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Synthesis of Linoleic Acid Hydroperoxide Using Immobilized Lipoxygenase in Polyacrylamide Gel

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ABSTRACT

Lipoxygenase from soybean has been immobilized in polyacrylamide gel derivatized with glutaraldehyde as a means to facilitate obtaining hydroperoxy derivatives of linoleic acid. We have found that 10% acrylamide, 6% glutaraldehyde, and pH 8.0 are the best conditions for the coupling of enzyme to gel. Although catalytic efficiency is lowered by the coupling process, the stability of the system is maintained at a high level, and lipoxygenase products are not altered by the immobilization process. The use of the system in a reactor revealed that no effect of self-inactivation is produced by long-term reaction, thereby making feasible the use of a lipoxygenase bioreactor for the synthesis of lipoxygenase products.

Index Entries: Lipoxygenase; immobilization; polyacrylamide gel; bioreactor.

INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyze the dioxygenation of polyunsaturated fatty acids possessing a *cis,cis*-1,4-pentadiene unit to yield *cis,trans*-conjugated diene hydroperoxides (*1*). In animal tissues, lipid hydroperoxides function as precursors of specific tissue hormones, such as leukotrienes and lipoxins. These compounds play an important role in

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physiological reactions, such as the immune response and the inflammatory process, thereby making lipoxygenases of considerable medical interest (2).

The potential implication of leukotrienes and related lipoxygenase products as causative agents in various disease states has imposed a great demand for a supply of these naturally-occurring substances for proper evaluation of their biological importance. This demand, coupled with the difficulty of isolating quantities of these substances from natural sources, has led to preparing these products of the lipoxygenase pathway by totally organic synthesis (3). Lipoxygenase immobilization in solid supports may constitute an efficient and simpler way to obtain these lipoxygenase products of biological interest.

In this study we compare the activity of free enzyme with immobilized enzyme in polyacrylamide gel to evaluate the possibility of obtaining a suitable system for the synthesis of hydroperoxy derivatives of polyunsaturated fatty acids.

MATERIALS AND METHODS

Chemicals

Acrylamide, glutaraldehyde, linoleic acid, and soybean lipoxygenase were purchased from Sigma (St. Louis, MO). Toluene, chloroform, and Tween-20 were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Polymerization of Gels

A method based on that described by Mosbach et al. (4) was used. Gels were polymerized by mixing an aqueous phase (10% w/v acrylamide, 5% w/w methylenbisacrylamide, 0.5% v/v (TEMED), and 0.1% w/w ammonium persulfate in 100 m*M* phosphate buffer, pH 7.4) with an organic phase (toluene:chloroform, 73:20, and 0.25% v/v Tween-20). Both solutions were mixed by magnetic stirring for 30 min under nitrogen atmosphere. At the end of the incubation period, the gel was thoroughly washed with 200 vols of distilled water by the use of a vacuum filtration system. The gel polymerized as 100-μm-diameter microspheres. Microscopic observation of the gel revealed a high grade of uniformity in the preparation.

Activation of the Gel

Gel microspheres were incubated at 37°C for 48 h in the presence of 3 vols of 6% w/v glutaraldehyde in 100 m*M* potassium phosphate buffer, pH 7.0. At the end of the incubation period, unbound glutaraldehyde was removed by washing the gel with 100 vols of distilled water.

Enzyme-Gel Binding

Binding of protein to activated gel takes place during incubation of both components for 12 h at +4°C with gentle shaking. Protein concentration and buffers used in the incubation are indicated in the Results and Discussion section. Unbound protein was removed by washing the gel with 100 vols 100 m*M* borate or phosphate buffer.

Activity Measurements

Lipoxygenase activity was assayed by following the dissolved O_2 content using a Clark-type electrode and 0.63 m*M* sodium linoleate as substrate (*5*). The oxygen concentration in the medium corresponded to an air-saturated aqueous solution (0.276 μmol O_2/mL at 20°C). Enzymatic activity was also assayed spectrophotometrically by following the increase in absorbance at 234 nm produced by the transformation of the *cis,cis*-1,4 pentadiene system of linoleic acid into the conjugated hydroperoxydiene *cis,trans* derivative (*6*).

Protein Measurement

Protein concentration was determined by the method described by Lowry (*7*).

R-P HPLC Measurements

Reversed-phase HPLC of the lipoxygenase products was carried out by using a Milton Roy HPLC instrument equipped with a LiChrospher® RP-18 (5 μm) column (150 × 46 mm) operating at a flow rate of 0.5 mL/min. The solvent system is indicated in the text.

