Relationship between the Activity of Soybean Lipoxygenase 1 and the Physicochemical Characteristics of Model Food Emulsion Systems

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The paper describes the relationship between the physicochemical characteristics of model oi-in-water emulsion systems containing oil droplets and soluble fragments (monomers and micelles) and the activity of pure soybean lipoxygenase 1. The method of emulsification, as well as the chemistry and concentration of surfactant and substrate molecules, was manipulated to modify the overall physicochemical properties of ternary emulsions. The effect of these experimental factors on the size and relative concentration of oil droplets and soluble fragments was examined with static light scattering and turbidity measurements. Lipoxygenase 1 activity was assayed polarographically. Experimental factors that increase the critical micelle concentration of the monomers and facilitate their transport out of micelles, as well as across the oil−water interfaces and into the aqueous phase, were shown to improve the activity of the enzyme and vice versa.

Keywords: *Lipoxygenase; emulsions; soybean; laser light scattering*

INTRODUCTION

One of the current specific priorities of industry is to develop and produce food emulsions with enhanced nutritional and sensory attributes (Dickinson et al., 1994; De Cindio and Cacace, 1995; Guyot et al., 1996). Lipoxygenase enzymes (lox) are of particular interest to food scientists because of their involvement in the biogenesis of both objectionable and desirable flavor and aroma compounds, the co-oxidation of vitamins, and the degradation of pigments in many plant products (O'Connor and O'Brien, 1991; Hsieh, 1994). The design of foodstuffs with improved quality, that is, the prevention or stimulation of lipoxygenase activity, depends on a better understanding of the physicochemical mechanisms behind the enzymatic reaction in model emulsion systems (Coupland and McClements, 1996).

Many publications report the measurement of lipoxygenase activity. Results are usually interpreted without too much concern about the physical and structural properties of the systems in which the reaction takes place. Therefore, the wide range of assay conditions has caused some confusion regarding optimal pH and reaction kinetics (Whitaker, 1991). Galpin and Allen (1977) and Verhagen et al. (1978) were the first workers to propose that some of the variations in the lipoxygenase reaction might be ascribed to changes in the physicochemical state of the test media. Structural considerations were also highlighted in a number of recent studies, which described soybean lipoxygenase 1 (lox-1) activity in micellar environments (Perez-Gilabert et al., 1992; Schilstra et al., 1994; Rodakiewicz-Nowak et al., 1996). These model systems, however, are less complex than real food products, which also contain fats dispersed as emulsion droplets well above critical micelle concentration (cmc) levels (Dickinson, 1992).

The objective of this work is to measure the activity of soybean lox-1 in systems containing oil droplets and soluble fragments (monomers and micelles) (Figure 1) and so gain an appreciation of lox-1 activity in complex emulsions. The method of emulsification, as well as the chemistry and concentration of surfactant and substrate molecules, was manipulated to modify the overall physicochemical properties of the ternary model systems. The effect of these experimental factors on the size and relative concentration of oil droplets and soluble fragments was examined with static light scattering and turbidity measurements. Lox-1 activity was assayed polarographically and related to the physicochemical state of the model emulsion systems. Variations in the activity of lox-1 are discussed in terms of the availability of effective substrate for the enzyme to react with, as well as direct activating/inhibitory effects on the protein itself.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, all of the reagents (analytical grade) were purchased from Sigma Chemical Co. (Poole, U.K.). Methyl linoleate and linoleic acid were 99% pure and used without further purification. The substrates were stored under nitrogen, protected from light, and frozen at −18 °C for up to 6 months. The surfactants Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 60 (polyoxyethylene sorbitan monostearate), Tween 85 (polyoxyethylene sorbitan trioleate), Brij 35 (polyoxyethylene 23 lauryl ether), and SDS (sodium dodecyl sulfate) did not contain added antioxidants and were stored under nitrogen, protected from light, and kept at room temperature for up to 6 months. Commercially sourced soybean lipoxygenase type 1-B (60% protein, 40% buffer salts) was used without further purification. The lyophilized enzyme was stored at −18 °C for up to 1 year.

Preparation of Solutions. Potassium phosphate (KH_2PO_4 + NaOH, pH 6−8), sodium borate (H_3BO_3 + NaOH, pH 8.5−

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10.1021/jf990822j CCC: $19.00
Published on Web 07/26/2000

