successful. Benzene probably lacks sufficient solvent polarity.

Recommendations. Field samples of runoff should be at room temperature and continuously stirred during subsampling. Oryzalin should be extracted with 3:1 to 9:1 benzene–methanol solution; however, based on present pesticide-grade solvent costs, 3:1 benzene–methanol is more economical than 9:1. The GC column should have at least the resolution of the one described in the Experimental Section.

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Separation of Soybean Lipoxygenase and Peroxidase by Hydrophobic Chromatography

Separation of lipoxygenase from peroxidase in legumes has posed some difficulties in using conventional ion-exchange chromatography. With hydrophobic chromatography, crude extracts of soybean flakes showed selective, reversible binding of the two enzymes onto Phenyl Sepharose CL-4B resins. Elution of the enzymes from the resin was performed by a stepwise decrease in the concentration of the equilibration buffer at pH 6.8. Lipoxygenase eluted from the hydrophobic column before peroxidase, resulting in clean separation, high yield, and substantial purification of both enzymes. Disc gel electrophoresis showed that lipoxygenase was considerably purer than peroxidase, and the two enzymes were clearly separated from one another without any cross contamination. An acid-shocking phenomenon was observed in the detection of the peroxidase isozyme forms.

Hydrophobic chromatography is a technique which allows the separation of proteins by making use of the differences in size and distribution of available hydrophobic pockets or regions on the proteins (Shaltiel, 1974). This technique has been used in studies characterizing food enzymes such as peach polyphenol oxidase (Flurkey and Jen, 1978). The resolution potential of hydrophobic chromatography is not exhausted by choosing the most appropriate spacer arms; further resolution can be achieved by specific elution conditions.

Lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.1.13.), isolated by Theorell et al. (1946), catalyzes the oxidation of various *cis,cis*-1,4-pentadiene systems into hydroperoxides by means of molecular oxygen (Holman and Bergström, 1951). Wolf (1975) and Rackis et al. (1972) have shown that the enzyme is closely related to flavor production and the off-flavor of soybean protein products.

Traditionally, soybean lipoxygenase was purified by conventional techniques such as ammonium sulfate fractionation, molecular filtration, and ion-exchange chromatography (Christopher et al., 1970; Johns et al., 1973). These techniques are reliable but the processes are usually slow and the yields are relatively low. Catsimpoolas (1969) achieved a 230-fold purification of soybean lipoxygenase using the isoelectric focusing technique. Grossman et al. (1972) applied affinity chromatography through a linoleyl aminoethyl agarose column and obtained near homogeneous soybean lipoxygenase. On the other hand, Allen et al. (1977) used a number of aminohexyl agarose derivatives of unsaturated fatty acids for a one-stage purification of soybean lipoxygenase-1 and obtained a purification factor of 16. They suggested that in their affinity chromatographs, the major specific substrate-binding force is not a hydrophobic interaction with the saturated hydrocarbon moiety of the substrate. Wheeler and Wallace (1978), using wheat germ lipoxygenase, have shown that the enzyme could be bound to Teflon or epoxy-coated surfaces through hydrophobic interactions.

In this report, we showed that soybean lipoxygenase can be bound to a hydrophobic resin. Further, we showed that in spite of the fact that both soybean peroxidase and lipoxygenase are retained on a hydrophobic column, they can be made to detach selectively from the column by changing the nature of the eluting solvent, thus resulting in clean separation and substantial purification of both enzymes.

MATERIALS AND METHODS

Materials. Defatted soybean flakes were obtained at a local market. Linoleic acid (99%) was purchased from Sigma Chemical Company. Phenyl Sepharose CL-4B was obtained from Pharmacia Fine Chemicals. All other materials used in this study were commercial products of reagent grade quality.

Enzyme Extraction. Lipoxygenase extraction was a modification of the method described by Christopher et al. (1970). Defatted soybean flakes (30 g) were homogenized with 300 mL of sodium phosphate buffer 0.05 M, pH 6.8) in a prechilled Virtis homogenizer. The soybean homogenate was stirred for 90 min at room temperature.

