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Stability of Immobilized Soybean Lipoxygenase in Selected Organic Solvent Media

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Abstract

The immobilization and biocatalysis of commercially purified soybean lipoxygenase (LOX) type I-B (EC 1.13.11.12) were investigated in organic solvent media. The results showed that the highest immobilization efficiencies of LOX, 30.6 and 29.3%, were obtained with DEAE-cellulose and modified Eupergit® C250L supports, respectively. The biocatalysis of free and immobilized (Eupergit® C250L/EDA) LOXs was investigated in different mixtures of hexane and a selected cosolvent (95:5 [v/v]). The results showed a 1.5 and a 1.6 increase in the activity of free and immobilized LOXs, respectively, using a mixture of hexane and 1,4-dioxane compared with that in hexane alone; however, cosolvents, including 2-octanone, 2-heptanone, 2-butanone, and cyclohexanone, displayed an inhibitory effect on LOX activity. In the mixture of 1,4-dioxane and hexane, LOX activity was dependent on the cosolvent concentration, which was increased with 1,4-dioxane up to 5% (v/v). The threshold 1,4-dioxane concentration (C_{50}) and the incubation period (T_{50}) at which 50% of the maximal enzyme activity was obtained for the free and immobilized LOXs were 6.7 and 8.9% (v/v) and 9.1 and 17.0 min, respectively.

Index Entries: Soybean lipoxygenase; immobilization; biocatalysis; organic solvent media; stability.

Introduction

Lipoxygenases (LOXs) (EC 1.13.11.12) are ubiquitously found in various animal tissues *(1)*, plant cells *(2)*, and microorganisms *(3)*. LOXs are an important class of nonheme iron enzymes that catalyze the specific dioxidation of polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene

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moiety to hydroperoxy fatty acids; these hydroperoxides are regarded as flavor precursors because they can be subsequently cleaved by hydroperoxide lyase and associated enzymes to flavor compounds such as ketones, aldehydes, and alcohols *(4)*. The production of aroma chemical has been recognized as one of the relevant applications of LOX *(5,6)*. In addition, hydroperoxide compounds have been considered as valuable intermediates for chemical synthesis and as important precursors to a number of physiologic effectors such as leukotrienes and lipoxins *(2,4)*.

Biocatalysis of LOX in organic solvent media (OSM) may decrease the possibility of rate-limiting depletion of oxygen cosubstrate, because oxygen is greater than 10 times more soluble in organic solvent than in water *(7)*. Furthermore, the biocatalysis of enzymes in nonaqueous media may provide a better solvation of the hydrophobic substrate, an easier recovery of the products and of the insoluble biocatalyst, and a higher thermostability of the active enzyme *(8)*. Over the past few years, the optimization of LOX biocatalysis in various nonconventional media, such as monophasic, biphasic, and ternary micellar systems, was investigated *(9,10)*; however, the biocatalysis efficiency of LOX in these systems was limited by its poor stability *(6)*.

Enzyme immobilization has been proven to be a convenient means of improving an enzyme's performance and stability *(11)*. In addition, it offers other advantages such as easy recovery of the enzyme, allowing repeated use of the biocatalyst, and the possibility of continuous catalysis in bioreactors *(12)*. Immobilization of soybean LOX has been studied using covalent binding *(6,13)*, physical adsorption *(14)*, and gel entrapment *(15,16)*. In addition to the fact that most of the reported research was carried out in aqueous media, the literature does not provide an overall conclusion for the stability of immobilized LOX in OSM *(9,17)*.

The present work is part of ongoing research in our laboratory aimed at the development of a biotechnological process for the use of LOX and associate enzymes for the production of natural flavors *(10,17)*. The specific aim of the present study was to evaluate selected supports for the immobilization of soybean LOX used as a model system and its biocatalysis in selected OSM in terms of immobilization efficiency as well as enzyme activity and stability.

