

## Isolation of Lipoxygenase Isozymes from Soybean Seeds

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### 대두 Lipoxygenase 이성효소의 분리

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#### Abstract

Soybean lipoxygenase isozymes were isolated from acetone-defatted soybean seeds (*Glycine max* [L.] Merr. variety AmSoy) by ammonium sulfate fractionation, gel filtration, and ion exchange chromatography. The final preparation of lipoxygenase-1 and -2 obtained was 19- and 32-fold purified, respectively, to the crude extract. But a considerable loss of total enzyme activity occurred during purification. On 7% polyacrylamide gel electrophoresis at pH 9.0, employing lipoxygenase specific staining technique, lipoxygenase-1, -2, and -3 showed distinctive Rf values of 0.38, 0.29, and 0.33, respectively.

#### Introduction

Lipoxygenase (E.C.1.13.11.12; linoleate: oxygen oxidoreductase) is a dioxygenase containing non-heme iron. It catalyzes the oxidation of polyunsaturated fatty acids having a *cis, cis* 1,4-pentadiene system to produce conjugated hydroperoxydiene derivatives through the insertion of molecular oxygen into the substrate.

The properties of lipoxygenase and the methods for its purification have been studied extensively in soybeans<sup>1-4</sup>. Since the indication of the existence of lipoxygenase isozymes by Kies<sup>5</sup>, evidence has been mounting to show that lipoxygenase is not a single entity but a complex system in soybeans. However, the evidence on the number of isozymes in soybeans is not consistent. Yamamoto et al.<sup>1</sup> and Verhúe and Francke<sup>2</sup> reported the existence of two isozymes, whereas Christopher<sup>6</sup> isolated four isozymes from hexane-defatted soybean meals

by incorporating hydroxyapatite column with ion-exchange chromatography.

In this study, isozymes of lipoxygenase were isolated from acetone-defatted soybean powder by employing traditional protein purification technique and identified by lipoxygenase specific staining technique in disc gel electrophoresis.

#### Materials and Methods

##### Materials

Soybean seeds (*Glycine max* [L.] AmSoy) were obtained from the Department of Agronomy, Iowa State University, Ames, Iowa, U.S.A.

##### Purification of Lipoxygenase isozymes

The purification procedure was a modification of the methods of Yoon and Klein<sup>7</sup> and Axelrod et al.<sup>8</sup>. All steps in the procedure were performed at 4°C unless otherwise stated.

Sixty grams of acetone-defatted soybean powder

was stirred for 2 hrs with 10 volumes of 50mM sodium phosphate buffer (pH 6.8). The slurry was forced through two layers of cheesecloth and centrifuged at 16,000g for 15 min in a Beckman model J-21 centrifuge (Palo Alto, U.S.A.). The supernatant was brought to 25% saturation with ammonium sulfate, then the solution was allowed to stand at least 1 hr and precipitate was spun down at 16,000g for 15 min. The supernatant was adjusted to pH 6.8 with 0.2N NaOH and was brought to 60% saturation with ammonium sulfate. The resulting precipitate was collected, which was resuspended in 50mM sodium phosphate buffer (pH 6.8) and dialyzed overnight against 2 changes of 50 volumes of each of 50mM sodium phosphate buffer (pH 6.8).

Approximately 50ml of the dialyzate (15mg/ml) was applied to the sephadex G-100 column (5.0×100 cm). The gel bed was eluted by gravity flow with 1,500 ml of 50mM sodium phosphate buffer (pH 6.8) at a rate of 50–75ml/hr. Ten ml of fractions were collected using a fraction collector. Lipoxygenase activity and protein concentration were determined on alternate fractions from the columns. Those with lipoxygenase activity were pooled and concentrated by using a 70 ml S-series Stirred Cell (Nucleopore, Pleasanton, U.S.A.) under nitrogen pressure of 60 psi. The membrane cut-off molecular weight was 50,000. This concentrated enzyme solution was dialyzed against three changes of 50 volumes of 50mM sodium phosphate buffer (pH 6.8).

Ten ml of enzyme solution (15mg/ml) was loaded on a column of DEAE-sephadex (2.6×70 cm), which had been pre-equilibrated with 50mM sodium phosphate buffer (pH 6.8). The enzyme was eluted with 2,000 ml of a linear gradient formed from 1,000 ml each of 50 and 220mM sodium phosphate buffer (pH 6.8), and alternate fractions from the column were assayed for lipoxygenase activity and protein concentration.

