*Food Chemistry* **31** (1989) 57–71

# The Behaviour of Green Bean Lipoxygenase on Chromatography and Isoelectric Focusing

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(Received 13 January 1988; revised version received 4 March 1988)

*ABSTRACT*

*A crude enzyme preparation was isolated from green beans (*Phaseolus vulgaris *cv. Cascade) by buffer extraction and subsequent fractionation with ammonium sulphate. The properties of lipoxygenase were investigated by three chromatographic methods: hydrophobic interaction chromatography followed by isoelectric focusing, ion-exchange and gel filtration chromatography.*

*Three forms of the enzyme were found which could be separated by chromatography on DEAE-Trisacryl M. The major form, having an isoelectric point at pH 7 and a molecular weight of 100 000, was hydrophobically bound to the gel. An ionic form having a higher molecular weight than the hydrophobic form was found to be present in ammonium sulphate precipitates but not in the original extracts. A third form of the enzyme which did not bind to the DEAE-gel was also observed. Its properties were not investigated. The implications of these findings in relation to the work of others is discussed.*

## INTRODUCTION

Lipoxygenase (linoleate : oxygen oxidoreductase EC 1.13.11.12) catalyses the oxygenation of the *cis,cis*-1,4-pentadiene moiety in polyunsaturated fatty acids to conjugated *cis,trans*-hydroperoxides. The latter then degrade to

yield a variety of secondary products which are involved not only in the production of acceptable flavours of fresh fruits and vegetables but also in the unacceptable off-flavours which sometimes arise, usually as a result of incorrect processing (Schwimmer, 1981). The hydroperoxides may also be involved in the loss of the characteristic colour of some products as a result of the co-oxidation of pigments (Klein *et al.*, 1984). Much of the literature on lipoxygenase has been concerned with the enzyme from soybeans where it is known to be involved in the formation of 'beany' off-flavours in processed products (Sessa, 1979). With the possible exception of peas (Chen & Whitaker, 1986), very little work has been carried out on the lipoxygenases of vegetables commonly processed by the frozen food industry.

Frozen green beans are a commercially important vegetable commodity which are sensitive to under- or over-blanching with serious losses in colour, flavour and texture during storage. In order to improve quality, and save energy it has been suggested that green beans should not be blanched to destroy all of the peroxidase activity but only blanched sufficiently to inactivate lipoxygenase (Adams, 1983). This has been shown to be adequate for a shelf life of 18 months at $-18°C$.

The purpose of the study was to investigate the purification of green bean lipoxygenase with a view to understanding its role in the deterioration of quality in food systems. Other workers have investigated the purification of lipoxygenase from other sources by means of ion-exchange, affinity, hydrophobic-interaction and gel-filtration chromatographies and by electrophoresis and isoelectric focusing. In most instances a major fraction of enzymic activity has been lost during ion-exchange chromatography and only the ionic forms subjected to study. This paper reports the behaviour of the green bean enzyme during purification and highlights some major differences with other published work.

## MATERIALS AND METHODS

Whole green beans (*Phaseolus vulgaris* cv. Cascade) grown at Luddington Experimental Horticultural Station, were harvested at a maturity suitable for commercial freezing. The lipoxygenase enzyme was extracted both from fresh beans and from frozen, unblanched beans held at $-18°C$.

All reagents were of analytical grade and unless stipulated below, were purchased from BDH Chemicals. Linoleic acid (99% pure), Tween 20, soybean lipoxygenase (Type I), lactoperoxidase, bovine serum albumin, horseradish peroxidase (Type VI), carbonic anhydrase, cytochrome c, *n*-octyl β-D-glucopyranoside (*n*-octyl glucoside) and Tris(hydroxymethyl)-aminomethane were purchased from Sigma Chemical Company Ltd.

Ultrogel AcA44, DEAE-Trisacryl M and the preparative isoelectric focusing equipment, fraction collector and UV detector were purchased from LKB Instruments. Pharmalyte (pH 3–10) and Phenyl Sepharose CL-4B were purchased from Pharmacia, protein assay kits from Bio-Rad, and polyethylene glycol 20000 (Aquacide) was purchased from Calbiochem. Dialysis tubing was obtained from Medicell International and was treated prior to use with 0·05% sodium carbonate (30 min at 60°C; four times), washed with 95% industrial methylated spirit (30 min at 50°C) and rinsed with distilled water.