Materials and Methods

LOX, Chemicals, and Supplies

Commercial purified soybean LOX type I-B (394,100 U/mg of protein, or 47.29 μmol of oxidized linoleic acid/[mg of protein·min]) and DEAE-cellulose were purchased from Sigma (St. Louis, MO). Linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid) was purchased from Nu-Chek Prep (Elysian, MN). Silica gel support was purchased from Silicycle (Quebec City, Quebec, Canada), and AffiGel® 10 (active ester agarose) was purchased from Bio-Rad (Hercules, CA). Eupergit® C and Eupergit® C250L (oxirane acrylic

beads) were a kind gift from Rohm Pharma (Darmstadt, Germany). Dowex® 50WX4-200 (anionic ion-exchange resin), ethylenediamine (EDA) and iminodiacetic acid (IDA) additives, and Tris were obtained from Aldrich (Milwaukee, WI). All solvents used were of high-performance liquid chromatography (HPLC) grade or better and were purchased from Aldrich except for hexane, isooctane, and 1,4-dioxane, which were purchased from Fisher (Fair Lawn, NJ).

Preparation of Modified Epoxy Supports

Different epoxy supports were prepared according to a modification of the method reported by Mateo et al. *(18).* One gram of wet Eupergit C or Eupergit C250L support was suspended in 10 mL of EDA aqueous solution (5% [v/v]) or in 5 mL of a 1.8 *M* IDA suspension at room temperature under continuous stirring. The modified epoxy/EDA and epoxy/IDA supports were washed with an excess of deionized water after 15 min and 5 h of stirring, respectively.

Determination of Protein

Protein concentration for free and immobilized LOXs as well as other fractions was determined according to a modification of the Lowry method *(19).* Bovine serum albumin (Sigma) was used as a standard for the calibration curve.

Immobilization of LOX

Prior to immobilization, the enzyme was dialyzed in order to remove the stabilizer and salts from the commercial preparation. The investigated supports, including DEAE-cellulose and modified Eupergit, were washed twice with Tris-HCl buffer (0.1 *M*, pH 9.0). Immobilization of LOX was carried out by adding 1 mL of the enzyme suspension prepared in Tris-HCl buffer (0.1 *M*, pH 9.0) to the wet support to obtain final loadings ranging from 10 to 100 mg of protein/g of wet support. Enzyme-support mixtures were stirred at 4°C until protein equilibrium was reached, which corresponded to incubation times of 2, 0.5, 3, 0.75, and 16 h for AffiGel 10, DEAE-cellulose, Dowex 50W, silica gel, and Eupergit supports, respectively. The supports, containing the adsorbed enzyme, were recovered by centrifugation (2000*g*, 2 min), washed with 1 mL of buffer to remove unbound proteins, and stored wet at 4°C. To evaluate the immobilization yield and efficiency, the supernatants and wash solutions were subjected to determination of protein content and LOX assay.

The immobilized protein yield (IPY) and the immobilized LOX yield (ILY) were calculated from the immobilized protein and immobilized LOX activity, respectively, divided by total initial protein and total LOX activity before immobilization, respectively, multiplied by 100. The selectivity factor was defined as the specific activity of the free enzyme over that of the supernatant recovered after immobilization. Immobilization efficiency was

calculated by dividing the specific activity of the immobilized LOX over that of the free one, and multiplied by 100.

LOX Assay in Aqueous Medium

LOX activity was measured, in triplicates, according to the modified procedure of Perraud and Kermasha *(3)*. Prior to each enzymatic reaction, a stock solution of linoleic acid (4 m*M*) was freshly prepared in Tris-HCl buffer as described previously *(20)*.

For the free LOX, enzyme suspensions (0.05–0.10 mg of protein/mL) were prepared in Tris-HCl buffer solution. The reaction medium contained 150 µL of the substrate stock solution, which was adjusted to 980 µL with the Tris-HCl buffer solution, and preincubated at 25°C. The reaction was initiated by adding 20 µL of the enzymatic suspension (1 to 2 µg of protein/mL of reaction). The absorbance was monitored continuously over a period of 3 min. LOX activity was calculated from the slope of the linear portion of the plot of absorbance vs time of hydroperoxides of linoleic acid (HPODs) products and expressed in micromoles of conjugated diene hydroperoxides/(milligram of protein·minute).

On the other hand, for the immobilized enzyme, LOX assay was performed as for the free one with certain modifications. The reaction medium contained 150 mL of substrate stock solution and 550–750 µL of the Tris-HCl buffer and was preincubated to 25°C. The enzymatic reaction was initiated by adding 100–300 µL of enzyme suspension containing 0.07–2.00 mg of wet immobilized LOX preparation (0.1–7.3 µg of protein). The reaction mixture was stirred for 5 min and centrifuged (2000*g*, 2 min) to halt the reaction and to remove the enzyme. Supernatant absorbance was measured at 234 nm. Immobilized LOX activity was calculated from the slope of the plot of hydroperoxide concentration vs the amounts of solid immobilized enzyme and expressed as micromoles of conjugated diene hydroperoxides/(milligram of immobilized enzyme·minute).

