Kinetic and Structural Studies on Thermal Inactivation of Lipoxygenase L1: Effect of Nonionic Surfactants

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Lipoxygenase 1 (LOX1) from soybean was irreversibly inactivated at 54 °C at pH 9.0, the midpoint of the thermal inactivation temperature (T_m) being 44 ± 1 °C. The kinetics of thermal inactivation of the enzyme followed the first-order kinetics with an estimated rate constant of 1.5×10^{-4} s^{-1} and a half-life of 36 min at 42 °C. The irreversible inactivation of the enzyme involved the oxidation of cysteine residues resulting in the intermolecular cross-linking. However, the conformation of the enzyme molecule remained practically unaltered. The nonionic surfactants Brij 35 and Tween 20 enhanced the thermal stability. The enhanced thermal stability could be due to strengthening of hydrophobic interactions around the cysteine residues.

Keywords: *Lipoxygenase; nonionic surfactants; thermal inactivation*

INTRODUCTION

Lipoxygenase 1 (LOX1) from soybean is a single polypeptide chain with 839 amino acids consisting of two domains and devoid of disulfide bridges (Ramachandran et al., 1992; Boyington et al., 1993). There are four cysteine residues in the lipoxygenase molecule, and they are embedded in a hydrophobic environment (Spaapen et al., 1980). The molecular properties, enzymology, and kinetics of both animal and plant lipoxygenases have been studied and extensively characterized (Vliegenthart and Veldink, 1982; Schewe et al., 1986). The lipoxygenases from plant sources have been implicated in the biogenesis of jasmonoic acid which has a role in plant growth, senescence, and defense against infection (Gardner, 1991). These enzymes from animal resources are involved in the synthesis of leucotrienes and lipoxins, which regulate cellular response in inflammation and immunity (Samuelsson et al., 1987).

During the process of unfolding and refolding of LOX1 by urea/guanidine hydrochloride, irreversible aggregation is observed (Srinivasulu and Rao, 1996). The denatauration of oligomeric/multidomain proteins in vitro is often an irreversible process due to nonspecific aggregation (Jaenicke, 1987). Among the approaches that help in preventing the aggregation are use of (i) surfactants (Tandon and Horowitz, 1986, 1987), (ii) stabilizing proteins such as molecular chaperones (Ellis, 1987; Ellis and Van der vies, 1991), and (iii) other factors (Jaenicke and Rudolph, 1989). Surfactants assist in refolding of proteins by stabilizing the folding intermediates in the refolding pathway.

In an earlier paper, we reported the interaction of surfactants with LOX1 by both kinetic and structural measurements and explained the kinetic behavior of enzyme activity in nonionic surfactant solutions in terms of altered v/k ratio (Srinivasulu and Rao, 1993). The effect of surfactants on the kinetics of oxidation of fatty acid by LOX1 had also been interpreted as the enzyme preferentially interacting with free linoleate and not with linoleate included in a matrix of nonionic amphiphiles (Schilstra et al., 1994). The present paper reports the mechanism of thermal inactivation and the effect of surfactants on thermal stability of LOX1.

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MATERIALS AND METHODS

Soybean LOX1 was purified according to the method of Axelrod et al. (1981) with an additional step of molecular sieve chromatography on Sephadex G-75. The enzyme had a purity of more than 95% and a specific activity of 150 units/mg of protein. The homogeneity of the preparation was ascertained by SDS−PAGE (Laemmli, 1970) and sedimentation velocity measurements. The SDS−PAGE pattern suggested a purity of better than 95%. The enzyme sediments appear as a single symmetrical peak with an $s^0_{20,w}$ value of 5 S, calculated using standard procedures (Schachman, 1959).

