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Biofunctional membranes Part IV. Active-site structure and stability of an immobilized enzyme, papain, on modified polysulfone membranes studied by electron paramagnetic resonance and kinetics

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Abstract

Biofunctional membranes are entities in which biological molecules (or cells) are attached to polymeric supports cast in the form of porous membranes. Such membranes are gaining increased importance in applications of enzymatic catalysis or synthesis (bioreactors), separations (affinity membranes), and chemical analysis (biosensors). However, fundamental studies of the active site of immobilized biomolecules have been rare. In this study, electron paramagnetic resonance (EPR) spin-labeling techniques using a short, active-site specific spin label have been employed to study the properties of a model enzyme, papain, immobilized on a fully-hydrated, modified polysulfone membrane. The EPR properties of the immobilized enzyme and reaction rate results using the amidase activity of papain and *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride as substrate are compared with that of the free enzyme in solution. The major findings in this study are: (1) Immobilization does change the active-site conformation of papain. The spin label at the active site of the immobilized papain has slower motion than that of the free papain in solution. (2) There are two major subpopulations of immobilized enzyme on modified polysulfone membranes: subpopulation *A* has faster spin-label motion at the active site than subpopulation *D*, suggesting that the enzyme in subpopulation *A* may have a more open active-site cleft than that of the subpopulation *D*. (3) The active-site conformation of subpopulation *D* of the immobilized papain is insensitive to the pH of the bulk solution, while that of subpopulation *A* has a similar response to pH changes as that of free papain in solution, suggesting that subpopulation *A* may be the active form of the immobilized enzyme while subpopulation *D* is the denatured form. (4) The pH-dependent curve of the amidase activity of the immobilized papain has a similar bell shape as that of the free papain in solution. (5) Subpopulation *A* is converted into subpopulation *D* upon addition of denaturants (urea or guanidine hydrochloride), which further confirms the assumption that the former subpopulation is the active form of the immobilized enzyme and the latter subpopulation is the denatured form. Moreover, denaturation at elevated temperature can also convert subpopulation *A* into subpopulation *D*. (6) By paramagnetic relaxation mechanisms, which require close proximity of the paramagnetic species and the spin label, $K_3Fe(CN)_6$ can broaden the EPR signal of spin label bound to subpopulation *A* but not that of subpopulation *D*, suggesting that subpopulation *A* is accessible to the substrate. More direct evidence that

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subpopulation *A* is the active form of the immobilized enzyme and more accessible to substrate than subpopulation *D* was obtained by immobilizing papain on modified polysulfone membranes first followed by spin labeling. In this way, only the active subpopulation *A* was labeled by the spin label. (7) The K_m(app) values of the immobilized papain are larger than that of papain in solution, while V_{max}(app) is smaller. (8) The enzyme is highly reusable and has very high storage stability after immobilization. (9) Papain immobilized on polysulfone membranes has much higher thermal stability and stability toward denaturants (urea or guanidine hydrochloride) than that of free papain in solution. In this study the polymeric membrane provided three functions: sites for covalent coupling of enzymes; enhanced enzyme stability; and substrate partitioning. This paper reports the first development of an effective method to acquire EPR spectra of fully-hydrated, spin-labeled enzymes immobilized on polymeric membranes. All these findings indicate that the EPR spin-labeling technique shows great promise as a powerful method for studying membrane-immobilized enzyme systems.

Key words: Biofunctional membranes; Spin-labeling; Membrane-bound enzymes

1. Introduction

The applications of enzymes immobilized on polymeric supports have increased substantially in recent years. Immobilized enzymes provide advantages over their corresponding soluble forms including their ease of recoverability and reusability. In order to most efficiently utilize such systems, it is important to know the relationship of the structure of the enzymatic active site to the immobilization. However, study of the conformation of immobilized enzymes is hindered by the spectroscopically opaque supports used in immobilization. One technique that can provide significant insights into the behavior of immobilized enzyme systems is electron paramagnetic resonance (EPR) spectroscopy. EPR measurements do not require optically transparent samples, a variety of spin labels for specifically labeling different protein active sites are either commercially available or can be often readily synthesized, and the EPR spectrum of a spin-labeled molecule is very sensitive to the label's microenvironment. Thus, the EPR spin-labeling technique has considerable potential for clarifying the behavior of the active site of enzymes in environments that deviate significantly from dilute aqueous solutions.