The absorbance of the reaction mixtures was measured spectrophotometrically at 234 nm *(21)*, using a Beckman DU-650 spectrophotometer (San Ramon, CA). The hydroperoxide product concentration was calculated from the molar extinction coefficient of 25,000 M^{-1} cm^{-1} for HPODs *(22)*. Control reactions without LOX were carried out in tandem with the assays.

LOX Assay in OSM

LOX biocatalysis in OSM was carried out, in triplicate, according to a modification of the method of Kermasha et al. *(17)*. Prior to the enzymatic assay in OSM, a stock solution of 70 m*M* linoleic acid was freshly prepared in hexane.

For the free enzyme, LOX suspensions (2.5–15 mg of protein/mL) were prepared in Tris-HCl buffer solution. Enzymatic reaction was initiated by adding 20 µL of the enzyme suspension (0.05–0.3 mg of protein) to

100 µL of substrate stock solution and 0.88 mL of hexane. For the immobilized LOX assay, the enzymatic reaction was initiated by adding 100 µL of substrate stock solution to the immobilized LOX (12.5–50 mg of wet support containing 34–134 µg of protein) suspended in 0.88 mL of hexane and 20 µL of the Tris-HCl buffer.

Control reactions without LOX were carried out in tandem with the enzymatic reactions under the same conditions. The reaction mixture was incubated at 25°C under gentle agitation for 30 min. The free and immobilized LOXs were then removed by centrifugation (2000*g*, 2 min), and the supernatant was subjected to HPLC analysis. LOX activity in OSM was calculated from the slope of the oxidized linoleic acid vs enzyme concentration.

HPLC Analysis

Quantification of residual linoleic acid was determined by HPLC analysis according to a modification of the method described by Perraud and Kermasha *(3)* using an Alphabond silica normal-phase column (300 × 3.9 µm, 5 mm; Alltech, Deerfield, IL) with a Beckman Gold HPLC system (Model 126) coupled to a laser light-scattering detector (LLSD) (Varex, Burtonsville, MD) and fitted with a computerized data-handling integrated delivery system. LLSD detection was performed at 75°C with a nitrogen flow rate of 40 mL/min. Injection was carried out with an automatic injector (Beckman Gold autosampler 507) fitted with a 50-µL loop. Elution was conducted with an isocratic mobile phase consisting of hexane:2-propanol: acetic acid (993:7:1 [v/v/v]) at a flow rate of 1 mL/min. Calibration curves were constructed using different concentrations of linoleic acid standards.

Effect of Cosolvent on LOX Activity in Organic Solvent Mixtures

A comparative study of the effect of selected cosolvents in hexane on the activity of free and immobilized LOXs was performed. The cosolvents were 2-butanone, cyclohexanone, 1,4-dioxane, 2-heptanone, and 2-octanone, with Log *P* values of 0.29, 0.96, –1.10, 1.80, and 2.40, respectively. LOX activity was assayed using the standard conditions described previously for the enzymatic assay in OSM.

Effect of 1,4-Dioxane Concentration on Activity of LOX in Organic Solvent Mixtures

The effect of 1,4-dioxane cosolvent concentration on the activity of free and immobilized LOXs was investigated by varying its proportion from 0 to 10% in hexane medium. LOX activity was assayed using the standard conditions described previously for the enzymatic assay in OSM. A quantitative determination of LOX inactivation by 1,4-dioxane was obtained by calculating the first-order inactivation constant, k_c, and the concentration of cosolvent required to decrease the LOX activity by 50%, C_{50}, using the following linear equation:

$$\ln(A/A_0) = -k_c C \quad (1)$$

in which, C is the concentration of 1,4-dioxane in hexane medium; and A and A_0 are the LOX activity at C concentration of 1,4-dioxane and without 1,4-dioxane, respectively.

