

Immobilization of lipoxygenase in an alginate-silicate solgel matrix: formation of fatty acid hydroperoxides

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A method for the immobilization of lipoxygenase (LOX) in an alginate-silicate gel matrix was developed. In this method, a mixture of calcium alginate beads and LOX in borate buffer are dispersed into a hexane solution of tetramethoxy-ortho-silicate (TMOS). Hydrolysis of the TMOS gives products that permeate and co-polymerize with the alginate gel to form a colloid within the beads that entraps the LOX. Optimum reaction conditions for sol-gel entrapment of LOX are at pH 9.0 in 0.2M borate buffer. The composite gel, after isolation and vacuum drying, had excellent protein retention that has good enzyme activity and stability at room temperature. The activity of the entrapped LOX was less than the activity of the free enzyme. However, the activity of the immobilized LOX can be restored by the addition of borate buffer and glycerol, or borate buffer saturated with an organic solvent. In contrast to the free enzyme in solution, which loses its activity in less than one day, sol-gel entrapped LOX retains its activity at ambient temperature for at least 25 days and can be recycled. This report demonstrates that the sol-gel entrapment method for immobilizing LOX can be useful in developing a process for the oxidation of polyunsaturated fatty acids.

Introduction

Lipoxygenase (EC1.13.11.12) (LOX) catalyzes the positional and specific dioxygenation of polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. Reduction of the latter compounds gives the corresponding hydroxy acids, which are prospective replacements for ricinoleic acid, an industrially important fatty acid. Lipoxygenase has been extensively studied (Siedow, 1991; Bombard *et al.*, 1993) for many years. Since lipoxygenase is a labile protein, techniques for the immobilization have been sought to improve its stability. Conventional methods of enzyme immobilization include physical adsorption or covalent binding to a suitable carrier matrix. For example, LOX has been immobilized by adsorption on to glutenin, glass wool, talc, polymer beads and ion exchange supports or by covalent linkage to matrices such as CNBr-activated Sepharose, agarose, oxirane acrylic beads (Cuperus *et al.*, 1995; Battu *et al.*, 1994) or a carbonyl diimidazole activated polymer (Piazza *et al.*, 1994; Parra-Diaz *et al.*, 1993). Alternatively, enzymes have been physically entrapped in polymeric matrices to improve their biostability and reaction efficiency. Excellent stabilization of enzymes by non-covalent bonds

has been achieved in enzyme-silicate gels made by the sol-gel process (Brinker and Scherer, 1990; Bakul *et al.*, 1994). Extension of this technique to industrial catalysis has been limited by two well-known shortcomings of sol-gel ceramics: 1) their brittleness and 2) their narrow mesopore network, which imposes diffusional limitations in macroscopic catalyst reaction. Recently, a method for immobilizing β -glucosidase in an alginate-silicate sol-gel matrix was developed that overcame the above-cited limitations (Heichal-Segal *et al.*, 1995). In this study, we report that soybean lipoxygenase entrapped by this novel immobilization method retained its enzymatic activity, had excellent thermal stability and was reusable.

Materials and methods

Materials

Soybean Lipoxidase Type I-B (lipoxygenase, LOX), linoleic acid, sodium alginate, sodium deoxycholate monohydrate (DOC) and cumene hydroperoxide were purchased from Sigma (St. Louis, MO). Tetramethoxy-ortho-silicate (TMOS) and the sodium salt of Xylenol Orange were purchased from Aldrich (Milwaukee, WI). All other reagents used were of the highest purity.

Immobilization of lipoxygenase

Sodium alginate (4%, w/v) in 0.2 M sodium borate buffer (pH 9.0) was mixed well with an equal volume

¹Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar brand not mentioned.

of soybean lipoxygenase (5 mg/ml in 0.2 M sodium borate buffer, pH 9.0). Droplets of this mixture were dispensed with a Pasteur pipette into a cold solution of 0.2 M CaCl_2 (100 ml) to form single beads. The beads were collected within 10 minutes and transferred to a beaker containing enough hexane to cover the beads. TMOS (1-1.5 vol/vol of the beads) was added and the mixture was left overnight at room temperature to complete the polymerization process. The beads were filtered and left to dry overnight under vacuum at room temperature. The beads then were soaked in 0.2M borate buffer (pH 9.0), or 75% glycerol in water, or borate buffer saturated with isooctane for 6 hours. Finally, the beads were filtered from the solution, air dried at room temperature and stored at room temperature or 4°C until needed.

