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Effectiveness of Cross-Linked Phyllosilicates for Intercalative Immobilization of Soybean Lipoxygenase

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ABSTRACT

A novel procedure was developed to intercalate enzymes into dispersed phyllosilicates that were cross-linked with silicate polymers formed by the hydrolysis of tetramethyl orthosilicate (TMOS). Lipoxygenase (LOX) intercalated into cross-linked phyllosilicates exhibited high enzymatic activity. The enzyme-phyllosilicate composite prepared by this procedure had an improved pore network. Alkylamines were used to occupy the charge sites of the phyllosilicate, which increased the hydrophobicity of the phyllosilicate and reduced charge–charge interaction between LOX and the phyllosilicate. The amount of macropores and the enzymatic activity of the lipoxygenase-phyllosilicate composites increased with an increase in the ratio of trimethylammonium (TMA)-phyllosilicate to cross-linking reagent TMOS. LOX intercalatively immobilized into phyllosilicates displayed good storage stability and reusability at ambient temperature.

Index Entries: Lipoxygenase; enzyme immobilization; cross-linked phyllosilicates; intercalation.

INTRODUCTION

Lipoxygenases (LOX, EC1.13.11.21) catalyze the oxidation of polyunsaturated fatty acids (PUFA) that contain a Z-1, Z-4 pentadiene structure to

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give a Z-1, E-3 conjugated diene-5-monohydroperoxy derivative. Hydroperoxy fatty acid derivatives can be exploited as chemical synthons in chemical and pharmaceutical applications. For example, hydroperoxides can be reduced to hydroperoxy compounds that can serve as replacements for ricinoleic acid and hence have potential in a number of industrial applications such as, lubricants, grease thickeners, and drying oils. Also, the hydroperoxy derivatives of linoleic and linolenic acid are claimed to be effective fungicides in several agriculture applications *(1)*.

Lipoxygenases are very labile proteins that lose most of their activity in aqueous solutions within a day. For practical considerations, an immobilized enzyme would enable a continuous process that gives high substrate conversions, good product recovery, and minimal loss of enzyme activity. Conventional methods of enzyme immobilization include covalently binding or adsorption of the enzymes onto a solid support. LOX has been immobilized by adsorption to glutenin, gliadin, glass wool, talc, polymer beads, and ion-exchange supports *(1,2)*. The matrices used in covalent binding of LOX include oxirane acrylic beads, CNBr-activated Sepharose and agarose, and carbonyldi-imidazole-activated polymer *(3)*. Although improving the stability of the enzyme, either covalent or ionic bonds formed by these methods can cause a decrease in enzyme activity. For example, the adsorption of *S. tuberosum* lipoxygenase on talc retained only 53% of its activity in immobilized form *(2)*. Immobilization of enzymes by entrapment has been achieved by encapsulating enzymes through sol-gel processes *(4)*. The entrapped enzymes retained much of their activity and had better stability in the sol-gel matrices. Extension of this technique, however, was limited by two shortcomings of sol-gel materials: their brittleness and narrow pore network *(5)*. Efforts were made to improve the activity of immobilized enzymes by introducing matrix-relaxing additives, such as algenate or polymers *(5,6)* into sol-gel matrices, or mixing alkyl-substituted silanes in a specific ratio *(7)*. Despite these improvements, however, alternative methods are still needed for enzyme immobilization.

Clay minerals are naturally occurring phyllosilicates (i.e., layered silicates) with good intercalative properties. Because their layered structures can be broken down into nanoscale building blocks, phyllosilicates can serve as a framework for intercalation. Metal hydroxyl polymeric cations, alkylammonium ions, polymers, and their combinations have been intercalated into phyllosilicates to form a broad spectrum of materials ranging from pillared clays, organoclay, to polymer-clay nanocomposites. The intercalated phyllosilicates exhibit good mechanical and thermal stability, controlled pore size (0.2–1 μm) and ion mobility, and high adsorption capacity *(8–12)*. We have developed a method to intercalatively immobilize enzymes within cross-linked phyllosilicates *(13)*. In this study,

soybean lipoxygenase was intercalatively immobilized in a phyllosilicate and the conditions for retaining the enzymatic activity, storage stability, and reusability of the intercalated LOX were investigated.