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All the following steps were performed at 4 °C. The extract was filtered through two layers of cheesecloth and the resulting filtrate was centrifuged at 17 500g for 15 min. The supernatant was filtered through glass wool to remove cell debris, and the pellet was discarded. The filtrate was assayed for lipoxygenase and peroxidase activities and was brought to 25% saturation with solid ammonium sulfate. This solution was allowed to sit overnight and centrifuged, and the pellet and supernatant were assayed for peroxidase and lipoxygenase activities. This supernatant was used for hydrophobic column chromatography.

Hydrophobic Chromatography. Preswollen Phenyl Sepharose CL-4B resin was degassed and equilibrated in 4 volumes of deaerated equilibration buffer (EB) consisting of 25% ammonium sulfate in 0.05 M potassium phosphate buffer, pH 6.8. Ten milliliters of the sample solution was layered onto each column (7.5 × 1.5 cm, 13 mL bed volume). The columns were eluted by a stepwise decrease in the concentration of the equilibration buffer (i.e., 0.8 × EB, 0.6 × EB, 0.2 × EB), followed by 5 mM potassium phosphate buffer (pH 6.8) and 50% ethylene glycol containing 1 M KCl. Six-milliliter fractions were collected and assayed for peroxidase and lipoxygenase activities.

Enzyme Assays. Lipoxygenase activities were determined by monitoring the increase in absorbance at 234 nm after the addition of 10 μL of enzyme to 2.5 mL of 0.1 mM linoleic acid in 0.2 M borate buffer, pH 9.0 (Chan, 1973). One unit of lipoxygenase activity was defined as a change of 1 absorbance unit/min at 30 °C. Peroxidase activities were assayed by adding 0.05 mL of enzyme extract into 2 mL of 0.45% guaiacol and 0.01% hydrogen peroxide in 0.2 M potassium phosphate buffer, pH 6.8, and following the increase in absorbance at 470 nm (Flurkey and Jen, 1978). One unit of peroxidase activity was defined as a change of 0.1 absorbance unit/min at 30 °C.

Protein Determinations. Protein determinations were performed using the Biorad Protein Assay (Biorad Laboratories, 1977) or the standard Lowry method (Lowry et al., 1951). The Biorad assay was used to measure the protein content of the sample prior to column application. All samples and column eluates were dialyzed against 0.05 M KCl solution before the Lowry measurements were made. During chromatographic runs, column eluates were monitored at 280 nm by an ISCO Model UA-5 monitoring system for approximate protein contents.

Disc Gel Electrophoresis. Disc gel electrophoresis was performed according to the method of Davis (1964). Peroxidase activity was located by incubation of the gels in 0.045% guaiacol and 0.001% hydrogen peroxide (Flurkey and Jen, 1978). Lipoxygenase activity was visualized by the method of Guss et al. (1967), with the exception that the substrate consisted of 0.1 mM linoleic acid in 0.2 M borate buffer, pH 9.0, instead of 2 mM linoleic acid in 0.05 M Tris-HCl buffer, pH 8.3. The running gels contained 1% soluble potato starch and electrophoresis was performed at 4 °C for 2 h at 5 mA/tube. Duplicate gels were stained for protein using Coommassie Blue R-250.

RESULTS AND DISCUSSION

Phenyl Sepharose CL-4B Column. A typical elution pattern for soybean extract chromatographed on a Phenyl Sepharose CL-4B column is shown in Figure 1. A considerable amount of protein (280 nm absorbing material) was not bound to the column, while some proteins appeared to be weakly bound and were eluted with 0.8 × EB and 0.6 × EB step gradients. The major portion of lipoxygenase eluted in the 0.2 × EB step, while peroxidase was eluted in the ethylene glycol-KCl step. Johns et al.

