ELSEVIER

Food Chemistry 70 (2000) 199–204

Food Chemistry

www.elsevier.com/locate/foodchem

Role of tryptophan residues of lipoxygenase-1 in activity, structure and stability: chemical modification studies with *N*-bromosuccinimide

Sonati Srinivasulu, A.G Appu Rao *

Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore-570 013, India

Received 18 November 1999; accepted 4 January 2000

Abstract

The tryptophan residues of lipoxygenase-1 (LOX1) from soybeans (*Glycine max*) were modified using *N*-bromosuccinimide under both native and denaturing conditions. The accessibility of tryptophan residues was pH-dependent. Only one tryptophan residue was accessible at the optimum pH of enzyme activity and with a decrease in pH from 9 to 2, the accessibility increased. Modification of the accessible four tryptophan residues at pH 4.0 under non-denaturing conditions resulted in complete loss of enzyme activity; one tryptophan residue was critical for enzyme activity. Modification of the tryptophan residues did not alter the substrate binding affinity; the presence of the substrate during modification did not alter the extent of modification. Modification of the surface-exposed tryptophans did not affect (i) the conformation or structural integrity but (ii) decreased the stability.

Keywords: Lipoxygenase-1; *N*-Bromosuccinimide; Tryptophan residues; Structure and stability

1. Introduction

Lipoxygenases (linoleate oxygen oxido reductase EC 1.11.13.12) catalyze the oxygenation of the *cis*, *cis* 1-4 pentadiene moiety of polyunsaturated fatty acids (Axelrod, Cheesebrough & Laakso, 1981). In animals, these enzymes are involved in the formation of leukotrienes and lipoxins which mediate inflammatory response (Samuelsson, Dahlen, Lingren, Rouzer & Serhan, 1987). In plants, these enzymes are involved in the synthesis of compounds which play a dominant role in plant growth, senescence and defence mechanisms (Gardner, 1991; Grechkin, 1998). Recently the crystal structure of LOX1 was solved at high resolution and shown to possess two domains (Boyington, Gaffney & Amzel, 1993; Minor, Steczko, Otwinowski, Bolin, Walter & Axelrod, 1996). Because of its higher stability and ease of preparation, LOX1 from soybean is extensively characterized (Schewe, Rapoport & Kuhn, 1986) and could serve as a model for mammalian lipoxygenases in general.

Chemical modification of proteins has been a valuable technique to study structure and function of enzymes (Feeney, 1987; Lundblad & Noyes, 1984). Modification of certain residues can result in either stabilization or destabilization (Cupo & Pace, 1983; Klibanov, 1983). Changes in enzyme structure through selective modification can lead to altered catalytic activity. Among the 13 tryptophan residues in LOX1, only two tryptophan residues are located in the 'N' terminal domain at positions 87 and 130, and the remaining 11 residues are in the 'C' terminal domain (Shibata, Steczko, Dixon, Hermodson, Yazdanparast & Axelrod, 1987). Based on fluorescence-quenching measurements, LOX1 is reported to have a large hydrophobic active site which contains the tryptophan residues of the protein and iron (Finazzi-agro, Avigliano, Egmond, Veldink & Vliegenthart, 1975). The modification of tryptophan residues resulted in complete loss of activity. Based on spectral studies in the UV region with the enzyme, it has been reported that at least two tryptophan residues are essential for catalytic activity (Klein, Cohen, Grossman, King, Malovany & Pinsky, 1985). In this present investigation, tryptophan residues on the enzyme molecule were modified with NBS to assess the role of these residues in catalytic/substrate binding, enzyme structure

* Corresponding author. Tel.: +91-821-520131; fax: +91-821-517233.

E-mail address: appu@cscftri.ien.nic.in (A.G. Appu Rao).

0308-8146/00/$ - see front matter
PII: S0308-8146(00)00080-7

and thermal stability of the molecule. The results obtained by activity and spectral studies are interpreted in the light of the available crystal structure data.

