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Biocatalysis by immobilized lipoxygenase in a ternary micellar system

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Abstract

Biocatalysis by immobilized soybean lipoxygenase (LOX) (EC 1.13.11.12) in selected organic solvent systems, including monophasic, biphasic and ternary micellar systems, was investigated using linoleic acid as a model substrate. The highest specific activity for the immobilized LOX was observed in a ternary micellar system composed of Tris–HCl buffer solution containing 4% *iso*-octane and 10 μM Tween 40. The immobilized LOX had optimum activity at a pH of 9.5 and showed better stability than its free counterpart at high temperatures, exhibiting maximum activity at a temperature of 70 °C. The highest catalytic efficiency value for the immobilized LOX was found to be 4.20 in the monophasic reaction medium of octane, followed by 0.59 and 0.44 obtained in the biphasic systems composed of buffer and containing either 3.5% octane or 4% *iso*-octane, respectively. The immobilized LOX showed higher specificity toward linoleic acid, followed by arachidonic acid. Characterization of the enzymatically catalyzed end products indicated that the proportions of hydroperoxide (HPOD) isomers produced from linoleic acid were dependent on the free and immobilized states of LOXs. The immobilized LOX was recovered and recycled up to four times before a complete loss of activity.

Keywords: Lipoxygenase; Immobilization; Biocatalysis; Organic solvent media

1. Introduction

Lipoxygenase (LOX) (linoleate: oxygen reductase; EC 1.13.11.12) is an enzyme that catalyzes the oxidation of linoleic acid and other polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene moiety to hydroperoxy fatty acids by hydrogen abstraction from the methylene carbon and antarafacial insertion of molecular oxygen [1]. These fatty acid hydroperoxides (HPODs), considered to be flavor precursors [2], are subsequently converted by enzymatic cleavage into flavor compounds, including ketones, aldehydes and alcohols.

Biocatalysis by LOX has been widely investigated in aqueous media and characterized in terms of optimum pH and temperature, kinetic parameters, substrate specificity as well as the nature of HPODs produced [3–6]. Recently, Zaks and Klibanov [7] investigated the alteration of LOX specificity and enhancement of its stability in organic solvent media. A keen interest in developing non-conventional media for LOX biocatalysis has also been reported [8–12]. Recent work in our laboratory investigated the effect of organic solvent media on the specificity

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of LOX, in particular, the nature of end products [13].

Enzyme immobilization has become a major application for the use of enzymes in biotechnological processes [14]. Immobilization facilitates the dispersion, recovery and reusability of enzymes [15] as well as provides better stability compared to their free counterparts [16]. Numerous methods have been developed for the immobilization of LOX, including its adsorption to various supports such as glutenin [17] and talc [18]. LOX has also been immobilized by covalent binding onto a commercially available carbonyl diimidazole activated support [19]. In addition, LOX has been immobilized by entrapment in agarose gel [20], polyacrylamide gel [21], alginate gel [22] and by cross-linking to phylosilicates [23].

Immobilization by ionic binding onto a carrier presents interesting features including low cost, simplicity and preservation of the native form of the enzyme [24]; however, the main drawback of this method is the weak bond between the support and enzyme, which can result in leakage of the enzyme [25]. In organic solvent media, however, the enzyme remains bound to the support due to its limited solubility thereby minimizing leakage from the support [15]. Using a wide range of ionic supports, the immobilization and characterization of selected enzymes, including lipases [26,27] and chlorophylase [28,29], were investigated in our laboratory.

The aim of this work was to optimize the immobilization of LOX onto an ion-exchange support in an organic solvent system. The specific objectives of this work were to investigate the activity by the immobilized enzyme in terms of pH, temperature, substrate specificity, storage temperature stability and effect of recycling as well as to characterize the LOX-catalyzed end products.

2. Material and methods

2.1. Enzyme preparation

Purified soybean LOX type I-B (110,600 units/mg solid) 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 [30]. Bovine serum albumin (Sigma) was used as a standard for calibration.

2.3. Immobilization of lipoxygenase

An ion-exchange resin Dowex 50WX4-200 (Sigma) was used as a support throughout this study. Immobilization of LOX was carried out by the addition of 1 ml of the enzyme suspension into various media, including deionized water and a wide range of buffer solutions (0.1 M) consisting of sodium phosphate (pH 7.0–8.0), Tris–HCl (pH 8.5–9.0) and sodium carbonate (pH 9.5–10.5), followed by the addition of selected amounts of the support. After gentle stirring at 4 °C for 3 h, the suspension was filtered (Whatman # 541; Sigma), and the filtrate and precipitate were lyophilized. To evaluate the immobilization efficiency, the precipitate was washed three times with 0.5 ml of the selected reaction medium and the protein contents of the washing medium and the filtrate were measured. The effect of different enzyme to support ratios (1:1, 1:2, 1:3 and 1:4) on the immobilization efficiency and activity of LOX were investigated.

