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# Activity of immobilized lipoxygenase used for the formation of perhydroxyacids

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**Abstract**

Lipoxygenase-produced oxidized fatty acids may serve as intermediates in chemical and pharmaceutical syntheses. For practical applications the enzyme should be immobilized to prevent its loss at product retrieval and to enable a continuous process. In this study the immobilization of soybean lipoxygenase on various neutral and charged supports was investigated. The best results were obtained using cellulose-based anion exchangers. The activity of the immobilized lipoxygenase was lower than the activity of the free enzyme, which was possibly caused by diffusion limitation of the fatty acids to or from the support beads.

## 1. Introduction

Lipoxygenases catalyze the oxygenation of fatty acids which contain non-conjugated double bonds. During the process the double bonds are conjugated and perhydroxy fatty acids are formed. The mechanism of this reaction has been studied in detail [1–3] and involves essentially two steps. First, electron transfer takes place from the pentadiene towards the enzyme–$Fe^{3+}$ and a radical is formed. The radical electron shifts towards one of the end-carbons leading to a conjugated diene. Second, the radical reacts with molecular oxygen yielding the perhydroxy fatty acid, whereby the enzyme–$Fe^{2+}$ is re-oxidized to $Fe^{3+}$. The enzyme is probably only involved in the first step as its iron is of a non-heme type: only iron coordinated in porphyrins and other conjugated chelates are known to bind molecular oxygen. At low $O_2$ concentration the second step proceeds differently, leading to dimerized fatty acids, ketones and terminal aldehydes by chain cleavage.

Other substrates without the pentadiene group are unsaturated ketones [4] and catecholamines [5], but the principal substrates are unsaturated fatty acids like linoleic and γ-linolenic acid. Both are readily available, linoleic acid occurring in safflower oil (77% content) and γ-linolenic acid in linseed oil (67% content).

Recent interest in lipoxygenase is two-fold. On the one hand, from the medical side efforts are directed towards inhibiting lipoxygenase activity because this enzyme contributes to the inflammation of the airways in asthma [6,7]. On the other hand perhydroxy fatty acids can be used in pharmaceutical and chemical syntheses. Perhydroxides of arachidonic acid (C18:4) are precursors for prostaglandine and leukotriene synthesis and perhydroxides of linoleic and linolenic acid are effective as fungicide for agricultural applications [8–10].

The feasibility of commercial applications will be greatly enhanced if the enzyme can be immo-

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bilized and stabilized. In this case a continuous production process is possible with retention of the enzyme. In general, enzyme immobilization may be classified into three kinds, hydrophobic adhesion, electrostatic adhesion on charge supports like ion exchangers and covalent binding. Recently, lipoxygenase has been immobilized by covalent binding to carbonyl-diimidazole activated polymer [11,12]. The immobilized enzyme was active in water and in biphasic water–organic media. Initial reaction rates were not determined, therefore the activity of the immobilized enzyme relative to that of the free enzyme was uncertain. Also, activated Mg-silicate has been applied [13] for immobilization.

However, covalent binding is a multi-step process, relatively expensive and regeneration of the support, once the enzyme has lost its activity, does not seem to be practical. With hydrophobic or ionogenic binding the latter is more easy, but this also implies that the enzyme may potentially desorb during the production process itself.

In the present study the binding of soybean lipoxygenase on several neutral polymer beads and on ion-exchange supports was investigated. Activities of the free and immobilized enzyme, and also enzyme desorption from the supports were measured at aerobic conditions with linoleic acid as substrate.

## 2. Materials and methods

Linoleic acid was obtained from Sigma. Anion exchangers were obtained from Whatman (DEAE cellulose; capacity 0.7 meq/g), Serva (Dowex) and Sigma (Ecteola cellulose; capacity 0.32 meq/g). Lipoxygenase was obtained from Fluka. In this preparation, the relative amounts of lipoxygenase-1, -2 and -3 were not known. However, the measurements were done at pH 9.0 which is the optimal pH of lipoxygenase-1. Types 2 and 3 have pH optima around pH 7 thus it was assumed that during the measurements the activity of type 1 prevailed.

The activity of purified lipoxygenase is 180–190 $\mu$mol $min^{-1}$ $mg^{-1}$ [14]. The commercial preparation had an activity of 7–11 $\mu$mol $min^{-1}$ $mg^{-1}$, thus corresponding to 4–6% enzyme content.