MATERIALS AND METHODS

Immobilization Procedure

A phyllosilicate (montmorillonite SWy-1 from Source Clay Minerals Repository, Columbia, MO) was Na-saturated by three washes with 1*M* NaCl solution, followed with three washes with deionized water to remove excess salt. The Na-saturated SWy-1 was fractionated for particle-size ≤ 2 μm and dispersed in water. The concentration of the phyllosilicate suspension was 3.3 % (w/v). The Na ions were exchanged subsequently with alkylammonium ions by treatment of the Na-phyllosilicate with trimethylammonium chloride (TMA) ($HN[CH_3]_3Cl$) or cetyltrimethylammonium chloride (HDTMA) ($CH_3[CH_2]_{15}\ N[CH_3]_3Cl$) obtained from Aldrich (Milkwaukee, WI). The intercalation of soybean lipoxygenase (type I-B obtained from Sigma, St. Louis, MO) into the galleries of phyllosilicate was accomplished by mixing the enzyme with the dispersed phyllosilicate in buffer solution (0.2 *M* Na_3BO_3, pH 9.0). Cross-linking of enzyme-phyllosilicate mixture was accomplished by adding tetramethyl orthosilicate (TMOS) ($Si[OCH_3]_4$) from Aldrich, to the mixture and vortexed from 1 min. The volume ratios of buffer solution, phyllosilicate suspension, and TMOS are listed in Table 1. For comparison, TMOS was added to LOX in buffer solution (volume ratio 1:1) as the sol-gel treatment *(14)*. The cross-linked enzyme-phyllosilicate complex was kept at room temperature overnight for completion of the polymerization reaction. After freeze-drying or vacuum-drying at room temperature for 24 h, the enzyme-phyllosilicate complex was ready for use.

Measurement of Enzymatic Activity

The activity of lipoxygenase was assayed by measurement of hydroperoxide formation *(3)*. An aliquot of the substrate (5 μmoles of linoleic acid) dissolved in methylene chloride was placed in a 10-mL flask and evaporated to dryness under a stream of nitrogen. The reaction medium containing 0.2 mL of 100 m*M* deoxychlorate (DOC) and 1.8 mL of sodium borate buffer (0.2 *M*, pH 9.0) was added to the substrate, and the mixture was then shaken at 250 rpm for 0.5 h at 15°C. The reaction was initiated by adding a suitable amount (approx. 0.15 mg) free or immobilized LOX. Oxidation was conducted at 15°C with agitation at 250 rpm for 2 h. The reaction was quenched by adding 400 μL of 1 *M* citric acid. Linoleic acid hydroperoxide was isolated by extracting the reaction mixture twice with

Table 1
Enzymatic Activity (% of Freshly Prepared Free LOX) of Immobilized Soybean Lipoxygenase After Vacuum-Drying

	Fresh-prepared free LOX	Frozen free LOX[b]	CTS1[a]	CTS2[a]	CTS3[a]	CTS4[a]	CHS[a]	CS1[a]	CS2[a]	S[a]
Nonground	100	33.9	26.2 ± 2.4	45.9 ± 2.7	—	—	—	9.04 ± .6	3.12 ± .03	0.04 ± .002
Ground	—	—	31.7 ± 2.1	95.9 ± 5.8	133.5 ± 8.0	161.6 ± 8.5	152.3 ± 12.9	25.6 ± 1.9	6.47 ± .04	3.64 ± .03
SWy-1/ TMOS volume ratio	—	—	1	5	10	5	5	1	5	0
Buffer/ TMOS volume ratio	—	—	2	10	10	5	5	2	10	1

[a]Treatment codes: C, Clay SWy-1 (montmorillonite from Source Clay Minerals Repository, Columbia, MO); T, TMA (trimethylammonium); H, HDTMA (cetyltrimethylammonium); S, TMOS (tetramethyl orthosilicate).

[b]Free LOX in aqueous solution stored frozen for 2 d.