Lipoxygenase assay

The assay for lipoxygenase activity was conducted by measuring the hydroperoxide formation from linoleic acid, as described previously (Piazza and Nunez, 1995). In a typical assay, the substrate (5 μmol linoleic acid) dissolved in methylene chloride was placed in an Erlenmeyer flask (10 ml), and the methylene chloride evaporated under a stream of N_2 . The reaction medium containing 0.2 ml 100 mM deoxycholate (DOC) and 1.8 ml sodium borate buffer (0.2 M, pH 9.0) was added to the linoleic acid, and the mixture was shaken at 250 rpm for 0.5 h at 15°C. Reaction was initiated by the addition of 0.5 mg of entrapped LOX (in order to obtain the maximum lipoxygenase activity, beads containing entrapped LOX were ground with a mortar and pestle in all experiments unless otherwise noted). Oxidation was carried out at 15°C with agitation at 250 rpm for two hours. The reaction mixture was quenched with 400 μl of 1M citric acid, and linoleic acid hydroperoxide was extracted with 2 ml of chloroform: methanol (2:1, v/v) twice. The extracts were combined and dried under a stream of N_2 . The residue was redissolved in 3 ml of absolute ethanol and assayed for hydroperoxide content.

Hydroperoxide assay

The level of hydroperoxide was determined spectrophotometrically by the xylenol orange method previously described (Jiang *et al.*, 1991). Standards, made from dilution of commercial cumene peroxide, were prepared daily to construct the calibration curve. All results were corrected by subtracting the reading from controls without enzymes. The data are the averages of two or three experiments with three to five replications.

Results and discussion

pH Optimum for immobilization of LOX

The activity of LOX immobilized in an alginate-silicate sol-gel matrix was studied over the pH range 7–10. Figure 1 shows the pH profile of LOX entrapped in calcium-alginate beads in borate buffer. The formation of the linoleic acid oxidation products (13-hydroperoxy-cis-9-trans-11-octadecadienoic acid, HPOD) was maximal at pH 9.0. The oxidation rate decreased about 20–30% at pH's away from this optimum.

Immobilized lipoxygenase

The enzymatic activity of immobilized LOX in calcium alginate beads was affected by different drying treatments (Figure 2). The average activity of entrapped LOX relative to that of the free LOX (solution) varied from 15 to 30%. Vacuum drying was the most efficient method for removing water from samples and this preparation also retained the highest LOX activity. Freeze drying caused the samples to shrink and also resulted in greater loss of LOX activity. Air dried samples had poor enzymatic activity (approximate 15–25% of free enzyme activity). Grinding the beads resulted in the best activity for entrapped LOX. Without grinding the beads, LOX activity did not attain the maximum activity, perhaps because of the diffusion limitations of

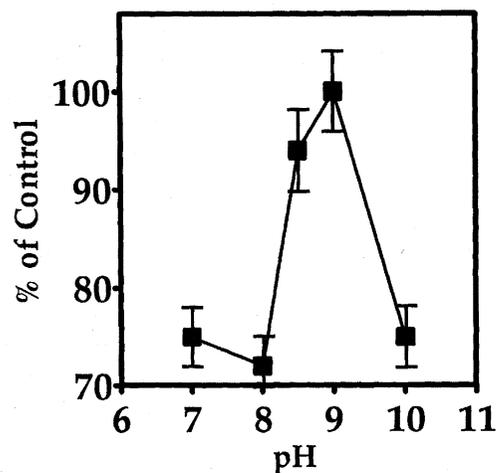


Figure 1 Influence of buffer pH on entrapped lipoxygenase activity. Sodium alginate (4%) in sodium borate buffer (at pH indicated) was mixed well with an equal volume of lipoxygenase suspended in sodium borate buffer (0.2 M, pH 9.0). The immobilization procedure and the measurement of hydroperoxide formation are described in 'Materials and Methods.' Data are expressed as percentage of control (pH 9.0 of sodium borate buffer containing sodium alginate and an equivalent amount of lipoxygenase). The control value (100%) is 650 nmoles of oxidized linoleate.