Thermal Stability of LOX in OSM

The thermal stability of LOX activity was investigated by incubating 20 µL of free and immobilized LOX suspensions with 0.88 mL of a 1,4-dioxane/hexane mixture (5:95 [v/v]) for a wide range of incubation times (5–40 min) at 40°C. The residual LOX activity was then measured according to the standard assay conditions. The inactivation constant, k_t, and the half-life, T_{50}, of the free and immobilized LOXs were determined from the semilogarithmic plots of the inactivation kinetics according to the following equation:

$$\ln(A/A_0) = -k_t T \quad (2)$$

in which, T is the incubation time at 40°C; and A and A_0 are the LOX activity with a defined incubation time T and without incubation, respectively.

Kinetics Parameters of LOX in OSM

The effect of substrate concentration on the specific activity of free and immobilized LOXs in OSM was investigated using a wide range of linoleic acid concentrations (0–14 m*M*).

Results and Discussion

Immobilization of LOX

Commercially purified soybean LOX was immobilized by physical adsorption and covalent binding on different types of supports. AffiGel 10, Eupergit C, and Eupergit C250L as well as the modified epoxy supports were used for their covalent chemical bonding ability, whereas DEAE-cellulose, Dowex 50W, and silica gel were used for their ionic and hydrophobic adsorption. The results (Table 1) show that the IPY and ILY were strongly dependent on the nature of the support. The immobilization of LOX on silica gel resulted in the lowest IPY (11.9%), and the lowest ILY (13.6%) was obtained with the use of Eupergit C/EDA. The highest IPY (57.5%) was obtained when Eupergit C250L was used as support, and the highest ILY (69%) was obtained with AffiGel 10. The experimental findings also indicate that the immobilization of LOX on the investigated supports resulted in a lower ILY than IPY except for AffiGel 10, Eupergit C/IDA, and silica gel; the latter supports showed a capacity to immobilize the LOX preferentially compared to the other proteins present in the enzymatic preparation. The differences between the IPY and ILY values may be owing to differences in the adsorption selectivity onto the support between the soybean LOX and the other proteins.

The selectivity factor can be used as a measure of the adsorption selectivity *(18)*. Among all the investigated supports, AffiGel 10, Eupergit C/IDA,

Table 1
Soybean LOX Immobilization Parameters
Using Selected Covalent and Adsorption Supports

Support	IPY (%)[a]	ILY (%)[b]	Selectivity factor[c]	Immobilization efficiency (%)[d]
AffiGel 10	51.0	69.0	1.66	4.26
DEAE-cellulose	46.0	43.9	0.87	30.6
Dowex 50W	34.1	21.7	0.80	0.64
Silica gel	11.9	31.7	1.13	1.14
Eupergit C	40.2	20.6	0.69	5.24
Eupergit C/IDA	25.0	39.6	1.20	9.86
Eupergit C250L	57.5	33.1	0.59	5.19
Eupergit C250L/EDA	33.8	13.6	0.80	29.3
Eupergit C250L/IDA	49.6	33.5	0.77	6.39

[a]IPY was determined as the relative percentage of immobilized protein to that of the total initial protein. An enzyme-to-support ratio of 1:100 was used.

[b]ILY was calculated as the relative percentage of the total activity of the immobilized enzyme to that of the free enzyme treated under the same conditions.

[c]The selectivity factor was defined as the ratio of the specific activity of the free enzyme to that of the supernatant, obtained by filtration and centrifugation after immobilization.

[d]The immobilization efficiency was defined as the relative percentage of specific activity of the immobilized enzyme compared with that of the free one.

and silica gel showed a selectivity factor >1.0 (Table 1), which confirmed the higher affinity of soybean LOX for those supports compared to the other proteins. Similarly, the differences in affinity and/or selectivity might be related to different supports *(23)*. From the values of the selectivity factor, it can also be seen that the pretreatment of the epoxy supports with EDA and IDA resulted in an increase in the adsorption selectivity of LOX onto those supports. These results are in agreement with those reported by Mateo et al. *(18)*, who showed that the selectivity of adsorption could be increased when the concentration of the chelate groups, such as the epoxy groups, was low.

Although AffiGel 10, Eupergit C/IDA, and silica gel showed a preferential capacity for the immobilization of LOX compared to other proteins, these supports did not yield high immobilization efficiency (Table 1). The highest immobilization efficiency was obtained with Eupergit C250L/EDA (29.3%) and DEAE-cellulose (30.6%). Petrus Cuperus et al. *(14)* investigated LOX stability in aqueous medium by its immobilization on DEAE-cellulose. In addition, epoxy supports were used previously for the immobilization of soybean *(6,13)* and potato LOXs *(24)* as well as for other enzymes such as acylases *(11,18)*. Our results (Table 1) also indicate that the immobilization of LOX by adsorption on silica gel and Dowex 50W altered greatly the enzyme activity, with an immobilization efficiency of 1.14 and 0.64%, respectively; the low recovered LOX activity on immobilization may be

attributed to the changes in enzyme conformation, steric hindrance at the active sites, and substrate diffusion limitations *(23)*.