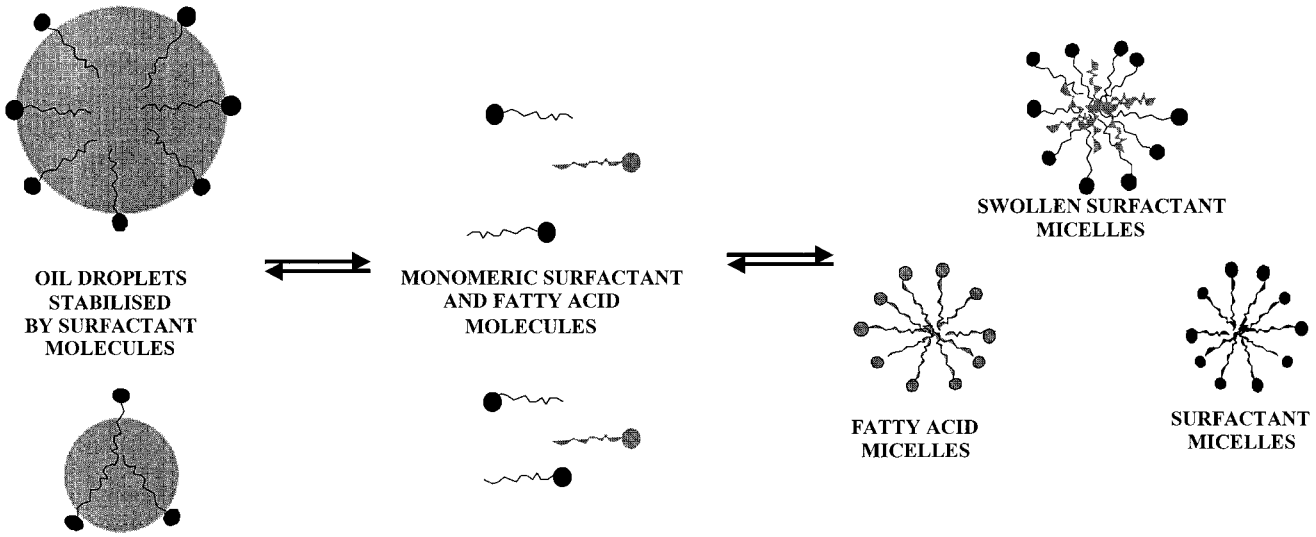

Figure 1. Schematic representation of the different entities present in the model emulsion systems. Emulsion systems, which contain substrate and surfactant levels greater than their cmc values, can be described as a collection of the following: oil droplets (diameter from ~0.1 to 100 μm), the kinetic stabilization of which is associated with the adsorption of surfactant molecules to the oil−water interface; surfactant and substrate molecules in the monomeric form (length of molecules at ~2.5 nm); and mixed micellar aggregates as well as single-species micelles (diameter of micelles at ~5 nm). An aqueous micellar solution in equilibrium with an excess oil phase is a dynamic system in which oil substrate molecules are continually exchanged between droplets and micelles (Dickinson and McClements, 1996).

10.5), and sodium carbonate ($NaHCO_3$ + Na_2CO_3, pH 8−11) buffers (0.1 M) were prepared weekly and stored at room temperature.

The agent in oil and agent in water methods of emulsification were both applied for the daily preparation of substrate (20 mM)/surfactant (3.4−26.8 mM)/deionized water stock solutions. Substrate/surfactant stock solutions needed for assaying lox activity were divided into small aliquots (1.5 mL). These were stored at 25 °C under nitrogen and protected from light. For any other experiment, the stock solutions were kept protected from light at room temperature.

Soybean lox-1 working solutions consisted of the appropriate amount of lyophilized enzyme diluted in sodium borate buffer (0.1 M, pH 9). These solutions were frozen at −18 °C until further use. They were stored for up to 2 weeks. Because lipoxygenase specific activity changes on storage and between batches, the quantities of enzyme in the assays were corrected to keep the number of units constant.

Physicochemical Characterization of the Model Emulsion Systems. Substrate/surfactant stock solutions prepared with the agent in oil method of emulsification were gently hand-shaken for 15 s, rested for 10 s, and then diluted with buffer to match concentrations used in the enzyme assays. The resulting reaction mixtures were immediately assessed with laser light scattering and turbidity measurements. Substrate/surfactant stock solutions prepared with the agent in water method of emulsification were vigorously hand-shaken for 15 s and directly diluted with buffer just prior to their examination. Light scattering and turbidity experiments were carried out at levels of surfactants well above the cmc (Helenius et al., 1979).

Laser light scattering experiments were performed at 20 °C on a Malvern Instruments Mastersizer μ+ particle sizer (Malvern, Worcs., U.K.) fitted with a 120 mL sampling cell and integral stirrer. Buffer (60 mL) was first circulated in the apparatus to measure the background light scattered. Up to 56 mL of newly prepared reaction mixtures (substrate/surfactant stock solutions diluted with buffer) were added to produce obscuration levels of 10−20%. The scattering from the sample was determined using a 5NED presentation. The data were analyzed using the polydisperse analysis model of the Mastersizer μ+ PSW0005 software (version 2.15). The result of the analysis was a volume-based particle size distribution characterized over the size limits of the optical configuration used (0.1−1000 μm). The distribution was characterized with its specific surface area (SSA), which is defined as the total area of the particles divided by the total weight (m^2/g). The software calculates the weight from total scattering assuming that all of the substrate scatters and that the presentation (5NED) is correct for the sample. Hence, it should be emphasized that SSA values will refer only to the oil particles having sizes within the range of 0.1−1000 μm; it does not incorporate the micelles and droplets outside the size limits that are also contained in the emulsion. Figure 2 shows a typical result and includes a guide to interpreting the data.

Turbidity measurements per se take the form of a relative light intensity transmitted through an emulsion sample (Faratino and Rowell, 1983). The percentage transmittance (% T) of freshly prepared reaction mixtures was measured without dilution, against water (100% T) and at 20 °C using Beckman plastic cuvettes (1 cm light path) in an LKB Biochrom Ultrospec 4050 spectrophotometer set at a wavelength of 610 nm. The polydispersity (0.3−100 μm) and relatively high substrate concentration (5 mM) of the reaction mixtures meant that transmittance results were useful only in detecting gross changes in the size distribution and concentration of the oil droplets.

