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Biocatalysis of lipoxygenase in selected organic solvent media

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Abstract

The biocatalysis of purified soybean lipoxygenase (LOX) (EC 1.13.11.12), using linoleic acid as a substrate model, was investigated in selected organic solvent media, including chloroform, dichloromethane, hexane, iso-octane, octane and toluene. The results indicated that there was a 2.6-fold increase in LOX activity in the monophasic iso-octane medium compared to that obtained in the aqueous medium. The results also showed that there was an increase of 2.2- and 1.8-fold in LOX activity in the monophasic reaction media of octane and hexane, respectively. However, an inhibitory effect on enzyme activity was observed when the monophasic reaction media of toluene, chloroform and dichloromethane were used. In addition, the results showed that the optimum concentration of octane and iso-octane in the biphasic medium containing the organic solvent and Tris–HCl buffer solution, was determined to be 3.5% and 4%, respectively, for LOX activity. Moreover, the biocatalysis of LOX in a ternary micellar system, containing either 3.5% octane or 4% iso-octane, Tris–HCl buffer solution and an emulsifier, resulted in an overall increase in enzyme activity. The K_m and V_{max} values, substrate specificity, optimum protein concentration, optimum reaction temperature as well as the enzymatically catalyzed end-products were investigated for LOX biocatalysis in both ternary micellar systems.

Keywords: Lipoxygenase; Biocatalysis; Ternary micellar system

1. Introduction

Lipoxygenase (LOX) (linoleate: oxygen reductase; EC 1.13.11.12) is a dioxygenase, which catalyzes the oxidation of linoleic acid and other polyunsaturated fatty acids, containing a *cis,cis*-1,4-pentadiene moiety, into fatty acid hydroperoxides, considered to be flavor precursors [1]. These hydroperoxides are then converted after enzymatic cleavage into ketones, aldehydes and alcohols.

The discovery that enzymes can function in organic solvent media has expanded the range of reactions, which can be approached through biocatalysis. Biocatalysis in organic solvent media is very useful when one or more components of the enzymatic reaction are poorly water-soluble [2]. Several approaches have been used to investigate enzymatic behavior in water-restricted environments, including the use of water miscible organic solvent systems [3,4], biphasic aqueous–organic solvent systems [5,6], reversed micelle systems [7] and monophasic organic solvent systems [8].

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The ultimate challenge in the area of biocatalysis is the alteration of enzyme specificity [9]. Recently, it has been reported that depending on the organic solvent system used for biocatalysis, the substrate specificity, enantioselectivity, chiral selectivity and regioselectivity of enzymes can be manipulated [10]. An enormous interest in developing a non-conventional medium for LOX biocatalysis has been reported [2,11–13]. Piazza [2] investigated the bioconversion of linoleic acid into hydroperoxides by soybean LOX in a microemulsion system consisting of surfactant, water and organic solvent, with air as a source of oxygen. Pourplanche et al. [11] reported that the addition of water-soluble co-solvents such as sorbitol in the aqueous reaction medium of typeI-B soybean LOX could modify the selectivity of the enzyme in the hydroperoxide synthesis reaction. Gargouri et al. [12] investigated the behavior of soybean LOX in a biphasic system, where the poorly water-soluble substrate was dissolved in an apolar solvent, octane. In addition, research work was carried out on the biogeneration of natural flavor compounds using microbial LOXs [13].

This work was aimed at the optimization of the use of non-conventional reaction media for LOX activity in the production of selected fatty acid hydroperoxides, considered to be flavor precursors. The specific objectives of this work were to investigate LOX biocatalysis in selected organic solvent media and to optimize the enzyme activity in terms of protein concentration, temperature, substrate specificity as well as to characterize the LOX-catalyzed end products.

2. Materials and methods

2.1. Enzyme preparation

Purified soybean LOX type I-B (110,600 units/mg) was purchased from Sigma (St. Louis, MO). The enzyme suspension (1 mg protein/ml) was prepared in Tris–HCl buffer solution (0.1 M, pH 9.0).

2.2. Protein determination

The protein content of the LOX preparation was assayed using a modification of the Lowry method [14]. Bovine serum albumin (Sigma) was used as a standard for calibration.

