

Characterization of soybean lipoxygenase immobilized in cross-linked phyllosilicates

Lipoxygenase (LOX) is an enzyme that regioselectively introduces the hydroperoxide functionality into polyunsaturated fatty acids, such as linoleic acid (LA). Hydroperoxide derivatives of polyunsaturated fatty acids are of interest because they can serve as important intermediates in the synthesis of chemical and pharmaceutical compounds. In this study, LOX was immobilized in dispersed phyllosilicate layers that were cross-linked with silicate polymers formed by the hydrolysis of tetramethyl orthosilicates. The effects of substrate concentration, reaction temperature and solvent participation were studied on the oxidation of LA by LOX. The temperature optimum for the oxidation of LA by immobilized LOX was 25 °C and values of K_m and V_{max} for this reaction were 1.7 mM and 0.023 $\mu\text{mol}/\text{min}$ respectively. Enzymic activity was stimulated by the addition of 10% (v/v) iso-octane to the reaction mixture. The immobilized LOX preparation showed a degree of substrate preference that demonstrated that 1,3-dilinolein was a better substrate than LA in the oxidation reaction, followed in order by 1-monolinolein, methyl oleate and trilinolein. In general, LOX immobilized in cross-linked phyllosilicates retained the physical and chemical characteristics of free LOX.

Introduction

Lipoxygenase (LOX) (EC 1.13.11.12) selectively oxidizes polyunsaturated fatty acids that contain a (1Z),(4Z) pentadiene structure to give a (1Z),(3Z) conjugated diene-5-monohydroperoxy derivative. Such hydroperoxy derivatives of polyunsaturated fatty acids are of interest because they can serve as useful components in pharmaceutical and chemical syntheses. For example, simple reduction gives hydroxy fatty acids (via ricinoleic acid analysis) that may have potential in a number of industrial applications such as lubricants, grease thickeners and drying oils [1,2]. Perhydroxides of arachidonic acid ($C_{18:4}$) are precursors for prostaglandin and leukotriene syntheses and perhy-

droxides of linoleic acid (LA) and linolenic acid are effective as fungicides in agriculture application [3–6].

The feasibility of commercial applications will be greatly enhanced if the enzyme can be immobilized and stabilized. Numerous methods for the immobilization of LOX are described in the literature [7–9]; however, most of these methods do not provide an immobilized LOX with a storage stability of 1 month at room temperature. Recently we used a cross-linked alkylammonium phyllosilicate for the immobilization of soybean LOX [10]. This procedure was developed to intercalate LOX into dispersed phyllosilicates, which were subsequently cross-linked with silicate polymers formed by the hydrolysis of tetramethyl orthosilicate (TMOS). Phyllosilicates are layered silicates with large surface areas. These layered structures can be broken down to nanoscale building blocks, which makes them good matrices for intercalation. The LOX–phyllosilicate composite prepared by cross-linking to phyllosilicates had an improved pore network. Alkylamines such as trimethylammonium chloride or cetyltrimethylammonium chloride (HDTMA) were used to occupy the charged sites on the phyllosilicate surfaces, to increase the hydrophobicity of the phyllosilicates, which decreases the charge–charge interactions between LOX and the phyllosilicate. LOX immobilized into phyllosilicates by intercalation displayed excellent retention of enzyme activity and improved stability at ambient temperature.

The purpose of this paper was to characterize LOX immobilized in cross-linked phyllosilicates. The behaviour of LOX intercalated into phyllosilicates was studied in terms of temperature dependence, solvent requirement, substrate specificity and dissociation behaviour. The kinetic factors K_m and V_{max} for this preparation also are reported.

Materials and methods

Materials

Soybean LOX type I-B, LA, 1-monolinolein, 1,3-dilinolein and trilinolein were obtained from Sigma (St. Louis, MO, U.S.A.). HDTMA, trimethylammonium chloride, TMOS and Xylenol Orange sodium salt were purchased from Aldrich Chemical (Milwaukee, WI, U.S.A.). The phyllosilicate clay (montmorillonite from Wyoming, U.S.A.) was obtained from Source Clay Minerals Repository (Columbia, MO, U.S.A.). All other reagents were obtained from commercial sources and were of analytical grade.