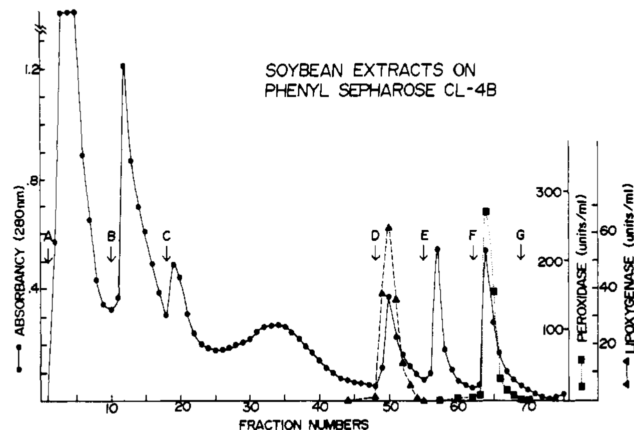

Figure 1. Hydrophobic chromatography of soybean extract on Phenyl Sepharose CL-4B. Elutions were with a decreasing step gradient: (A) equilibration buffer, EB, 50 mL; (B) 0.8 × EB, 50 mL; (C) 0.6 × EB, 200 mL; (D) 0.2 × EB, 50 mL; (E) 5 mM phosphate buffer, 50 mL; (F) 50% ethylene glycol in 1 M KCl, 50 mL; (G) H_2O, 50 mL; (●—●) protein content, A_{280} units/mL; (▲—▲) lipoxygenase activity at pH 9.0, A_{234} units/mL; (■—■) peroxidase activity at pH 6.8, A_{470} units/mL.

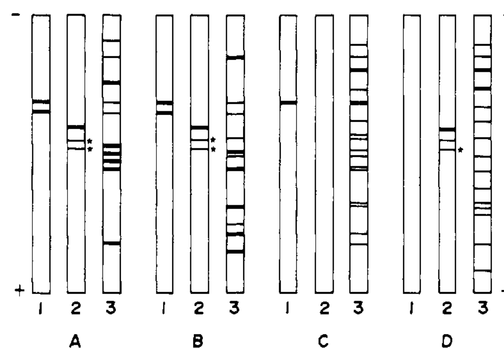

Figure 2. Starch polyacrylamide gel electrophoresis. Electrophoresis was carried out for 2 h at 4 °C and 5 mA/tube. Two hundred micrograms of protein was layered onto each gel: (A) crude soybean supernatant, (B) 25% ammonium sulfate cut supernatant, (C) fraction 49-52 of column eluates, (D) fraction 64-66 of column eluates; (1) lipoxygenase stain, (2) peroxidase stain, (3) protein stain. (*) These bands appear after acetic acid incubation.

(1973) found considerable contamination of the lipoxygenase fractions with peroxidase when purifying soybean extracts by DEAE Sephadex. Eriksson and Svensson (1970), using ion-exchange chromatography, encountered similar peroxidase contamination in the purification of pea lipoxygenase. We have observed a clean separation between these two enzymes using hydrophobic interaction chromatography. The distinct separation indicates that these two soybean enzymes probably possess different affinities for the hydrophobic resin used here. Biospecific and pseudo-affinity effects as well as hydrophobic effects have been found to operate in the binding of peach polyphenol oxidase and tomato peroxidase to Phenyl Sepharose CL-4B resin (Flurkey et al., 1978). Therefore, it is not unlikely that soybean peroxidase may show a pseudo-affinity for the resin due to structure similarities between the resin and many of the peroxidase substrates.

The peak of lipoxygenase activity (fractions 49-52) represented 76% recovery from the ammonium sulfate fraction and a 9.3-fold of purification in comparison with the crude extract (Table I). Allen et al. (1977) had mentioned that with a purification factor of 16 from 45 to 50% ammonium sulfate precipitates, they obtained an almost pure lipoxygenase-1 tested by isoelectric focusing