The following chemicals were used, linoleic acid (99%) from Nucheck Inc.; Elysian MN, Brij 35, dithiothreitol (DTT), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) from Sigma Chemical Co., St. Louis, MO; Tween 20 from Aldrich Chemical Co., Milwaukee, WI; DEAE Sephadex A50 and Sephadex G-75 from Pharmacia Fine Chemicals, Uppsala, Sweden; and all other chemicals were of analytical grade.

Enzyme Assay. The enzyme was assayed according to the method of Axelrod et al. (1981) by following the absorption changes at 234 nm due to the formation of conjugated dienes using Tween 20-solubilized substrate. One unit of enzyme activity is defined as the amount of the enzyme required to form 1 μmol of product/min at 25 °C under the assay conditions. The enzyme concentration was determined by measuring the optical density at 280 nm and using a value of $E^{1mg/mL}_{1cm} = 14.0$.

Transition Temperature Measurements. The LOX1 was incubated in both the presence and absence of surfactants at different temperatures from 27 to 55 °C for 15 min, and the remaining activity was measured at 27 °C. The midpoint of transition (T_m), where 50% of the activity remained, was calculated from the plot of percent remaining activity vs temperature.

Kinetics of Thermal Inactivation Measurements. The kinetics of inactivation at different temperatures in the presence and absence of surfactants were measured at pH 9.0 in 0.2 M borate buffer by incubating the enzyme at the desired temperature. The remaining activity at each time interval was measured at 27 °C. From a plot of log percent residual activity vs time, the rate contansts were calculated. To determine the activation energy values (E_a) for thermal inactivation, an Arrhenius plot was constructed, with the experimental values by means of linear regression analysis. Activation parameters were then calculated as described (Moore, 1962). Activation

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enthalpy values (ΔH^*) were calculated according to the equation

$$\Delta H^* = E_a - RT \quad (1)$$

where R is the universal gas constant and T is the absolute temperature. Activation free energy values (ΔG^*) were calculated according to the equation

$$\Delta G^* = -RT \ln \frac{k_r h}{kT} \quad (2)$$

where k_r is the rate constant, h is the Planck constant, and k is the Boltzmann constant. Activation entropy values (ΔS^*) were then calculated according to the equation

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (3)$$

Circular Dichroism (CD) Measurements. The circular dichroism spectra were measured at 25 °C on a Jasco J20C spectropolarimeter using 10 and 1 mm path length quartz cells, in the near-UV and far-UV regions, respectively. Slits were programmed to yield 10 Å bandwidth at each wavelength. Mean residue elipticity values were calculated using a mean residue weight of 112.

Fluorescence Measurements. Fluorescence measurements were made at pH 9.0 in 0.2 M borate buffer at 25 °C with a Shimadzu RF 5000 automatic recording spectrofluorometer with 5 and 10 nm bandwidth for excitation and emission monochromators, respectively. Temperature was maintained by circulating water through the cell holder. The emission spectra of enzymes were recorded after excitation at 285 nm in the region 300−400 nm before and after heating the sample; the enzyme concentration was 7.6×10^{-7} M.

Fluorescence Quenching Measurements. The quenching of enzyme fluorescence by acrylamide or potassium iodide was made before and after heating the sample. The enzyme emission at 333 nm was monitered. The enzyme fluorescence was quenched by progressive addition of acrylamide or potassium iodide. Sodium thiosulfate (0.1 mM) was added to KI solution to prevent I^{3-} formation. Since the absorbance of potassium iodide at the excitation wavelength (285 nm) was not detectable, no correction was given for inner filter effect. The absorption of acrylamide at 295 nm was corrected using the equation (Lehrer and Leavis, 1978)

$$F_{corr} = F_{abs} 10^{\Delta A/2} \quad (4)$$

where ΔA is the increase in absorbance at the center of the cuvette by the addition of acrylamide. The quenching data were analyzed with the Stern−Volmer equation

$$F_o/F = 1 + K_{SV}(Q) \quad (5)$$

where Q is quencher concentration and K_{SV} is the dynamic quenching constant.