Lipoxygenase-1 active fractions from DEAE-sephadex column were collected, dialyzed against distilled water for 6 hrs, and checked for purity by 7% polyacrylamide gel electrophoresis (PAGE).

Fractions containing lipoxygenase-2 and -3 were pooled, and the solution was made up to 70% saturation with ammonium sulfate while maintaining the pH 6.8 with 0.2N NaOH. An additional 1 hr was allowed for complete precipitation of lipoxygenase-2 and -3. After centrifugation at 16,000g for 15 min, the precipitate was dissolved in 25 ml of 50mM sodium phosphate buffer (pH 6.8), and it was dialyzed against 2 changes of 50 volumes each of 50mM sodium phosphate buffer (pH 6.8).

The dialyzed protein solution was loaded on a second DEAE-sephadex column. Elution was carried out with an increasing linear gradient formed from 750 ml each of 50mM and 220mM sodium phosphate buffer (pH 6.8). The flow rate was adjusted to 25 ml/hr, and fractions of 6 ml were collected. Lipoxygenase-2 and -3 active fractions were pooled, and dialyzed, and checked for purity by 7% PAGE.

#### Protein Determination

Protein concentrations were determined by optical density at 280nm and by the Lowry method<sup>9)</sup>. The absorbance of protein at 280nm was used as an index of protein concentration in the column eluants. The Lowry method was used to determine protein concentration of sample except those of column eluants.

#### Lipoxygenase Assay

Lipoxygenase was determined polarographically using a YSI Oxygen Monitor model 53 with Clark electrode (Yellow Spring Inc., U.S.A.). Throughout purification, an aqueous linoleate substrate was used<sup>10)</sup>. The stock substrate solution was diluted with 50mM borax buffer (pH 9.0) and 50mM

sodium phosphate buffer (pH 6.8) for lipoxygenase -1 and -2/3 assay, respectively, giving a final concentration of 2.57mM linoleic acid. Values for oxygen solubility in the reaction medium were obtained from Chappell<sup>11)</sup>. One unit of lipoxygenase activity corresponds to the consumption of 1 $\mu$ M of oxygen per minute.

#### Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed to check the purity of lipoxygenase-1, -2 and -3 according to the methods of Davis<sup>12)</sup> and Guss et al.<sup>13)</sup> All the PAGE chemicals were obtained from Bio-Rad, Richmond, U.S.A.

The electrophoresis was performed at 3 to 5mA per tube until the tracking dye was approximately 5mm from the bottom of the gel. For lipoxygenase specific staining, the gels were incubated in the substrate solution containing 2.57mM linoleic acid in 50mM tris-HCl buffer (pH 8.3) for approximately 30 min with frequent inversion to assure adequate aeration. The gels were then removed from the substrate solution, rinsed with distilled water, transferred to test tubes containing 0.02% 3,3'-dimethoxybenzidine hydrochloride (Eastman Kodak Co., Rochester, U.S.A.), and stained for 2 hrs at room temperature<sup>14)</sup>. Reddish-brown bands were identified as the site of lipoxygenase migration. The distance of the dye and lipoxygenase band migration and the length of the gel were measured.

The protein staining was done by immersing the gels in 0.025% coomassie brilliant blue R-250 in 5% methanol, 10% acetic acid for 10 hrs at room temperature.

## Results and Discussion

The purification of lipoxygenase isozymes from acetone-defatted soybean powder was accomplished by employing the traditional procedures for the separation of proteins: ammonium sulfate fractionation, sephadex G-100 gel filtration, and DEAE Sephadex A-50 ion exchange chromatography.

Specific activity and fold purification during purification of lipoxygenase from soybean seeds are summarized in table 1. About 19-fold purification for lipoxygenase-1 was obtained after DEAE Sephadex chromatography, while 32-fold purification of lipoxygenase-2 was obtained after the rechromatography of the pooled fractions of lipoxygenase -2 and -3, resulting in a considerable loss of total enzyme activity. After ammonium sulfate fractionation, 39% of lipoxygenase-1 was recovered, while 81% of lipoxygenase-2 was recovered, which indicates that lipoxygenase-2 is more stable than lipoxygenase-1. The recovery of total isozyme activity after DEAE Sephadex column remained between 10 and 14%, which has been reported by a few researchers<sup>8,15)</sup>.