Immobilization of LOX

LOX was immobilized in phyllosilicates by the procedure reported by Shen et al. [10]. In brief, 3.3% (w/v) of phyllosilicate clay (2 ml) suspended in water (the clay was sodium-saturated by washing three times with 1 M NaCl solution) was added to 100 μ l of trimethylammonium chloride (0.5 M) or 50 μ l HDTMA (1 M) and LOX solution (2 ml, 10 mg/ml in 0.2 M sodium borate buffer, pH 9.0). Cross-linking of the LOX/phyllosilicate mixture was done by adding TMOS (10% of the total volume of incubation solution). The LOX–phyllosilicate–TMOS composite was kept at room temperature overnight to complete the polymerization and cross-linking process. After being air-dried for 24 h, the composite of LOX and cross-linked phyllosilicate was ready for use.

Assay for LOX activity

LOX activity was conducted by measuring hydroperoxide formation from LA as described previously [11]. In a typical assay, LA (5 μ mol) was dissolved in methylene chloride and placed in an Erlenmeyer flask (10 ml); the methylene chloride was evaporated under a stream of N_2 . To the dry residue was added 0.2 ml of 100 mM deoxycholate and 1.8 ml of sodium borate buffer (0.2 M, pH 9.0) and the mixture was shaken at 250 rev./min for 0.5 h. The oxidation reaction was initiated by the addition of immobilized LOX (1.5 g containing 0.3 mg of protein) at 25 °C for 2 h. The reaction mixture was quenched with 400 μ l of 1 M citric acid and the mixture was extracted twice with 2 ml of chloroform/methanol (2:1, v/v). The combined extracts were dried over anhydrous sodium sulphate, filtered and the filtrate evaporated under a stream of nitrogen. The residue was dissolved in 3 ml of ethanol and assayed for hydroperoxide content.

Hydroperoxyoctadecadienoic acid (HPOD) assay

HPOD levels were measured spectrophotometrically with the Xylenol Orange technique [12]. The Xylenol Orange

reagent was composed of 100 μ M Xylenol Orange, 250 μ M ammonium ferrous sulphate, 25 mM H_2SO_4 and 4 mM 2,6-di-(*t*-butyl)-4-methylphenol in methanol/water (90:10, v/v). The reagent (2 ml) was added to the sample (10–50 μ l) and the volume adjusted to 2.1 ml with ethanol. The mixture was incubated at room temperature for 45 min and the absorbance at 560 nm measured against a blank mixture of 2.0 ml of Xylenol Orange and 100 μ l of ethanol. Fresh solutions of cumene hydroperoxide were used for the construction of the calibration curve of the dye each day.

Kinetic procedures

Immobilized LOX was assayed as described above. In most experiments, the amount of LA used was 5 μ mol unless noted otherwise in the figure legend. The conditions used in the kinetic studies are listed in the legend of the figure for each experiment. All experiments were conducted at least in duplicate with a triplicate determinations. The standard error was approx. 5%.

Results and discussion

Effect of substrate concentration

The rate of HPOD formation was measured at LA concentrations ranging from 0 to 20 μ mol (Figure 1, upper panel). Higher concentrations of LA were not used to avoid inactivation of the enzyme by HPOD. Figure 1 (upper panel) shows that LA oxidation was catalysed by LOX immobilized in cross-linked phyllosilicates; HPOD formation increased with increasing LA concentration. The Lineweaver–Burk plot (Figure 1, lower panel) was linear, demonstrating that the reaction followed Michaelis–Menten kinetics. Estimated values of K_m and V_{max} were 1.7 mM and 0.023 μ mol/min respectively. Under similar conditions the K_m and V_{max} values of free LOX are 2.5 mM and 0.056 μ mol/min respectively (results not shown). Results of analysis of K_m in terms of substrate affinity for the active sites of LOX imply that when the enzyme is immobilized it still retains its affinity for LA; however, compared with free LOX, K_m and V_{max} values were decreased when the enzyme was immobilized in cross-linked phyllosilicate.

It is known that the deactivation of the LOX reaction is due to product inhibition, HPOD accumulation and partial anaerobic conditions that developed during the reaction [13]. To study reaction kinetics, the amount of protein used in the experiment has to be small enough to avoid this inhibition. In a previous study [14] it was found that when LOX was immobilized in the sol–gel matrix, the oxidation reaction was less sensitive to HPOD inhibition. Therefore it can be assumed that the immobilized state of