2 mL chloroform: methanol (2/1, v/v). After removing the solvent under a stream of nitrogen, the hydroperoxide was redissolved in 3 mL ethanol. The amount of hydroperoxide was determined spectrophotometrically by the xylenol orange method *(15)*. Standards were prepared by diluting a commercial cumene peroxide. All results were corrected by subtracting reading from controls without enzymes. All measurements were in triplicate.

Scanning Electron Microscopy (SEM)

A small fragment of the samples was immersed in 1 mL 10% glutaraldehyde for protein fixation, washed in 0.1 *M* imidazole buffer solution, dehydrated in 50% ethanol and then pure ethanol, and frozen in liquid N_2 for 5 min. The frozen fragments were thawed into pure ethanol and critical-point dried from CO_2. A few chunks of the treated samples were glued to aluminum stubs with colloidal silver adhesive paint and coated with a thin layer of gold. The images were collected with a JSM-840A SEM at a magnification of 10,000X. The instrument conditions were 15 kV, 3×10^{-11} A, and 70 μm objective aperture.

RESULTS

Enzymatic Activity of Immobilized LOX

The lipoxygenase-phyllosilicate composite was prepared by intercalating soybean lipoxygenase into dispersed Na-, TMA-, or HDTMA-phyllosilicate, and then cross-linking the phyllosilicate with silicates from TMOS hydrolysis. LOX was efficiently immobilized in the cross-linked phyllosilicate as demonstrated by the enzymatic activity of enzyme-phyllosilicate composites. Intercalatively immobilized LOX exhibited higher activity than LOX immobilized by the simple sol-gel entrapment (Fig. 1). Both freeze-drying and vacuum-drying were used to remove water after the immobilization treatments. The vacuum-dried samples retained much of their enzymatic activity, whereas the freeze-dried samples lost most of their activity (Fig. 1). The phyllosilicate suspended in buffer solution served as the framework and introduced macropores (0.2–1 μm) to the composite (Fig. 2). Vacuum-drying removed excess water from the composites but still kept them in a hydrated state, which preserved the original structure of the composite. In contrast, freeze-drying removed most of water from the composites, causing the samples to shrink, presumably by collapse of the framework. This shrinkage of the silicate framework, along with dehydration of the protein might cause denaturing of the intercalated LOX. Accordingly, samples were vacuum-dried in all subsequent experiments.

For the same amount of SWy-1, LOX intercalated in the phyllosilicate saturated with TMA had higher activity than LOX immobilized in Na-

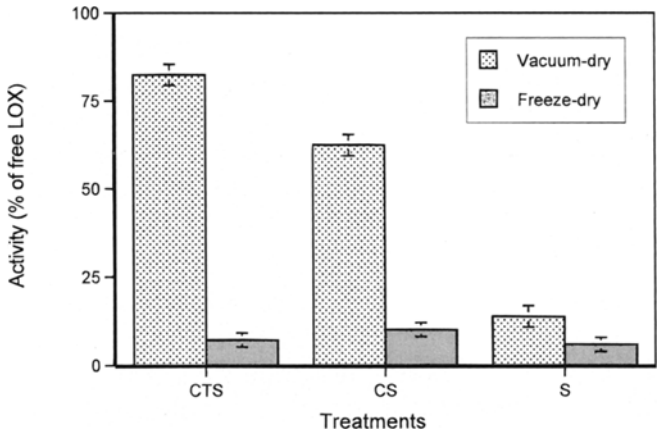

Fig. 1. Yield of the immobilized LOX. The treatment codes are the same as those in Table 1. CTS, LOX intercalated in TMA-phyllosilicate; CS, LOX intercalated in Na-phyllosilicate; S, LOX entrapped in silicate so-gel matrix.