The overall results show that the most appropriate supports for immobilization in terms of LOX residual activity were Eupergit C250L/EDA and DEAE-cellulose. However, experimental work (data not shown) indicated that the effect of support on the partitioning of the substrate was more important with DEAE-cellulose than with Eupergit C250L/EDA. On the basis of these findings, Eupergit C250L/EDA was used for the immobilization of LOX throughout this study.

Effect of Cosolvent on LOX Activity

One of the limitations of LOX biocatalysis in OSM is the insolubility of hydrophobic fatty acids and the relatively more hydrophilic hydroperoxide end products in the same reaction medium. In addition, Siedow *(25)* reported that the accumulation of hydroperoxides in the microenvironment of the enzyme has an inhibitory effect on LOX activity. To overcome the solubility restriction, the effect of the presence of selected cosolvents in hexane medium (5:95 [v/v]) on LOX activity was investigated (Table 2). Cosolvents were chosen on the basis of their capacity to solubilize hydroperoxides and their miscibility with hexane.

Using Eupergit C250L/EDA as support, the specific activity of free and immobilized LOXs in hexane medium was found to be 0.20 and 0.36 μmol/(mg of protein·min), respectively, whereas in aqueous buffer medium it was 18.02 and 5.279 μmol/(mg of protein·min), respectively (Table 2). These experimental findings indicate that the ratio of activity (ROA) of LOX in organic solvent to that in aqueous medium was 0.01 and 0.05, respectively, for the free and immobilized LOXs. Pencreac'h and Baratti *(26)* reported a wide range of ROA values for a wide range of commercial lipase preparations, with most of them <1.0. The experimental results (Table 2) also indicate a similar ROA for LOX, with values higher for the immobilized enzyme compared with the free one; these findings could be explained by an underestimation of the activity of the immobilized enzyme in aqueous medium, as a result of the diffusion limitations of the hydrophobic substrate *(26)*.

Table 2 also shows that the addition of most cosolvents, including butanone, cyclohexanone, heptanone, and octanone, to the hexane medium resulted in a decrease in the specific activity of LOX, which could indicate their denaturating effect on enzyme activity *(27)*. However, the denaturating effect of cosolvents was less pronounced with the immobilized LOX than with the free one, which may be owing to the multipoint covalent attachment *(11)* of the immobilized enzyme onto the support, leading to lesser conformational changes. On the other hand, the results (Table 2) demonstrate that the addition of 1,4-dioxane as cosolvent increased the specific activity of soybean LOX by 1.6-fold. These findings suggest that the presence of 1,4-dioxane may have decreased the end-product partitioning effect

Table 2
Activity of Free and Immobilized LOX
in Different Mixtures of Cosolvent and Hexane

Organic cosolvent[a]	Specific activity[b]		Log *P*[d]
	Free LOX	Immobilized LOX[c]	
Aqueous medium[e]	18.02 (±2.59)[f]	5.279 (±0.506)[f]	—
Hexane only	0.204 (±0.028)[f]	0.363 (±0.043)[f]	3.50
Octanone	0.121 (±0.015)[f]	0.210 (±0.010)[f]	3.45
Heptanone	0.090 (±0.010)[f]	0.213 (±0.016)[f]	3.41
Butanone	0.031 (±0.005)[f]	0.260 (±0.025)[f]	3.25
Dioxane	0.304 (±0.041)[f]	0.577 (±0.084)[f]	3.12
Cyclohexanone	—[g]	0.037 (±0.460)[f]	3.33

[a]The organic phase consisted of a mixture of the defined cosolvent with hexane (5:95 [v/v]); the reaction mixture was composed of the organic phase and a limited amount of Tris-HCl buffer (0.1 *M*, pH 9.0) at 98:2 (v/v).

[b]Specific activity is expressed as micromoles of HPODs/(milligram of protein·minute).