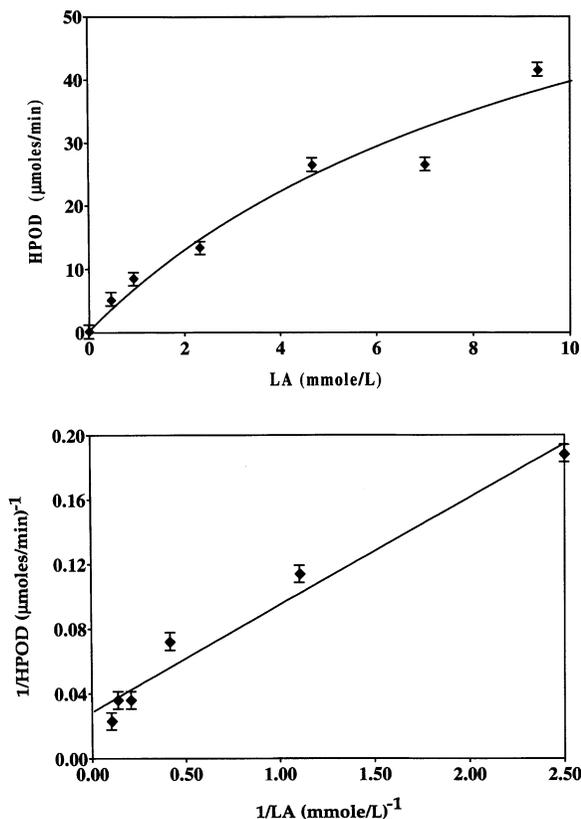


Figure 1 Influence of LA concentration on HPOD formation by LOX immobilized in cross-linked phyllosilicates

Upper panel: the HPOD formation was measured as described in the Materials and methods section. The lower panel is the double-reciprocal plot of the upper panel. The results were plotted to determine the fit to simple Michaelis-Menten kinetics. The linearity of the lines was better than 99%. Results are the average of two experiments with three replicates.

the enzyme remains active longer than the free enzyme. Shen et al. [10] demonstrated that LOX immobilized by intercalation, crossed-linked with phyllosilicate, retained its enzymic activity for more than three months.

Temperature dependence of oxidation of LA by immobilized LOX cross-linked with phyllosilicates

The enzymic activity of LOX immobilized in cross-linked phyllosilicates was studied over the temperature range 0–50 °C (Figure 2). Results show that the optimal temperature for the oxidation of LA by this immobilized LOX preparation was 25 °C, which is also the optimal temperature for free LOX (results not shown). In contrast with our results, previous studies [9] that used LOX covalently linked to a carbonyl di-imidazole-activated polymer indicated that the maximum production of HPOD was at 15 °C. The authors suggested that the decreased yields of HPOD at temperatures above 15 °C was a result of decomposition of HPOD or anaerobic

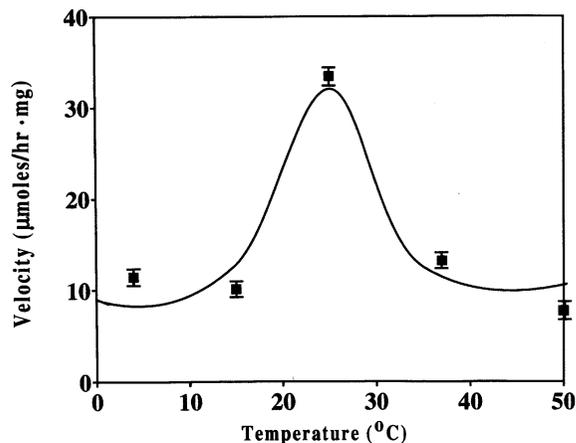


Figure 2 Influence of temperature on HPOD formation catalysed by LOX immobilized in cross-linked phyllosilicates

Assays were performed at 25 °C for 2 h in an incubation mixture containing 100 μg of protein and 40 mg of LA at various temperatures (0, 4, 15, 25, 37 and 50 °C). The formation of HPOD was determined as described in the Materials and methods section. Results are the average of two experiments with three replicates.

conditions caused by decreased oxygen solubility at higher temperatures. In the present study, all buffers used in the experiments were presaturated with oxygen, which could account for the shift to a higher optimum reaction temperature (from 15 to 25 °C). Another reason for the difference in temperature optima could be the different immobilization methods used. Our study indicated that the HPOD product generated during the oxidation reaction at 25 °C did not undergo significant decomposition (results not shown).