Polyacrylamide Gel Electrophoresis (PAGE). Nondenaturing PAGE was performed using 7.5% gels using an acrylamide:bisacrylamide ratio of 1:30 in 50 mM glycine−NaOH buffer (pH 9.0). The enzyme samples were prepared by heating at 50 °C for 15 min in both the presence and absence of surfactants except for the control.

Sedimentation Velocity Measurements. These measurements were made using a Spinco Model-E analytical ultracentrifuge equipped with a phase plate schlieren optics and rotor temperature indicator control (RTIC) unit. Standard duraluminum center piece of 12 mm Kel-F cell was used for sedimentation velocity measurements. Sedimentation velocity experiments were carried out at room temperature using 7.5 mg/mL protein solution and in 0.2 M borate buffer, pH 9.0. Centrifugation was done at 59 800 rpm. The $s^0_{20,w}$ was calculated using the standard procedure (Schachman, 1959).

Estimation of Free Cysteine Groups. Free cysteine residues in LOX1 were estimated by the DTNB method. Measurements were made in 1 mL of protein solution in 6 M guanidine hydrochloride, pH 9.0, 50 mM borate buffer containing 1 mg of protein. Small aliquots (10 μL) of stock (10 mM) DTNB reagent were added and stirred for 2−3 min, and absorbance was recorded at 412 nm. The number of free cysteine residues was calculated using a molar extinction coefficient value of 13 600 cm^{-1} M^{-1} (Ellman, 1959).

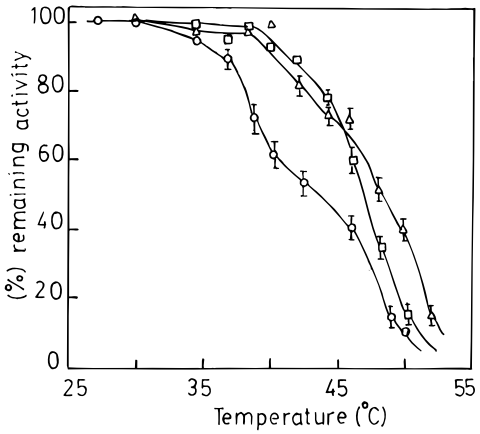

Figure 1. Effect of temperature on the irreversible thermal inactivation of lipoxygenase in the presence and absence of nonionic surfactants. The protein concentration was 4.3×10^{-7} M: (○) control, (△) 10 mM Brij 35, and (□) 10 mM Tween 20.

Table 1. Thermodynamic Activational Parameters for Thermal Inactivation of Lipoxygenase L1

sample	temp (K)	rate constant k_r (10^{-5} s^{-1})	ΔG^* (kcal)	ΔH^* (kcal)	ΔS^* (eu)
control	303	9.0	23.3	12.6	−35
	309	12.5	23.6	12.6	−36
	315	15.6	23.9	12.6	−36
	319	21.2	23.8	12.6	−35
10 mM Tween 20	303	2.7	24.0	24.4	1
	309	4.0	24.3	24.4	3
	315	7.0	24.4	24.4	0
	319	15.6	24.3	24.4	0
10 mM Brij 35	303	2.4	24.1	19.4	−16
	309	4.5	24.2	19.4	−16
	315	9.9	24.2	19.4	−15
	319	20.0	24.1	19.4	−15

RESULTS

The thermal stabilities of LOX1 in the presence of two nonionic surfactants, Brij 35 and Tween 20, were measured by determining the midpoint of the irreversible thermal inactivation temperature (T_m). With the increase in temperature from 25 to 55 °C, lipoxygenase lost its activity completely at 54 °C. The midpoint of transition was 44 ± 1 °C at pH 9.0 (Figure 1). With the addition of 10 mM Brij 35 or Tween 20, the T_m shifted to 48 ± 1 °C. The extent of the shift in T_m was similar for both Tween 20 and Brij 35.