2.4. Enzyme assay

Biocatalysis by the free and immobilized LOXs was carried out using selected organic solvent reaction media. Enzymatic assays were performed in monophasic, biphasic and ternary micellar organic solvent systems developed in our laboratory [13]. The monophasic organic solvent system consisted of 940 μl of one of the selected organic solvents, including hexane, *iso*-octane and octane containing a limited amount (2%) of Tris–HCl buffer solution (0.1 M, pH 9.0). The immiscible biphasic system was composed of buffer solution containing either 3.5% octane, 4% hexane or 4% *iso*-octane. The ternary micellar system was composed of either a mixture (96.5:3.5, v/v) of the buffer solution and octane with 50 μM Tween 80 or a mixture (96:4, v/v) of the buffer solution and *iso*-octane with 10 μM Tween 40. A volume of 40 μl of pure linoleic acid was added as substrate. The total reaction volume for all the organic solvent systems was 980 μl.

The enzymatic reaction was initiated by the addition of 20 μl of the free or immobilized LOX (1 mg protein/ml) to the reaction mixture, which was then stirred for 3 min at 25 °C. Aliquots of 10–25 μl were withdrawn from the reaction medium and analyzed for the presence of HPODs. Controls, containing all the components of the reaction medium except the enzyme, were run in tandem. All assays were performed in triplicate.

The formation of the LOX-catalyzed HPODs of linoleic acid was determined spectrophotometrically using the Xylenol Orange method [31]. Two milliliters of Xylenol Orange reagent were 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 against 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 Inc., San Raman, CA) was used. A freshly prepared diluted cumene HPOD solution (Sigma) was used to establish a calibration curve.

2.5. Effect of pH

The effect of pH on the activities of free and immobilized LOXs was investigated using a wide range of buffer solutions (0.1 M) consisting of sodium phosphate (pH 7.0–8.0), Tris–HCl (8.5–9.0) and sodium carbonate (9.5–10.5). The assays were performed in a ternary micellar system composed of the selected buffer solution containing 4% *iso*-octane and 10 μM Tween 40.

2.6. Effect of incubation temperature

The effect of temperature on the activities of free and immobilized LOXs was determined by incubating the enzymatic assays at a wide range of temperatures (20–100 °C). The enzymatic reactions were performed in the ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM Tween 40.

2.7. Effect of substrate concentrations

The effect of linoleic acid concentration (1.61–16.1 mM) on the specific activity of the immobilized LOX was investigated in the monophasic, biphasic and ternary micellar organic solvent systems using selected organic solvents, such as hexane, octane and *iso*-octane. Sodium carbonate buffer solution (0.1 M, pH 9.5) was used for the preparation of the enzyme solution.

2.8. Substrate specificity

The substrate specificity of the immobilized enzyme was investigated. Selected 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-eicosatetraenoic 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 Inc. (Elysian, MN). A 40 μl volume of the pure fatty acid was added to the reaction medium of the ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane with 10 μM Tween 40. The relative activity was defined as the percentage of specific activity obtained with each substrate compared to that obtained with linoleic acid.

2.9. Effect of temperature on the stability of the enzyme during the assay

The effect of temperature on the stability of free and immobilized LOXs was investigated. Enzymatic assays were performed in the ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM Tween 40. Each assay was incubated for different periods of time (3–15 min) and at various temperatures (20–70 °C).

2.10. Effect of storage temperature on the stability of the enzyme

The free and immobilized LOXs were suspended in the ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM

Tween 40. The enzyme mixtures were stored at 4, 25, 50 and 70 °C for different periods of time (0–12 h). The LOX assay (70 °C, 3 min) was performed at the end of the storage periods using linoleic acid as substrate.

2.11. Effect of enzyme recycling

The effect of recycling on the immobilized LOX activity in the ternary micellar reaction medium composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM Tween 40 was investigated. The enzymatic reaction was carried out at 70 °C for 3 min. The immobilized enzyme was then recovered and reused in a freshly prepared ternary micellar reaction medium. The procedure was repeated until the immobilized LOX showed no more activity. The relative LOX activity was expressed as the percentage of the specific activity obtained after each cycle to that of the initial one.

2.12. Recovery of end products

Immobilized LOX (45 mg sample) or its free counterpart (1 mg protein/ml) was introduced into 25 ml of the ternary micellar reaction medium containing sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane at a proportion of 96:4 (v/v) with 10 μM Tween 40. A 400 μl volume of one of the various selected substrates, including linoleic acid, linolenic acid and arachidonic acid, was subsequently added. After 1 h at 70 °C, the enzymatic reaction was halted by lowering the pH to 3.0 with a solution of 4 M HCl.

The HPODs obtained by the LOX-catalyzed reaction were recovered according to the method described by Hamberg and Samuelson [32]. The HPODs were extracted by diethyl ether and washed with water to eliminate all traces of protein and surfactant. The diethyl ether layer was then evaporated using a gentle stream of nitrogen. The polar oxygenated HPODs products were separated from the non-oxidized fatty acids by a SPE silica column (Supelclean LC-Si 6 ml, Supelco Inc., Bellefonte, PA) according to the procedure described by Toschi et al. [33]. The HPODs were re-dissolved in methanol and reduced to the corresponding hydroxides of linoleic acid with the use of sodium borohydride [3].