2. Materials and methods

Soybean LOX1 was purified by the method of Axelrod et al. (1981) with an additional step of molecular sieve chromatography as reported earlier (Srinivasuu & Rao, 1995). The purified enzyme was over 95% pure as characterized by SDS-PAGE electrophoresis and sedimentation velocity measurements; the specific activity was 150 units/mg protein. The following chemicals were used : linoleic acid (>99%), Nucheck Prep. Inc. (MN), *N*-bromosuccinimide (NBS) from Sigma Chemical Company, M.O. DEAE Sephadex A50 and Sephadex G75 were from Pharmacia Fine Chemicals, Sweden and all other chemicals were of analytical grade.

2.1. Enzyme assay

The enzyme was assayed by following the increase in absorption at 234 nm, due to the formation of conjugated dienes using Tween 20-solubilized substrate (Axelrod et al., 1981). The enzyme concentration was determined by measuring the optical density at 280 nm and using a value of $E_{1cm} = 14.0$ (Axelrod et al., 1981). The remaining activity is expressed as (%) remaining, due to the modification, in comparison to that of control. The reaction was followed up to 3 min. For modification studies, the enzyme samples were incubated with varying concentrations of *N*-bromosuccinimide at 27°C for 30 min. For activity measurements, 10 μl sample was withdrawn and diluted to 3000 μl with 0.2 M borate buffer (pH 9.0) containing 100 M sodium linoleate.

2.2. Estimation of tryptophan groups modified

The extent of modification of tryptophan residues in LOX1 was estimated by titrating the protein with NBS (4.15×10^{-3} M). The reagent was added in small aliquots to the sample and the decrease in absorbance at 280 nm was followed. The number of residues modified was calculated using a molar extinction coefficient of 5500 $cm^{-1}M^{-1}$ (Spande & Witkop, 1967). These measurements were done in 50 mM acetate buffer, (pH 4.0).

2.3. Determination of essential tryptophan residues

The statistical method of Tsou (1962) was used to calculate the number of essential tryptophan residues for enzyme activity. The relationship between remaining activity against tryptophan residues modified is as follows:

$$(A/A_0)^{1/i} = (n-m)/n$$

The number of essential tryptophan residues is that value of i, which gives a straight line when remaining activity $(A/A_0)^{1/i}$ is plotted against m, the number of tryptophan residues modified. Circular dichroism and fluorescence measurements were made as reported earlier (Srinivasulu & Rao, 1993, 1995). Fluorescence quenching by acrylamide was analyzed with the Stern–Volmer equation (Lehrer & Leavis, 1987). All measurements were made in 20 mM acetate buffer (pH 4.0).

2.4. Substrate binding studies

Substrate binding measurements were made by following the quenching of the enzyme fluorescence by the addition of small increments of Tween-20-solubilized linoleic acid. The emission at 333 nm was followed after being excited at 285 nm and the band widths for excitation and emission monochromators were 5 and 10 nm, respectively. Fluorescence-quenching measurements with modified enzyme were made after dialyzing the samples against acetate buffer to remove excess of reagent. The data were analyzed in terms of binding of the substrate to the enzyme using established procedures (Rao & Cann, 1981).

2.5. Thermal unfolding measurements

The thermal unfolding of LOX1 was measured in acetate buffer pH 4.0 (5 mM)in a Gilford response II spectrophotometer with an integrated temperature program. The thermal transition was followed by the decrease in absorbance at 287 nm. The heating rate was 1°C /min and the path length was 1 cm.

2.6. Urea induced unfolding measurements

The urea-induced unfolding of LOX1 before and after modification was followed by changes in far UV CD (mean residue ellipticity values at 222 nm) and fluorescence emission (332 nm) due to unfolding. The enzyme was incubated with different urea concentrations at pH 4.0, for 30 min, for unfolding. The extent of unfolding was calculated as percentage native at each concentration of urea. These calculations were made with the assumption that the difference in any parameter between native and 8M urea was 100 %.

3. Results

The accessibilities of tryptophan residues of LOX1 to NBS as a function of pH, both in the presence and absence of denaturant (8 M urea) are shown in Fig. 1. Under non-denaturing conditions, the number of

groups accessible increased from one residue at pH 9.0 to 7 residues at pH 2.0. In the presence of denaturant at all the pH values, the accessibility of tryptophan increased by three more residues.