Viscosity measurements were carried out on a Bohlin CS Rheometer fitted with a double-gap geometry. All measurements were performed at 25 ± 0.1 °C, over a shear rate range of 1−100 s^{-1}. Results were recorded on a PC control system running Bohlin CS software and expressed as viscosity (Pascal seconds) versus shear rate (s^{-1}).

Lipoxygenase Assays. The activity of soybean lox-1 was determined at 25 °C in a Clark-type oxygen electrode (Rank Brothers Ltd., Cambridge, U.K.). Reaction mixtures (3 mL) consisted of substrate/surfactant stock solution (0.75 mL), buffer (2.05 mL), and lipoxygenase working solution (0.2 mL). Enzyme activity was never limited by the concentration of fatty acid molecules in the reaction mixtures. Oxygen levels in the reaction mixtures were not saturating but were standardized to 195 ± 5 μM by incubating the buffers and substrate/surfactant stock solutions for at least 7 h at 25 °C. Lipoxygenase activity was derived from the gradient of oxygen consumption in the system, and the results were expressed as nanomoles of oxygen consumed per minute per milliliter.

RESULTS AND DISCUSSION

Effect of pH on the State of the Emulsions and on Lox-1 Activity. Methyl linoleate (5 mM)/Tween 20

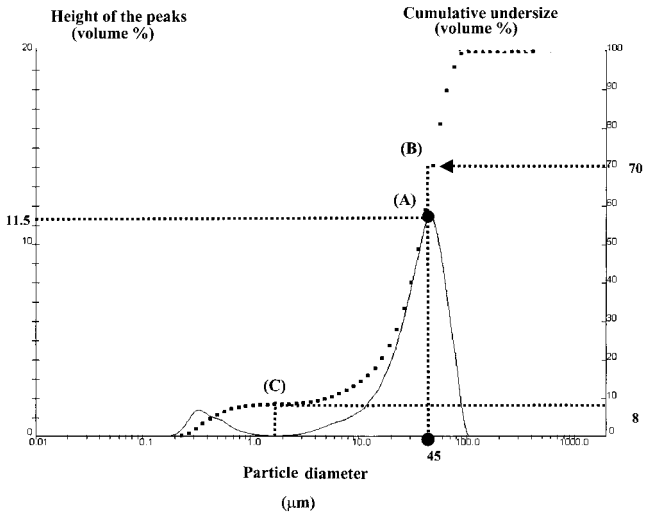

Figure 2. Schematic of a typical result produced by the Mastersizer software. The result from the analysis is the relative volume distribution of particles characterized over the size limits of the configuration. The peaks of the frequency curve (line) give the modal diameters, that is, the most commonly occurring diameters of the discrete droplet populations. For example, the mean diameter of the largest particles is 45 μm (A). The actual relative volume concentration of droplets displaying this particular diameter is 11.5%. Symbols (■) represent a cumulative undersize curve. Point B shows that 70% of the total volume of observed sample corresponds to particles having diameters of ≤45 μm. Point C indicates that the smallest particles from the distribution account for 8% of the total sample volume. By difference, the relative volume concentration of the largest particles is found to be 100 − 8 = 92%.

(1.70 mM) dispersions were prepared using the agent in oil method of emulsification, and the effect of pH on their physicochemical state was examined from pH 6 to 8 with laser light scattering. The particle size distribution of the emulsions indicated three distinct oil droplet populations having mean diameters (1.4, 5.5, and 45 μm), as well as relative volume concentrations (3.5, 4.0, and 92.5%), that were independent of the pH of the continuous phase. The SSA of the dispersions remained unchanged at ∼0.4−0.5 m^2/g. The transmittance of light through the methyl linoleate emulsions remained constant at ∼20% over the same pH scale. The turbidity of polydisperse emulsion systems is complex as it is not only proportional to the concentration and size of the scatterers but it is also strongly influenced by the optical properties of both the oil and the dispersant (Dickinson and Stainsby, 1988). Transmittance data in such systems can only be used to detect gross changes in concentration and size distribution. Both techniques showed that the chemistry of methyl linoleate and, hence, the physicochemical state of the emulsions were not significantly affected by the pH range selected for this study because pK_a values for esters are normally well above 11.

The activity of soybean lox-1 in the methyl linoleate/Tween 20 emulsion systems was measured over a pH range from 6 to 10.5 (Figure 3). The pH activity profile of lox-1 with the ester produced a narrow bell-shaped curve. Maximum activity was observed at pH ∼9−9.2, after which the reaction rate decreased quite dramatically. Glickman and Klinman (1995) devised a series of experiments to explain the pH dependence of the lox-1 reaction with linoleic acid. The involvement of two pK_a values was suggested. A first pK_a at 7.72 arising from

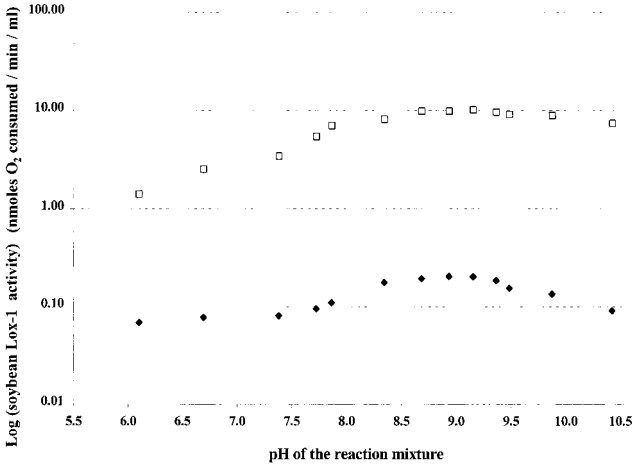

Figure 3. pH activity profiles of soybean lox-1 with linoleic acid and methyl linoleate. The effect of pH on the specific activity of lox-1 with linoleic acid (□) and methyl linoleate (◆) was recorded at 25 °C. Substrates were emulsified with Tween 20 (1.70 mM) in potassium phosphate (pH 6−8) and sodium carbonate (pH 8−10.5) buffers (0.1 M). Results, normalized to the concentration of lipoxygenase (13.33 and 290 μg/mL in linoleic acid and methyl linoleate emulsions, respectively), are plotted on a log scale.