**Lipoxygenase assay**

Lipoxygenase assays were carried out as described by Surrey (1964) with the following modifications. A stock substrate solution was first prepared containing $8 \times 10^{-3}$M linoleic acid and 0·25% Tween 20 and then this was diluted with 0·18M phosphate-citrate buffer (pH 7·0) to give a working substrate containing $1{\cdot}34 \times 10^{-4}$M linoleic acid and 0·004% Tween 20. The lipoxygenase activity was determined by adding either 0·05 ml or 0·1 ml of enzyme preparation to 2·5 ml of the working substrate at 30°C. The rate of increase in absorbance at 234 nm due to the formation of hydroperoxides containing the conjugated diene chromophore, was measured using a Pye Unicam PU 8800 UV/VIS recording spectrophotometer. The activity of the enzyme was taken to be the mean rate at the completion of 3 min reaction (rates every 20 s) and expressed as $A_{234\,nm}\,min^{-1}$ per volume of enzyme preparation assayed. The stock substrate was stable for 1–2 weeks at 4°C whilst the working substrate, which was held at room temperature, was only stable for 2–3 h.

**Protein determination**

Protein determinations were carried out using the commercial form of the dye-binding method of Bradford (1976). The assays were performed as recommended by the suppliers (Bio-Rad). A standard curve was constructed using soybean lipoxygenase (Type I) in the concentration range 200–1400 $\mu g\,ml^{-1}$. It was shown to be non-linear.

**Preparation of enzyme extract and ammonium sulphate precipitation**

All extractions, pH measurements and subsequent chromatographic steps were carried out at 4°C. 100 g of green beans were thoroughly blended for 30 s with 200 ml 0·1M Tris-HCl buffer (pH 8·0 at 4°C) and the brei squeezed

through two layers of muslin. The filtrate was centrifuged for 20 min at 21 000*g*. Sufficient ammonium sulphate was added to the supernatant to give 40% saturation. After standing for 10–15 min, the precipitate was removed by centrifugation for 20 min at 21 000*g*. The pellet was discarded and sufficient ammonium sulphate added to the supernatant to give 60% saturation. The precipitate was removed by centrifugation for 20 min at 21 000*g* and the pellet held at −15°C prior to dissolving it in the appropriate buffer for subsequent purification steps.

**Hydrophobic interaction chromatography**

Hydrophobic interaction chromatography was carried out on Phenyl Sepharose CL-4B according to the method of Flurkey *et al.* (1978) with the following modification. The ammonium sulphate precipitate was suspended in 20 ml of a buffer consisting of 0·05M potassium phosphate (pH 6·8) which was 25% saturated with ammonium sulphate. After centrifugation, the supernatant was applied to a column of Phenyl Sepharose CL-4B (5·7 × 2·6 cm; 30 ml bed volume), pre-equilibrated with the same buffer. The loaded column was washed with 150 ml of equilibration buffer to remove unbound protein and then eluted with 50 ml of equilibration buffer which had been diluted 5-fold with 0·05M potassium phosphate (pH 6·8), followed by 75 ml of $1{\cdot}33 \times 10^{-2}$M glycine. Finally, the column was eluted with 144 ml of $1{\cdot}33 \times 10^{-2}$M glycine and this eluate was used for isoelectric focusing.

**Isoelectric focusing**

The manufacturer's instructions were followed with regard to the filling and running of the 440 ml isoelectric focusing column. Pharmalyte (Type, pH 3–10) was mixed with the glycine eluate from the Phenyl Sepharose CL-4B and sucrose was then dissolved in this mixture to form a gradient solution of higher density. The top electrode was made the anode and the power was applied in two stages, 10 W for 30 min and then 30 W for 18 h.

**Ion-exchange chromatography**

*Crude extract*

A 0·1M Tris buffer extract of 100 g of fresh green beans was centrifuged and the resulting supernatant applied, without ammonium sulphate fractionation, to a column of DEAE-Trisacryl M (4·7 × 2·6 cm; 25 ml bed volume), equilibrated with 0·1M Tris HCl. Elution was carried out at 1 ml $min^{-1}$ with 0·1M Tris buffer, followed by 0·1M Tris-1M NaCl and finally 0·1M Tris-0·01M *n*-octyl glucoside. All eluants were pH 8·0.