RESULTS AND DISCUSSION

Immobilization of the Enzyme

With the aim of finding the optimum conditions for polyacrylamide gel activation and protein immobilization, gel beads synthesized at several acrylamide concentrations, ranging from 2.5% to 30%, were derivatized with glutaraldehyde (6%) and incubated for 24 h in soybean lipoxygenase at a final concentration of 0.45 mg/mL for 24 h. Gel beads synthesized with 10% acrylamide and derivatized with 6% glutaraldehyde bound proteins most efficiently.

The effect that pH has on coupling of lipoxygenase to the gel was evaluated. Enzymatic activity was measured in the gel bead and in the solution before and after the period of incubation (24 h). The evaluation of the coupled protein at several pH values reveals that maximum coupling

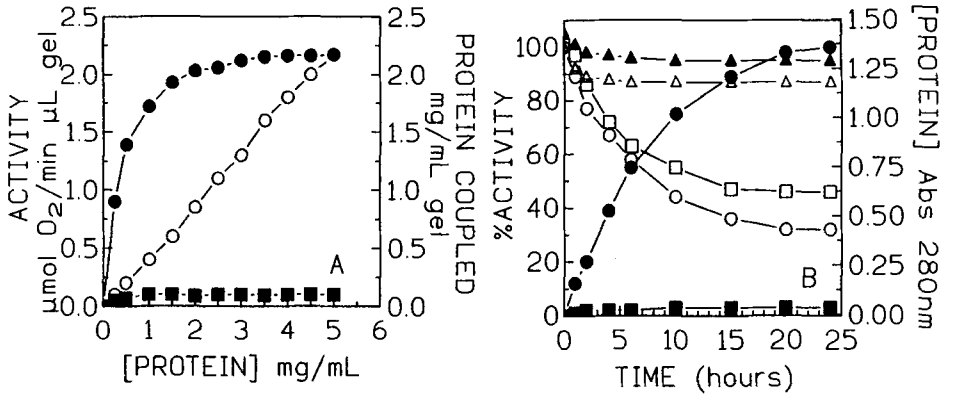

Fig. 1. (**A**) Enzymatic activity (gel beads activated [○] and without activation [■]) and protein concentration of lipoxygenase coupled to gel (●) as a function of the protein concentration in the medium. Two mL enzymes were incubated at the concentration indicated in 100 m*M* borate buffer, pH 8.0, for 24 h at 20°C in the presence of 2 mL polyacrylamide gel (10% acrylamide, 6% glutaraldehyde). Protein concentration of lipoxygenase coupled to gel was determined as the difference between protein concentration in the medium before and after the incubation process. (**B**) 2 mL of soybean lipoxygenase (1.6 mg/mL) were incubated in the presence of 2 mL 10% acrylamide gel beads in 100 m*M* borate buffer, pH 8.0, at 4°C. Lipoxygenase activity was measured by oxygen consumption. One hundred percent activity corresponds to 0.3 μmol O_2/min/μL gel for immobilized enzyme (●) and 2.0 μmol O_2/min/μL solution for enzyme in the medium (○). Enzymatic activity was measured, as well, in the medium (△) and in the gel beads (■) for gel without activation. The time course of protein concentration in the medium was followed in the presence of activated gel (□) or in the presence of gel without activation (▲).

efficiency was achieved in the pH 8.0–9.0 range. Typically, in the experimental conditions described in Materials and Methods, 49% of the initial concentration of protein was coupled to gel. As results of these findings, 10% acrylamide, 6% glutaraldehyde, and pH 8.0 were chosen as the best conditions for immobilization.

The effect of protein concentration in the incubation medium on the final amount of protein coupled to gel is shown in Fig. 1A. It is to be noted that, although the incorporation of protein into the gel appears as a linear pattern, the shape of the activity profile is hyperbolic, showing a $K_{0.5}$ value near 0.4 mg/mL.

The time course of the coupling reaction was followed for 24 h (Fig. 1B). Protein concentration in the medium and enzymatic activity was determined. It is apparent that loss of activity results by the coupling reaction, because a decrease in protein concentration in the absence of glutaraldehyde is minor, compared with that resulting in the presence of glutaraldehyde. The loss of the lipoxygenase activity in the medium and the increase in the enzymatic activity of the gel is shown in Fig. 1. Both processes exhibit a $T_{1/2}$ of approximately 6 h.