Table 1. Effect of pH on the Characteristics of Linoleic Acid/Tween 20 Emulsions[a]

droplet population	pH	mean diameter (μm)	rel vol concn (%)
1	6	35.1 ± 0.9	87.6 ± 0.5
	7	39.8 ± 0.6	93.6 ± 0.0
	8	54.4 ± 4.4	94.5 ± 0.3
2	6	0.3 ± 0.0	12.4 ± 0.5
	7	0.3 ± 0.0	6.4 ± 0.0
	8	0.3 ± 0.0	5.5 ± 0.3

[a] Linoleic acid (5 mM) was emulsified with Tween 20 (1.70 mM) over the pH range 6−8. Results show a series of values (means ± standard deviations) obtained from triplicate experiments.

the ionization of the substrate was found to control the binding to the enzyme active site. A second pK_a at 6.95 arising from the ionization of the enzyme was thought to govern substrate release. Methyl linoleate does not ionize, making the first pK_a irrelevant. Instead of being directly related to the chemistry of the methyl linoleate emulsions, the steep increase in the dioxygenation kinetics from pH 6 to pH 9 was probably associated with the second pK_a mentioned by Glickman and Klinman (1995). The dramatic decrease in the rate of the reaction after pH 9.2 could be due to progressive enzyme denaturation following the weakening of the forces stabilizing protein conformation (Stauffer, 1989).

The physicochemical properties of linoleic acid (5 mM)/Tween 20 (1.70 mM) model systems (agent in oil method of emulsification) were also investigated with laser light scattering and turbidity techniques. The dispersions contained two distinct oil droplet populations, the characteristics of which were significantly affected by pH (Table 1). The increase in the relative volume concentration and size of the larger particles with increasing pH was probably caused by three concomitant mechanisms:

(1) Progressive solubilization of the oil into monomers. The smaller oil droplets were expected to dissolve fastest due to their greater surface area-to-volume ratio. This removed the smallest particles, shifting the median size to higher values.

Table 2. Chemical Designation and HLB of a Selection of Nonionic Surfactants

trade name	chemical designation[a]	abbreviation	HLB[b]
Tween 85	POE (20) sorbitan trioleate	$C_{(18:1)_3}$ sorbitan E_{20}	11.0
Tween 60	POE (20) sorbitan monostearate	$C_{18:0}$ sorbitan E_{20}	14.9
Tween 20	POE (20) sorbitan monolaurate	$C_{12:0}$ sorbitan E_{20}	16.9
Brij 35	POE (23) lauryl alcohol	$C_{12}E_{23}$	16.9

[a] POE stands for polyoxyethylene. The average number of polyoxyethylene units in the molecules is indicated in parentheses. [b] Becher and Schick (1987).

Table 3. Effect of a Range of Surfactants on the Physicochemical Properties of Methyl Linoleate Emulsion Systems and on the Activity of Soybean Lox-1[a]

surfactant concn (mM)	Tween 20	Brij 35	Tween 60	Tween 85
	(A) SSA of Methyl Linoleate/Surfactant Emulsions at pH 9 (m^2/g)			
0.85	0.58 ± 0.01	ND	0.65 ± 0.06	4.61 ± 0.10
1.70	0.75 ± 0.00	0.41 ± 0.01	0.68 ± 0.04	9.20 ± 0.29
2.55	0.55 ± 0.09	ND	0.76 ± 0.06	9.76 ± 0.16
3.35	0.98 ± 0.05	0.43 ± 0.01	0.84 ± 0.06	10.43 ± 0.04
5.00	ND	0.40 ± 0.02	1.03 ± 0.15	15.19 ± 0.61
6.70	0.79 ± 0.01	0.45 ± 0.00	1.10 ± 0.07	15.46 ± 0.61
	(B) Lox-1 Activity with Methyl Linoleate at pH 9 (nmol of O_2 consumed min^{-1} mL^{-1})			
0.85	57.18 ± 1.68	ND	ND	ND
1.70	64.96 ± 2.78	72.60 ± 3.98	44.79 ± 2.34	14.85 ± 1.94
2.55	78.46 ± 2.65	ND	ND	ND
3.35	80.95 ± 3.90	85.85 ± 4.25	54.65 ± 2.88	23.40 ± 2.14
5.00	76.34 ± 3.70	ND	ND	ND
6.70	71.00 ± 3.04	ND	ND	ND

[a] The activity of soybean lox-1 (300 μg/mL) with methyl linoleate (5 mM) was recorded at 25 °C. Methyl linoleate was emulsified with Tween 20, Brij 35, Tween 60, and Tween 85 (0.85−6.70 mM) in sodium carbonate buffer (0.1 M, pH 9). Results are means and standard deviations calculated from triplicate experiments. The SSA (m^2/g) of the corresponding model emulsion systems was determined from triplicate experiments; values are means ± standard deviations.