*Ammonium sulphate precipitate*
The 40–60% ammonium sulphate precipitate was dissolved in 5 ml of 0·1M Tris-HCl and dialysed against the same buffer until equilibrium had been reached. Any precipitate at this stage was removed by centrifugation and the sample applied to a column of DEAE-Trisacryl M (4·7 × 2·6 cm; 25 ml bed volume) previously equilibrated with 0·1M Tris-HCl. Initial elution was carried out with 0·1M Tris-HCl and then with the following alternative regimes: A 0·1M Tris-1M NaCl then 0·1M Tris-1M NaCl-0·01M *n*-octyl glucoside. B. 0·1M Tris-0·01M (or 0·025M) *n*-octyl glucoside then 0·1M Tris-1M NaCl. All buffers were pH 8·0. Flow rates were 1 ml $min^{-1}$.

*Post-gel filtration chromatography*
0·1M Tris-1M NaCl eluants from gel filtration on Ultrogel AcA44 were applied directly to 25 ml columns of DEAE-Trisacryl M freshly prepared and equilibrated with 0·1M Tris-1M NaCl. Elution was performed at 1 ml $min^{-1}$ with 0·1M Tris-1M NaCl followed by 0·1M Tris-0·01M *n*-octyl glucoside. All eluants were pH 8·0.

**Gel filtration chromatography**

*Ammonium sulphate precipitate*
The 40–60% ammonium sulphate precipitate was dissolved in 5 ml of 0·1M Tris-1M NaCl (pH 8·0) and applied to a column of Ultrogel AcA44 (45·2 × 2·6 cm; 240 ml bed volume) previously equilibrated with 0·1M Tris-1M NaCl (pH 8·0). The column was eluted at a flow rate of 1 ml $min^{-1}$.

*Post ion-exchange chromatography*
A 0·1M Tris-0·01M *n*-octyl glucoside eluant from a column of DEAE-Trisacryl M was concentrated by dialysis against solid Aquacide to give 1·4 mg protein in 5 ml. This was then applied to the Ultrogel AcA44 column as described above.

The column was calibrated using the following proteins: soybean lipoxygenase (molecular weight, 100 000), lactoperoxidase (molecular weight, 79 000), bovine serum albumin (molecular weight of monomer, 66 000), carbonic anhydrase (molecular weight, 29 000) and cytochrome c (molecular weight, 12 000). 2 ml of each protein was applied at a concentration of 1 mg $ml^{-1}$ except for lactoperoxidase which was applied at 0·5 mg $ml^{-1}$. The proteins were applied individually in order to avoid any interactions which may occur in a mixed sample and were detected in the column eluate using an ultraviolet detector set at 280 nm. Enzyme assays were also performed on the lipoxygenase and peroxidase standards.

## RESULTS AND DISCUSSION

Major losses of lipoxygenase activity have been found on ion-exchange chromatography of the enzyme from soybeans (Stevens *et al.*, 1970), peanuts (Sanders *et al.*, 1975) and from a variety of fruits and vegetables (Haydar & Hadziyev, 1973; Klein, 1976; Bonnet & Crouzet, 1977; Truong & Mendoza, 1982; Truong *et al.*, 1982; Hidaka *et al.*, 1986; Chen & Whitaker, 1986). Losses occur at all stages of purification, however, and an important part of our study was to examine the behaviour of the green bean lipoxygenase using a range of enzyme purification procedures in order to understand some of the factors involved.

### Extraction and precipitation of lipoxygenase

Preliminary experiments showed that the optimum extraction of lipoxygenase from green beans occurred at pH 8·0 in 0·1M Tris-HCl at a ratio of 2 parts buffer to 1 part beans. This gave the highest activities as measured by oxygen consumption (Adams & Ongley, 1984). Various additions to the buffer were evaluated. Sodium chloride (0·1-1M) was included in order to improve the extraction of ionic forms of the enzyme. Calcium chloride (0·001–0·1M) was included as other workers have shown that calcium ions stabilise the activity of lipoxygenase under certain conditions (Klein, 1976). Sucrose (10% and 40%) was added as Wardale & Galliard (1975) had found that it increased the stability of the enzyme in subcellular organelles. The additions had little effect on the amount of lipoxygenase extracted although the sucrose addition did result in a higher retention of enzyme activity on storage of the extracts at 4°C. In agreement with previous findings on frozen snap beans (Klein, 1976), the precipitate obtained between 40% and 60% saturation of the extract with ammonium sulphate was found to contain the majority of the lipoxygenase activity.