[c]Enzyme was immobilized on Eupergit C250L (Rohn Pharma) support treated with EDA with an enzyme-to-support ratio of 1:10.

[d]Log *P* of the mixtures was calculated according to the empirical formula log *P* mixture = $X_1 \log P_1 + X_2 \log P_2$, in which X_1 and X_2 are the mole fractions of solvents 1 and 2 *(36)*.

[e]The aqueous medium was composed of Tris-HCl buffer (0.1 *M*, pH 9.0).

[f]Standard deviation of triplicate trials.

[g]Absence of activity for this sample.

by accelerating its removal rate from the microenvironment of the enzyme, which is a limiting step in the conversion of linoleic acid into its HPODs. The presence of 1,4-dioxane in the reaction medium may prevent the deactivation of LOX by the HPOD product *(25)*. Although the Log *P* values of the solvent mixtures are close, LOX showed variable specific activity. Higher enzyme activity is usually expected with a concomitant increase in solvent hydrophobicity, which is owing to a decrease in the solvent's ability to strip the water layer from the surface of the enzyme *(27)*. In addition, the molecules of the more polar solvents are more likely to be present in the microenvironment of the enzyme *(28)*. Bell et al. *(8)* reported that in addition to the physical properties of the solvent, their molecular structure played an important role in enzyme activity. Enzyme-solvent interaction may alter the active conformation of the enzyme by a direct molecular binding of the solvent onto or near the active site of the enzyme, hence resulting in a denaturating effect *(27)*.

Effect of Concentration of 1,4-Dioxane Cosolvent on LOX Activity

Figure 1 shows the effect of the proportion of 1,4-dioxane in hexane medium on LOX activity. The results indicate that the specific activity of free and immobilized LOXs was increased to its maxima of 0.304 and 0.577 µmol of oxidized linoleic acid/(mg of protein·min), respectively, with a concomitant increase in 1,4-dioxane concentration up to 5% (v/v).

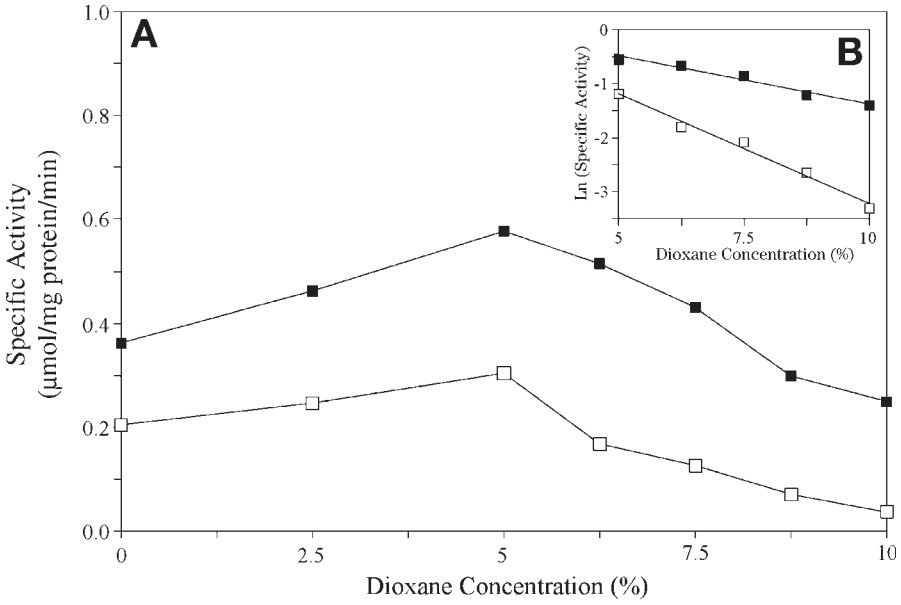

Fig. 1. Effect of 1,4-dioxane cosolvent on activity of (□) free and (■) immobilized soybean LOX enzyme in hexane medium: **(A)** specific activity measurements; **(B)** first-order kinetics behavior of deactivation effect of cosolvent concentration.

The increase in LOX activity could be explained by an increase in the hydroperoxide end-product solvation, which may decrease the microenvironmental effect and hence their inhibition role *(25)*. However, a further increase in the proportion of 1,4-dioxane to 10% resulted in an 88 and a 57% decrease in the specific activity of free and immobilized LOXs, respectively; this decrease in enzyme activity may be owing to changes in the conformation of LOX by stripping off the essential water layer surrounding the enzyme molecule *(28)*. The presence of specific interactions between cosolvent molecules and the enzyme may also account for the low specific activity in higher proportions of 1,4-dioxane *(29)*.