(2) Ostwald ripening (Weiss et al., 1997).

(3) A reduction in the hydrogen bonding capability of the droplets surface with the surfactant molecules simultaneous with the progressive dissociation of the fatty acid molecules (Hough and Thompson, 1987).

The changes in the concentration and size of the particles can be described by a decrease in the specific surface area of the emulsion from 2.71 to 0.87 m^2/g as pH was changed from 6 to 8. The decrease in SSA was concomitant with a change in the turbidity of the reaction mixtures. There was a neat transition from opaque emulsions to clear solutions as pH was increased on the same scale. Bild et al. (1977) found apparent pK_a values in the range of 7−8 for linoleic acid. The progressive solubilization of linoleic acid in the emulsion systems is, therefore, expected to occur between pH 6 and 9. The chemical change $RCOOH \rightleftharpoons RCOO^-$ is usually accompanied by a dramatic increase in water solubility (Mead et al., 1986). Thus, the decrease in turbidity with increasing pH was explained by a progressive solubilization (into micelles and monomers) of the fatty acid molecules in the aqueous phase.

The pH−activity profiles of lox-1 with linoleic acid and methyl linoleate differed markedly in two respects (Figure 3). As already reported by Bild et al. (1977) when they examined the behavior of lox-1 against a variety of linoleic acid derivatives, the enzyme was considerably more active with the fatty acid than with the ester. At pH 9, lox-1 activity with linoleic acid was ~50 times greater than with methyl linoleate. The position of the maximum on the pH−activity profiles was not affected by the nature of the substrate. However, the activity of lox-1 with linoleic acid was noticeably extended on the acid side of the peak. The ratio of enzyme activity with linoleic acid and methyl linoleate varied significantly with pH, increasing from 20 to 50 as pH was changed from 6 to 9. The relative activity of soybean lox-1 with linoleic acid was compared to the transmittance of light in the corresponding reaction mixtures. The two curves were correlated at 99.7% up to pH 9.2. Hence, the pH−activity profile of lox-1 with linoleic acid did not seem to be a simple reflection of pH effects on the ionic state of the enzyme. Changes in the physicochemical properties of the fatty acid/surfactant model systems upon pH increase would seem to be responsible for the observed variations in the activity ratio. The results confirmed previous work from Perez-Gilabert et al. (1992).

Effect of Surfactant Concentration and Type on the State of the Emulsions and on Lox-1 Activity. The influence of surfactant concentration and type on the physicochemical characteristics of methyl linoleate and linoleic acid emulsions (pH 9) was examined with laser light scattering. The dispersions were prepared using the agent in water method of emulsification. Tween 20 was investigated first as it is most commonly used for in vitro lipoxygenase assays. The nonionic surfactants Brij 35, Tween 60, and Tween 85 were also considered. These surfactants were chosen to produce emulsions of varying physicochemical properties due to their diversity of HLB numbers (Table 2). HLB numbers characterize the balance between the hydrophilic and lipophilic properties of nonionic amphiphilic emulsifiers (Schott, 1995). Surfactants with HLB numbers between 8 and 15 are commonly employed as detergents, whereas those in the range of 15−18 are normally good solubilizers (Adamson, 1990).

Increasing the concentration of Tween 20 from 0.85 to 6.70 mM had a modest effect on the kinetic stabilization of the methyl linoleate droplets (Table 3A); the SSA was increased from about 0.5 to 0.8 m^2/g. Tween 20 and Brij 35 share the same HLB number, but the SSA of

Table 4. Effect of a Range of Surfactants on the Physicochemical Properties of Linoleic Acid Emulsion Systems and on the Activity of Soybean Lox-1[a]

surfactant concn (mM)	Tween 20	Brij 35	Tween 60	Tween 85
	(A) SSA of Linoleic Acid/Surfactant Emulsions at pH 9 (m^2/g)			
0.85	1.11± 0.14	2.40 ± 0.54	0.70 ± 0.02	4.55 ± 0.05
1.70	0.80 ± 0.03	2.02 ± 0.32	0.65 ± 0.06	8.83 ± 0.52
2.55	0.43 ± 0.01	1.20 ± 0.13	0.63 ± 0.05	13.78 ± 0.40
3.35	0.30 ± 0.01	NM	0.13 ± 0.00	16.61 ± 0.81
5.00	NM	NM	0.12 ± 0.00	17.83 ± 0.33
6.70	NM	NM	0.12 ± 0.00	23.44 ± 0.20
	(B) Lox-1 Activity with Linoleic Acid at pH 9 (nmol of O_2 consumed min^{-1} mL^{-1})			
0.85	148.30 ± 2.30	132.22 ± 1.92	125.42 ± 4.84	62.41 ± 0.88
1.70	144.52 ± 2.02	131.11 ± 1.92	122.37 ± 2.03	54.16 ± 0.00
2.55	133.07 ± 0.26	120.97 ± 2.81	94.20 ± 4.97	49.07 ± 0.92
3.35	124.45 ± 2.12	112.31 ± 3.67	91.17 ± 2.38	45.12 ± 1.61
5.00	111.65 ± 2.44	105.47 ± 3.23	87.12 ± 0.63	39.42 ± 0.87
6.70	110.25 ± 2.65	97.12 ± 5.10	79.50 ± 4.30	38.81 ± 1.59