Using a 40–60% ammonium sulphate precipitate dialysed against Tris buffer, it was shown that the specific activity of the lipoxygenase apparently increased as the protein concentration was decreased on dilution with Tris (Table 1). Thus, any chromatographic procedure which causes dilution of the sample may appear to purify the enzyme. Similar results have been obtained by Klein (1976) on preparation of an ammonium sulphate precipitate from green beans, by Grossman *et al.* (1969) with a crude extract from alfalfa and by Christopher *et al.* (1972) using a highly purified isoenzyme from soybeans. This phenomenon could be explained if green bean lipoxygenase was a Type 2 enzyme requiring a high substrate concentration to achieve maximal activity. Under such conditions it may not be possible to obtain maximal activity, either because the available substrate

**TABLE 1**
Effect of Dilution on Lipoxygenase Activity

| *Dilution factor (fold)* | *Lipoxygenase activity* ($A_{234\,nm}\ min^{-1}\ ml^{-1}$) | *Specific activity (lipoxygenase activity per mg of protein)* |
|---|---|---|
| 0 | 1·87 | 1·2 |
| 5 | 4·71 | 3·1 |
| 10 | 6·83 | 4·5 |
| 20 | 9·43 | 6·3 |

has diminished as a result of its having exceeded its critical micelle concentration or because of product inhibition. However, a reduction in enzyme concentration could lead to a higher proportion of enzyme–substrate complexes and, therefore, to a relatively high activity. Other explanations are that high molecular weight inhibitors, remaining after dialysis or chromatography, have a lower affinity for the lipoxygenase enzyme when the sample is diluted. Alternatively, the enzyme may form aggregates with itself or other hydrophobic proteins, which disproportionate on dilution, leading to a relatively high concentration of available enzyme to react with the linoleic acid substrate.

*Isoelectric focusing*

Isoelectric focusing of the glycine eluate from hydrophobic interaction chromatography resulted in the separation of two isoenzymes focusing at pH 7 and pH 9, respectively. The purification achievable by this procedure was affected by the presence of a yellow–green protein component which co-eluted with the lipoxygenase fraction from the hydrophobic gel and precipitated on the isoelectric focusing column (Fig. 1). The less active of the two lipoxygenase isoenzymes, focusing at pH 9, gave a distinctly different progress curve from that of the main isoenzyme when assayed using linoleic acid under the standard conditions given. The rate of increase of absorbance at 234 nm, which was rapid over the first 20 sec with no observable lag, fell off quickly and approached zero in 1–2 min. The main isoenzyme gave a typical sigmoidal curve with the rate falling off slowly. Further study of the minor isoenzyme showed it to be an artefact of the isoelectric focusing procedure which could be generated in a control column containing no applied sample. It is suggested that the basic ampholytes are reacting with the linoleic acid to give compounds which absorb at 234 nm. Thus, under the conditions used in this study, only one isoenzyme has been established and this has an isoelectric point at about pH 7.