saturated SWy-1 (Fig. 1). A more significant difference in LOX activity was noted between Na- and TMA-phyllosilicate with increasing amounts of phyllosilicates used for the immobilization (Table 1). For TMA-saturated SWy-1, the activity of immobilized LOX increased with the amount of phyllosilicate used (from treatment CTS1 to CTS3), whereas an opposite effect was observed for LOX immobilized in Na-saturated SWy-1 (treatment CS1 to CS2). One possible explanation for this observation is that charge–charge interactions between LOX and SWy-1 inhibited the enzyme activity. The SEM images of CS1 and CS2 (not shown) were similar to those of CTS1 and CTS2 (Fig. 2B,C), indicating pore networks for Na- and TMA-saturated SWy-1 were similar. Exchange of Na ions with TMA reduced the cation-exchange sites and thus the net charge of the phyllosilicate because TMA strongly binds to phyllosilicates. This process also increases the hydrophobicity of the phyllosilicate *(10)*. For the same LOX/SWy-1 ratio, treatment CTS4 (Table 1) in which Na ions were exchanged with TMA ions before intercalation of the enzyme exhibited a higher enzymatic activity than treatment CTS3 in which TMA and LOX were intercalated at the same time. Compared to TMA, the long-chain alkylammonium ions, HDTMA, showed no significant difference in the effect on the activity of immobilized LOX (Table 1).

Structure of Enzyme-Phyllosilicate Composite

The inhibition of LOX activity from enzyme-phyllosilicate interaction was insignificant for the LOX intercalated in TMA-saturated SWy-1. The restriction on substrate diffuse into the composites was reduced because of

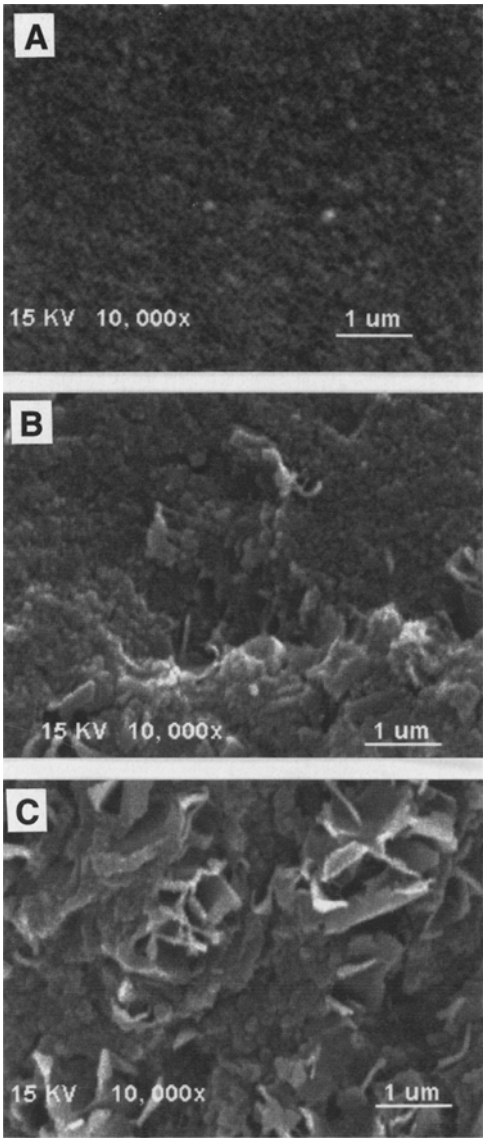

Fig. 2. SEM image of the immobilization composites. (**A**) sol-gel treatment S; (**B**) intercalative immobilization treatment CTS1; (**C**) intercalative immobilization treatment CTS2. The treatment codes are the same as those in Table 1. The volume ratios of buffer solution, phyllosilicate suspension and TMOS for the treatments are listed in Table 1.

the increase in macropores in the composites that was observed by scanning electron microscopy (SEM). The SEM image showed uniform silicate aggregates in the form of fused globules, ranging in size between 0.05 and 0.1 μm in the sol-gel composite. The pore network formed in this composite was also in that size (micropore and mesopore, <0.13 μm) *(6)* range (Fig. 2A). On the other hand, for the cross-linked phyllosilicate, SEM images showed turbulent clay layers immersed into the silicate network with some macropores ranging in size from 0.2 to 0.8 μm between clay layers (Fig. 2B). For a larger volume-ratio of phyllosilicate suspension to TMOS liquid, SEM images clearly showed that more macropores were introduced into the lipoxygenase-phyllosilicate composite (Fig. 2C). With macropores in the enzyme-phyllosilicate composite, substrates could diffuse more easily into the composite for reaction with the immobilized LOX. Accordingly, the intercalatively immobilized LOX had higher activity when a larger volume-ratio of TMA-saturated SWy-1 to TMOS was used for immobilization (Table 1). Another possible reason for higher enzyme activity in the composites made with lower amount of TMOS might be the smaller amounts of methanol produced from TMOS hydrolysis, which denatures most enzymes.