[a] The activity of soybean lox-1 (13.33 μg/mL) with linoleic acid (5 mM) was recorded at 25 °C. Linoleic acid was emulsified with Tween 20, Brij 35, Tween 60, and Tween 85 (0.85−6.70 mM) in sodium carbonate buffer (0.1 M, pH 9). Results are means and standard deviations calculated from triplicate experiments. The SSA (m^2/g) of the corresponding model emulsion systems was determined from triplicate experiments; values are means ± standard deviations.

methyl linoleate/Brij 35 emulsions was very slightly lower (0.4−0.5 m^2/g). One rationale is that the kinetic stabilization of nonpolar surfaces decreases with increasing number of polyoxyethylene units (Hough and Thompson, 1987). A more significant event in these systems was the likely incorporation of ester molecules into Tween 20 or Brij 35 micelles because the rate of oil solubilization into micellar solution is known to be proportional to the concentration of surfactant micelles and, hence, to the amount of surfactant in the reaction mixtures (Saito et al., 1993). Increasing levels of Tween 60 (HLB of 14.9) from 0.85 to 6.70 mM had a slightly more noticeable impact on the physical state of the methyl linoleate emulsions as the SSA was almost doubled from 0.6 to 1.1 m^2/g. Adsorbed layer coverage of nonpolar surfaces, hence, droplet stability, increases with extending alkyl chain length (Hough and Thompson, 1987). The addition of Tween 85 (HLB of 11.0) to the methyl linoleate emulsions significantly affected the stabilization of small droplets at the expense of the larger ones, shifting the SSA from 4.6 to 15.5 m^2/g. This effect can be partly explained by an improved interaction between the surface of methyl linoleate droplets and the emulsifier but mostly by an increase in the shearing stress to which the larger droplets were submitted. The shearing stress is proportional to the viscosity of the emulsions. The zero shear viscosity of the methyl linoleate/Tween 85 dispersions was found to increase from 1.1 to 530 mPa·s as levels of emulsifiers were changed from 0.85 to 6.70 mM.

The effect of Tween 20 concentration (0.85−6.70 mM) on the activity of lox-1 with methyl linoleate (5 mM) was investigated at pH 9 (Table 3B). Enzyme activity with the nonpolar substrate was enhanced by relatively low concentrations of Tween 20. However, surfactant levels >3.35 mM reduced the rate of the reaction. The conformation of lox-1 was possibly affected by Tween 20, which could have progressively inhibited the enzyme (Srinivasulu and Appu Rao, 1993). When both enzyme activity and SSA increased with the concentration of detergent, the data did not correlate well ($R^2 = 0.23$). The activity of lox-1 with methyl linoleate and the specific surface area of the model emulsions were both enhanced by increasing the concentration of the nonionic surfactants Brij 35, Tween 60, and Tween 85 from 1.70 to 3.35 mM (Table 3). These increases were disproportionate, which weakens the case for lox-1 acting at the oil/water interface. A possible explanation for these results can be proposed if one considers the HLB values of the surfactants used. The lower the HLB value, the better the emulsifier generally is at stabilizing oil droplets, but the less efficient it is at incorporating nonpolar material into micelles and vice versa. Hence, the activity of lox-1 in methyl linoleate systems emulsified with Tween 20, or Brij 35, could be strongly influenced by the incorporation of substrate inside surfactant micelles. However, the activity of the enzyme in emulsions stabilized with Tween 60, or Tween 85, could be stongly dependent on the surface of oil exposed to the continuous phase. Although the reaction mixtures emulsified with Tween 85 displayed very large SSA, the activity of lox-1 in these systems was fairly low. The diffusion of the reactants in these emulsions was possibly hindered by the high viscosity of the continuous phase.

Linoleic acid model sytems (agent in water method of emulsification) containing Tween 20 or Brij 35 were optically clear at pH 9 for surfactant concentrations >2.55 mM. They were not suitable for examination with laser light scattering. Emulsions containing Tween 60 and Tween 85 could be studied over a larger range of emulsifier levels as they were more opaque (Table 4A).

Increasing the amount of surfactant from 0.85 to 2.55 mM reduced the SSA of linoleic acid emulsions from 1.1 to 0.4 with Tween 20 and from 2.4 to 1.2 m^2/g with Brij 35. This decrease in SSA was concurrent with a decrease in the turbidity of the dispersions. Greater concentrations of both of these surfactants probably led to the micellar solubilization of oil from the droplets with the largest surface areas exposed to the aqueous medium. With Tween 60, the SSA was reduced from 0.7 to 0.1 m^2/g and the turbidity of the dispersions increased as the emulsifier concentration was changed from 0.85 to 6.70 mM. This reflects a balance between a better stabilization of the emulsion and the solubilization of substrate into the aqueous phase. Increasing surfactant concentrations changed the volume weighted mean diameter of the particles from 32 to 42 μm with Tween 20, from 15 to 31 μm with Brij 35, and from 50 to 76 μm with Tween 60. This can be explained by Ostwald ripening (Weiss et al., 1997) and by the reduction in the hydrogen-bonding capability of the droplets' surface

with the surfactant molecules simultaneous with the progressive dissociation of the fatty acid molecules (Hough and Thompson, 1987). SSA and mean diameter characteristics for the three types of dispersions showed that, as expected from the HLB theory, both Tween 20 and Brij 35 (HLB = 16.9) are both better solubilizers and emulsifiers for linoleic acid (HLB = 16.2; Griffin, 1954) than Tween 60 (HLB = 14.9). Larger amounts of Tween 85 increased the SSA of the linoleic acid dispersions from 5 to 23 m^2/g. As with the methyl linoleate emulsions, this dramatic increase in SSA was probably due to the increase in the shearing stress, which larger oil droplets were submitted to, as the viscosity vastly increased with surfactant concentration.