2.3. Substrate preparation

The substrate standards used throughout this study, including linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid), linolenic acid (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid), arachidonic acid (*cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatraenoic acid), monolinolein (1-mono[(*cis*,*cis*)-9,12-octadecadienoyl]-rac-glycerol), dilinolein (1,3-di[(*cis*,*cis*)-9,12-octadecenoyl]-rac-glycerol) and trilinolein (1,2,3-tri[(*cis*,*cis*,*cis*)-9,12,15-octadecadienoyl]-rac-glycerol), were purchased from Nu-Check-Prep (Elysian, MN).

For enzymatic assays in aqueous media, the aqueous substrate stock solutions were prepared at a concentration of 4×10^{-3} M according to the procedure described previously [15]. For enzymatic assays in monophasic, biphasic and ternary micellar organic solvent systems, pure substrate was directly introduced into the reaction medium.

2.4. Enzyme assay

For the enzymatic assays in aqueous media, a 200-μl volume of the aqueous substrate stock solution of linoleic acid was added to 780 μl of Tris–HCl buffer solution (0.1 M, pH 9.0), whereas for those in monophasic, biphasic and ternary micellar organic solvent systems, 10 μl of pure linoleic acid was added as substrate. The monophasic organic solvent system consisted of 970 μl of one of the selected organic solvents; the monophasic system also contained a limited amount (2%) of Tris–HCl buffer solution with or without the LOX suspension for the enzymatic and control assays, respectively. The immiscible biphasic system consisted of a mixture of a wide range of proportions (0–10%) of one of the selected organic solvents and the Tris–HCl buffer solution (0.1 M, pH 9.0), whereas the ternary micellar system was composed of Tris–HCl buffer solu-

tion (0.1 M, pH 9.0), a selected organic solvent and a specific surfactant; the total reaction volume for both biphasic and ternary micellar systems was 970 μl.

The enzymatic reaction was initiated by the addition of 20 μl of the LOX suspension (1 mg protein/ml) to the reaction mixture, which was then stirred for 3 min at 25°C. Aliquots of 10 to 25 μl were withdrawn from the reaction medium and analyzed for the presence of hydroperoxides. All assays were performed in triplicate.

The formation of LOX-catalyzed hydroperoxides of linoleic acid (HPODs), was determined spectrophotometrically using the Xylenol Orange method [16]. A total of 2 ml of the Xylenol Orange reagent was added to the extracted HPOD aliquot (10–25 μl), and the volume was adjusted to 2.1 ml with ethanol. The assay was allowed to react at room temperature for 15 min, after which the absorbance at 560 nm was measured vs. a blank consisting of a mixture of 2.0 ml of the Xylenol Orange reagent and 100 μl of ethanol. A Beckman DU-650 spectrophotometer (Beckman Instruments, San Raman, CA) was used. In addition, a freshly prepared diluted cumene hydroperoxide solution (Sigma) was used to establish a calibration curve.

2.5. Effect of organic solvent concentration on LOX activity

Selected organic solvents (ACS grade), including chloroform, dichloromethane, hexane, iso-octane, octane and toluene were individually used as a reaction medium for LOX biocatalysis. The optimization of the solvent concentration for LOX activity was performed for each organic solvent. A range of organic solvent concentrations (0–10%) was investigated by mixing the organic solvent with Tris–HCl buffer solution (0.1 M, pH 9.0).

2.6. Effect of surfactants on LOX activity

LOX activity was assayed in the presence of a variety of Spans, including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60), sorbitan monooleate (Span 80) and sorbitan trioleate (Span 85), as well as Tweens, including polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60) and polyoxyethylene sorbitan monooleate (Tween 80) (ICI Americas, Wilmington, DE). A surfactant suspension (0 to 100 μM) of each surfactant was prepared in Tris–HCl buffer solution (0.1 M, pH 9.0). The effect of the surfactants on LOX activity was studied in two reaction media; the first one consisted of a mixture (96.5:3.5, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and octane, while the second one was composed of a mixture (96:4, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and iso-octane.

2.7. Effect of enzymatic protein on LOX activity

The effect of enzymatic protein concentration on LOX activity was determined by varying the amount of protein between 1 and 25 μg/1 ml assay.

2.8. Effect of temperature on LOX activity

The effect of temperature on LOX activity was determined by incubating the enzymatic reaction at a wide range of temperatures between 5°C and 70°C.

2.9. Effect of substrate concentration on LOX activity

The effect of substrate concentration on LOX activity was assayed using a wide range of linoleic acid concentrations, between 26.8 and 1000 μM in the aqueous medium, and between 1.61 and 16.1 mM in the monophasic, biphasic and ternary micellar systems.