2.13. High-performance liquid chromatography analysis of the reduced end products

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

3. Results and discussion

3.1. Immobilization of LOX using selected media and enzyme/support ratios

The immobilization of LOX on an ion-exchange support using various media was investigated. The results (Table 1) show that the highest immobilization efficiency (74.6–81.1%) was observed when LOX was immobilized onto the support in a medium consisting of deionized water. Moreover, the results indicate that the highest immobilization efficiency of 81.1% and specific activity of 0.47×10^{-3} μmol HPODs/mg protein/min were obtained in the deionized water medium when the ratio of enzyme to support was 1:3. Table 1 also shows that an overall lower immobilization efficiency of 27.3–48.8% was observed when LOX was immobilized in buffer solutions (0.1 M) consisting of sodium phosphate (pH 7.0) and sodium borate (pH 9.0). The use of a 1:4 ratio of enzyme to support resulted in the highest specific activities of 1.9, 1.9 and 1.6 μmol HPODs/mg protein/min for the immobilized enzyme in deionized water, sodium phosphate and sodium borate, respectively. The overall higher immobilization efficiencies of LOX obtained in deionized water could be explained by the cationic state of the protein, possessing a calculated isoelectric point

Table 1
The effect of immobilization of lipoxygenase by ionic binding using different ratios of enzyme to Dowex 50WX4-200 in selected media

Enzyme/support ratio	Immobilization efficiency (%)[a]	Specific activity[b]	Relative activity (%)[c]
Free enzyme	–	25.5 (±0.04)[d]	100 (0.06)[e]
Enzyme suspended and washed in deionized water			
1:1	74.6 (±11.7)[d]	0.46 (±0.04)[d]	1.8 (0.10)[e]
1:2	74.8 (±2.96)[d]	0.45 (±0.03)[d]	1.8 (0.09)[e]
1:3	81.1 (±6.61)[d]	0.47 (±0.03)[d]	1.8 (0.09)[e]
1:4	70.9 (±10.4)[d]	0.50 (±0.02)[d]	1.9 (0.08)[e]
Enzyme suspended and washed in sodium phosphate buffer solution (0.1 M, pH 7.0)			
1:1	48.8 (±2.26)[d]	0.13 (±0.01)[d]	0.5 (0.02)[e]
1:2	38.3 (±10.9)[d]	0.14 (±0.01)[d]	0.5 (0.02)[e]
1:3	27.3 (±8.43)[d]	0.31 (±0.03)[d]	1.2 (0.06)[e]
1:4	35.9 (±6.61)[d]	0.48 (±0.09)[d]	1.9 (0.19)[e]
Enzyme suspended and washed in borate buffer solution (0.1 M, pH 9.0)			
1:1	42.5 (±4.09)[d]	0.08 (±0.01)[d]	0.3 (0.01)[e]
1:2	34.7 (±8.58)[d]	0.19 (±0.02)[d]	0.8 (0.04)[e]
1:3	27.4 (±10.4)[d]	0.31 (±0.04)[d]	1.2 (0.07)[e]
1:4	34.0 (±15.9)[d]	0.40 (±0.01)[d]	1.6 (0.06)[e]

[a] The immobilization efficiency was determined as the relative percentage of immobilized enzymatic protein compared to that of the total free enzymatic protein.

[b] The specific activity was expressed as micromoles of hydroperoxides (HPODs) of linoleic acid per milligrams of protein per minute. The enzymatic reaction was carried out in the ternary micellar system composed of a mixture (96:4, v/v) of Tris–HCl buffer (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40.

[c] The relative activity was defined as the percentage of specific activity compared to that obtained with the free enzyme.

[d] Standard deviation of samples was performed in triplicate.

[e] Standard deviation was calculated from the percent relative standard deviation values of the specific activity with the free enzyme (R.S.D.1) and the specific activity with the immobilized enzyme (R.S.D.2) according to the equation $[(\text{R.S.D.1})^2 + (\text{R.S.D.2})^2]^{1/2}$relative specific activity [34].

(Ip) of 6.2, in the acidic water medium of pH 5.0–5.5 compared to that obtained in buffer media at higher pH values, thereby making LOX more interactive with the negatively charged Dowex 50WX4-200 support. On the basis of these findings, immobilization of LOX using an enzyme to support ratio of 1:4 in deionized water was selected as the most appropriate condition for further characterization studies.

The results also indicate that an overall lower specific activity was obtained for the immobilized LOX compared to that exhibited by the free enzyme and that different immobilization efficiencies were demonstrated in our study with the use of different media for immobilization. The overall findings could be explained by the fact that pH, electrostatic interactions and steric hindrance play an important role in the activity of the immobilized enzyme by modifying the three dimensional structure of the enzyme [35]. A loss in activity during the immobilization process could also be derived due to the processes involved in the enzyme-support interactions [36]. Dinelli [37] reported that immobilization may induce a loss in enzymatic activity depending on the nature of the enzyme and the immobilization method used; however, cases where the enzymatic activity increased were also reported. Chikere et al. [38] reported increases in enzyme activity when immobilization was carried out with short incubation periods in low ionic strength buffers.