3.1. Activity measurements

The (%) remaining activity, with increase in molar ratio of NBS to protein is shown in Fig. 2A. The activity of LOX1 decreased with an increase in the number of modified tryptophan residues: the addition of 50 μM linoleic acid in the incubation mixture did not provide any protection against modification of the tryptophan residues. The extent of modification was very similar.

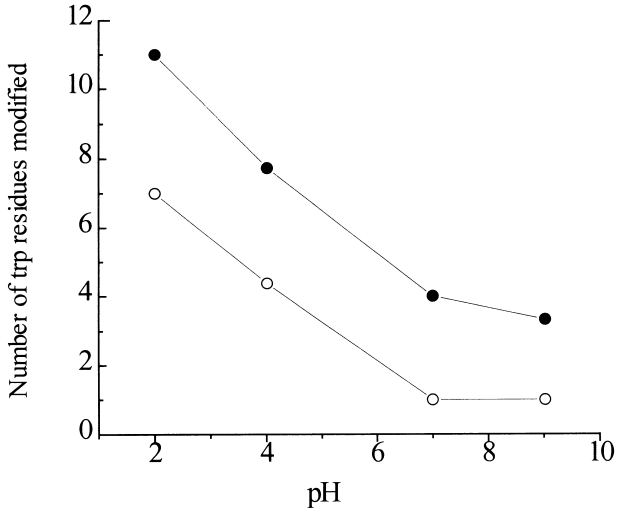

Fig. 1. The accessibility of tryptophan residues as a function pH both in the presence and absence of denaturants. (○) Native, (●) 8 M urea. The protein concentration was 5.3×10^{-6}M.

The statistical method of Tsou (1962) was used to calculate the number of essential tryptophan residues for inactivation of LOX1. The data could be fitted to a straight line with $i=1$, suggesting that modification of one tryptophan residue is critical for activity (Fig. 2B).The kinetics of fatty acid oxidation before and after modification of LOX1 are also studied. The lag period in the fatty acid oxidation increased with the modification of tryptophan residues of LOX1 (data not shown).

3.2. Substrate binding measurements

The binding of substrate linoleic acid to LOX1 was measured by following the quenching of protein fluorescence by the addition of substrate linoleic acid, before and after modification at pH 4.0. The binding constant of substrate for LOX1 at pH 4.0 was 4×10^4 M^{-1}. After modification of four tryptophan residues on the enzyme molecule, the binding constant was not altered, indicating that the binding of the substrate to the enzyme was not affected by the modification (data not shown).

3.3. Fluorescence measurements

The fluorescence emission spectra of LOX1 before and after modification at pH 4.0 are shown in Fig. 3. The emission maximum at 333 nm for the control did not change with the modification, but there was a decrease in the relative fluorescence intensity. Modification of the first residue did not affect the emission intensity significantly; with subsequent modification the quantum yield decreased (Fig. 3 inset). To quantitate subtle changes in protein conformation due to modification, the quenching of protein fluorescence by acrylamide was measured before and after modification of