The activity of lox-1 with linoleic acid decreased with increasing concentrations of the nonionic surfactants (Table 4B). As with the methyl linoleate systems, there was no correlation between SSA data and enzyme activity results. The rate of the reaction, however, increased with the HLB number of the surfactant. This further argued that the physical chemistry of the reaction mixtures, that is, the partition of substrate between the oil and water phases, could be an important parameter for lipoxygenase activity.

Several workers have proposed that the lox-1 reaction is not interfacial and that the enzyme interacts preferentially with monomeric substrates dissolved in the water phase (Galpin and Allen, 1977; Schilstra et al., 1994). The decrease in enzyme activity brought about by the presence of the surfactants is presumably complex and may not be completely explained by variations in the relative amounts of solubilized and interfacial substrate. The high viscosity of the continuous phase (in systems containing Tween 85) and the thickness of the surfactant layers adsorbed to the oil−water interface could have hindered the diffusion of the reactants. Enzyme activity could also have been limited by inhibitory surfactant effects on the enzyme itself.

Effect of the Emulsification Process on Enzyme Activity. The interpretation of the relationships between the activity of soybean lox-1 and the physicochemical properties of model emulsion systems is often complicated by the action of pH and detergents on the enzyme itself. This was overcome by working with emulsion systems having physicochemical characteristics that had been altered while the concentration of possible activating/inhibitory factors was kept constant. This was achieved by selecting emulsions with different shear histories (mixing times and techniques). The activity of lox-1 was examined in methyl linoleate and linoleic acid (5 mM)/Tween 20 (1.70 mM) emulsion systems, which were either hand mixed or emulsified with a rotor-stator blender (Ultra Turrax, Janke und Kunkel GmbH and Co., Staufen, Germany) at low and high speeds, for times varying between 10 and 60 s.

The effects of mixing time and technique on the physicochemical properties of methyl linoleate/Tween 20 emulsions were examined with laser light scattering. Emulsification processes imply the deformation of the interface between dispersed and continuous phases to such an extent that large droplets are formed, which are subsequently broken into smaller ones (Becher, 1959). The formation of small methyl linoleate droplets at the expense of larger ones was concomitant with greater SSA values. The increase in SSA was related to the strength and the duration of the mixing (Table 5).

Table 5. Effect of Emulsification Time and Force on the Activity of Soybean Lox-1 and the Physicochemical Properties of Methyl Linoleate Emulsion Systems[a]

mixing technique	mixing time (s)	lox-1 specific activity [nmol of O_2 consumed min^{-1} mL^{-1} $(mg\ of\ protein)^{-1}$]	SSA (m^2/g)
gentle hand mixing (reference)	10	0.08 (0.09, 0.08)	0.39 ± 0.02
vigorous hand mixing	10	0.13 (0.12, 0.13)	1.62 ± 0.12
	30	0.12 (0.11, 0.12)	1.23 ± 0.09
	60	0.11 (0.11, 0.11)	1.56 ± 0.07
low-speed Ultra Turrax	10	0.12 (0.11, 0.14)	1.77 ± 0.39
	30	0.17 (0.16, 0.18)	7.19 ± 0.04
	60	0.23 (0.25, 0.21)	8.78 ± 0.01
high-speed Ultra Turrax	10	0.14 (0.13, 0.15)	3.61 ± 0.24
	30	0.22 (0.21, 0.22)	9.05 ± 0.17
	60	0.25 (0.23, 0.26)	9.46 ± 0.41

[a] Methyl linoleate (5 mM)/Tween 20 (1.70 mM)/potassium phosphate buffer (0. 1 M, pH 7) emulsion systems were either hand mixed or emulsified with an Ultra Turrax set on low or high speed, for 10, 30, or 60 s. Lox-1 activity was immediately assayed in 3 mL of the resulting emulsions. Reaction mixtures contained 250 μg of enzyme/mL and 240 μM O_2. Experiments were carried out in duplicates. Values in parentheses represent individual measurements. The SSA (m^2/g) of the corresponding model emulsion systems was determined from triplicate experiments; values are means ± standard deviations.

The influence of the emulsification process on the activity of lox-1 with methyl linoleate was pH independent. Therefore, results are presented at only pH 7 (Table 5). Prolonged hand-mixing of the emulsions did not appear to affect enzyme activity. Extended mixing time with the Ultra Turrax, however, introduced some noticeable modifications in the rate of the reaction. Lox-1 activity was enhanced by ~80−90% when the duration of blending was increased from 10 to 60 s. Overall lox-1 activity in methyl linoleate emulsions increased with the force of the emulsification process. Enzyme activity results and SSA data were correlated at 96.8%, with a proportionality constant of ~7.