Kinetics of Thermal Inactivation. The kinetics of thermal inactivation of LOX1 in the presence and absence of surfactants at 42 °C are shown in Figure 2. The kinetics of thermal inactivation at different temperatures followed first-order kinetics. The rate constants for thermal inactivation and activational parameters are shown in Table 1. For LOX1 in the absence of surfactants with the increase in temperature the rate constant increased. With the addition of either 10 mM Brij 35 or Tween 20, the rate constants for thermal inactivation decreased and the half-life of the enzyme increased (Figure 2). From the rate constants as a function of temperature, the Arrhenius plots were

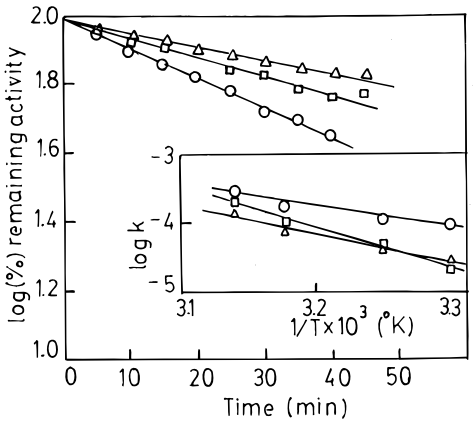

Figure 2. Kinetics of thermal inactivation of lipoxygenase in the presence and absence of nonionic surfactants at 42 °C. Inset shows the Arrhenius plot of thermal inactivation. The protein concentration was 4.3×10^{-7} M: (○) control, (△) 10 mM Brij 35, and (□) 10 mM Tween 20.

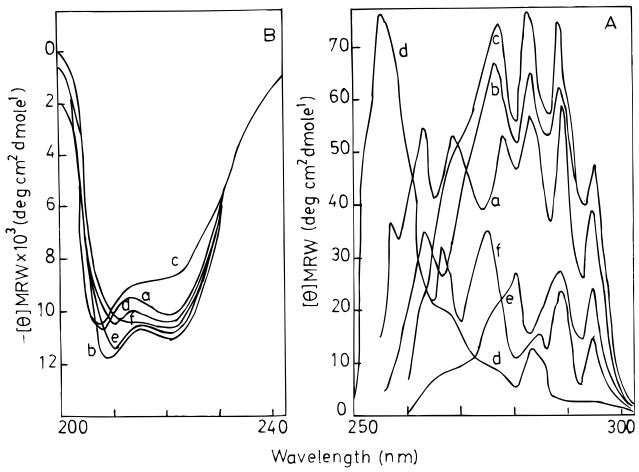

Figure 3. (A) Near-UV CD spectra of lipoxygenase before and after heat inactivation (protein concentration, 1.14×10^{-5} M). (B) Far-UV CD spectra of lipoxygenase before and after heat inactivation (protein concentration, 2.66×10^{-6} M) (curves a and d before and after heat inactivation of control, curves b and e for Brij 35, curves c and f for Tween 20).

constructed (Figure 2, inset). For the thermal inactivation of LOX1, activational free energy (ΔG^*), activational enthalpy (ΔH^*), and activational entropy (ΔS^*) did not change significantly in the temperature range stuided. With the addition of Tween 20 or Brij 35, ΔG^* did not change, but ΔH^* and ΔS^* increased.

CD Measurements. In order to understand the mechanism of thermal inactivation and the significance of activational parameters, the solution conformation of the enzyme was followed by both near- and far-UV CD measurements, before and after heating the sample. The near-UV CD data are shown in Figure 3A. The native enzyme had CD bands at 293, 288, 283, 274, and 266 nm.

