The proportion of phosphatidic acid in the phospholipids in the extracted oil calculated from Fig. 2B increased from 27 to 39% in 6 months. These values were higher than those in soybeans, which were 8% at 0 time and 19% after 6 months (Fig. 2A).

Considerable amounts of phospholipids were removed by the degumming procedure. However, only phosphatidic acid remained constantly at a high level in the degummed oil, regardless of the phosphatidic acid content in the extracted oil before degumming (Fig. 2C). After 6 months, phosphatidic acid was the only phospholipid dissolved in the degummed oil.

### Fatty Acid Composition of Phospholipids

As described above, the proportions of phosphatidic acid in the phospholipids in the oils were higher than those in the seeds. Phosphatidic acid was assumed to be preferentially dissolved in the extracted oil. This suggests the possibility that specific fatty acyl chains are attached to phosphatidic acid. The fatty acid compositions of phospholipids were determined. Linoleic and palmitic acids were the major fatty acids of phosphatidic acid (Fig. 3). The fatty acid composition of phosphatidic acid in soybean did not differ from that in the extracted oil and did not change during storage. Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine were similar to that of phosphatidic acid (Figs. 4 and 5). However, the fatty acid composition of phosphatidylinositol was different from those of the three phospholipids (Fig. 6). Thus, phosphatidic acid has a diacylglycerol moiety similar to those of phosphatidylcholine and phosphatidylethanolamine as shown by the phospholipase D reaction.

### Phospholipase D Activity

Phospholipase D activity in the soybean extract required $Ca^{2+}$ and ethyl ether (Table I). Under the assay conditions described, the reaction proceeded linearly for at least 30 min. The optimum pH for the reaction was 4.8 (Fig. 7).As the crude extract was diluted, specific activity increased (Table II). This result suggests that a phospholipase D inhibitor may be present in the crude extract. As storage continued, specific activity of the diluted extract decreased from 344 to 189 nmole/mg/min (Table II), whereas that of crude extract increased from 41 to 47 nmole/mg/min. Because the ratio of specific activities of the crude extract and the diluted extract increased (Table II), phospholipase D activity can be assumed to become less inhibited the longer the period of storage.

## DISCUSSION

The amounts of phospholipids in the extracted oil were proportional to those in soybean (Fig. 1). However, the amounts and classes of phospholipids in the degummed oil were not comparable to those in the extracted oil. Phosphatidic acid remained in the degummed oil. The preferential solubility of this phospholipid was independent of its fatty acid composition. Since phosphatidic acid is produced from phosphatidylcholine and phosphatidylethanolamine by phospholipase D reaction, the increase in phosphatidic acid in soybean decreases the yield of "lecithin" after the degumming process.

Phospholipase D activity was found in a buffer extract of homogenized soybeans. The specific activity of the crude extract at 0 time was 41 nmole/mg/min. However, the more the extract was diluted with 10 m*M* Tris-HCl buffer, pH 7.0, the higher the specific activity became. This suggests the presence of a phospholipase D inhibitor in soybean. The crude extract was prepared by homogenizing 1 g of soybean in 10 ml of buffer. Thus, the enzyme and inhibitor are assumed to be more concentrated in soybean, and the activity may be effectively inhibited. As storage proceeded, inhibition decreased (Table II). On the other hand, the pH dropped during storage (Saio et al 1980).This change may have produced a better environment for the phospholipase D activity in soybean (Fig. 7). By these changes during storage, membrane phospholipids initially may have been degraded to phosphatidic acid. On the other hand, a small amount of phospholipids was hydrolyzed to lysophospholipids. These degraded phospholipids may have further decomposed to water-soluble compounds, because the increase of phosphatidic and lysophosphatidic acids was not comparable to the decrease of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol as storage proceeded (Fig. 2A). Therefore, degradation of phospholipids may bring about disintegration of organelle membranes, and as the result, the oils and proteins in spherosomes and protein bodies are exposed to cytoplasm and undergo deterioration.

**Table I**
**Effect of Activators on Phospholipase D Activity[a]**

| Substance | Choline (nmole/min) |
|---|---|
| Complete reaction | 4.5 |
| Reaction without | |
| Enzyme | 1.2 |
| Phosphatidylcholine | 0 |
| $Ca^{2+}$ | 1.1 |
| Ethyl ether | 1.5 |

[a] 10 μg of enzyme protein was used for the assay.

**Table II**
**Effect of Storage and Dilution of the Extract on Enzyme Activity**

| Time (months) | Dilution | Choline (nmole/min) | Protein (μg) | Specific Activity: Amount (nmole/mg/min) | Specific Activity: Ratio (amt. at 1/amt. at 1:100) |
|---|---|---|---|---|---|
| 0 | 1 | 19.56 | 480 | 41 | 0.12 |
| | 1:100 | 1.65 | 4.8 | 344 | |
| 2 | 1 | 17.85 | 430 | 42 | 0.17 |
| | 1:100 | 1.05 | 4.3 | 244 | |
| 4 | 1 | 17.59 | 390 | 45 | 0.19 |
| | 1:100 | 0.90 | 3.9 | 231 | |
| 6 | 1 | 17.73 | 380 | 47 | 0.25 |
| | 1:100 | 0.72 | 3.8 | 189 | |

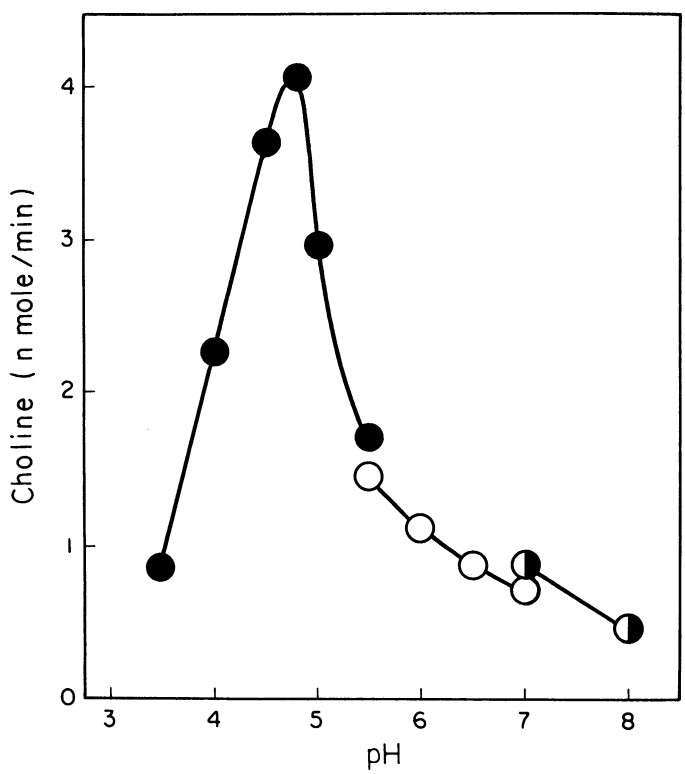


**Fig. 7.** Effect of pH on phospholipase D activity. Buffers: • = acetate, o = malate, ◑ = tris-HCl. Five micrograms of enzyme protein was used for the assay.

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