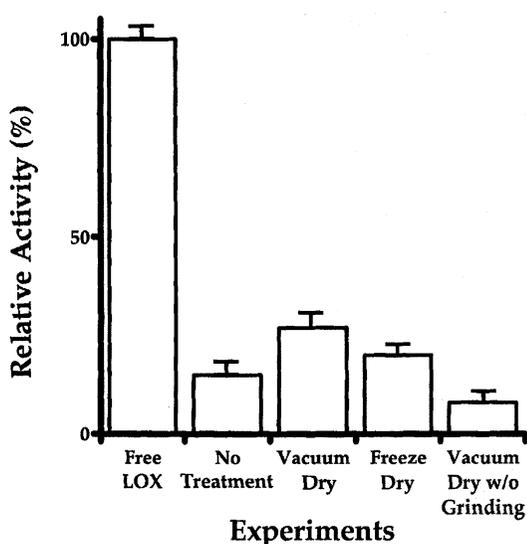


Figure 2 The effect of drying method on entrapped lipoxygenase activity. Beads of entrapped lipoxygenase were dried under conditions (as indicated) overnight and the enzyme activity was measured (all experiments used ground samples except the last entry) as described in 'Materials and Methods.' Free lipoxygenase activity is expressed as 100% which represents 2.8 μ moles of oxidized linoleate. All other activity are calculated as relative percentage to the free enzyme.

linoleic acid. Although the loose polymer network of Ca^{2+} alginate gel supported the silica gel inclusion, the mesopore network of the particles was not sufficiently large enough to overcome the limitation of substrate diffusion in samples that were not ground.

The enzymatic activity of entrapped LOX after immobilization could be enhanced by various treatments. This was shown by soaking the beads in various solutions for six hours. The beads then were removed from the solutions and air dried at room temperature for 12 h and 48 h (Figure 3). The borate buffer-treated beads after 12 h had enzymatic activity 40% greater than that of the enzyme in solution. Further air drying of the samples for 48 hours resulted in the buffer-soaked samples having significantly decreased activity to the extent that activity was about the same as that of the samples receiving no treatment (Figure 3). Beads soaked in 75% glycerol or in borate buffer saturated with iso-octane had enzyme activity 10–15% greater than the samples receiving no treatment. For long term storage, samples were soaked in 75% glycerol. Samples not stored in glycerol lost more than 50% of their activity after one week of storage at room temperature.

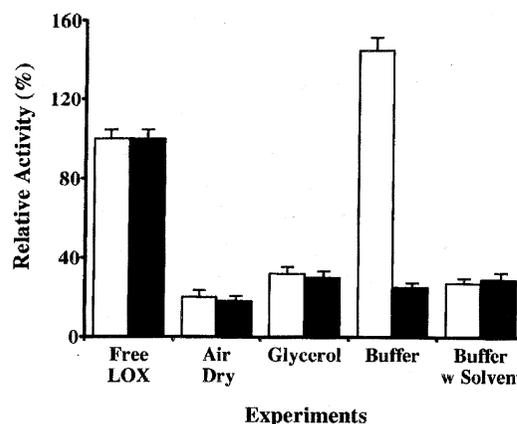


Figure 3 Recovery of enzyme activity after immobilization. Alginate – silicate beads were prepared as described under 'Materials and Methods.' The beads were left to air dry or soaked for 6 hours in either 75% glycerol, 0.2 M sodium borate buffer (pH 9.0), or sodium borate buffer saturated with iso-octane for 6 hours. Lipoxygenase activity was then measured after 12 hours or 48 hours of air drying. □ 12 h, ■ 48 h.