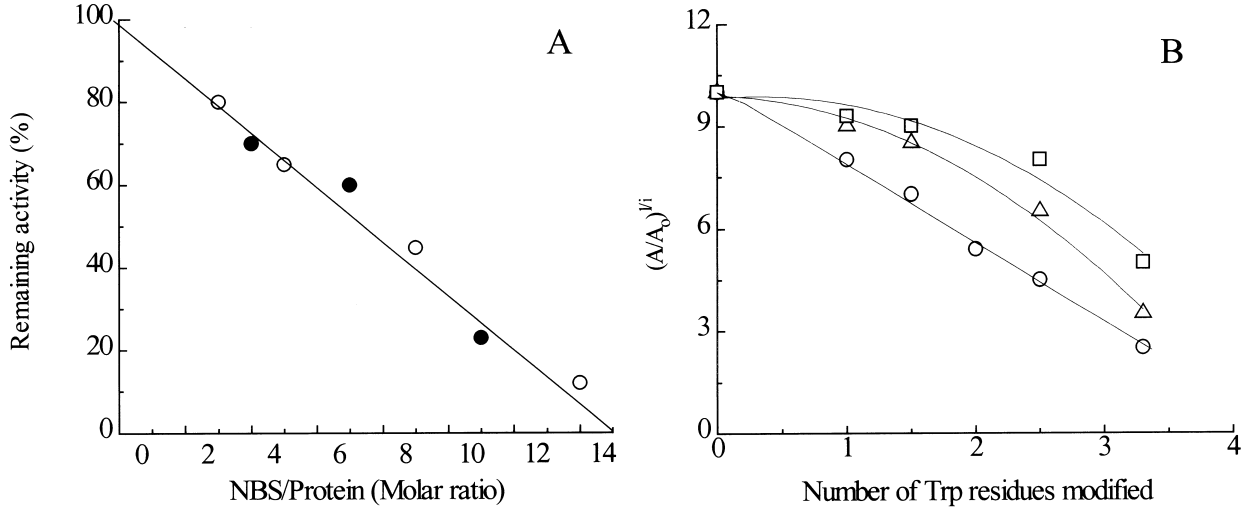

Fig. 2. (A) The (%) remaining activity of lipoxygenase-1 as a function of NBS/enzyme molar ratio (○) native and (●) in the presence of 50 μM sodium linoleate. (B) Correlation between the number of tryptophan residues modified and the remaining enzyme activity (Tsou Plot) (○) $i=1$ (Δ) $i=2$ (□) $i=3$.

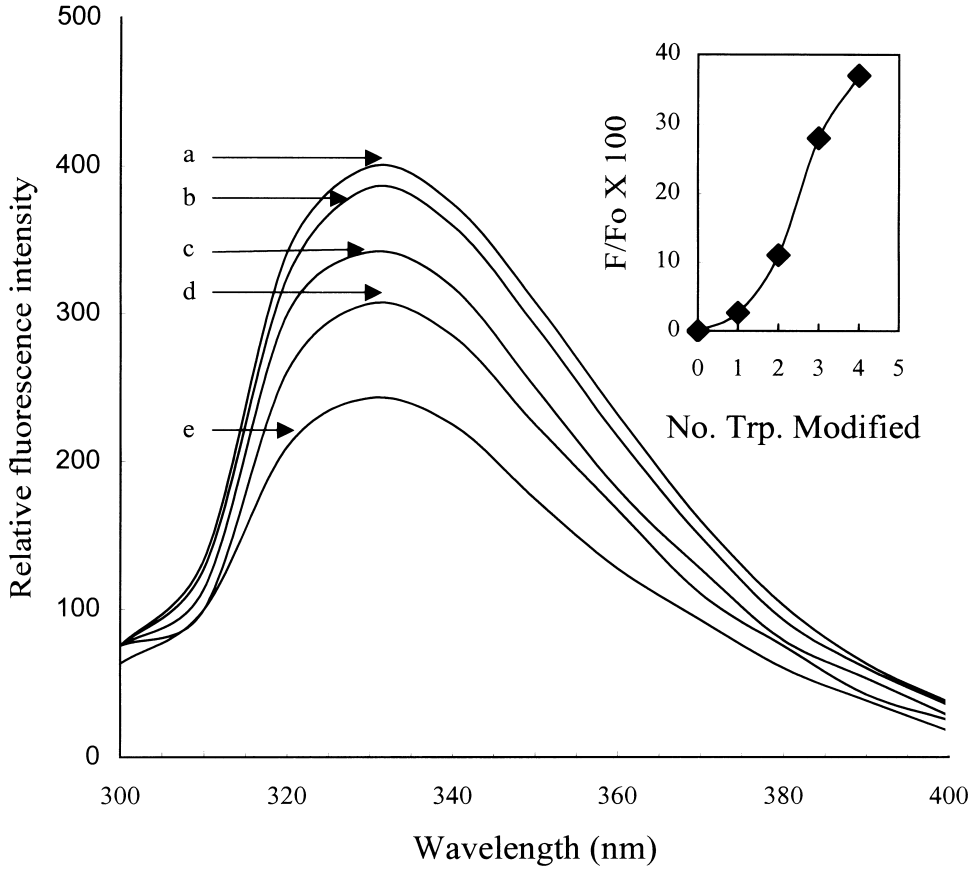

Fig. 3. Fluorescence emission spectra of LOX1 with varying extent, of modification of tryptophan residues (a) native, (b) 1 residue (c) 2 residues, (d) 3 residues and (e) 4 residues. inset: (%) change in fluorescence emission intensity as a function of extent of modification is expressed as number of residues modified. Modification is expressed to the nearest integer.