The use of phyllosilicates in immobilization treatments improved substrate diffusion by introduced macropores into the composites, but failed to eliminate all limits on substrate diffusion to the immobilized enzymes. This was noted since the activity of the ground samples was higher than that of nonground samples from the same preparation (Table 1).

Table 2
Storage Stability of the Intercalative Immobilized LOX at 4°C or Room Temperature[a]

Temperature (°C)	Storage time (d)	Treatment CTS3[b]	Treatment CTS4[b]	Treatment CHS[b]
	9	95 ± 6	97 ± 6	103 ± 6
	15	77 ± 5	93 ± 6	77 ± 5
4	29	77 ± 5	68 ± 5	64 ± 5
	91	46 ± 4	39 ± 4	24 ± 4
	181	44 ± 9	25 ± 2	15 ± 9
	6	80 ± 5	93 ± 6	85 ± 5
22	13	82 ± 5	94 ± 6	74 ± 4
	28	61 ± 4	72 ± 4	68 ± 4
	91	34 ± 4	30 ± 4	11 ± 3

[a]Residual activity: percentage of the original immobilized activity.

[b]The treatment codes are the same as those in Table 1. The volume ratios of buffer solution, phyllosilicate suspension and TMOS for the treatments are listed in Table 1.

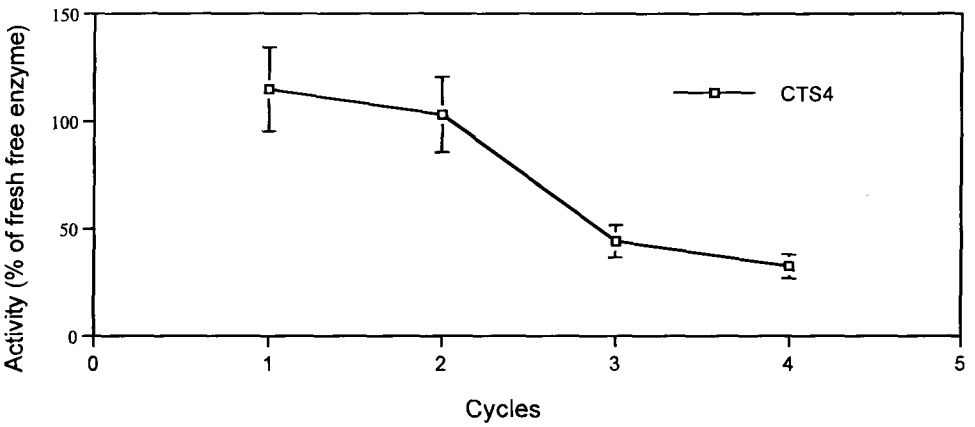

Fig. 3. Reusability of the intercalatively immobilized LOX (treatment CTS4 in Table 1, unground samples, $n = 12$). The volume ratios of buffer solution, phyllosilicate suspension, and TMOS for the treatment are listed in Table 1.

Storage Stability and Reusability

To assess storage stability, intercalatively immobilized LOX composites were stored with or without buffer solution at different temperatures for up to 3 mo. Stability of immobilized LOX stored in buffer solution showed no significant difference from that stored without buffer solution. Accordingly, the data for both sample sets were combined as shown in Table 2.

After 3 mo at 4°C, the activity of intercalatively immobilized LOX decreased from 75 to 54% (Table 2). Immobilized LOX stored at room temperature showed a similar loss (88–66%) in activity (Table 2). The effect of storage temperature on the stability of intercalatively immobilized LOX was less significant than that of LOX immobilized by other methods *(2,3)*. Drying was the major factor that caused the immobilized LOX to lose activity during storage. This observation was more prominent at room temperature than at 4°C. By plotting the activity data vs storage time and using regression analysis, the half-life of intercalatively immobilized LOX was estimated to be 77, 69, and 55 d for samples CTS3, CTS4, and CHS at 4°C, respectively. At room temperature, samples CTS3, CTS4, and CHS had respectively a half-life of 57, 63, and 47 d, respectively. The half-life of intercalatively immobilized LOX was similar to that of LOX adsorbed on talc at 4°C *(2)*, but much longer than that of LOX covalently bound to carbonyldi-imidazole-activated polymer *(3)*.