The effects of mixing time and technique on the physicochemical properties of linoleic acid/Tween 20 emulsions were examined at pH 7 and 9, that is, below and above the apparent pK_a of the fatty acid (Bild et al., 1977). At pH 7, the linoleic acid dispersions responded similarly to methyl linoleate emulsions to the shearing action of the mixing (Table 6). The formation of small fatty acid droplets at the expense of the larger ones was concomitant with greater SSA values and could be related to the strength and the duration of the emulsification process. Large amounts of linoleic acid molecules were expected to be ionized at pH 9 and, hence, to be dissolved in the aqueous phase. The enhanced solubilization of fatty acid droplets due to prolonged and vigorous mixing was accompanied by the formation of air bubbles stabilized by the fatty acid and surfactant molecules. The presence of foam in samples submitted to extended vigorous mixing could explain the variability of the overall SSA characteristics of the model systems.

The patterns of lipoxygenase activity with linoleic acid at pH 7 (Table 6) were similar to those described for methyl linoleate model systems (Table 5). The duration of hand mixing did not affect lox-1 activity in linoleic acid systems at pH 9. The emulsification of the model systems with the Ultra Turrax enhanced the rate of the reaction as the blending time increased from 10 to 30 s. Longer mixing times, however, reduced the activity

Table 6. Effect of Emulsification Time and Force on the Activity of Soybean Lox-1 and the Physicochemical Properties of Linoleic Acid Emulsion Systems[a]

emulsification process		pH 7		pH 9	
mixing technique	mixing time (s)	lox-1 specific activity [nmol of O_2 consumed min^{-1} mL^{-1} (mg of protein)$^{-1}$]	SSA (m^2/g)	lox-1 specific activity (nmol of O_2 consumed min^{-1} mL^{-1} (mg of protein)$^{-1}$]	SSA (m^2/g)
gentle hand mixing (reference)	10	3.44 (3.44)	2.51 ± 0.24	9.85 (9.85)	0.87 ± 0.03
vigorous hand mixing	10	5.97 (5.81, 6.13)	4.21 ± 0.25	9.76 (9.90, 9.62)	0.74 ± 0.04
	30	6.14 (6.30, 5.98)	5.23 ± 0.06	10.06 (10.32, 9.80)	1.02 ± 0.13
	60	6.40 (6.60, 6.20)	6.63 ± 0.63	10.11 (10.42, 9.80)	1.00 ± 0.08
low-speed Ultra Turrax	10	7.47 (7.37, 7.57)	4.98 ± 0.34	10.06 (10.45, 9.67)	1.24 ± 0.24
	30	8.13 (8.17, 8.09)	6.59 ± 0.16	10.93 (10.93, 10.93)	1.05 ± 0.06
	60	8.72 (9.27, 8.17)	9.61 ± 0.63	9.17 (9.16, 9.18)	0.48 ± 0.01
high-speed Ultra Turrax	10	8.28 (8.17, 8.39)	6.34 ± 0.33	8.62 (8.39, 8.84)	1.18 ± 0.10
	30	8.72 (9.34, 8.06)	10.25 ± 0.55	12.14 (13.03, 11.26)	0.78 ± 0.00
	60	9.85 (9.14, 10.55)	11.04 ± 0.35	9.73 (10.61, 8.85)	1.34 ± 0.08

[a] Linoleic acid (5 mM)/Tween 20 (1.70 mM)/buffer (0.1 M, pH 7 or 9) emulsion systems were either hand mixed or emulsified with an Ultra Turrax at low and high speeds, for 10, 30, or 60 s. Lipoxygenase-1 activity was immediately assayed in 3 mL of the resulting emulsions. Reaction mixtures contained 13 μg of enzyme/mL. Experiments were carried out in duplicate. Values in parentheses represent individual measurements. The SSA values (means and standard deviations) of the model emulsion systems were obtained from triplicate experiments.

of lox. The stabilization of foam particles by surfactant and linoleate molecules probably denatured lox and deprived the enzyme from some of the substrate. Although the results were noisy, the dioxygenation of linoleic acid by lox-1 appeared to decrease with the duration and strength of the emulsification process. Statistical analysis of the oxygen electrode and light scattering results showed that, although enzyme activity and SSA both increased with the duration and strength of the emulsification process, the data were not well correlated at pH 7 ($R^2 = 0.85$).

Conclusions. The state of linoleoyl molecules within an emulsion system is significantly affected by the chemistry of the linoleoyl groups (free or esterified) and by the physicochemical parameters that determine the behavior of surfactant molecules. This has profound effects on lox-1 activity. Limitations on the resolution of particles <0.05 μm by light scattering prevents a direct measurement of micelles; considerable indirect evidence from this work, however, supports the theory that lox-1 acts most efficiently on linoleoyl moieties in the monomeric state. This finding has implications for understanding the action of lox enzymes in vivo and for the control of lox-1 activity in the food industry. The procedures that are currently applied in the food industry to control lipoxygenase activity in foodstuffs, are not completely satisfactory. A novel approach for inhibiting lox-1 activity could be proposed from this work involving the promotion of oil droplet formation.

Observations from this work will provide workers measuring the activity of purified lox enzymes with a broad appreciation of factors that may explain fluctuations in results within and between laboratories.

ABBREVIATIONS USED

lox, lipoxygenase; lox-1, isoenzyme 1 of lipoxygenase; cmc, critical micelle concentration; O/W, oil-in-water; SDS, sodium dodecyl sulfate; SSA, specific surface area; (ES), enzyme−substrate complex; RCOOH, carboxylic acid; $RCOO^-$, carboxylate anion; HLB, hydrophile−lipophile balance, NM, not measurable; ND, not determined.

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Received for review July 23, 1999. Revised manuscript received June 2, 2000. Accepted June 2, 2000.

JF990822J