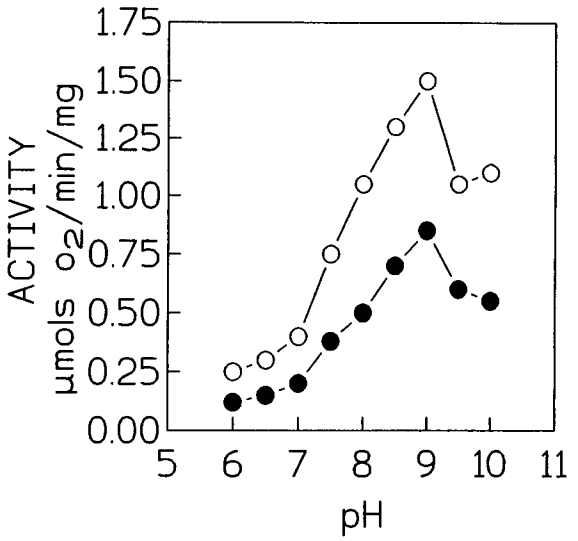

Fig. 2. Effect of pH on the activity measurement. Lipoxygenase activity was measured by oxygen consumption, as is indicated in the Methods section. Filled and open symbols stand for immobilized and free enzymes, respectively. Buffers used were 100 m*M* borate, pH 8.0 to pH 10.0; m*M* phosphate, pH 6.0 to pH 8.0.

Characterization of the Immobilized Lipoxygenase-Support System

To determine if our experimental conditions of immobilization modify the activity-pH profile, lipoxygenase activity for free and immobilized enzyme was measured (Fig. 2). Significant changes do not appear to be introduced by the immobilization process, since activity-pH profiles for free and immobilized enzymes are unchanged. In contrast, the evaluation of the comparative effect of the temperature (Fig. 3) reveals that the immobilization process provides protection against thermal inactivation at temperatures over 35°C. On the other hand, the energy-of-activation values obtained (19.3 kJ/mol for immobilized enzyme and 22.8 kJ/mol for free enzyme) strongly suggest that difussion effects are minimized; thus, the system would be mainly under kinetic control (*4*).

The effect that linoleic acid has on the coupling process (Table 1), and the kinetic parameters obtained for the immobilized enzyme (Table 2), suggest that the coupling of enzyme-matrix takes place near the catalytic center; however, this interaction does not induce a serious unstability in the system.

Characterization of the Products

Soybean lipoxygenase produces 9- and 13-hydroperoxy derivatives from linoleic acid in a 96:4 ratio (*8*). This ratio has been determined by

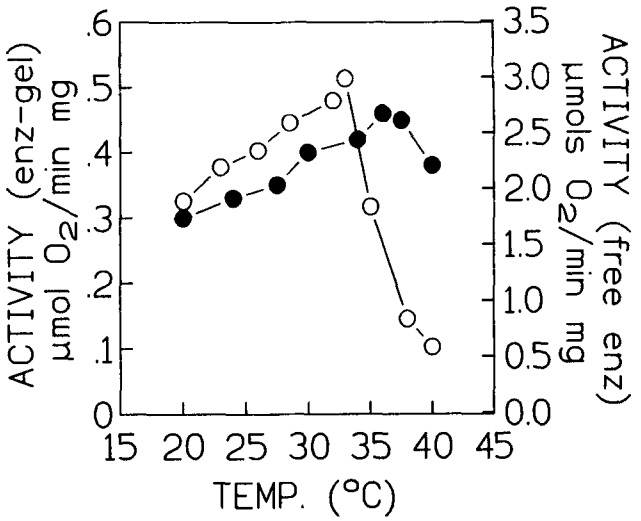

Fig. 3. Effect of temperature on immobilized lipoxygenase activity. Open and filled symbols correspond to free and immobilized enzymes, respectively.

Table 1
Effect of Linoleic Acid Concentration on the Coupling Lipoxygenase Gel[a]

Linoleic acid, μM	0	9	18	90	900
Coupled protein, mg/mL gel	0.30	0.26	0.21	0.18	0.15

[a]0.7 mg/mL of soybean lipoxygenase were incubated, as is described in Materials and Methods, in the presence of polyacrylamide gel activated with 6% glutaraldehyde in 100 m*M* borate buffer, pH 8.0.