Influence of iso-octane concentration on HPOD formation

The enzymic activity of LOX immobilized in cross-linked phyllosilicates was examined at 25 °C in reaction mixtures containing iso-octane and aqueous buffer. Figure 3 shows that the maximum oxidation of LA occurred in the reaction mixture containing 15% (v/v) iso-octane. At this concentration of iso-octane the rate of HPOD formation was approx. 3.5-fold greater than the reaction without the addition of iso-octane. The rate of LA oxidation decreased at iso-octane concentrations higher or lower than 15%. When free LOX was used to catalyse the oxidation of LA, HPOD production increased approx. 2-fold with the addition of 25–30% (v/v) iso-octane (results not shown). This stimulation effect by the addition of iso-octane was also reported by Piazza et al. [9]. In that study, the addition of 35% (v/v) iso-octane to the reaction mixture resulted in a 3-fold increase in LA oxidation rate compared with the reaction without the addition of iso-octane. As noted above, LOX was immobilized by covalent linkage to

carbonyl-di-imidazole-activated polymer; in the present study LOX was entrapped in phyllosilicates. It seems from both studies that the addition of iso-octane increases the oxidation rate of LA for all types of immobilized LOX. Recently [14] we reported that a similar solvent effect occurred in the oxidation reaction catalysed by LOX immobilized in a sol-gel matrix. In that study the oxidation rate was also increased (approx. 40% stimulation) compared with the reaction in the absence of iso-octane.

Substrate specificity for LOX immobilized in cross-linked phyllosilicates

Figure 4 shows the relative rates of the immobilized LOX-catalysed oxidation reactions for LA and acylglycerols containing linoleoyl residues. The relative oxidation rates were determined by the Xylenol Orange method [12] with the substrates LA methyl ester, 1-monolinolein, 1,3-dilinolein and trilinolein. Oxidation rates are normalized to the LA oxidation rate (setting LA to 100%). The results in Figure 4 show that 1,3-dilinolein was oxidized to a greater extent than LA (40% more); LA methyl ester and 1-monolein were oxidized at a rate of 60% of LA and trilinolein was oxidized at a rate 40% that of LA. In a similar study with free LOX, LA was the best substrate and trilinolein was the poorest (18% of LA). Relative substrate oxidation production by free LOX followed a similar order to that of immobilized LOX cross-linked with phyllosilicates (results not shown). Previous work by Piazza et al. [15] indicated that 1,3-dilinolein was the poorest substrate for the oxidation reaction catalysed both by free LOX and by LOX covalently immobilized on a polymer. In contrast, we reported that LOX immobilized in a sol-gel matrix oxidized 1,3-dilinolein at a rate 93% of that of LA. From this study and the work on LOX immobilized in a sol-gel matrix [14], it seems that LOX immobilized by entrapment methods has a greater substrate preference for 1,3-dilinolein than the oxidation reaction catalysed by free LOX or covalently linked LOX.

Protein loss from LOX-phyllosilicates

Figure 5 shows the loss of protein from the immobilized preparation over time. Protein loss from the matrix was determined by measuring the protein content of the supernatant in the incubation mixture containing immobilized LOX in 0.2 M sodium borate buffer. Figure 5 also shows that the rate of oxidation of LA by the LOX-phyllosilicates increased with increasing incubation time. After 20 h of incubation, both protein and HPOD formation reached their maximum level. The results show that approx. 40% of the protein leaked out from the immobilized matrix and that 60% of the enzyme activity

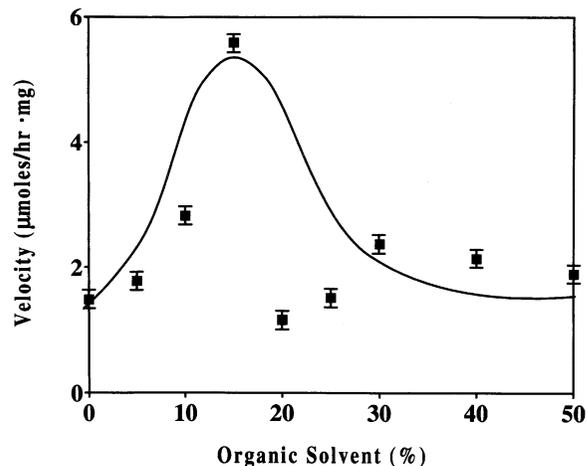


Figure 3 Influence of the amount of iso-octane on HPOD formation catalysed by LOX immobilized in cross-linked phyllosilicates

Each assay contained 0.2 M sodium borate buffer, pH 9.0, 100 µg of protein, 40 mg of LA and different amounts of iso-octane as indicated. The reaction was performed at 25 °C for 2 h. The iso-octane concentrations were calculated as volume of iso-octane/(volume of buffer plus volume of iso-octane) × 100. Results are means of four determinations.