2.10. Substrate specificity of LOX activity

The substrate specificity of LOX was studied using standards, including linoleic acid, linolenic acid, arachidonic acid, monolinolein, dilinolein and trilinolein. For assays in the aqueous medium, stock solutions were prepared at a concentration of 4×10^{-3} M, as described previously, while for assays in the ternary micellar system, 10 μl of the pure substrate was directly added to the reaction medium.

2.11. Hydroperoxide recovery

The LOX-catalyzed HPODs were recovered according to the method described by Hamberg and Samuelsson [17]. The HPODs were extracted with diethyl ether and all traces of protein and surfactant were eliminated by washing the ether extract with water. The diethyl ether was then evaporated using a gentle stream of nitrogen. The polar oxygenated products, including the HPODs, were further separated from the non-oxidized linoleic acid with the use of a SPE silica column (Supelclean LC-Si 6 ml, Supelco, Bellefonte, PA) according to the procedure described by Toschi et al. [18].

The HPODs were then re-dissolved in methanol and reduced to the corresponding hydroxides of linoleic acid (HODs) by the addition of sodium borohydride ($NaBH_4$) [13].

2.12. High-performance liquid chromatography (HPLC) analysis of the reduced HPODs

The HPLC system used for the analyses of the reduced HPODs was Beckman Gold (Beckman Instruments) equipped with a computerized integration and data handling system (Beckman Model 126), and a Beckman diode-array UV detector (Model 128). Injection was performed using an automatic injector (Varian model 9095, Varian Associates, Walnut Creek, CA) fitted with a 50-μl loop. The reduced HPODs were separated on a normal-phase Alphabond silica column (300 × 3.9 mm, 5 μm) (Altech Associates, Deerfield, IL) with an eluant system consisting of a mixture of hexane/2-propanol/acetic acid (1000:10:1, v/v/v) at a flow rate of 1.0 ml/min [19]. The elution of the reduced HPODs was monitored by their specific absorption at 234 nm.

3. Results and discussion

3.1. Selection of solvents

LOX activity was investigated in various selected monophasic systems containing a 2% water content that consisted of a enzyme suspension in Tris–HCl buffer solution for the enzymatic assays and only the buffer for the control assays. The results (Table 1) show that there was a 2.6-fold increase in activity when the enzymatic reaction was performed in the iso-octane monophasic system compared to that obtained with the aqueous medium. Moreover, the results also show that there was a significant increase of about 2.2- and 1.8-fold in LOX activity with the use of the reaction media of octane and hexane, respectively. However, an inhibitory effect on enzyme activity was observed when the reaction media of toluene, chloroform and dichloromethane were used. These results show that enzymes have the ability to function in nearly anhydrous media [20]. In addition, these findings suggest that in the reaction media of iso-octane, octane and hexane, the water needed for enzymatic activity was tightly bound to the enzyme molecules and remained bound even when the bulk water was replaced by organic solvent [21].

The overall results may be explained by the logarithm of partition coefficient (log P), used to define the degree of solvent polarity [22]. In general, biocatalysis in organic solvents has been reported to be relatively low in hydrophilic solvents having a log $P < 2$, moderate in solvents having a log P between 2 and 4, and high in hydrophobic solvents having a log $P > 4$. Moreover, high biocatalytic rates have also been reported in organic solvents having a log $P < 4$ when the essential water layer around the enzyme is stabilized by a hydrophilic support [23].

Table 1
LOX activity in selected monophasic organic solvent systems

Monophasic system[a]	Specific activity[b]
Aqueous	2.64 (±0.18)[c]
Hexane	4.88 (±0.31)[c]
iso-Octane	6.89 (±0.11)[c]
Octane	5.87 (±0.09)[c]
Toluene	1.49 (±0.02)[c]
Chloroform	0.84 (±0.11)[c]
Dichloromethane	0.18 (±0.23)[c]

[a] The monophasic system was composed of a selected organic solvent and 0.1 M Tris–HCl buffer at pH 9.0 (98:2, v/v).

[b] Specific activity was defined as μmol of hydroperoxides of linoleic acid (HPODs)/mg protein/min.

[c] Standard deviation of samples, performed in triplicate.

These findings are in agreement with our results as the highest specific LOX activity was obtained with the reaction media of octane and iso-octane having a log *P* value of 4.5, followed by that of hexane possessing an intermediate log *P* value of 3.5. The use of the reaction media of toluene, dichloromethane and chloroform, whose log *P* values are 2.5, 2.0 and 2.0, respectively, resulted in a lower specific activity than the one obtained in the aqueous medium, thereby suggesting that the hydrophilicity of these organic solvents could have stripped away the essential water layer bound around the enzyme active site.