Table 1. Summary of purification of lipoxygenase-1 and -2 from soybeans

Fractions	Specific activity (units / mg)		fold purification		Recovery (%)	
	LOX-1	LOX-2	LOX-1	LOX-2	LOX-1	LOX-2
Crude extract	0.43	1.23	1.00	1.00	100	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 25-60% ppt.	1.15	6.83	2.67	5.55	39.00	80.97
Gel filtration	4.37	16.71	10.16	13.59	37.75	50.39
DEAE-Sephadex	8.04	—	18.70	—	10.36	—
2nd DEAE-Sephadex	—	39.11	—	31.80	—	13.83

A typical elution pattern from a Sephadex G-100 column is shown in figure 1. The activities at pH 9.0 and pH 6.8, optimum pH of lipoxygenase -1 and -2/3, respectively, were both eluted at the same fractions. The elution profile from DEAE Sephadex column is shown in figure 2. Lipoxygenase -1 (peak 111) was sharply separated from lipoxygenase -2 and -3 (peak 11 and 1, respectively),

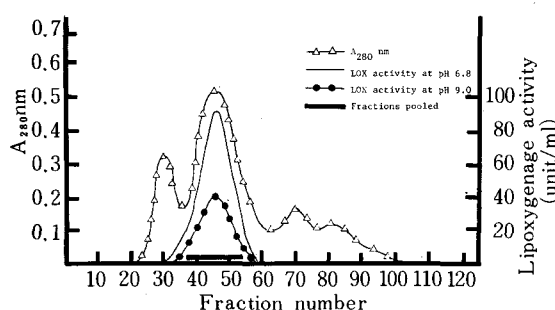


Fig. 1. Gel filtration chromatography of soybean lipoxygenase

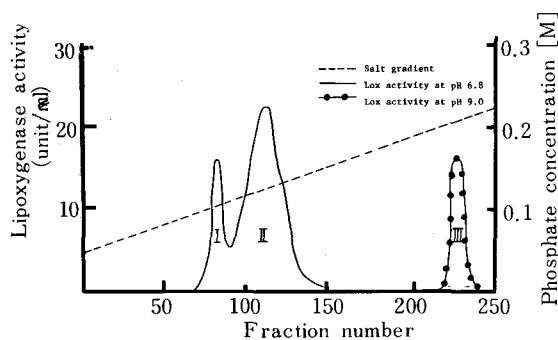


Fig. 2. DEAE-sphadex chromatography of soybean lipoxygenase

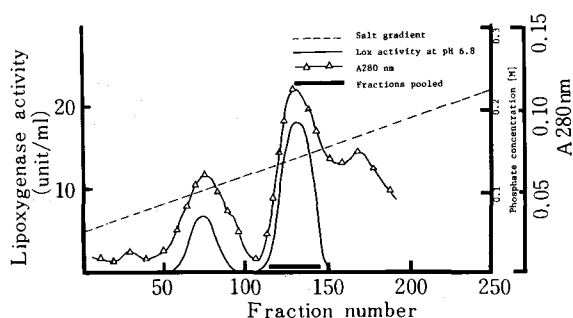


Fig. 3. Rechromatography of fractions rich in lipoxygenase -2 and -3 obtained from first DEAE-sphadex chromatography

and appeared last, eluted at a concentration of 200–200mM phosphate. The fractions of lipoxygenase -2 and -3 appeared to overlap. These fractions were pooled and then passed through the second DEAE Sephadex column again. The elution profile from the second DEAE Sephadex column is shown in figure 3, where lipoxygenase -2 appears immediately after lipoxygenase-3.