Hale *et al.* (1969) and de Lumen *et al.* (1978) have found one major

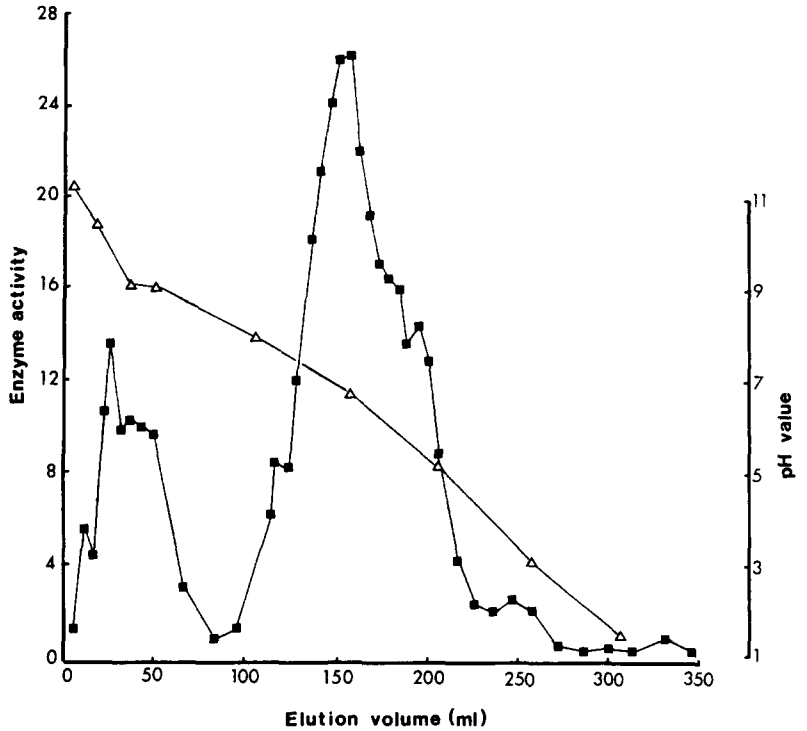


**Fig. 1.** Isoelectric focusing of the glycine eluate from Phenyl Sepharose CL-4B. 2·5 ml fractions were collected at 4 ml $min^{-1}$. A yellow–green precipitate formed during focusing in the pH region 4–6·5. ■ Lipoxygenase activity × $10^2$ ($A_{234\,nm}$ $min^{-1}$ 0·05 $ml^{-1}$); △ pH value (at 4°C).

lipoxygenase isoenzyme in green beans and one minor isoenzyme by means of polyacrylamide gel electrophoresis. Delincée *et al.* (1975) has shown a greater multiplicity of isoenzymes using analytical thin-layer isoelectric focusing. He reported ten isoenzymes with isoelectric points between pH 5 and pH 9. However, many were minor components and only two or three isoenzymes were present in significant quantities. Of these, a broad band of activity focusing at about pH 6 appeared to be similar to the isoenzyme found in the present work.

*Ion-exchange chromatography*

DEAE-based ion-exchangers have been used in many schemes for purifying lipoxygenase. In the present work, DEAE-Trisacryl M was employed, initially with a view to separating the two lipoxygenase isoenzyme groups with isoelectric points at pH 7 and pH 9. By equilibrating with 0·1M Tris buffer at pH 8·0, it was expected that the minor isoenzyme, focusing at pH 9, would be positively charged overall and would not bind to the column

**TABLE 2**
Purification of Green Bean Lipoxygenase on DEAE-Trisacryl M

| *Sample/eluant* | *Total lipoxygenase activity* ($A_{234\,nm}\,min^{-1}$ *per total volume*) | *Total protein concentration (mg per total volume)* | *Specific activity (lipoxygenase activity per mg of protein)* |
|---|---|---|---|
| 40–60% $(NH_4)_2SO_4$ precipitate | 11·2 | 175·0 | 0·064 |
| 0·1M Tris buffer | 11·6 | 38·3 | 0·30 |
| 0·1M Tris-1M NaCl | 4·0 | 80·7 | 0·050 |
| 0·1M Tris-1M NaCl-0·01M *n*-octyl glucoside | 64·0 | 0·8 | 80·0 |

whereas the major isoenzyme, focusing at pH 7, would have an overall negative charge and would bind ionically. Using ammonium sulphate precipitates, it was observed that a small amount of lipoxygenase activity, together with a relatively large amount of protein, was eluted during loading and subsequent washing stages (Table 2). The amount of unbound form varied from sample to sample and was undetectable on some occasions. Assay progress curves were sigmoidal, unlike those for the minor isoenzyme. This was taken as further evidence that the minor isoenzyme was an artefact of the isoelectric focusing procedure.