3.2. Effect of solvent concentration

LOX activity was further investigated in biphasic systems, which contained Tris–HCl buffer solution and one of the selected organic solvents at a wide range (0–10%) of concentrations. The results (Fig. 1) show that in the presence of 4% iso-octane, the enzymatic activity of LOX increased by 4.7-fold. The results also show that the use of dichloromethane, hexane and octane at a concentration 1%, 1% and 3.5% produced a 1.5-fold increase in LOX activity. However, the presence of chloroform and toluene at a concentration of 1% resulted in a dramatic decrease in LOX activity. Based on these findings, the LOX biocatalysis was further investigated in the biphasic Tris–HCl reaction medium containing 4% iso-octane and compared to that obtained in the aqueous environment and the biphasic Tris–HCl reaction medium containing 3.5% octane.

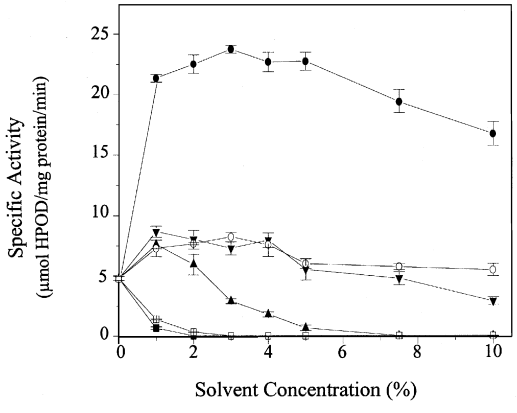

Fig. 1. Effect of solvent concentration on LOX activity in the reaction medium containing Tris–HCl buffer (0.1 M, pH 9.0) and organic solvent: chloroform (■—■), dichloromethane (▲—▲), hexane (▼—▼), iso-octane (●—●), octane (○—○) and toluene (□—□).

The overall results suggest that the presence of a small amount of organic solvent in the aqueous medium yielded better activity as it allowed better solubility of the substrate and created more interaction between enzyme and substrate. In addition, the overall findings suggest that a higher LOX activity was obtained in the biphasic medium as oxygen, a co-substrate in the LOX reaction, is more soluble in organic solvents than that in water; in the iso-octane medium equilibrated with air, the oxygen concentration was reported to be 3.5 mM, while in water, it was only 0.25 mM [24].

3.3. Effect of surfactant concentration

Figs. 2 and 3 show the LOX activity in the ternary micellar systems of octane/Tris–HCl (3.5:96.5, v/v) and iso-octane/Tris–HCl (4:96, v/v), respectively, containing the surfactants Tween or Span at a wide range (0–100 μM) of concentrations. The results were obtained in triplicate trials, with a relative standard deviation of 15–17%.

Fig. 2A shows that the presence of 50 μM of Tween 80 in the reaction medium of octane/Tris–HCl (3.5:96.5, v/v) was the optimum concentration of surfactant required to enhance LOX activity; higher concentrations of surfactant did not result in significant further increases of LOX activity. The results also indicate that the presence of low concentrations of Tweens 20, 40 and 60 showed an increase in LOX activity, whereas at higher concentrations, enzyme activity was inhibited. Fig. 2B also indicates that an increase of 1.1-fold in the LOX biocatalysis was obtained using 25 μM of Span 20 while a decrease or no change in LOX activity was observed in the presence of Spans 40, 60, 80 and 85. In addition, Fig. 3A indicates that when using the reaction medium of iso-octane and Tris–HCl buffer solution (4:96, v/v), the highest specific activity was obtained in the presence of 10 μM of Tween 40; a further increase in surfactant concentration resulted in little change in LOX activity. Fig. 3A also shows that in the presence of low concentrations of Tweens