LOX1. The data were analysed by the Stern–Volmer equation. The Stern–Volmer constants for acrylamide quenching did not change significantly due to the modification of tryptophan residues (data not shown).

3.4. Circular dichroism (CD) measurements

The CD measurements were made both in the near UV and far UV region before and after modification at pH 4.0. The near UV CD spectra of LOX1 did not change significantly by the modification of one to three tryptophan residues. With the modification of the fourth residue, there was a slight decrease in intensity of all the bands. LOX1 at pH 4.0 exhibited two minima, one at 220 and another at 208 nm. After modification, there was a slight decrease in the ellipticity values below 230 nm. The native enzyme at pH 4.0 had 24% α helix; after modification of tryptophan residues, the value decreased to 22% α helix. The difference CD (control against modified), computed by subtracting the ellipticity values for the sample from the control at each wavelength, also showed minima at 220 and 208 nm, suggesting a marginal loss in helix content.

3.5. Stability measurements

The apparent stabilities of LOX1 and various modified derivatives were assessed by determining the mid-point of thermal unfolding transition (Tm), as followed by a decrease in absorbance at 287 nm. Due to the modification of tryptophan residues, the stability decreased as reflected in a decrease in Tm (Fig.4A).

3.6. Urea induced unfolding transition curves

The urea-induced unfolding transition curves of LOX1, before and after modification, are shown in Fig. 4B. At pH 4.0, native LOX1 unfolded completely in 8.0 M urea. The mid point concentration of urea induced unfolding transitions (Cm) for two different parameters were different. The Cm for far UV CD measurements was 4.0 M urea and, for fluorescence measurements, 3.3 M. The non-superposability of the transition curves was suggestive of intermediates in the unfolding of LOX1. After modification of four accessible tryptophan residues at pH 4, the mid-point concentrations for both the transitions were 2.7 and 1.8 M, respectively, suggesting

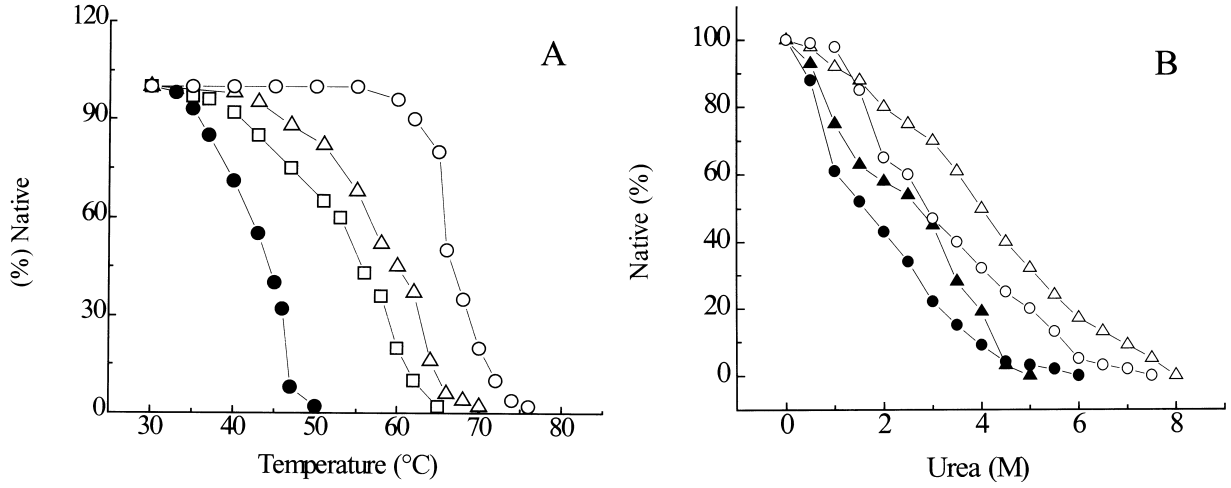

Fig. 4. (A) Temperature induced unfolding of LOX1 (○) native, (△) 1 residue, (□) 2 residues, (●) 4 residues, Measurements were made by following the decrease at 287 nm in 5 mM acetate buffer, pH 4.0 before and after modification. (B) Percentage of native LOX1 as a function of urea concentration before and after modification. (△) ellipticity at 222 nm for native, (▲) ellipticity at 222 nm for modified LOX1, (○) fluorescence emission at 332 nm for native and (●) fluorescence emission at 332 nm for modified LOX1. Measurements were made in 0.05 M acetate buffer, pH 4.0.