A second small peak of lipoxygenase activity was eluted with buffer containing 1M NaCl. This ionic form of the enzyme was closely associated with a large amount of protein and with the yellow–green pigment observed during hydrophobic chromatography and isoelectric focusing. However, the major fraction of lipoxygenase remained bound to the column and could be removed only by the addition of *n*-octyl glucoside, a non-ionic detergent, to the buffer. The protein concentration of this fraction was very low whilst the total level of lipoxygenase activity was far in excess of that applied to the column in the first instance. This phenomenon is most likely related to that shown on dilution of ammonium sulphate precipitates and discussed earlier. The elution profile was tailed, indicating a strong hydrophobic interaction between the enzyme and the gel. However, increasing the concentration of the *n*-octyl glucoside to the critical micelle value (0·025M) did not reduce the tailing significantly. Confirmation of independent mechanisms of binding of the ionic and hydrophobic forms of lipoxygenase to the DEAE-gel was obtained by reversing the above elution sequence; that is, eluting with Tris-*n*-octyl glucoside in the first instance followed by Tris-NaCl subsequently. The

hydrophobic form eluted with the detergent containing buffer whilst the ionic form eluted with the salt-containing buffer (Fig. 2). The ratio of activities in the two forms was approximately the same whichever elution regime was employed.

Differences in behaviour, on DEAE-Trisacryl M, were observed between ammonium sulphate precipitate and a fresh buffer extract of green beans. In the latter case, a substantial amount of unbound lipoxygenase activity was measured whilst little or no ionic fraction was detected. The hydrophobic form of the enzyme eluting with *n*-octyl glucoside was found to be present in both cases.

Application of the major isoenzyme band to DEAE-Trisacryl M, resulted in binding of the hydrophobic fraction only, and no ionic form was

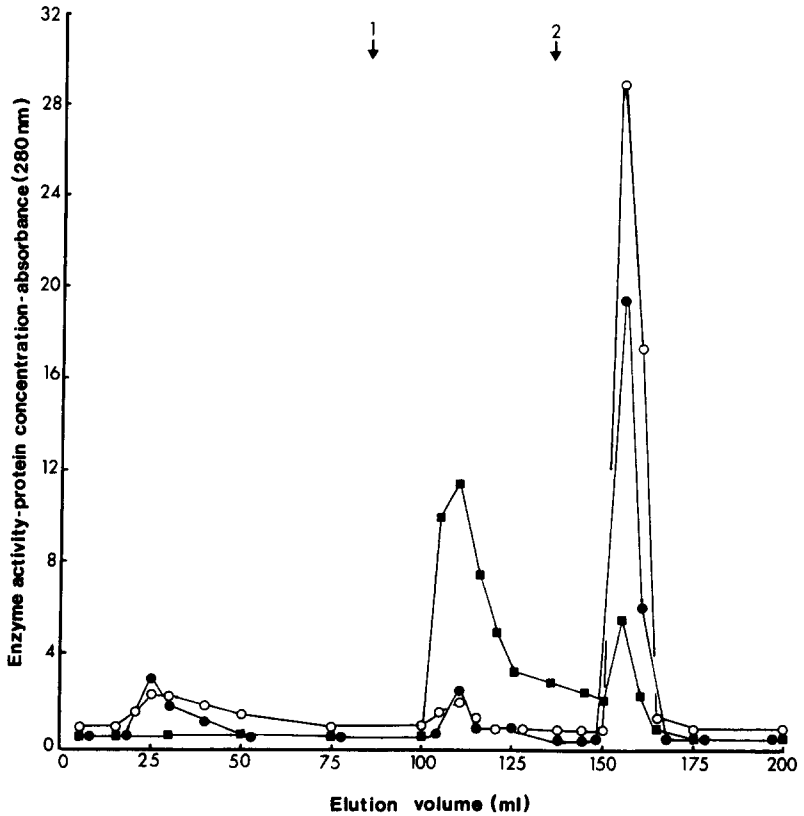


**Fig. 2.** Chromatography of an ammonium sulphate precipitate from green beans on DEAE-Trisacryl M. Approximately 30 mg protein applied. Total protein recovered = 64%. Initial elution was with 0·1M Tris buffer, pH 8·0. Change of eluates: 1. 0·1M Tris-0·025M *n*-octyl glucoside (pH 8·0) 2. 0·1M Tris-1M NaCl (pH 8·0). 5 ml fractions were collected at 1 ml $min^{-1}$. ■ Lipoxygenase activity × $10^2$ ($A_{234\,nm}$ $min^{-1}$ 0·1 $ml^{-1}$); ● Protein concentration × $10^2$ (mg 0·1 $ml^{-1}$); ○ Absorbance at 280 nm × 10.

observed. Presumably, the ionic form of the enzyme had co-precipitated with other proteins during isoelectric focusing (see Fig. 1).