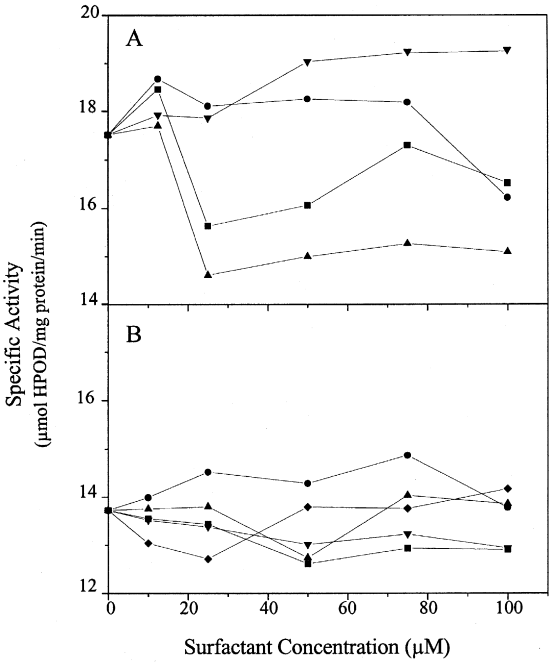

Fig. 2. Effect of surfactant concentration on LOX activity in the reaction medium composed of 0.1 M Tris–HCl buffer at pH 9.0 containing 3.5% octane and surfactant: (A) Tweens: Tween 20 (●—●), Tween 40 (■—■), Tween 60 (▲—▲), Tween 80 (▼—▼); (B) Spans: Span 20 (●—●), Span 40 (■—■), Span 60 (▲—▲), Span 80 (▼—▼) and Span 85 (◆—◆).

20, 60 and 80, a decrease in LOX activity was observed followed by a gradual increase at higher concentrations. The results (Fig. 3B) show that there was an overall dramatic decrease in LOX activity at low concentrations of Spans 20, 40, 60, 80 and 85; however, LOX activity was restored at higher surfactant concentrations.

On the basis of these experimental findings, the selected surfactant concentration was 50 μM of Tween 80 and 10 μM of Tween 40 in the reaction media of octane/Tris–HCl buffer solution (3.5:96.5, v/v) and iso-octane/Tris–HCl buffer solution (4:96, v/v), respectively, for further characterization studies.

The overall results suggest that the difference in the degree of hydrophobicity of the Tweens and Spans affected LOX biocatalysis as lower LOX activity was observed when Spans were used as emulsifiers in the reaction medium compared to that obtained in the presence of Tweens. In addition, the experimental findings suggest that the enhancement of enzyme activity in the presence of the surfactant may be due to an increased solubility of both enzyme and substrate at the interface. The length of the hydrophobic tail of the surfactant may also influence the LOX activity, although a correlation between the alkyl chain length of the surfactant and the LOX activity is not obvious. A decrease in LOX activity may be due to the denaturation of the enzyme, or the disruption of the enzymatic protein into inactive sub-units by the surfactant [25].

3.4. Effect of protein concentration

The effect of protein content on enzymatic activity (not shown) was investigated in the two ternary micellar systems. The results indicated that for the ternary micellar systems containing octane and iso-octane, the optima protein concentrations were 2.5 and 5 μg, respectively, which showed respective specific LOX activities of 19.0 and 27.5 μmol HPODs/mg protein/min, whereas in the aqueous buffer system, the highest specific activity of 18 μmol HPODs/mg protein/min was obtained using 7.5 μg of enzymatic protein. In addition, the overall results showed that further increases in protein concentrations resulted in an overall decrease in specific activity.

These findings suggest that at low protein concentrations, the gradual increase in specific activity was dependent on the formation of the E–S complex in order to shift the equilibrium towards the formation of the product [26]. At higher protein concentrations, a decrease in specific activity was observed, which could be due to protein aggregation thereby resulting in diffusion limitation and/or the inactivation of enzymes.

3.5. Effect of temperature

Fig. 4 shows that the optimum temperature for LOX activity in the aqueous buffer system was 20°C, above which a steady decrease in enzyme activity was observed. The results also show that the optimum temperature for LOX activity in both ternary