The activities of free and immobilized enzyme were measured by recording the oxygen consumption in a thermostatted closed vessel (110 ml). A polarographic $O_2$ electrode (Consort Belgium) was used. The reactions were performed at 10°C in 10 mM Na-borate buffer, pH 9.0. The buffer was pre-equilibrated with oxygen to ensure aerobic conditions throughout the reaction. After filling the vessel with buffer and (immobilized) enzyme, the reaction was started by bubble-free injection of 100 $\mu$l K-linoleate (0.45 M in MeOH/water: 50/50), giving an initial linoleate concentration of ca. 410 $\mu$M in the reaction vessel. From the initial slope, allowing for the lag phase [15,16], the $V_{max}$ of the reaction was determined. The total amount of oxygen consumed corresponded to the amount of linoleate injected, indicating that indeed only the aerobic reaction took place. Activities were also determined by measuring HPOD (hydroperoxy-octadecadienoic acid) formation spectrophotometrically at 234 nm ($\epsilon_{234} = \pm 25500\ M^{-1}\ cm^{-1}$).

To determine the shelf-life of dissolved lipoxygenase, solutions of 2 mg/ml concentration were kept at 8 different conditions: pH 7.0 (10 mM phosphate buffer) and 9.0 (10 mM borate buffer), 5°C and 25°C and in the presence or absence of 20 $\mu$M linoleate, which converts to HPOD. The activity (at pH 9.0, 25°C) of these solutions was monitored during three days.

All immobilizations were performed at low temperature ($\pm 5$°C) in Na-acetate buffer, 10 mM, pH 7.0. The lipoxygenase solution was stirred with the support for about 3 hours. Hydrophobic materials were prewetted with ethanol and washed with water. Prior to the immobilization, the anion exchange supports were brought either in the $Cl^-$, $OH^-$ or $Ac^-$ (acetate) form and washed with water. The weights of these support are moist weights after centrifugation at $2000 \times g$.

After the immobilization, activity of the immobilized enzyme was measured. Additionally, the activity of the remaining dissolved, not immobilized, lipoxygenase was measured. To test the binding strength between the support and the enzyme, a number of supports with immobilized enzyme were washed with the borate buffer at pH 9.0.

Stock solutions of K-linoleate in MeOH/water were made freshly each week. The absorbance at 234 nm slowly increased suggesting oxidation of the linoleate and the formation of dienes. However, this was not more than 2% in a week at pH 9.0, either at 25°C or at 10°C. The concentration of oxidized linoleate in the reaction vessel at the start of an experiment was about 5%.

## 3. Results and discussion

### *3.1. Stability of freely dissolved lipoxygenase*

The activity decrease of freely dissolved lipoxygenase stored at different conditions was as follows:

- At pH 7.0, the activities decreased linearly with about 35% in three days both at 5°C and at 25°C. The presence of 20 $\mu$M HPOD — produced after addition of 20 $\mu$M linoleic acid —led to a decline with 50% at 25°C but not at 5°C.
- At pH 9.0, the activities decreased with about 20% in two days but more rapidly in the third day. Like at pH 7.0, the presence of 20 $\mu$M HPOD decreased the activity only at 25°C but not at 5°C.

## 4. Immobilized lipoxygenase

Immobilization on neutral microporous polymer beads showed that in contrast to e.g. lipase, lipoxygenase is rather sensitive towards immobilization. On the one hand, on hydrophobic polymers like polypropylene (Accurel, Akzo) or on more hydrophilic polyamide and also on activated charcoal the enzyme adsorbed completely but became inactive. On the other hand, hydrophylized polyethersulfone beads did not bind the enzyme at all.

However, it was found that ionogenic binding is possible with anionic but not with cationic ion