The information in the literature on the behaviour of green bean lipoxygenase on ion-exchange chromatography is sparse. Klein (1976) observed a low recovery of the enzyme from DEAE-Sephadex when eluting with a 0·05–0·5M NaCl gradient in 0·05M Tris-HCl (pH 7·2). However, the use of detergents to improve enzyme recovery, was not investigated. Very large losses of lipoxygenase activity have also been found at the DEAE chromatography stage when purifying the enzyme from soybeans (Stevens *et al.*, 1970), peanuts (Sanders *et al.*, 1975) tomatoes (Bonnet & Crouzet, 1977), potatoes (Sekiya *et al.*, 1977), cowpeas (Truong & Mendoza, 1982), winged beans (Truong *et al.*, 1982), pumpkin (Hidaka *et al.*, 1986) and peas (Haydar & Hadziyev, 1973; Chen & Whitaker, 1986) and the enzyme which eluted with the salt-containing buffers represented only a minor proportion of the total activity. However, none of the groups evaluated detergent-containing buffers as eluants. The first reported hydrophobic chromatographic separations involved the adsorption of protein to a variety of matrices, including DEAE-cellulose, by 1·5–3M ammonium sulphate or 1M potassium phosphate followed by desorption with a decreasing salt gradient (Mevarech *et al.*, 1976). In the present case, ammonium sulphate was not required to induce the binding of green bean lipoxygenase to DEAE-Trisacryl M which implies that this enzyme form is extremely hydrophobic. As lipoxygenase is probably associated with lipid bilayers in cellular membranes, *in vivo*, and functions at polar/apolar interfaces, it is perhaps not surprising that native forms of the enzyme bind hydrophobically to the DEAE-gel rather than ionically.

*Gel filtration chromatography*

The behaviour of a 40–60% ammonium sulphate precipitate from green beans on a column of Ultrogel AcA44 eluted with 0·1M Tris-1M NaCl is shown in Fig. 3. The main peak of lipoxygenase activity had an elution volume corresponding to a molecular weight of approximately 100 000, which compares well with that of the soybean enzyme (Stevens, 1970; Christopher *et al.*, 1972). There was also a shoulder on the leading edge of the lipoxygenase peak which corresponded to a protein of higher molecular weight.

A sample from the central part of the peak was shown to contain only the hydrophobic form of lipoxygenase as all activity bound to a DEAE-Trisacryl M column, pre-equilibrated with Tris-NaCl buffer, and *n*-octyl glucoside was required for its elution. A sample representing the shoulder lipoxygenase activity from the Ultrogel AcA44 column was also applied to DEAE-Trisacryl M when part of the activity eluted in Tris-NaCl and part in