Table 2
Kinetic Parameters of Free and Immobilized Lipoxygenase[a]

	K_m, m*M*	V_{max}, μmol O_2/min/mg	K_{cat}, s^{-1}	K_{cat}/K_m, $s^{-1}\ M^{-1}$
Free enzyme	0.077	6.25	10.40	1.35×10^5
Immobilized enzyme	0.145	2.08	3.46	2.30×10^5

[a]Enzymatic activity was measured by oxygen consumption by the incubation of 0.5 mg/mL of protein, using linoleic acid as substrate in 100 m*M* borate buffer, pH 9.0.

several methods, including high-performance liquid chromatography of underivatized forms of isomeric fatty acids (*9*). Figure 4 shows the chromatograms of products from free (4A) and immobilized (4B) soybean lipoxygenase. When peaks from 4-A and 4-B were collected, pooled, and chromatographed, only one peak was obtained (data not shown). These data suggest that the immobilization process does not modify the position of the insertion of the hydroperoxy group in the linoleic acid molecule and, additionally, that the ratio of 9-:13-hydroperoxy derivative is unchanged with regard to free enzyme.

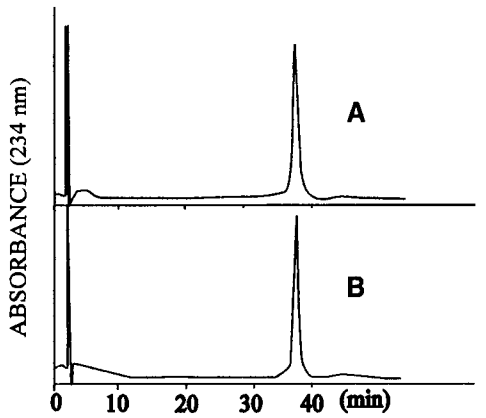

Fig. 4. High-performance liquid chromatogram of the hydroperoxides produced by free (**A**) and immobilized (**B**) lipoxygenase during the incubation in the presence of linoleic acid. Free lipoxygenase (0.01 mg/mL) or immobilized enzyme (0.5 mg/mL) was incubated in the presence of 0.8 m*M* linoleic acid in 100 m*M* borate buffer, pH 9.0, at room temperature for 15 min. The reaction was stopped by adding 0.5 vols of ice-cold methanol to the incubation mixture. In the case of immobilized enzyme gel, microspheres were removed from the medium by centrifugation. In both cases, the products of the reaction were obtained by acidification to pH 3.0 with HCl and extraction with diethyl ether followed by evaporation under nitrogen stream. The sample was chromatographed into a C-18 reversed-phase column using methanol:water:acetic acid (75:25:0.1) as solvent system.

Stability of the System

A self-catalyzed inactivation of lipoxygenase has been reported by several investigators. They found that lipoxygenase activity decreases before oxidation of all the substrate and that fresh enzyme was needed to restore activity. This process is dependent on the type of substrate used. The dienoic fatty acids, 18:2n-6 and 20:2n-6, are the least effective "suicide substrates," and 20:5n-3 and 22:6n-3 are the most effective (*10*). The possible occurrence of this process in our experimental conditions has been checked by determining the lipoxygenase activity of gel beads by oxygen consumption measurements. When the substrate was exhausted, the gel beads were removed from the medium by filtration and then resuspended in fresh buffer. Immediately after, new substrate was added and enzymatic activity was measured. This process was repeated up to 20 times and no significant differences in the enzymatic activity were detected. When immobilized lipoxygenase was loaded into a tubular bioreactor, no enzyme self-inactivation was detected. It can be seen that the slopes of the increase of absorbance at 234 nm, when substrate is added to the medium, do not change (Fig. 5).

Finally, we have checked the stability of the free and immobilized enzyme in different storage conditions (Table 3). The immobilization process does not significantly affect the stability of the enzyme when stored