decrease in the conformational stability of the LOX1 due to the modification of tryptophan residues. The unfolding process of LOX1 was irreversible, which precluded the determination of thermodynamic parameters.

3.7. Discussion

Lipoxygenase-1 contains more hydrophobic amino acids with a calculated average hydrophobicity index of 1340 cal using the Tanford scale (Nozaki & Tanford, 1971). The hydropathy plot of LOX1 suggested the presence of hydrophobic clusters in the molecule (data not shown). In the "amino" terminal domain of LOX1, there are three regions of hydrophobic interior and two regions of hydrophilic exterior. In the "carboxy" terminal domain, there are 13 segments of hydrophobic and hydrophilic regions each. From the hydropathy plot, it can be inferred that the tryptophan residues that are likely to be exposed to the solvent are at positions, 340, 648, 649, 664, 665 and 771 and hence may be accessible for modification.

Of the 13 tryptophan residues in the molecule, at pH 9.0,the optimum pH for enzyme activity, only one tryptophan residue was accessible to NBS. Even in the presence of 8 M urea, the number of accessible groups increased to four, suggesting a partial exposure of tryptophan groups to the reagent. The presence of 8 M urea increased the accessibility by three more groups than its absence at any given pH.

The modification of one tryptophan residue at pH 4.0 did not affect the enzyme activity. From the hydropathy plot and non-essential nature of the first tryptophan being modified for activity, it can be inferred that the first likely tryptophan susceptible to modification is at position 771 of the 'C' terminal domain, which is far away from the active site. Modification of the first residue had little effect on activity, fluorescence quantum yield or the overall conformation of the protein. The presence of 50 μM linoleic acid, during the course of modification, did not affect the extent of modification. This is consistent with the thinking that none of the tryptophan residues which is in the hydrophobic interior is modified by NBS. The apparent thermal stability of LOX1 with the modification of one group decreased considerably. At pH 4.0, modification of four tryptophan residues resulted in a complete loss of activity. There were no major changes in the conformation of the enzyme as reflected in CD and fluorescence measurements. The fluorescence emission maximum remained at 333 nm, suggesting that none of the tryptophan residues in the interior was modified.

The statistical analysis of essential tryptophan residues for activity suggested that the modification of one critical residue resulted in loss of activity. The ability of the substrate fatty acid to bind to the enzyme was not affected by the modification. The conformational changes of the enzyme at the active site were minimal due to modification of tryptophan residues, as reflected in its ability to bind fatty acid substrate. Among the six tryptophan residues that are susceptible to modification, tryptophan at position 340 which is surrounded by hydrophilic amino acids, and is conserved in all lipoxygenases, is located at the beginning of the fatty acid binding cavity (Boyington, et al., 1993; Minor et al., 1996). We speculate that modification of this critical residue could be leading to complete inactivation of enzyme. The complete loss in activity could have resulted in a change in the orientation of the substrate fatty acid binding which may be critical for enzyme activity. The enhanced kinetic lag in the oxidation of fatty acid could be attributed to the state of iron in the presence of

NBS. The apparent thermodynamic stability of LOX1 decreased with the modification of tryptophan residues as reflected in urea induced unfolding of LOX1. The thermal stability of the enzyme molecule also decreased as reflected in decreased apparent mid point of transition temperature (Tm).

In conclusion, it can be stated that modification of one critical residue out of the four that was accessible for modification resulted in a complete loss of activity. Presence of substrate did not affect the extent of modification. Modification of these residues resulted in decreased apparent stability of the molecule, implicating their role in stability of the enzyme molecule. The apparent irreversibility of thermal unfolding of LOX1 and modified derivatives could be due to the multidomain nature of the enzyme molecule.