Figure 1B shows a straight line with a correlation coefficient of 0.99 and 0.97 for the free and immobilized LOXs, respectively, indicating hence that enzyme inactivation by 1,4-dioxane followed first-order kinetics behavior. First-order kinetics of enzyme inactivation by cosolvents in monophasic aqueous mixtures has been reported for aminotransferase *(30,31)* and acylase *(29)*.

A quantitative determination of LOX inactivation by the cosolvent 1,4-dioxane was obtained by calculating the inhibition factor (C_{50}). C_{50} (Table 3) was estimated to be 6.7 and 8.9% for the free and immobilized LOX, respectively; these findings suggest that 1,4-dioxane may display a higher denaturating effect on the free enzyme than on the immobilized one. The low denaturating effect of 1,4-dioxane on immobilized LOX may be

Table 3
Inactivation Constants and Half-Life Parameters
for Free and Immobilized LOX in Dioxane:Hexane Mixtures

Parameter	Free LOX	Immobilized LOX[a]
Cosolvent inactivation[b]		
k_c (%$^{-1}$)[c]	0.4064	0.1786
C_{50} (%)[d]	6.7	8.9
Thermal inactivation[e]		
k_t (min^{-1})[f]	0.0759	0.0408
T_{50} (min)[g]	9.1	17.0

[a]Enzyme was immobilized on Eupergit C250L (Rohn Pharma) support treated with EDA, with an enzyme-to-support ratio of 1:10.

[b]Parameters were obtained from the linear equation $\ln(A/A_0) = -k_cC$, in which A is the activity at the concentration of dioxane, C; A_0 is the activity at the optimal 5% dioxane concentration; and k_c is the constant of inactivation of the dioxane effect.

[c]Constant of inactivation as determined from the first-order kinetics behavior of the inactivation effect of increasing the concentration of 1,4-dioxane cosolvent.

[d]Defined as the concentration of solvent required for half of the initial activity.

[e]Parameters were obtained from the linear equation $\ln(A/A_0) = -k_tT$, in which A is the activity at incubation time, T; A_0 is the activity without deactivating incubation period; and kt is the constant of inactivation of the incubation at 40°C.

[f]Constant of inactivation as determined from the first-order kinetics behavior of the inactivation effect of increasing the incubation time at 40°C.

[g]Defined as the incubation time at 40°C in 5:95 (v/v) dioxane:hexane required to report a 50% decrease in the initial activity.

explained by multipoint covalent attachment of the immobilized LOX on the support, which may have led to fewer conformational changes *(11)*.

Thermal Stability of LOX in OSM

The thermal stability of LOX in the 5:95 (v/v) 1,4-dioxane/hexane mixture was investigated at 40°C. The results (data not shown) indicated that after 40 min of incubation, the free and immobilized LOXs retained only 4.4 and 28.5% of their initial activity, respectively. From the semilogarithmic plots, the inactivation constant and half-life of free and immobilized LOXs were estimated (Table 3). The half-life (T_{50}) of LOX increased by a factor of 2 upon immobilization on Eupergit C250L/EDA. The results (Fig. 2) also indicate that the thermal inactivation of both enzyme preparations followed first-order kinetics behavior, as indicated by the linearity of the semilogarithmic plots. Similarly, Chikere et al. *(6)* reported that soybean LOX type I-B immobilized on epoxy supports in borate buffer (0.2 *M*, pH 9.0) showed first-order kinetics. By contrast, the kinetic inactivation of LOXs from tomato *(32)*, beans *(33)*, and potatoes *(34)* did not follow first-order kinetics. Park et al. *(34)* demonstrated that the non-first-order kinetics behavior of a crude enzyme preparation was owing mainly to the presence of more than one LOX species with different thermal stabilities.