3.2. Effect of selected organic solvent systems on the activity of free and immobilized LOXs

Table 2 shows the immobilization efficiency and specific activity of the immobilized LOX in different organic solvent reaction media. The results indicate that when the enzymatic reaction was performed in a ternary micellar system composed of a mixture (96:4,

Table 2
Enzymatic activity of the free and immobilized LOXs in selected organic solvent media

Organic solvent system	Specific activity in selected organic solvent media[a]					
	Hexane		Octane		*iso*-octane	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
Monophasic[b]	55	8.6	43	2.5	57	4.8
Biphasic[c]	188	8.5	180	8.8	246	13.8
Ternary[d]	n.a.[e]	n.a.[e]	188	10.4	272	17.1

[a] Specific activity was expressed as nmoles hydroperoxides (HPODs) of linoleic acid/(mg protein min).
[b] The monophasic system was composed of Tris–HCl buffer solution (0.1 M, pH 9.0) and the selected organic solvent at a proportion 98:2.
[c] The biphasic system was composed of Tris–HCl buffer solution (0.1 M, pH 9.0) containing either 4% hexane, 3.5% octane or 4% *iso*-octane.
[d] The ternary micellar system was composed of either a mixture (96.5:3.5, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and octane containing 50 μM Tween 20 or a mixture (96:4, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40.
[e] Non applicable.

v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM of Tween 40, the highest activities for the free and immobilized LOXs were 272.0 and 17.1 nmoles HPODs/(mg protein min), respectively. Slightly lower activities of 188.0 and 10.4 nmoles HPODs/(mg protein min) were observed for the free and immobilized LOXs, respectively, in the ternary micellar system composed of a mixture (96.5:3.5, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and octane with 50 μM Tween 80.

In the biphasic organic solvent reaction media, the highest LOX activities for the free and immobilized enzymes were also obtained in the system composed of buffer and *iso*-octane, followed by slightly lower activities obtained in the reaction media composed of buffer and either octane or hexane.

For the monophasic systems investigated, an overall decrease in enzyme activity was observed; the highest activity for LOX in its immobilized state was found in the hexane environment followed by those obtained in *iso*-octane and octane media while similar activities were exhibited for LOX in its native state in all three monophasic media.

Piazza et al. [9] reported that the addition of buffer at a concentration of 35% to octane produced more than a three-fold increase in the activity of LOX immobilized by covalent binding to a carbonyl diimidazole-activated support.

The overall results indicate that the highest specific activities for both free and immobilized LOXs were exhibited in the ternary micellar system composed of a mixture (96:4, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40; this ternary micellar system was subsequently used for further characterization studies.

3.3. Effect of pH on the activity of immobilized LOX

Fig. 1 shows the effect of pH on the specific activity of the free and the immobilized LOXs. The results show that the optimum pH values for the enzyme activity of the free and immobilized LOXs were 9.0 and 9.5, respectively. In addition, the overall results show that further increases or decreases in the pH value of the reaction medium resulted in an overall decrease in the specific activity of LOX.

Hsu et al. [22] reported that the optimum pH for the enzymatic activity of LOX immobilized by entrapment in calcium alginate beads was 9.0. In general, enzymes attached to a positively charged matrix exhibit a shift in optimum pH towards the acidic side [39], whereas those attached to a negatively charged matrix exhibit a shift towards the alkaline side. In agreement, our findings show that the optimum pH for LOX activity shifted from 9.0 for the free enzyme to 9.5 for the immobilized one. These findings may be explained by Hartmeier [40] who reported that the position of the active center of the protein, the size of the enzyme molecule and the site at which the enzyme is bound to the support are all important factors that will influence the optimum pH for activity

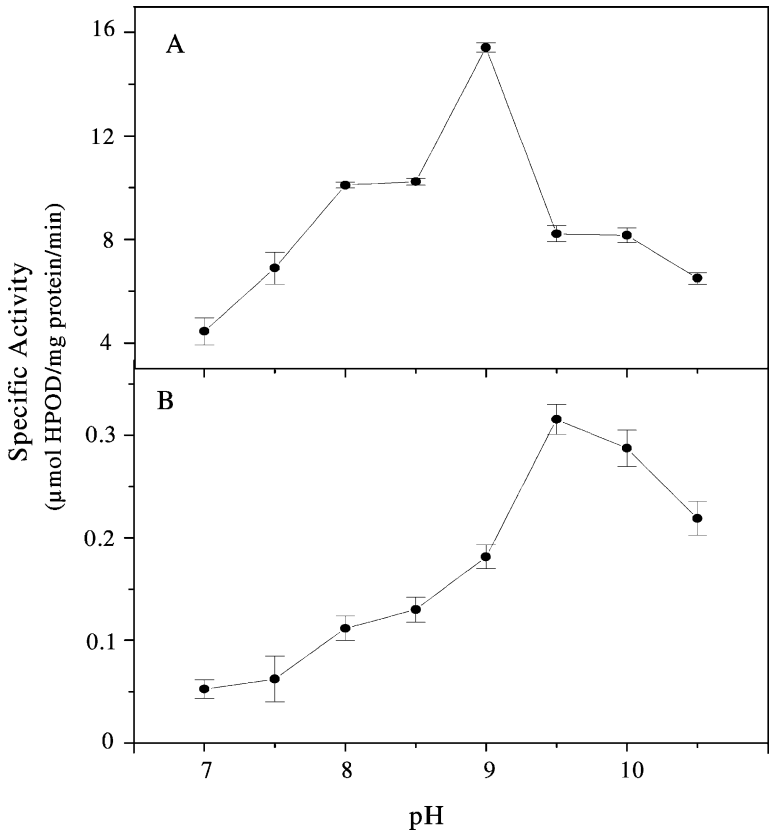

Fig. 1. Effect of pH on the enzyme activity of free (A) and immobilized (B) LOXs in reaction media composed of one of the selected buffer solutions, including sodium phosphate (0.1 M, pH 7.0–8.0), Tris–HCl (0.1 M, pH 8.5–9.0), and sodium carbonate (0.1 M, pH 9.5–10.5), and 4% *iso*-octane containing 10 μM Tween 40.

of an immobilized enzyme. Berset [35] reported that shifts in the optimum pH values for the enzyme activities of several immobilized enzymes were due to the presence of charges depending on the support used. Immobilization can affect the conformation as well as the state of ionization and dissociation of an enzyme and its environment so that changes in the relationship between pH and optimal activity are not uncommon.