Berliner et al. pioneered the application of EPR methods for studying immobilized enzymes [1]. Trypsin was spin-labeled with 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate either before or after covalent immobilization on porous glass beads. The most striking feature found was that the active-site conformations were identical whether the enzyme was labeled before or after the immobilization process which was an indication of equivalent spin-label accessibility to the enzyme active site in both conditions. This is also true for the study of immobilized glutamate dehydrogenase on a sepharose support [2].

Clark and his co-workers extended the EPR methods in studying immobilized enzyme systems [3–10]. These workers studied the structure–function relationships of immobilized α-chymotrypsin on CNBr-activated sepharose beads [3–5,7]. Two distinct subpopulations of immobilized enzymes with more restricted motion of spin labels in the active site were revealed by the EPR spin-labeling technique. Both subpopulations were relatively inaccessible to solvent which was reflected by the difficulty in broadening the EPR signals with $K_3Fe(CN)_6$ [4]. The specific activity of the more constricted immobilized enzyme active form was shown to be ~15 times smaller than that of the other class of immobilized enzyme molecules, and variations in overall specific activity of formulations with different loadings and different supports resulted entirely from changes in the proportions of the same two subpopulations of immobilized enzyme molecules [4]. Clark and Bailey [5] also showed that immobilization greatly enhanced the stability of α-chymotrypsin in 50% *n*-propanol. The deactivation kinetics were complex and

could not be described by a simple first-order model. A model with three immobilized enzyme states (*A*, *B*, and catalytically inactive forms) was proposed to explain the deactivation kinetics [5]. EPR methods were used by Clark and co-workers to examine other bead-immobilized enzyme systems as well, including immobilized bovine liver β-galactosidase [6] and immobilized horse liver alcohol dehydrogenase (LADH) [6,7,9,10]. The immobilized forms of both β-galactosidase and LADH on either CNBr-activated sepharose 4B or Amberlite CG-50 had the characteristics of extremely restricted spin-label motion and lower specific activity than the soluble forms of the enzyme [6]. However, immobilization enhanced the thermostability of LADH [8]. Covalent and non-covalent immobilization methods were also compared: absorption of LADH to octyl-sepharose CL-4B (a hydrophobic matrix in which eight-carbon spacer arms are attached through ether linkages to the surface of sepharose CL-4B) perturbed at least two separate regions of the enzyme's site, whereas covalent immobilization to CNBr-sepharose influenced only one of these [8–10].

Thermal inactivation of immobilized enzymes (α-amylase, chymotrypsin, and trypsin covalently bound to silica, polystyrene, or polyacrylamide) showed biphasic kinetics, which distinctly differed from the first-order inactivation kinetics of the corresponding soluble enzymes [11]. These kinetics data combined with EPR findings indicated that there were two major subpopulations in each immobilized enzyme systems [11].

Asakura and co-workers also used spin-labeling methods to study silk fibroin membranes and glucose oxidase entrapped in silk fibroin membranes by different entrapment methods [12,13]. It was found that the conformation and the thermal and pH stabilities were essentially the same among glucose oxidase immobilized silk fibroin membranes entrapped by different methods [12,13].

Clark et al. found that the active site of α-chymotrypsin was relatively inaccessible to solvent based on $K_3Fe(CN)_6$ broadening of EPR signals [4]. However, Song and co-workers used two methods to reinvestigate solvent accessibility to the active site of the immobilized α-chymotrypsin: the first was the same as that used by Clark et al., the second approach utilized a kinetic method, i.e., ascorbate reduction of the nitroxide group [14]. Though the first approach yielded similar results as that obtained by Clark et al., the kinetics of ascorbate reduction from the second approach suggested that both spectral subpopulations had identical accessibilities to the bulk solvent [14].

Immobilized papain on porous silica beads, with spacers of two different lengths (1,6-diaminohexane, spacer 6; 1,2-diaminoethane, spacer 2), was also studied by Telo et al. using *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide as the spin label [15]. Though specific activity and mobility decrease strongly with immobilization, the polarity of the active site was shown to be similar to that of water. Within immobilized papain, a lower specific activity corresponded to a higher mobility suggesting unfolding of the protein [15]. This is exactly opposite to the results of Clark and Bailey on α-chymotrypsin [4].