Heating the enzyme at 50 °C for 15 min resulted in complete loss of activity and the intensity of the near-UV CD bands decreased in the region 260−300 nm with an appearance of a predominant band at 255 nm. The effect of addition of Brij 35 or Tween 20 to the enzyme before and after heating is shown in Figure 3A. The intensity of the near-UV CD bands was enhanced due to the addition of these surfactants before heating (Figure 3A, spectra b and c). The heated sample in the presence of these surfactants showed lesser intensity

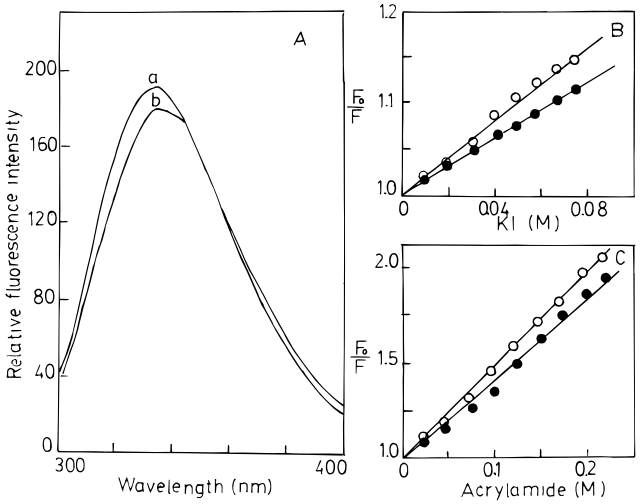

Figure 4. (A) Fluorescence emission spectra of lipoxygenase: (a) control and (b) heat-inactivated control. (B and C) Stern−Volmer plots for the fluorescence quenching by potassium iodide and acrylamide: (●) control and (○) heat-inactivated control.

of these bands. The 255 nm band was not found with the heated sample in the presence of surfactant.

The effect of heating on the far UV-CD spectra of LOX1 in the presence and absence of surfactants is shown in Figure 3B. The secondary structure of LOX1 was not significantly affected due to the heating (compare spectra a and d). The addition of Brij 35 enhanced the intensity of the 222 and 210 nm bands. Heating the enzyme solution in the presence of Brij 35 did not significantly affect the far-UV CD spectrum. In the case of Tween 20, there were subtle changes in the far-UV CD spectra, and heating of the enzyme in the presence of Tween 20 altered the secondary structure (spectra c and f in Figure 3B).

Fluorescence Measurements. The fluorescence emission spectra of native and heat-inactivated LOX1 are shown in Figure 4A. The fluorescence emission maximum did not change after thermal inactivation, but fluorescence intensity decreased slightly. Fluorescence quenching by potassium iodide or acrylamide is shown in Figure 4B,C. The Stern−Volmer constant for potassium iodide and acrylamide changed from 1.3 to 1.7 and from 5.2 to 5.4, respectively.

Sedimentation Velocity Measurements. Sedimentation velocity pattern of LOX1 gave a single peak of 5.2 ± 0.2 S value (Figure 5A). The sedimentation velocity pattern of the protein after heating at 50 °C for 15 min showed two peaks, one with higher sedimentation coefficient of 15.0 ± 1.5 S and the other with a sedimentation cofficient of 6.0 ± 0.5 S value. Thus, thermal inactivation involved the formation of higher aggregates.

Polyacrylamide Gel Electrophoresis. The PAGE pattern of native and thermally inactivated LOX1 is shown in (Figure 5C). From the mobility of the sample, it is apparent that there were higher molecular weight aggregates in the thermally inactivated sample. However, the aggregates were not found in the presence of the surfactant-heated sample.

Estimation of Free Cysteine Residues. The cysteine residues in native LOX1 were not accessible with sulfhydryl reagents. Hence, the estimation of cysteine residues was made after unfolding of LOX1 in 6 M guanidine hydrochloride (pH 9.0, 0.05 M borate). In LOX1 3.3 cysteine residues were titrated with DTNB.