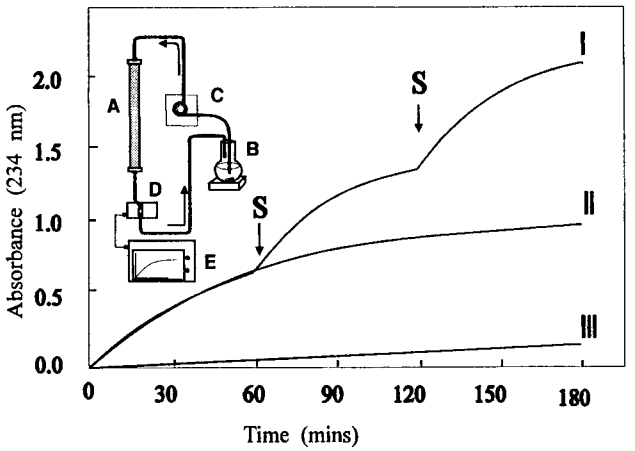

Fig. 5. Time course of hydroperoxide production by a bioreactor of immobilized soybean lipoxygenase. Gel beads, 2.6 mL, were loaded into a column (0.7 × 7.0 cm). The system (shown in the insert) was used in continuous mode at a flow rate of 2.5 mL/min at 20°C in the presence of 0.035 m*M* linoleic acid in 50 m*M* borate buffer, pH 9.0. Substrate (plus the product formed) is loaded from recipient (B) into the column (A) by a peristaltic pump (C). The absorbance at 234 nm of the eluent from the column is continously checked by a UV monitor (D) coupled to a recorder (E). The process was followed with (I) or without (II) the addition of 0.035 m*M* substrate (S). Auto-oxidation of substrate (III) has been determined using gel beads without lipoxygenase coupled.

Table 3
Effect of the Days of Storage on Enzymatic Activity
of Free or Immobilized Lipoxygenase at Different Temperatures

	Lipoxygenase activity, %			
Days of storage	Free (+4)[a]	Free (−70)[a]	Coupled (+4)[b]	Coupled (−70)[b]
0	100	100	100	100
5	80	90	95	98
10	62	87	80	90
20	50	80	68	85
45	28	70	60	83

[a]Lipoxygenase activity measured as % for samples of free lipoxygenase in solution stored at +4°C or −70°C.

[b]Lipoxygenase activity measured as % for samples of immobilized enzyme stored at +4°C or − 70°C.

at −70°C; however, at +4°C, the immobilized enzyme shows a higher level of activity than free enzyme. It is noted that the enzymatic activity of immobilized enzyme is 22% higher than free enzyme after 10 d storage, and 32% higher after 45 d storage. This behavior may be explained by the existence of a stabilization induced by the interaction between the enzyme and the support, which protects against a possible alteration of the three-dimensional structure of the protein (*11*).

CONCLUSIONS

The first aim of this work has been to establish the best conditions for lipoxygenase immobilization and the characterization of the lipoxygenase-support system, which allows us to obtain lipoxygenase products in a bioreactor.

The best conditions for protein coupling were obtained at pH 8.0, using 6% glutaraldehyde and 10% arcylamide. In these conditions, 49% of the initial protein concentration in the medium is bound to gel, and the specific activity of gel beads when the incubation period finished was 42% of the initial value. A possible cause of the observed loss of activity may be that gel is coupled to the enzyme in a position near the catalytic site. To check this possibility, we have performed lipoxygenase-gel coupling in the presence of linoleic acid. Table 1 shows that linoleic acid causes an evident interference in the coupling process. These data suggest that the residue of *Lys* which bind glutaraldehyde may be placed near the catalytic site. Although the specificity of the coupling reaction is low, because it can take place at any free amino group, the binding near the catalytic site is supported by the protective effect that immobilization induces on the inhibitory effect of nordihydroguaiaretic acid (NDGA). We have found (data not shown) that enzymatic activity of immobilized lipoxygenase in the presence of 5 m*M* NDGA is 40% higher than obtained with the same concentration of free enzyme in the presence of 5 m*M* NDGA. Considering that this inhibition is produced by a reduction of the active ferric enzyme to the inactive ferrous form (*9*), a possible blockade of the access of inhibitor to the catalytic site may be produced by a coupling enzyme-support through an amino acid near the catalytic site.

On the other hand, the possible interaction enzyme-matrix does not appear to modify the ratio between 13- and 9-hydroperoxy derivatives from linoleic acid. In experimental conditions similar to those used in our case (pH 9.0 and RP-HPLC of underivatized products), this ratio previously has been established as 96:4 (*1*). Our data shows that RP-HPLC chromatograms of enzymatic products from free and immobilized enzyme are totally coincidental, and, consequently, no major changes should be introduced to the immobilization process in the ratio or chemical structure of the products.

Finally, we have found that lipoxygenase activity is not modified when subject to repeated cycles of substrate-product conversion. Likewise, when gel beads were loaded into a column, as a model of a tubular reactor operating in continuous mode, the initial rates obtained were very similar to the rates when fresh substrate is added to the system. This suggests that there is no effect of the product on lipoxygenase activity.

In conclusion, the data show that soybean lipoxygenase is suitable to be immobilized in a polyacrylamide support, maintaining kinetic characteristics close to that of free enzyme and a stability level that allows

its use in a bioreactor for the preparation of hydroperoxy derivatives from polyunsaturated fatty acids.

ACKNOWLEDGMENTS

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