Based on the experimental findings, the ternary micellar reaction media consisting of either a mixture (96:4, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM of Tween 40 or a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM of Tween 40 were selected for further characterization of the free and immobilized LOXs, respectively.

3.4. Effect of temperature on the activity of immobilized LOX

The effect of temperature on the activity of free and immobilized LOXs was investigated. Fig. 2 shows that the highest specific activities for the free and immobilized LOXs were at 40 and 70 °C, respectively. The results also show that further increases or decreases in temperature produced a gradual decrease in the specific activity of LOX. However, the immobilized LOX showed greater stability at higher temperatures than its free counterpart, which was more stable at lower temperatures. The findings also indicate that at 100 °C, the immobilized LOX retained 50% of its enzymatic activity, whereas the free one displayed a complete loss of activity at 90 °C.

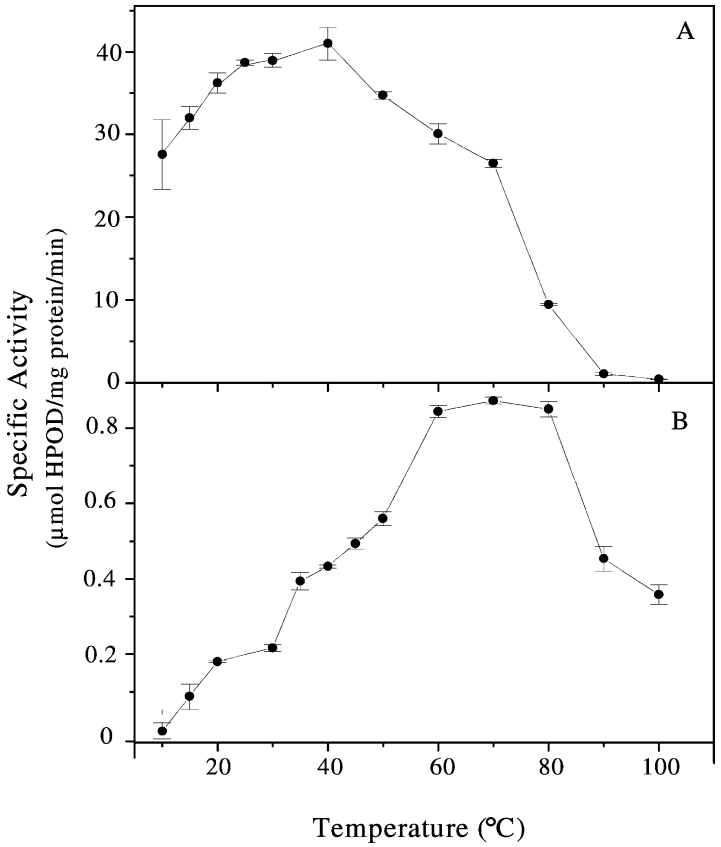

Fig. 2. Effect of temperature on the activity of free (A) and immobilized (B) LOXs in a ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40.

Immobilization has been described as a technique that increases enzyme rigidity due to the fact that the movement of the enzyme molecule is restricted by its contact with the support. Enzyme stability is therefore enhanced with respect to extremes of pH and temperature [36]. Klibanov [41] reported that an increase in the temperature of an enzymatic reaction medium could lead to a dramatic increase in its reaction rate; a temperature increase from 25 to 70 °C can result in a two-order magnitude of rate enhancement for an average enzymatic reaction.

Hsu et al. [23] reported that the optimal temperature for the activity of LOX immobilized by cross-linking to phyllosilicates was 25 °C, while Parra-Diaz et al. [19] reported an optimum temperature of 15 °C for the enzymatic activity of LOX covalently linked to a carbonyl diimidazole-activated polymer. These findings suggest that the differences in the optimal temperature for LOX activity obtained in the literature and in this study could be due to the different immobilization methods used.

Based on these findings, further kinetics studies were performed at 40 and 70 °C for the free and immobilized LOXs, respectively.

3.5. Effect of substrate concentration

Table 3 summarizes the K_m and V_{max} values for the activity of LOX in its immobilized state obtained from Lineweaver–Burk plots of $1/v$ versus $1/[S]$, using linoleic acid as a substrate in the selected organic solvent reaction media, including monophasic, biphasic

Table 3
Kinetic parameters for the enzymatic activity of LOX in its immobilized state in selected organic solvent media

Medium	Hexane			Octane			*iso*-Octane		
	V_{max}[a]	K_m[b]	Catalytic efficiency[c]	V_{max}[a]	K_m[b]	Catalytic efficiency[c]	V_{max}[a]	K_m[b]	Catalytic efficiency[c]
Monophasic[d]	0.26	2.67	0.10	0.26	0.06	4.20	2.37	9.73	0.24
Biphasic[e]	0.87	9.02	0.10	1.41	2.38	0.59	1.48	3.39	0.44
Ternary system[f]	–	–	–	2.27	7.95	0.28	1.59	5.69	0.28