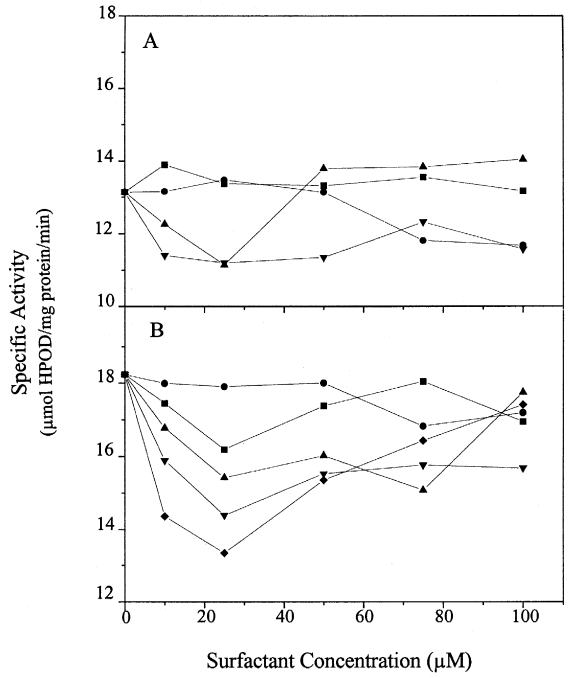

Fig. 3. Effect of surfactant concentration on LOX activity in the reaction medium composed of 0.1 M Tris–HCl buffer at pH 9.0 containing 4% iso-octane and surfactant: (A) Tweens: Tween 20 (●—●), Tween 40 (■—■), Tween 60 (▲—▲), Tween 80 (▼—▼); (B) Spans: Span 20 (●—●), Span 40 (■—■), Span 60 (▲—▲), Span 80 (▼—▼) and Span 85 (◆—◆).

micellar systems, containing octane and iso-octane, was 15°C and 40°C, respectively, beyond which a decrease in enzyme activity occurred. The results also show that in the ternary micellar system containing iso-octane, there was a higher LOX activity with an increase in temperature, whereas in the aqueous medium and the ternary micellar system containing octane, an overall lower activity was displayed. The overall findings suggest that the use of higher temperatures produced an activatory effect on the biocatalysis of LOX in the ternary micellar system containing iso-octane. These findings also suggest that the ternary micellar system containing iso-octane provided better enzyme stability at higher temperatures.

Overbeek et al. [27] stated that temperature plays an essential role in micellar behavior since the solubility of non-ionic surfactants is highly temperature dependent. Attwood and Florence [28] also indicated that the effect of temperature on enzyme activity could be related to the changes in the properties of micelles. In addition, Myers [29] reported that the effect of temperature on the properties of non-ionic surfactants was complex; an increase in the temperature could result in a decrease in the hydration of the hydrophilic group, which favors the formation of micelles, but could also disrupt the structured water surrounding the hydrophobic group thereby disabling micelle formation.

3.6. Substrate specificity

The specificity of LOX activity (Table 2) was assayed using a wide range of substrates, including polyunsaturated free fatty acids and polyunsaturated fatty acid acylglycerols. The substrate specificity was investigated in both ternary micellar systems, as well as in the aqueous medium system. The results show that LOX demonstrated an increase of 88% and 9% in relative activity with arachidonic acid as substrate in the ternary micellar system containing iso-octane and in the aqueous medium, respectively; in addi-

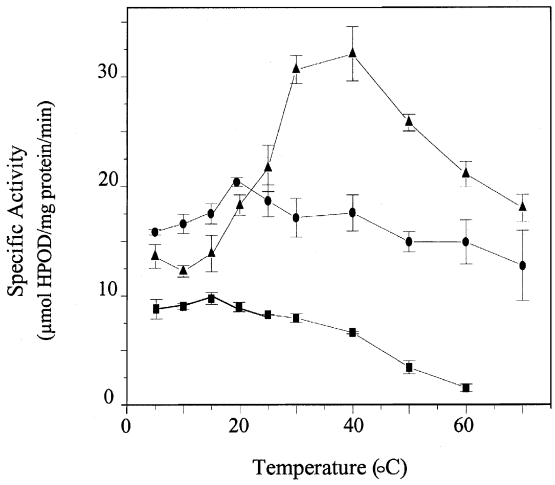

Fig. 4. Effect of temperature on LOX activity in an aqueous medium composed of 0.1 M Tris–HCl buffer at pH 9.0 (●—●), in a ternary micellar system composed of 0.1 M Tris–HCl buffer at pH 9.0, containing 3.5% octane and 50 μM Tween 80 (■—■), and in a ternary micellar system composed of 0.1 M Tris–HCl buffer at pH 9.0, containing 4% iso-octane and 10 μM Tween 40 (▲—▲).

tion, a 25% increase in the relative LOX activity was observed with linolenic acid as substrate in the former reaction medium. The results also show that the highest specific activity in the ternary micellar system containing octane was obtained with linoleic acid as a substrate. Table 2 indicates that for the three reaction media investigated, an overall lower relative activity (24–46.2%) was displayed towards monolinolein as a substrate whereas with dilinolein and trilinolein, a dramatic decrease (0.4–9.3%) was observed.