This profile is similar to that of Christopher et al.<sup>15). Who isolated a third enzyme of soybean lipoxygenase employing ammonium sulfate fractionation and DEAE Sephadex chromatography. It is clear from this profile that at least three isozymes of lipoxygenase occur in soybean extracts. Later, Christopher<sup>6)</sup> could resolve lipoxygenase-3 into lipoxygenase-3a and -3b by incorporating a hydroxyapatite column. However, the mixture can be treated as a single enzyme<sup>18)</sup>, since lipoxygenase -3a and -3b were alike in all aspects except chromatographic behaviors on columns of hydroxyapatite and CM Sephadex<sup>6)</sup>.</sup>

Purified lipoxygenase-1, -2, and -3 were subjected to 7% PAGE to check their purity. The proteins having lipoxygenase activity were localized by a specific staining technique based on the ability of enzymatically formed hydroperoxide to react with dye, 3,3'-dimethoxybenzidine hydrochloride, producing a reddish-brown color<sup>14)</sup>. This method is reported to be more sensitive and to have less background staining from one described by Guss et al.<sup>13)</sup> who employed acidic potassium iodide on polyacrylamide gels impregnated with starch, which acts through the reaction of enzymatically produced hydroperoxide with acidic potassium iodide producing the elementary form of iodine. The iodine then reacts with starch in the gel to produce brownish violet bands as the site of lipoxygenase activity.

Employing the above procedures, it was possible to obtain preparations of lipoxygenase-1, -2, and -3 that appeared homogenous on disc gel

electrophoresis in tris-glycine buffer at pH 9.0. The respective Rf values were 0.38, 0.29, and 0.33. Axelrod et al.<sup>8)</sup> reported that Rf values of lipoxygenase -1, -2, and -3, employing bromophenolblue as a standard in tris-glycine buffer (pH 9.5), were 0.30, 0.25, and 0.28, respectively. These differences could be ascribed to the different buffer systems employed.

For the purification and isolation of lipoxygenase, hexane-defatted soybean meal was used most commonly.<sup>1,2,6,16)</sup> In this experiment, acetone-defatted soybean powder was subjected to the extraction of lipoxygenase isozymes with 50mM sodium phosphate buffer (pH 6.8), which was useful for the isolation of both lipoxygenase-1 and -2.<sup>8)</sup>

A few nonconventional technique for the purification of lipoxygenase, such as isoelectric focusing and affinity chromatography, were reported to be efficient and specific.<sup>17,18)</sup> But these techniques suffer from the disadvantages that the one is one-step purification,<sup>17)</sup> and the other does not separate the isozymes of lipoxygenase.<sup>18)</sup> For the separation of isozymes, traditional purification procedures have been reported to be successful and very reliable.<sup>1,2,7,19)</sup>

Lipoxygenase activity was measured by a polarographic method, based on the measurement of oxygen uptake during the reaction throughout this experiment. Turbidity of the reaction mixture originating either from crude extracts or poorly solubilized substrate did not present any difficulties in measuring enzyme activity, since this method does not require an optically clear solution as does the spectrophotometric method.<sup>20)</sup> One disadvantage of this technique is that oxygen absorption is not specific for lipoxygenase since oxygen uptake can be caused by other systems such as heme proteins.<sup>8)</sup> Therefore, controls were employed when the activity of crude extracts was tested in this assay. No significant activity by heme proteins was detected. Linoleic acid, which is the best substrate

for lipoxygenase, is not soluble below pH 7.0, but become increasingly soluble at higher pHs.<sup>20)</sup> The substrate solution prepared for lipoxygenase-2 and -3 assay at pH 6.8 was turbid. Poor solubility of linoleic acid was improved by the inclusion of an emulsifier (tween 20), resulting in soluble substrate over a rather wide pH range. Allen<sup>21)</sup> also reported the successful use of water-soluble substrate, potassium octadeca-9,12-dienyl sulphate or potassium linoleyl sulphate in kinetic studies, and pH profile determinations of lipoxygenase. However, no further studies with these substrates have been reported, and they are not commercially available yet.

## 요 약

아세톤으로 탈지한 대두분으로부터 gel여과법과 이온 교환 크로마토그래피 등의 전통적인 단백질 추출 방법을 이용하여 Lipoxygenase 이성효소를 분리하였다. 이온 교환 후의 lipoxygenase-1과 -2는 Crude extract에 비해 각각 19배와 32배 정제 되어 졌으나, 상당한 효소활성의 손실을 초래하였으며, lipoxygenase 에 특이적인 염색기술을 이용하여 행한 7% PAGE에서 lipoxygenase-1, -2, 그리고 -3 은 각각 0.38, 0.29 그리고 0.33의 Rf치를 나타내었다.

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