[a] The maximum velocity V_{max} was expressed as μmol of hydroperoxides (HPODs) of linoleic acid/(ml min).
[b] Michaelis constant K_m was defined as μmol of HPODs of linoleic acid/ml.
[c] Catalytic efficiency (V_{max}/K_m) was defined as 1/min).
[d] The monophasic system was composed of either octane or *iso*-octane containing 2% sodium carbonate buffer solution (0.1 M, pH 9.5).
[e] The biphasic system was composed of sodium carbonate buffer solution (0.1 M, pH 9.5) containing either 4% hexane, 3.5% octane or 4% *iso*-octane.
[f] The ternary micellar system was composed of either a mixture (96.5:3.5, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and octane containing 50 μM Tween 20 or a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM Tween 40.

and ternary micellar systems. The results show that the highest V_{max} value of 2.37 μmol HPODs/(ml min) was obtained in the monophasic *iso*-octane system, followed by the V_{max} values of 2.27 and 1.59 μmol HPODs/(ml min) determined in the ternary micellar systems consisting of sodium carbonate buffer (0.1 M, pH 9.5) and either 3.5% octane containing 50 μM Tween 20 or 4% *iso*-octane containing 10 μM Tween 40, respectively. In addition, the results show that the lowest K_m value of 0.06 mM was obtained in the monophasic octane system, thereby suggesting a better affinity between the enzyme and the substrate. The overall results also indicate that among all the organic solvent systems investigated, the highest catalytic efficiency of 4.20 min^{-1} for the activity of LOX in its immobilized state was determined in the monophasic octane system, followed by that obtained in the biphasic octane or *iso*-octane systems, respectively. On the other hand, the lowest catalytic efficiency (0.10 min^{-1}) was found in the monophasic and biphasic systems that contained hexane.

These findings suggest that the differences between the kinetic values obtained for the activity of the immobilized LOX in the selected organic solvent media could be due to a change in the conformation of the enzyme upon interaction with the different components of the reaction environment. In addition, the kinetic values may also be affected by the increased diffusion limitation of the substrate into the pores of the immobilized enzyme [38].

3.6. Effect of temperature on the storage stability of LOX

Fig. 3 shows the effect of selected storage temperatures on the activity of the free and immobilized LOXs in the ternary micellar systems consisting of either Tris–HCl buffer solution (0.1 M, pH 9.0) and 4% *iso*-octane containing 10 μM Tween 40 or sodium carbonate buffer solution (0.1 M, pH 9.5) and 4% *iso*-octane containing 10 μM Tween 40, respectively. The results indicate that the free LOX exhibited almost no activity after 2 h of storage at 50 and 70 °C. However, the immobilized LOX displayed greater storage stability at 70 °C, retaining 50% of its initial activity after 9 h of storage. Fig. 3 also shows that the activity of the free LOX gradually decreased by approximately 80% after 12 h of storage at 4 and 25 °C, while that of the immobilized LOX decreased dramatically, losing 75% of its activity within the first hour of storage at 4, 25 and 50 °C. The overall findings suggest that the immobilized LOX was more stable than the free enzyme when stored at higher temperatures.

Improvements in the storage stability of immobilized enzyme preparations in comparison to free enzymes has also been reported in the literature. Pinto and Macias [21] reported that the enzymatic activity of LOX immobilized by entrapment in polyacrylamide gel beads was 22% higher than that of the free enzyme after 10 days of storage at 4 °C, and 32% higher

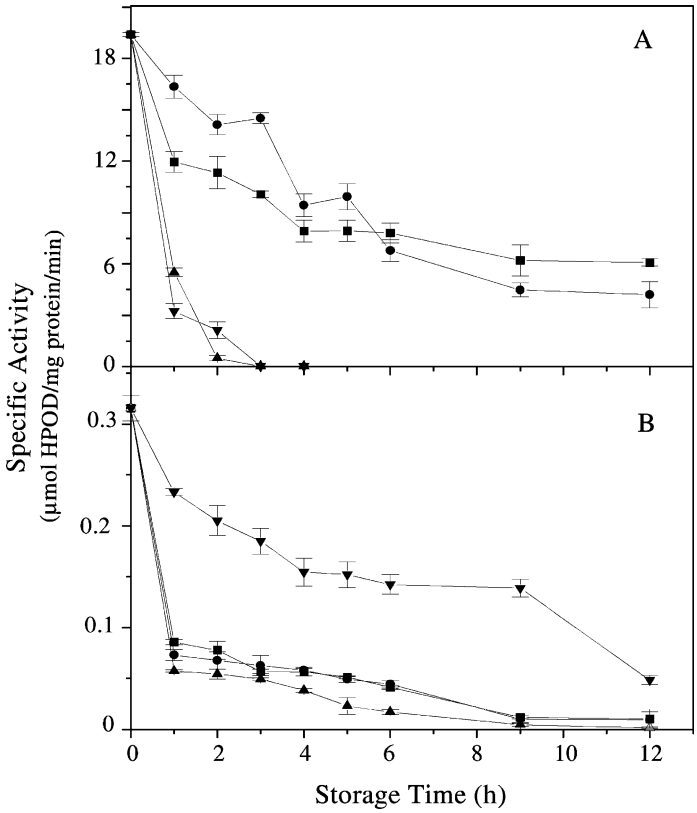

Fig. 3. Effect of selected temperatures 4 °C (■), 25 °C (●), 50 °C (▲) and 70 °C (▼) on the storage stabilities of the enzyme activity of free (A) and immobilized (B) LOXs in the reaction medium composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40.

after 45 days. This behavior may be explained by the occurrence of a stabilization induced by the interactions between the enzyme and the support, which protect the enzyme against a possible alteration of the three-dimensional structure of the protein.