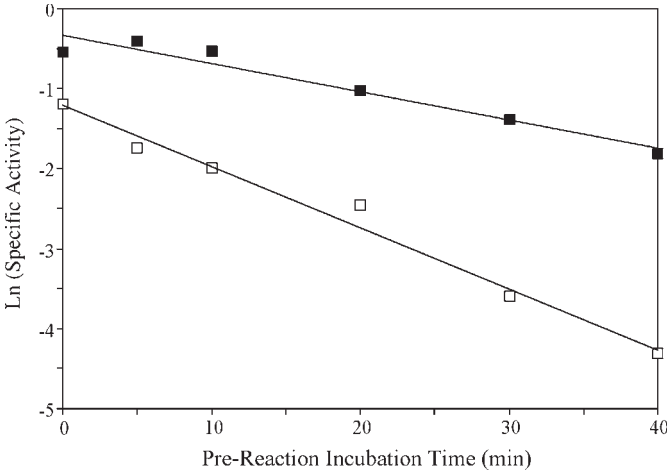

Fig. 2. Thermal inactivation of (□) free and (■) immobilized soybean LOX in reaction medium mixture of 5:95 (v/v) 1,4-dioxane/hexane.

Kinetics Parameters of LOX in OSM

To compare the catalytic efficiency of free and immobilized LOXs, their kinetic parameters were determined in a 1,4-dioxane/hexane mixture. The Lineweaver-Burk plots of $1/v$ vs $1/[S]$ (data not shown) displayed a linear relationship with a correlation coefficient of 0.97 and 0.98, respectively, for the free and immobilized LOXs, which indicates a Michaelis-Menten kinetics behavior.

The kinetic parameters K_m and V_{max} for the free and immobilized LOXs (Table 4) were estimated from the Lineweaver-Burk plots. The results indicate that the K_m values were determined as 14.4 and 36.0 m*M* for the free and immobilized LOXs, respectively. The immobilization of LOX onto Eupergit C250L/EDA increased the K_m value by approx 2.5. Bindhu and Abraham *(35)* has attributed the increase in K_m values for peroxidase to the increased hydrophobicity of the enzyme, which may have altered the interaction with the substrate. In addition, the decrease in enzyme affinity for the substrate may be owing to the immobilization process, which tends to cause conformational changes, steric hindrance, and substrate diffusion limitations *(23).* However, Kermasha et al. *(17)* reported lower K_m for LOX after its immobilization, with values as low as 0.06 m*M* in octane medium.

Our results (Table 4) show that the V_{max} values for the free and immobilized LOXs were, respectively, 5.84 and 4.94 μmol of HPODs/(mL·min). Kermasha et al. *(17)* obtained a relatively lower V_{max} value (2.37 μmol of HPODs/[mL·min]) for the immobilized soybean LOX on Dowex 50W in isooctane; however, they reported a much higher V_{max} (29.68 μmol of HPODs/[mL·min]) for the free enzyme. The discrepancy in V_{max} values of the free LOX between the present study and those reported by Kermasha et al. *(17)* may be explained by the apparent character of V_{max}. To compare

Table 4
Kinetics Parameters of LOX Activity in 5% 1,4-Dioxane in Hexane (v/v)

Parameter	Free LOX	Immobilized LOX[a]
K_m	14.40	36.00
V_{max}	5.84	4.94
Catalytic efficiency[b]	0.41	0.14

[a]Enzyme was immobilized on Eupergit C250L (Rohn Pharma) treated with EDA. The free LOX was treated under the same conditions as the immobilized LOX without support.

[b]Catalytic efficiency was defined as the ratio of V_{max} to K_m.

the kinetics data for both free and immobilized LOXs, the present study treated the free enzyme under the same conditions as for the immobilized one, which implied continuous stirring for 16 h. Because the free LOX is poorly stable in aqueous medium *(6)*, the lower V_{max} value may be owing to a partial denaturation of the enzyme.

The apparent catalytic efficiency, which is defined as V_{max}/K_m, was found to be 0.41 and 0.14 min^{-1} for the free and immobilized LOXs, respectively. Although Kermasha et al. *(17)* reported a higher affinity for the immobilized soybean LOX in octane medium, our experimental findings (Table 4) indicate that the V_{max} values are higher than those reported by these investigators. The catalytic efficiency of soybean LOX in octane medium (Table 4) is thus similar to that reported by Kermasha et al. *(10,17)*.

Conclusion

The experimental data obtained throughout this study showed that the immobilization of soybean LOX increased its activity and stability in monophasic OSM compared with free LOX. In addition, the use of cosolvent in the reaction medium increased enzyme activity. Moreover, the relationship between LOX activity and both cosolvent concentration and thermal inactivation of the enzyme followed first-order kinetics.

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