The reusability of intercalatively immobilized LOX was evaluated by repeating incubation cycles with the substrate solution. After each cycle, the substrate solution was removed for hydroperoxide analysis and the immobilized LOX samples were washed and stored in buffer solution

(0.2 *M* Na_3BO_3). The results showed that immobilized LOX retained most of its activity for the second cycle (Fig. 3), but after the third cycle, the activity decreased to approx 30% of the original immobilized activity. The activity of the forth cycle was still 33% of freshly prepared, free LOX. The breakdown of lipoxygenase-phyllosilicate composites had little effect on their reusability. Leaching of immobilized LOX from the enzyme-phyllosilicate composite seemed to be the major reason for the decrease in activity after the third cycle. In the leaching test, the enzyme-phyllosilicate composite was incubated in deionized water, 0.2 *M* Na_3BO_3 buffer solution, or the substrate solution used in LOX assay, and shaken at 70 rpm for 24 h. After centrifuge, the protein content of the incubation solution was measured by Lowry assay. Depending on the type of incubation solutions, 2–6% of total immobilized protein leached out during incubation.

DISCUSSION

The immobilization procedure described in this paper is an example of improved biocatalyst preparation using an innovative procedure that may have general applicability. The lipoxygenase-phyllosilicate composite prepared had excellent enzymatic activity and exhibited less restriction on the diffusion of substrates. Various phyllosilicates with surfaces modified by different organic and inorganic species can be used to provide an array of different enzyme-phyllosilicate composites. As demonstrated, the simple attempt to produce immobilized LOX described in this study could be useful for commercial applications.

An interesting aspect of these lipoxygenase-phyllosilicate composites is that the composites after silicate polymerization behaved like frozen suspensions of LOX and SWy-1. The LOX, after intercalation into dispersed phyllosilicates, should be restrained in the galleries (i.e., interlayers) of phyllosilicates. Yet the intercalated LOX was kept relatively free from tightly binding with the phyllosilicates that were pretreated with organic counter ions, e.g., TMA, and this treatment with organics was helpful in retaining of enzymatic activity.

The interactions between enzymes and phyllosilicates may include electrostatic interaction, hydrogen bonding, hydrophobic adhesion, or covalent bonding *(1,16)*. Electrostatic interactions between external charges on enzymes and phyllosilicates is the primary force governing adsorption. Factors controlling immobilization by electrostatic adsorption include active sites, pH, ionic strength, and relative amounts of enzyme and phyllosilicate *(2,16,17)*. The results from this study showed that LOX was denatured by electrostatic interactions (Table 1), whereas this effect was insignificant for peroxidases *(18)*. A similar observation was found for peroxidases and lipases adsorbed on talcs *(17)*. One possible explanation for

this difference may be the locations of charge sites in the enzymes. Enzyme activity may be inhibited if the electrostatic interactions of charge sites interfere with the catalytic sites of an enzyme *(16)*. Hydrophobic/hydrophilic balance in an immobilization matrix is another factor that can influence the catalytic activity of an immobilized enzyme *(17)*. A strong hydrophobic matrix may tightly adsorb and inactivate enzymes, whereas no enzyme may be adsorbed onto a hydrophilic support *(1)*. LOX, like lipases *(17)*, favors a hydrophobic/hydrophilic-balanced (amphiphilic) matrix because of lipophilicity. Incorporating alkylammonium ions on the phyllosilicate surface adjusts the amphiphilicity of phyllosilicates and might also disrupt hydrogen bonds, causing fast inactivation of the enzyme after it is adsorbed on the phyllosilicates *(19)*. Such enzyme-phyllosilicate interactions play an important role in the intercalative immobilization of enzymes.

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