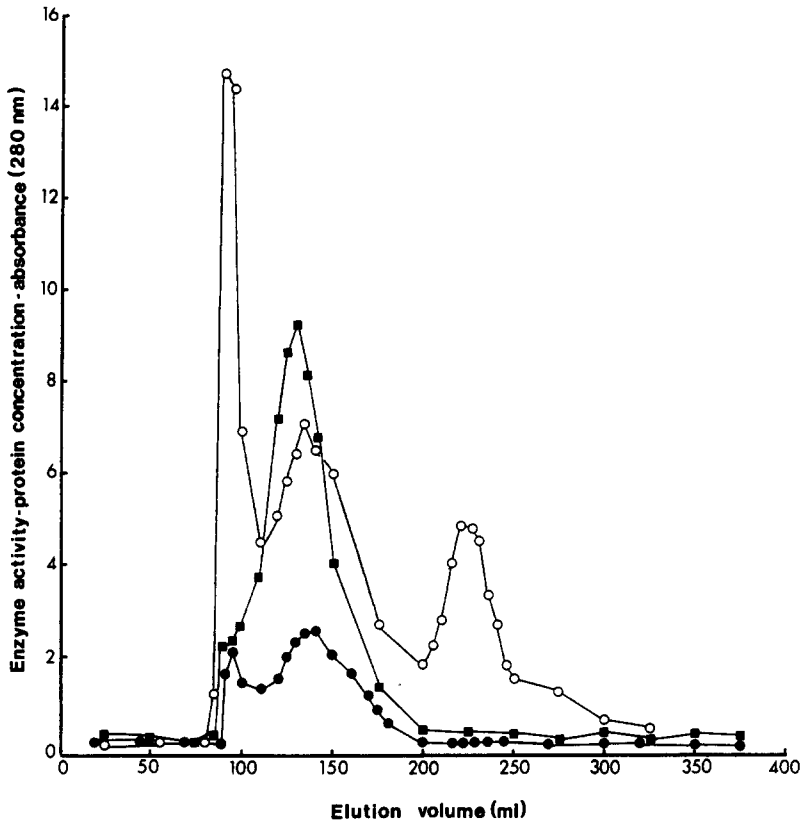


**Fig. 3.** Gel filtration chromatography of an ammonium sulphate precipitate from green beans on Ultrogel AcA44. Approximately 20 mg protein applied. Total protein recovered = 100%. Eluant was 0·1M Tris-1M NaCl (pH 8·0). 5 ml fractions collected at 1 ml $min^{-1}$. ■ Lipoxygenase activity × $10^2$ ($A_{234nm}$ $min^{-1}$ 0·1 $ml^{-1}$; ● Protein concentration × $10^2$ (mg 0·1 $ml^{-1}$); ○ Absorbance at 280 nm × 30.

Tris-*n*-octyl glucoside. This suggests that the higher molecular weight part of the lipoxygenase activity contained the ionic form of the enzyme.

The hydrophobic form of the lipoxygenase, extracted from fresh green beans and purified by chromatography on DEAE-Trisacryl M, was found to give a peak of activity on elution from the Ultrogel AcA44 column which corresponded to a molecular weight of 100 000. The high molecular weight protein was absent in this case and no shoulder was observed on the leading edge of the lipoxygenase peak. Thus, the purification on DEAE-Trisacryl M has removed the higher molecular weight components from the ammonium sulphate precipitate and has not caused any change in the apparent molecular weight of the lipoxygenase.

Some variation in the molecular weight of lipoxygenase is apparent in the literature. The soybean enzyme is generally accepted to have a molecular

weight of 100 000 but in the case of pea lipoxygenase, various values have been reported between 64 000 and 106 000 (Chen & Whitaker, 1986). Delincée *et al.* (1975) found the molecular weight of green bean lipoxygenase to be 50 000 using Sephadex thin-layer gel filtration, although additional zones of 100 000 and 500 000 were noted occasionally. The latter components were considered to be artefacts or labile forms of the enzyme. The discrepancy in molecular weights between the hydrophobic form of lipoxygenase in our work and the enzyme extracted by Delincée *et al.* cannot be explained at present. However, it may be related to the existence of different forms of bound enzyme in different cultivars and to different methods of extraction and separation.

In conclusion, this study suggests that there are three forms of green bean lipoxygenase which can be separated by DEAE-chromatography according to various properties of charge and hydrophobicity. The major form was hydrophobically bound to DEAE-Trisacryl M and was eluted only in the presence of the non-ionic detergent, *n*-octyl glucoside. It has an isoelectric point at pH 7 and a molecular weight of 100 000. This form of lipoxygenase does not appear to have been investigated by other workers.

The ionic form of the enzyme, which is eluted from DEAE-Trisacryl M with salt, has a higher molecular weight than the hydrophobic form. It appears to represent a relatively small proportion of the total lipoxygenase and was detected in ammonium sulphate precipitates but not in the original green bean extracts.

The amount of unbound form of the enzyme varied from sample to sample and its properties were not studied further.

Further work is required to determine the relative importance of the various forms of lipoxygenase for the deterioration of quality in frozen green beans.

## ACKNOWLEDGEMENTS

The authors thank the Ministry of Agriculture, Fisheries and Food for financial support for this work and the Director of Campden Food and Drink Research Association for permission to publish it.

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