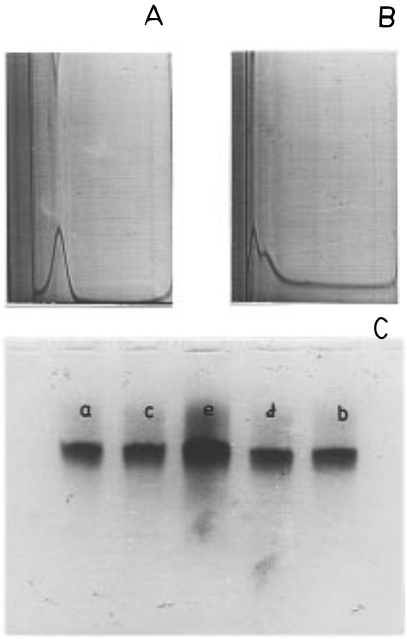

Figure 5. Sedimentation velocity pattern of lipoxygenase: (A) control and (B) heat-inactivated control (photographs taken at the end of 31 and 14 min, respectively). (C) Polyacrylamide gel electrophoresis pattern of lipoxygenase at various treatments: (a and b) control, (c) Tween 20, (d) Brij 35, and (e) heat-inactivated control.

After heating the LOX1 at 50 °C for 15 min, the value decreased to 1.8 residues indicating the decrease in titratable cysteine content of LOX1.

Effect of DTT. The effect of the reducing agent dithiothreitol on enzyme activity was checked. Reactivation of the thermally inactivated enzyme by incubating the sample with 10 mM DTT at 4 °C for 3 h was not successful. However, incubation of the enzyme with 10 mM DTT during heating completely prevented thermal inactivation.

DISCUSSION

The nonionic surfactants Brij 35 and Tween 20 enhanced the thermal stability of the LOX1 as evidenced from an increase in T_m and a decrease in thermal inactivation rate constants. The kinetics of thermal inactivation followed first-order kinetics in the presence and absence of surfactants implying a similar mechanism must be operative in either case. The Arrhenius plots were linear in the temperature range studied. The changes in values of ΔG^* and ΔH^* were small in the presence of surfactants. Similar values have been reported for the thermal inactivation of lipoxygenase from soybean and other sources (Baker and Mustakas, 1973; Brown et al., 1982; Esaka et al., 1986; Lopez et al., 1994).

Structural changes associated with thermal inactivation of LOX1 were probed by CD, fluorescence, PAGE, and sedimentation velocity measurements. The appearance of the 255 nm band in the near-UV CD spectra suggests the oxidation of free cysteine groups in the thermal inactivation process (Strickland, 1974). In the native LOX1, the cysteine residues are not accessible to DTNB (Ellman's reagent) suggesting that these residues were in a hydrophobic environment (Spaapen et al., 1980). Estimation of free cysteine residues using DTNB confirmed the oxidation of cysteine residues due to heating. The PAGE and sedimentation velocity measurements suggest the intermolecular cross-linking is due to oxidation. Reactivation of the thermally inactivated enzyme was not successful. Incubation of enzyme with DTT prevented thermal inactivation. The thermal inactivation process did not alter the secondary structure singnificantly. Fluorescence measurements confirmed that there were no major structural changes. The enhanced T_m and decreased rate constants in the presence of surfactants could be attributed to stabilizing effects of surfactants on the hydrophobic interaction.

It may be concluded that thermal inactivation of LOX1 at the optimum pH of enzyme activity (pH 9.0) is due to the oxidation of cysteine residues. The hydrophobic environment around cysteine residues is strengthened in nonionic surfactant solutions, which enhance the thermal stability of lipoxygenase. In the case of the LOX1 nonionic surfactants, Tween 20 and Brij 35 prevent the specific aggregation due to sulfhydryl oxidation. These surfactants may assist in preventing the nonspecific aggregation during unfolding and refolding of lipoxygenase. Further experimentation on the use of these surfactants in stabilizing the folded intermediates is in progress in this laboratory.

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