Piazza [30] reported that depending upon the source of LOX and the type of LOX isozymes present, different degrees of specificity were exhibited depending on whether the carboxylic acid moiety of the polyunsaturated fatty acid was free or esterified; the same author also indicated that LOX from soybean was highly specific towards free fatty acids under most conditions. However, Piazza et al. [31] also reported that soybean LOX could oxidize esterified fatty acids to a greater degree in the presence of deoxycholic acid in aqueous medium.

3.7. Effect of substrate concentration

Table 3 summarizes the K_m and V_{max} values of LOX activity obtained from Lineweaver–Burk plots of $1/v$ vs. $1/[S]$, using linoleic acid as a substrate in the selected reaction media, including the aqueous, monophasic, biphasic and ternary micellar systems. The results show that the lowest K_m value of 0.36

Table 2
Substrate specificity of LOX activity

Substrate	Specific activity[a]			Relative specific activity (%)[b]		
	Ternary micellar system[c]			Ternary micellar system[c]		
	Octane	iso-Octane	Aqueous[d]	Octane	iso-Octane	Aqueous[d]
Linoleic acid	7.33 (±0.10)[e]	51.33 (±0.30)[e]	11.93 (±0.76)[e]	100.0 (±0.14)[f]	100.0 (±0.42)[f]	100.0 (±1.08)[f]
Linolenic acid	6.00 (±0.20)[e]	64.19 (±0.25)[e]	11.22 (±0.70)[e]	81.8 (±0.18)[f]	125.0 (±0.49)[f]	93.2 (±0.96)[f]
Arachidonic acid	4.80 (±0.71)[e]	96.57 (±0.61)[e]	12.04 (±0.39)[e]	65.5 (±0.47)[f]	188.1 (±1.28)[f]	108.9 (±0.93)[f]
Monolinolein	3.39 (±0.51)[e]	12.33 (±0.83)[e]	3.68 (±0.16)[e]	46.2 (±0.24)[f]	24.0 (±0.21)[f]	0.5 (±0.24)[f]
Dilinolein	0.03 (±0.44)[e]	4.77 (±0.69)[e]	0.93 (±0.23)[e]	0.4 (±0.18)[f]	9.3 (±0.69)[f]	7.4 (±0.59)[f]
Trilinolein	0.25 (±0.23)[e]	0.44 (±0.37)[e]	0.80 (±0.21)[e]	3.4 (±0.80)[f]	0.9 (±0.40)[f]	6.4 (±0.50)[f]

[a] Specific activity was defined as μmol of hydroperoxides with the substrate (HPODs)/mg protein/min.
[b] The relative activity was defined as the percentage of specific activity compared to that obtained with linoleic acid as substrate.
[c] The system was composed of 0.1 M Tris–HCl buffer at pH 9.0 containing either 3.5% octane and 50 μM Tween 80 or 4% iso-octane and 10 μM Tween 40.
[d] The aqueous medium was composed of 0.1 M Tris–HCl buffer at pH 9.0.
[e] Standard deviation of samples, performed in triplicate.
[f] Standard deviation was calculated from the percent relative standard deviation values of specific activity with linoleic acid (RSD1) and specific activity with another substrate (RSD2) according to the equation $[(RSD1)^2 + (RSD2)^2]^{1/2} \times$ relative specific activity [35].

Table 3
Kinetic parameters of LOX activity in selected organic solvent media

Reaction medium	Organic solvent					
	Octane			iso-Octane		
	V_{max}^{a}	K_m^{b}	Catalytic efficiency[c]	V_{max}^{a}	K_m^{b}	Catalytic efficiency[c]
Aqueous[d]	18.85	0.36	52.36	–	–	–
Monophasic[e]	8.29	14.65	0.56	29.68	6.95	4.27
Biphasic[f]	47.96	21.42	2.24	23.52	3.16	7.44
Ternary system[g]	20.49	5.57	3.68	18.90	2.45	7.71

[a] The maximum velocity, V_{max}, was expressed as μmol of hydroperoxides of linoleic acid (HPODs)/ml/min.
[b] The Michaelis constant, K_m, was defined as mmol of linoleic acid.
[c] Catalytic efficiency was defined as the ratio V_{max}/K_m.
[d] The aqueous medium was composed of 0.1 M Tris–HCl buffer at pH 9.0.
[e] The monophasic system was composed of either octane or iso-octane and 0.1 M Tris–HCl buffer at pH 9.0 (98:2, v/v).
[f] The biphasic system was composed of 0.1 M Tris–HCl buffer at pH 9.0 containing either 3.5% octane or 4% iso-octane.
[g] The ternary micellar system was composed of 0.1 M Tris–HCl buffer at pH 9.0 containing either 3.5% octane and 50 μM Tween 80 or 4% iso-octane and 10 μM Tween 40.