Table I. Summary of Purification of Crude Soybean Lipoxygenase and Peroxidase on Phenyl Sepharose CL-4B Resin

			lipoxygenase				peroxidase			
	volume, mL	total protein, mg	total activity, units	sp act., units/mg	purification	step yield, %	total activity, units	sp act., units/mg	purification	step yield, %
crude	10	99.0	1080	10.9	1.00	100	3880	39.2	1.00	100
25% supernat.	10	84.9	982	11.6	1.06	91	3571	42.1	1.07	92
fractions 49-52	26.8	7.37	748	101.5	9.30	76				
fractions 64-66	20	3.4					2697	793.2	20.2	76

and specific activity. The peroxidase peak (fractions 64–66) also represented 76% recovery of the enzyme but showed a 20.2-fold of purification. The specific activity of peroxidase seemed to be higher than that of lipoxygenase, and these were in agreement with the findings of Rackis et al. (1972). Optimal conditions for separation and binding of these two enzymes to the Phenyl Sepharose CL-4B resin appear to be related to sample composition, total protein content applied onto the column, and the protein/gel volume ratio. Overloading the column with high protein content resulted in an incomplete separation of peroxidase from lipoxygenase activities. A low protein/gel ratio resulted in decreased yields of the two enzymes. We found that a ratio of 10 mg of protein/mL of gel, as estimated by the Biorad Protein Assay, resulted in good separation and high yields of both enzyme activities. We have consistently observed that the amount of protein applied onto the column did not equal the amount of protein eluted off the column; presumably, some material was firmly bound to the resin and must be eluted by more drastic methods. Allen et al. (1977) have noticed a large proportion of the applied protein bound more firmly than lipoxygenase when using linoleate derivatives as ligands in affinity chromatography.

Disc Gel Electrophoresis. Electrophoresis on potato starch–polyacrylamide gels of the crude extract, the 25% ammonium sulfate cut supernatant, the active lipoxygenase fraction (PS 49–52), and the active peroxidase fraction (PS 64–66) gave the results presented in Figure 2. The crude extract revealed two bands of lipoxygenase activity with R_f values of 0.32 and 0.35, respectively. The ammonium sulfate cut supernatant also showed two bands of similar R_f values. However, fractions 49–52 of the column eluates revealed only one of these bands with R_f 0.32, probably corresponding to that of lipoxygenase-1 as defined by Christopher et al. (1970). Guss et al. (1967) showed two major bands with R_f values of 0.33 and 0.40 and two minor bands with R_f values of 0.24 and 0.21 in crude soybean extracts and only one major band in commercially purified soybean preparations. Peroxidase, on the other hand, showed only one band in the crude extract and 25% ammonium sulfate cut supernatant, but two bands in fractions 64–66 of the column eluates. Acid shocking (i.e., gels placed in 7% acetic acid for 10 s) of the peroxidase gels resulted in the visualization of two more bands of peroxidase activity for the crude and 25% ammonium sulfate cut supernatant and one more band for the fractions 64–66 eluates, making a total of three peroxidase isozymes in all samples. This acid-shocking phenomenon has been observed in tomato peroxidase preparations as well (Flurkey et al., 1978).

Protein staining of the gels revealed many protein bands. For lipoxygenase, the activity bands corresponded well with the major protein bands particularly in the column eluated fraction 49–52 (Figure 2). The peroxidase bands, fractions 64–66, did not correspond to any major protein band. However, the background for these gels was quite dark due to the presence of the potato starch; therefore, only distinct protein bands could be clearly seen. It is, however, an evidence of clear separation between the two soybean enzymes eluted from this chromatographic column. Further, there was no evidence of any cross contamination of either of the active enzyme fractions.

Throughout this study we have found several advantages for the use of Phenyl Sepharose CL-4B resin for hydrophobic chromatography. The technique was easy to perform and high flow rates were normally obtained. The capacity of the resin for protein was high, and high yields together with significant purification can be achieved. In relation to other plant enzymes studied (Flurkey and Jen, 1978; Flurkey et al., 1978), this technique has been found to result in the separation of different isozymes, purification of desirable enzyme, removal of inhibitory substances, concentration of the desirable enzyme, and high yields of recovered enzyme activities in a single step. The potential use of hydrophobic chromatography to study other food enzymes, in our opinion, is very great.

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