Acknowledgements

The authors are thankful to the Department of Science and Technology, New Delhi for financial assistance to purchase a spectrofluorimeter. One of the authors (SS) thanks CSIR, New Delhi for providing a Senior Research Fellowship.

References

Axelrod, B., Cheesbrough, T. M., & Laakso, S. (1981). Lipoxygenase from soybeans. *Methods in Enzymology, 71*, 441–451.

Boyington, J. C., Gaffney, B. J., & Amzel, L. M. (1993). The three-dimensional structure of an arachidonic acid 15-lipoxygenase. *Science, 260*, 1482–1486.

Cupo, J. F., & Pace, C. N. (1983). Conformational stability of mixed disulfide derivatives of β-lactoglobulin B. *Biochemistry, 22*, 2654–2658.

Feeney, R. E. (1987). Chemical modification of proteins: comments and perceptives. *International Journal of Peptide and Protein Research, 29*, 145–161.

Finazzi-agro, A., Avigliano, L., Egmond, M. R., Veldink, G. A., & Vliegenthart, J. F. G. (1975). Fluorescence perturbation in soybean lipoxygenase-1. *FEBS Letters, 52*, 73–76.

Gardner, H. W. (1991). Recent investigations into the lipoxygenase pathway of plants. *Biochimca et Biophysica Acta, 1084*, 211–239.

Grechkin, A. (1998). Recent developments in biochemistry of the plant lipoxygenase pathway. *Progress in Lipid Research, 37*, 317–352.

Klein, B. P., Cohen, B. S., Grossman, S., King, D., Malovany, H., & Pinsky, A. (1985). Effect of modification of soybean lipoxygenase-1 with N-bromosuccinimide on linoleate oxidation, pigment bleaching and carbonyl production. *Phytochemistry, 24*, 1903–1906.

Klibanov, A. M. (1983). Stabilization of enzymes against thermal inactivation. *Advances in Applied Microbiology, 29*, 1–28.

Lehrer, S. S., & Leavis, P. C. (1978). Solute quenching of protein fluorescence. *Methods in Enzymology, 49*, 222–236.

Lundblad, R. L., & Noyes, C. M. (1984). Chemical reagents for protein modification. Boca Raton, FL: CRC Press (Vols. 1 & 11).

Minor, W., Steczko, J., Otwinowski, Z., Bolin, J. T., Walter, R., & Axelrod, B. (1996). Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. *Biochemistry, 35*, 10687–10701.

Nozaki, Y., & Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. *Journal of Biological Chemistry, 246*, 2211–2217.

Rao, A. G. A., & Cann, J. R. (1981). A comparative study of the interaction of chloropromazine, trifluoroperazine and promethazine with mouse brain tubilin. *Molecular Pharmacology, 19*, 295–301.

Samuelsson, B., Dahlen, S. E., Lingren, J. A., Rouzer, C. A., & Serhan, C. N. (1987). Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science, 237*, 1171–1176.

Schewe, T., Rapoport, S. M., & Kuhn, H. (1986). Enzymology of reticulocyte lipoxygenase: comparison with other lipoxygenases. *Advances in Enzymology and Related Areas in Molecular Biology, 58*, 191–272.

Shibata, D., Steczko, J., Dixon, J. E., Hermodson, H., Yazdanparast, R., & Axelrod, B. (1987). Primary structure of soybean lipoxygenase-1. *Journal of Biological Chemistry, 262*, 10080–10085.

Spande, T. F., & Witkop, B. (1967). Determination of tryptophan content of the protein with N-bromosuccinimide. *Methods in Enzymology, 11*, 498–506.

Srinivasulu, S., & Rao, A. G. A. (1993). Kinetic and structural studies on the interaction of surfactants with lipoxygenase-1 from soybean. *Journal of Agricultural and Food Chemistry, 41*, 366–371.

Srinivasulu, S., & Rao, A. G. A. (1995). Structure and stability studies on interaction of monohydric alcohols with lipoxygenase-1 from soybeans. *Journal of Agricultural and Food Chemistry, 43*, 562–567.

Tsou, C. L. (1962). Relation between modification of functional groups of proteins and their biological activity. 1. A graphical method for the determination of the number and type of essential groups. *Scientia Sinica, 11*, 1535–1538.