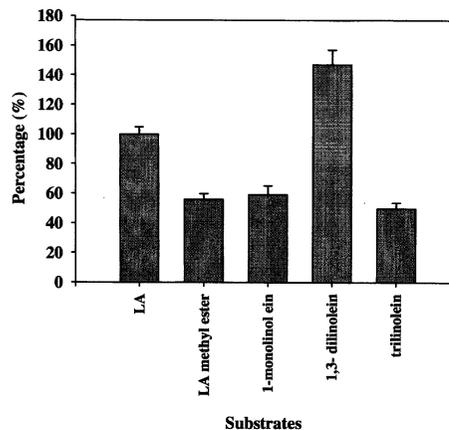


Figure 4 Relative substrate reactivity for HPOD formation catalysed by immobilized LOX

Assays contained 6 µmol of linoleoyl residues as substrate, 400 µl of 2% (w/v) deoxycholate, 1.6 ml of sodium borate buffer (pH 9.0, saturated with oxygen) and 100 µg of protein immobilized in phyllosilicates. Reaction was performed as described in the Materials and methods section. HPOD formation was determined by the Xylenol Orange method. The results are normalized to LA as 100%.

(as measured by HPOD formation) was detected in the supernatant of the incubation mixture. In general it would be expected that the amount of protein loss from the immobilized matrix should correlate with the amount of enzyme activity in the supernatant. In this study, protein loss and increased enzyme activity were not the same, but the results indicate that a significant amount of protein and associated enzymic activity was detected from the supernatant of LOX cross-linked with phyllosilicates. In con-

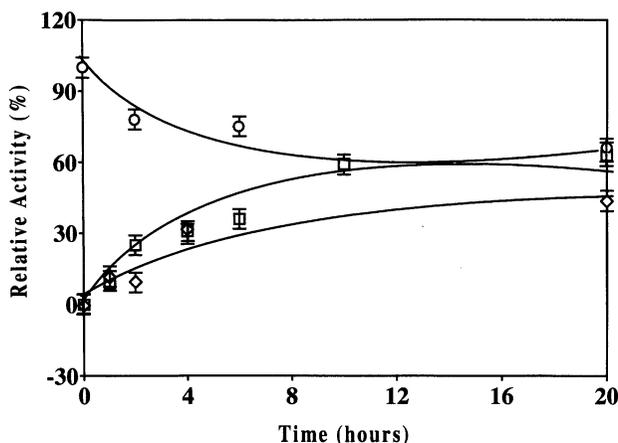


Figure 5 Loss of LOX from LOX-phylosilicate composites

LOX immobilized in cross-linked phylosilicates (containing 100 μg of protein) was incubated with 0.2 M sodium borate, pH 9.0, at 25 $^{\circ}\text{C}$ under constant shaking (200 rev./min). At selected times the mixture was centrifuged and the supernatant was assayed for protein content and LOX activity. In a parallel experiment under the same conditions the activity of the immobilized LOX was determined. Results are the average for two experiments with three replicates. Symbols: \diamond , protein content; \square , HPOD formation with supernatant solution, \circ , HPOD formation with immobilized LOX.

trast, assaying the residual enzymic activity remaining in the immobilized LOX cross-linked with phylosilicates indicated (Figure 5) that LOX activity decreased by approx. 40%. This decreased amount of enzymic activity was proportional to the amount of protein loss from LOX cross-linked with phylosilicate. Shen et al. [10] indicated that protein loss from LOX immobilized in cross-linked phylosilicates was 5.5% in the presence of water, increasing to 10% in citrate/phosphate buffer, pH 7.0. Similarly we have found that there is also significant protein loss in a buffer system containing sodium borate, pH 9.0.

In this study, LOX was immobilized by polymerization of TMOS with cross-linked phylosilicates. This immobilized LOX significantly enhanced and retained LOX activity while retaining the physical and chemical characteristics of free LOX. LOX cross-linked with phylosilicates might be a useful enzyme in industrial applications for fatty acid oxidation. In this study we found that protein loss from the matrix might be a problem when this immobilized LOX preparation is used industrially. Future research is needed to improve the nature of the cross-linkage in immobilized LOX with phylosilicates. This

improvement should be directed at decreasing the leakage of the entrapped enzyme from the matrix.

Acknowledgments

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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