Hsu et al. [22] reported that LOX immobilized by entrapment in calcium alginate beads showed no decrease in its activity after 25 days of storage at room temperature, whereas the activity of the free enzyme completely decreased in less than one day. Parra-Diaz et al. [19] investigated the stability of LOX immobilized by covalent binding to a carbonyl diimidazole-activated support and demonstrated that the stability of the immobilized enzyme at 15 °C was approximately 10-fold greater than that of the unbound LOX. Chikere et al. [38] reported that enzyme immobilization in buffers of high ionic strength is known to increase yield in bound activity since the protein molecules are more compact and therefore less susceptible to pH changes.

Fernandez-Lafuente et al. [36] reported that after multi-point immobilization of the enzyme, an enhanced stability of esterase activity was exhibited during storage with respect to temperature and exposure to potential denaturants, thereby suggesting that immobilization enhanced the overall conformational rigidity of the enzyme structure.

Although a correlation between enzyme stability and immobilization has not been established, it has been reported that the covalent binding and entrapment of enzymes usually lead to the improvement of their stability [42].

3.7. Effect of recycling on the activity of immobilized LOX

The effect of recycling on the activity of the immobilized LOX was investigated in the ternary micellar system consisting of sodium carbonate buffer solution (0.1 M, pH 9.5) and 4% *iso*-octane containing 10 μM Tween 40. The results (Fig. 4) show that after the first cycle, the immobilized LOX still retained 60% of its initial activity, while after the second cycle, 25% of the activity of the immobilized LOX was still present. The experimental findings also indicate that the relative activity of the immobilized LOX continued to decrease steadily reaching a minimum of 10% after four cycles.

Parra-Diaz et al. [19] investigated the reusability of LOX immobilized by covalent binding to a carbonyl diimidazole-activated support; in aqueous buffer, the immobilized LOX retained its activity after seven cycles, whereas in the biphasic octane/buffer (65:35, v/v) medium, the activity of the immobilized preparation decreased to 60% of its original activity after seven cycles. Hsu et al. [22] reported that LOX immobilized in calcium alginate beads could be reused at least five times without substantial loss of activity.

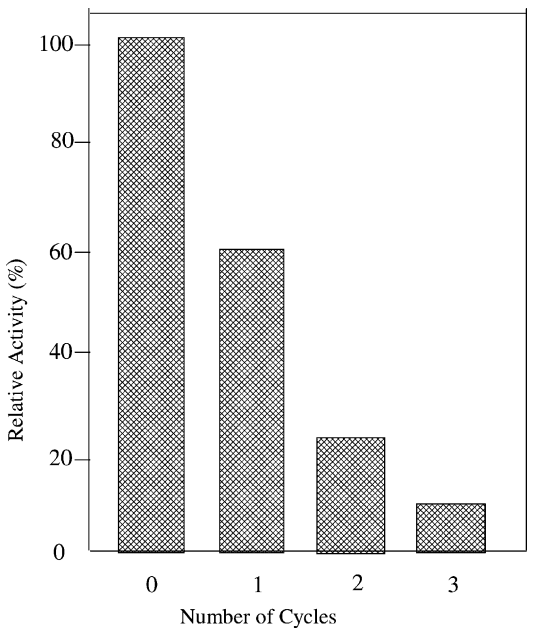

Fig. 4. Effect of recycling on the relative enzyme activity of immobilized LOX.

Siedow [43] stated that the free LOX is typically inactivated by HPOD accumulation and the partial anaerobic conditions that develop in reaction mixtures, thereby suggesting that immobilization can protect the enzyme against these factors.

Bickerstaff [44] also reported that immobilization restricts the movement of the backbone and side chains of the enzyme molecule thereby preventing intermolecular interactions and unfolding of the polypeptide chain; these advantages may result in an increase in the stability of the enzyme and retention of its stability after repeated use.

3.8. Substrate specificity

The specificity of the LOX activity of the immobilized enzyme (Table 4) was investigated in the ternary micellar system consisting of sodium carbonate buffer solution (0.1 M, pH 9.5) and 4% *iso*-octane containing 10 μM Tween 40. A wide range of substrates, including polyunsaturated fatty acids and polyunsaturated fatty acid acylglycerols were studied. The results show that the immobilized LOX demonstrated a 3.05, 2.67 and 1.57-fold increase in relative activity towards linolenic acid, arachidonic acid and dilinolein as substrates, respectively, compared to that obtained

Table 4
Substrate specificity of the immobilized lipoxygenase

Substrate	Specific activity[a]	Relative specific activity (%)[b]
Linoleic acid	0.42 (±0.14)[c]	100.0 (0.20)[d]
Linolenic acid	1.28 (±0.33)[c]	304.7 (1.09)[d]
Arachidonic acid	1.12 (±0.17)[c]	266.7 (0.59)[d]
Monolinolein	0.02 (±0.09)[c]	5.7 (0.10)[d]
Dilinolein	0.66 (±0.02)[c]	157.1 (0.22)[d]
Trilinolein	0.08 (±0.03)[c]	19.5 (0.03)[d]

[a] Specific activity was defined as micromoles of hydroperoxides (HPOD) of linoleic acid per milligrams of protein per minute, determined in a ternary micellar system composed of a mixture (96:4, v/v) of sodium carbonate buffer solution and *iso*-octane containing 10 μM Tween 40.