was obtained in the aqueous reaction medium, followed by those of 2.45 and 5.57 determined in the ternary micellar systems containing iso-octane and octane, respectively. The overall results indicate that among the organic solvent reaction media including the monophasic, biphasic and ternary micellar systems, the lowest K_m values were obtained with the ternary micellar systems, thereby suggesting the occurrence of a better affinity between the enzyme and the substrate, due to the presence of the surfactant. The discrepancy in the obtained K_m values could also be due to differences in the enzymatic assay methods used for LOX biocatalysis in the aqueous and organic solvent reaction media [32]. Similar V_{max} values of 18.85, 18.90, 20.49 μmol HPOD/ml/min for LOX activity were obtained in the aqueous medium, and in both ternary micellar systems containing iso-octane and octane, respectively. Moreover, the highest enzymatic catalytic efficiency for LOX activity, calculated as the ratio of V_{max}/K_m, was found to be in the aqueous medium followed by those obtained in the ternary micellar systems containing iso-octane and octane and then the biphasic and monophasic systems.

3.8. Characterization of the end-products

Table 4 shows the relative quantitative production of HPODs by LOX activity as determined by the HPLC analyses. The results indicate that both the 9- and 13-HPODs were produced by LOX activity in all the reaction media studied; similar findings have also been reported for LOX from plant sources by Gardner [33]. The results also show that LOX biocatalysis produced more of the 13-HPOD isomer, 67.7%, 69.7% and 87.1%, in the aqueous and monophasic octane and iso-octane reaction media, respectively, which could be explained by a change

Table 4
The HPLC analyses of linoleic acid hydroperoxide isomers produced by LOX in selected reaction media

Reaction medium	Relative hydroperoxide isomer (%)[a]			
	Octane		iso-Octane	
	13	9	13	9
Aqueous[b]	67.7	32.3		
Monophasic[c]	69.7	30.3	87.1	12.9
Biphasic[d]	42.0	58.0	52.9	47.1
Ternary system[e]	51.6	48.4	42.9	57.1

[a] The relative percent peak area was defined as the peak area of the hydroperoxide isomer of linoleic acid (HPOD) divided by the sum of the total isomers, multiplied by 100.
[b] The aqueous medium was composed of 0.1 M Tris–HCl buffer at pH 9.0.
[c] The monophasic system was composed of either octane or iso-octane and 0.1 M Tris–HCl buffer at pH 9.0 (98:2, v/v).
[d] The biphasic system was composed of 0.1 M Tris–HCl buffer at pH 9.0 containing either 3.5% octane or 4% iso-octane.
[e] The ternary micellar system was composed of 0.1 M Tris–HCl buffer at pH 9.0 containing either 3.5% octane and 50 μM Tween 80 or 4% iso-octane and 10 μM Tween 40.

in the surroundings of the enzyme, thereby modifying its specificity [34]. The overall findings also show that the production of the 9- and 13-HPODs was at an approximate ratio of 50:50 in the biphasic and ternary micellar systems containing octane and iso-octane.

Piazza et al. [34] investigated the end-products of immobilized soybean LOX in an octane biphasic reaction medium and found that the predominant isomer formed was the 13-HPOD, whereas in a hexane environment, the proportion of the same isomer reached 96.7%. Pourplanche et al. [11] reported that the addition of water soluble co-solvents to the reaction medium of type I-B soybean LOX resulted in a modification of the specificity of the enzyme in the hydroperoxide synthesis reaction by producing changes in secondary reactions such as the formation of carbonyl compounds.

Zaks and Klibanov [9] reported that the substrate specificity of chymotrypsin and subtilisin was significantly modified when placing them in organic solvents; for both these enzymes, a major driving force of substrate binding was the hydrophobic interaction between the side chain of the amino acid substrate and the binding pocket of the enzyme, so that in organic solvents, the substrate preference of chymotrypsin was reversed, resulting in a significant increase in reactivity towards hydrophilic substrates.

4. Conclusion

The experimental data obtained in this study showed that LOX exhibited greater activity when assayed in a ternary micellar system in comparison to that obtained in monophasic and biphasic media. In addition, the presence of only a small volume of organic solvent was necessary to enhance enzyme activity. Moreover, the substrate specificity and the end-product specificity of the LOX biocatalysis was altered depending on the reaction medium used.

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