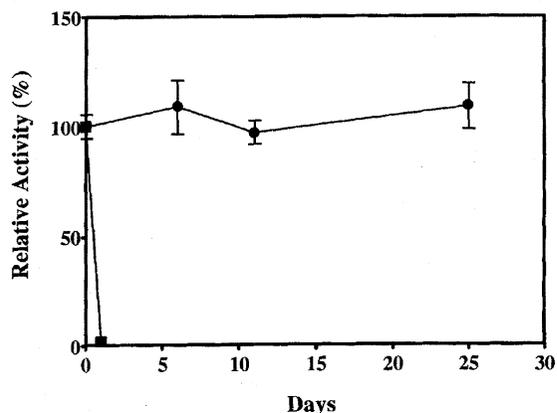


Figure 4 Comparison of immobilized (●—●) and free lipoxygenase (■—■) at room temperature. The immobilized beads were soaked with 75% glycerol solution as described in 'Materials and Methods,' and were stored at room temperature. At the times indicated, the samples were assayed to measure the lipoxygenase activity. Initial activity of fresh free or immobilized LOX is expressed as 100%.

Stability of immobilized LOX

The entrapped LOX was extremely stable at room temperature; there was no decrease in LOX activity even after 25 days of storage (Figure 4). In contrast, LOX in solution lost most of its activity after 24 hours at room temperature. A similar study of immobilized

β -glucosidase in alginate silicate gel reported by Heichal-Segal *et al.* (1995) also demonstrated the complete operational stability at ambient temperature for at least several months. Similarly, LOX covalently bonded to carbonyl-imidazole activated support (Piazza, 1994) had a half-life of only 75 hours at 15°C, but its stability at room temperature was not reported. Other immobilized LOX preparations had half lives on the order of only several hours (Cuperus *et al.*, 1995, Battu *et al.*, 1994).

Reusability of immobilized LOX

The reusability of LOX immobilized in calcium-alginate beads was determined by using the same beads for five reaction cycles with linoleic acid (LA) as the substrate (Figure 5). LOX activity was measured after each cycle of incubation, and the beads were recovered and washed with sodium borate buffer before reuse. To initiate the next cycle of oxidation, linoleic acid was added to the incubation mixture containing the

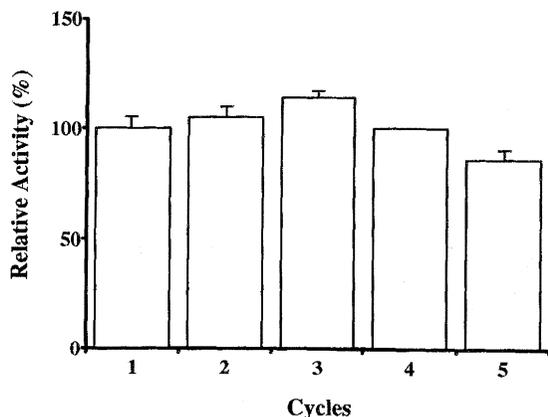


Figure 5 Reusability of the immobilized lipoxygenase. Glycerol-treated immobilized beads were incubated with a substrate (linoleic acid) for five cycles. For each cycle, the immobilized beads were washed with borate buffer and fresh linoleic acid added to conduct another oxidation cycle. The first cycle of lipoxygenase activity is expressed as 100%. Other activities are expressed relative to the first cycle.

recovered beads. The data (Figure 5) show that LOX immobilized in beads can be reused at least five times without substantial loss in enzyme activity. Free lipoxygenases are typically inactivated by product hydroperoxide accumulation and by the partial anaerobic conditions that develop in reaction mixtures (Siedow, 1991). The present study shows that entrapment of LOX in the alginate-silicate gel matrix somehow protects the enzyme from hydroperoxide inactivation which allows repeated use of the entrapped LOX.

Conclusion

In this study, lipoxygenase was successfully entrapped by a novel immobilization method within in a calcium-alginate silicate gel matrix. The entrapped lipoxygenase had stability better than LOX's previously reported methods. The immobilized preparation also was reusable. This procedure should enhance the potential of LOX to be used as a biocatalyst in the preparation of oxygenated fatty acid derivatives with unique properties.

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