[b] The relative activity was defined as the percentage of specific activity compared to that obtained with linoleic acid.

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

[d] Standard deviation was calculated from the percent relative standard deviation values of the specific activity with linoleic acid (R.S.D.1) and the specific activity with another substrate (R.S.D.2) according to the equation $[(\text{R.S.D.1})^2 + (\text{R.S.D.2})^2]^{1/2} \times$ relative specific activity [34].

Table 5
The HPLC analyses of hydroperoxide isomers[a] (%) produced by the free and immobilized LOXs in selected reaction media, using various substrates

	Linoleic acid		Linolenic acid		Arachidonic acid	
	13	9	13	9	13	9
Free LOX						
Ternary system[b]	51.6	48.4	59.4	40.6	53.0	47.0
Immobilized LOX						
Ternary system[c]	49.5	50.5	59.8	39.2	78.7	21.3

[a] The relative percent peak area was defined as the peak area of the hydroperoxide (HPOD) of the respective fatty acid isomer divided by the sum of the total HPOD isomers, multiplied by 100.
[b] The ternary micellar system for the free lipoxygenase was composed of a mixture (96:4, v/v) of Tris–HCl buffer solution (0.1 M, pH 9.0) and *iso*-octane containing 10 μM Tween 40.
[c] The ternary micellar system for the immobilized lipoxygenase was composed of a mixture (96:4, v/v) of sodium carbonate buffer solution (0.1 M, pH 9.5) and *iso*-octane containing 10 μM Tween 40.

with linoleic acid. The results also indicate that a dramatic decrease (79.5–94.3%) in relative activity was observed with the use of monolinolein and trilinolein, as substrates.

Hsu et al. [23] reported that the enzyme activity of LOX immobilized by cross-linking to phyllosilicates showed a substrate preference towards 1,3-dilinolein as opposed to linoleic acid in the oxidation reaction.

3.9. Characterization of end-products

LOX biocatalysis was investigated using linoleic acid, linolenic acid and arachidonic acid in the selected organic solvent reaction media, including monophasic, biphasic and ternary micellar systems. Table 5 shows the relative quantitative production of HPODs catalyzed by the LOX activities of the free and immobilized enzyme preparations in selected organic systems. The results indicate that both the 9- and 13-HPODs were produced in different proportions by the activities of both free and immobilized LOXs.

Using linoleic acid as substrate, the free LOX catalyzed the production of 13- and 9-HPODs at a proportion of 2:1 in the aqueous medium, while a proportion of 1:1 was obtained in the ternary micellar system.

Using linolenic acid as a substrate, the free enzyme produced predominantly the 13-HPOD isomer (86.7%) and a small portion of the 9-HPOD isomer (13.3%). In the ternary micellar systems, LOX activities of the free and immobilized enzyme preparations produced a slightly higher (59%) proportion of the 13-HPOD than the 9-HPOD isomer (40%) from linolenic acid.

The experimental findings also show that the production of the 9- and 13-HPOD isomers from arachidonic acid as substrate was at an approximate ratio of 1:1 for the free enzyme in both aqueous and ternary micellar systems, while the immobilized LOX predominantly produced the 13-HPOD isomer (78.7%) as opposed to the 9-HPOD isomer (21.3%) in the ternary micellar system.

These findings suggest that the environment used as a reaction medium for LOX biocatalysis could affect the type of HPODs produced as indicated by the different proportions of HPODs generated in the aqueous and ternary micellar reaction media. In addition, immobilization of the enzyme could also influence the ratio of HPOD isomers as demonstrated by the biocatalysis using arachidonic acid as substrate in the ternary micellar systems.

Piazza et al. [9] reported that LOX immobilized by covalent binding to a carbonyl diimidazole-activated support produced a 96.7% majority of the 13-HPOD isomer in a hexane/buffer medium. Pinto and Macias [21] found that the activity of LOX immobilized by entrapment in polyacrylamide gel beads produced the 9- and 13-HPOD isomers at a ratio of 96:4 in aqueous medium.

Clark [16] reported that modification of enzyme activity upon binding to a support could result due to structural alterations induced by immobilization, as the catalytic properties of an enzyme are crucially dependent upon the three-dimensional conformation of the protein and the precise functional alignment between the substrate and the active site of the enzyme. The attachment of an enzyme to a surface, either covalently or noncovalently, can therefore change the native configuration of the protein and thus influence enzyme activity.

4. Conclusion

The experimental data obtained in this study showed that although LOX exhibited a lower activity when

immobilized on an ion-exchange support, the immobilized LOX also displayed a higher stability at high temperatures than its free counterpart and could be recycled. Moreover, the substrate specificity and the end-product specificity of the immobilized LOX were altered by the immobilization method used.

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