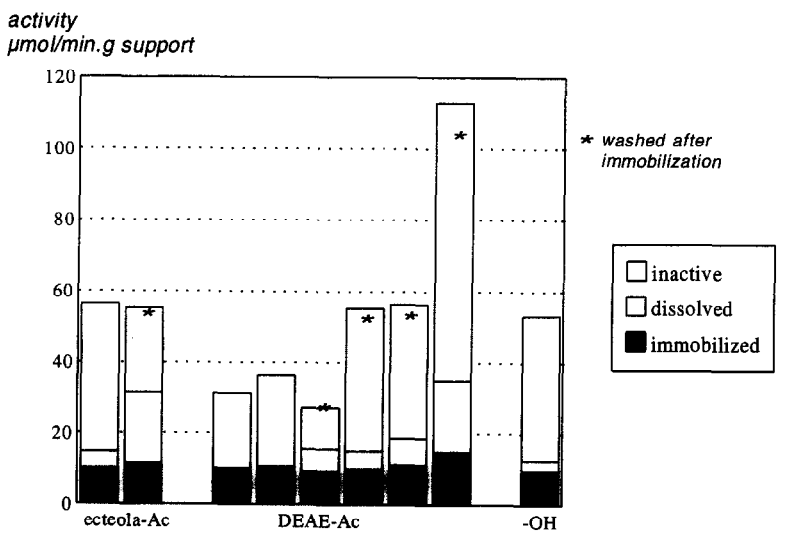


Fig. 1. Activity distribution of lipoxygenase immobilized on anion exchange supports. Although various amounts of lipoxygenase were immobilized, the activity of the module was the same. Ordinate: enzyme concentration expressed in activity per gram moist support. Inactive + dissolved + immobilized = total amount of lipoxygenase activity applied per gram carrier during immobilization. (1,2) ecteola-type carrier. (3–8) DEAE cellulose supports in $Ac^-$ form. (9) DEAE cellulose supports in $OH^-$ form. (*) Washed 6–8 times with Na-borate buffer, see text.

exchangers. The best performance was obtained with DEAE cellulose type supports. Fig. 1 shows the results of different experiments using different supports and different enzyme/support ratios. The ion-exchange supports were either in the acetate form or in the hydroxyl form, both yielding the same result. The chloride form did not bind lipoxygenase. Note, that the total added activity per mass immobilization matrix instead of amounts of enzyme are used at the ordinate.

In general, the degree of immobilization was fair as only 4–14% of the enzyme activity added initially remained in solution even after repeated washing with borate buffer. Only with ecteola-type support one-third of the enzyme activity was removed by washing. The sum of immobilized and non-immobilized activities was always less than the activity added initially to the support during the immobilization. In Fig. 1 the inactive, but immobilized, lipoxygenase is represented by the upper part of the bars. This enzyme (in) activity could be completely regained when the enzyme was desorbed from the immobilization matrix using concentrated salt solution. This suggests that the activity of lipoxygenase is lowered by mass transfer.

Surprisingly, in all preparations the immobilized activity was about the same, about 10–15 $\mu$mol $min^{-1}$ $g^{-1}$ carrier, irrespective of the enzyme/carrier ratio during immobilization. A partial deactivation of the bound enzyme would still lead to different immobilized activities at different enzyme loadings. However, a common explanation for both the decrease of the immobilized enzyme activity and the constant activity at various enzyme loadings might be diffusion limitation in the gel-like cellulose beads. This could lead either to a decrease of the substrate availability or to an increase of the product (HPOD) inhibition.

The stability of preparations 3–9 was measured again after 2 days. About 30–40% activity loss had occurred, indicating that the stability of the immobilized enzyme had not increased in these preparations.

## 5. Hybrid operation with packed bed and oxygenator

The immobilized enzymes were used in a packed bed and connected with a hydrophilized PSF membrane oxygenator (Fig. 2) (AGT Technology Corporation, Needham, USA, cutoff 30 kDa, surface area: 130 $cm^2$). The oxygenator provided mass transfer without the creation of inactivating gas–liquid interface (bubble formation). Initial reaction rates were approximately 2 mmol/g immobilized lipoxygenase. This is rather slower than the rates indicated in Fig. 1. From mass transfer considerations it is expected that mass transfer in the membrane does not limit the conversion. At the moment this set-up is being optimized and is under further investigation.

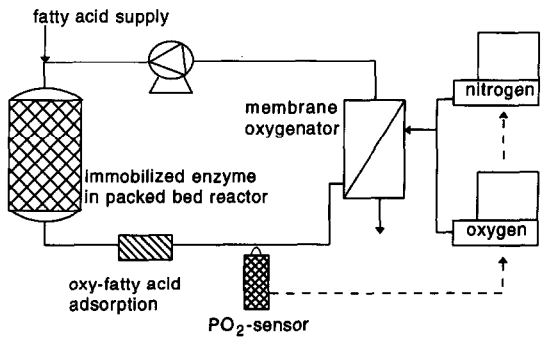


Fig. 2. Hybrid set-up for the continuous production of hydroperoxides. The oxy-fatty acid adsorber contains porous silica gel (No. 1, Crosfield Chemicals, UK [17]) and can be used for in situ recovery of hydroperoxides.

## 6. Conclusions

The inactivation of lipoxygenase on hydrophobic and moderately hydrophilic supports showed that the integrity of this enzyme is easily distorted. For immobilization the best results were obtained with DEAE anion exchange celluloses. The ionic form appeared to be important as the chloride form did not bind the enzyme but the hydroxyl and acetate form did. The maximal immobilized activity was 10–15 $\mu$mol $min^{-1}$ $g^{-1}$, and this figure was essentially independent of the amount of

enzyme added initially to the supports. This was possibly caused by the diffusion limitation, either of the substrate —linoleic acid —into the cellulose beads or of the product —HPOD —from the beads.

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