As far as we could determine, almost all immobilized enzymes studied by EPR methods were on supports in the form of beads. None of the studies mentioned above involved covalently immobilized enzymes on polymeric membranes. Yet, membrane-immobilized enzymes, antibodies, or other proteins are becoming increasingly important for separations (affinity membranes), detection of key analytes (biosensors), and catalysis (bioreactors). Consequently, it is important to understand how the conformation of the active site of the protein is affected by immobilization to the membrane in order to maximize structure–function efficiency. We have chosen a model system to begin this process. The thiol protease, papain, has been covalently immobilized onto porous polysulfone/aldehyde blend membranes.

We previously reported the first spin-labeling studies of papain immobilized on vinyl alcohol/vinyl butyral copolymer membranes [16–18], but these systems were not fully-hydrated, were largely composed of rather dense membranes,

and employed a lengthy spin label whose reporter nitroxide group was external to the active-site cleft of the enzyme. The current research is the first extensive study of enzyme behavior on fully-hydrated, porous polymeric membranes using the EPR spin-labeling technique. A short, cysteine-specific spin label (MTS) that labels the only SH group of papain located at the active site and whose nitroxide reporter group was within the active-site cleft was employed [19]. The effects of pH, denaturants (urea and guanidine hydrochloride) on the active-site conformation of papain immobilized on modified polysulfone membranes have been studied. Thermal stability and reusability of the immobilized papain, as well as the solvent accessibility to the active site of the immobilized papain, have also been investigated. The amidase activity of the immobilized papain was measured in order to determine if a correlation between the EPR results and the activity measurements exists.

2. Experimental

2.1. Materials

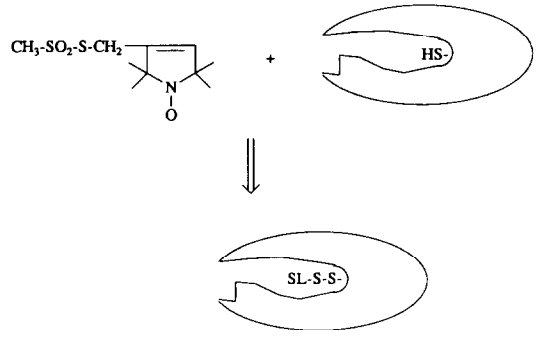
The modified polysulfone blend membrane, UltraBind™ US450, was purchased from Gelman Sciences. It has aldehyde functional groups on the membrane surface and, according to the manufacturer, has an average pore size of 0.45 μm and a thickness of 152.4 μm. Sucrose was obtained from ICN. Dried papaya latex, *N*-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA), DL-cysteine, and guanidine hydrochloride were obtained from Sigma Chemical Company. Urea was obtained from BDH Chemicals Ltd., while disodium ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were obtained from Aldrich. The spin label (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiosulfonate (MTS) was purchased from Reanal, Budapest, Hungary. The ultrafiltration membrane with a 10,000 MW cut-off, used for concentrating MTS-labeled papain, was obtained from Millipore Company. All other chemicals and solvents were reagent grade.

2.2. Methods

Immobilization of MTS-labeled papain/papain on polysulfone membrane

Papain was purified from dried papaya latex and then labeled with the MTS spin label at the active site as described previously [19]. In particular, the reaction of MTS with the single cysteine of papain to form a covalent disulfide is illustrated in Scheme 1. MTS-labeled papain was concentrated by ultrafiltration to ~3–4 mg/ml protein content, estimated by the method of Lowry et al. [20]. Modified, flat sheet, polysulfone membrane was used for all experiments. The membrane was cut into pieces each with an area of 4×4 cm. The protein was immobilized by reaction of free NH_2 groups of lysine and arginine on the enzyme with the aldehyde group of the modified polysulfone membranes resulting in a stable imine [18]. The extent of papain immobilization was ~0.5–0.6 g/m^2.

For EPR studies, one piece of membrane was incubated with 3 ml of MTS labeled papain solution for 24–36 h and then the membrane was washed extensively with 5P8 (5 m*M* phosphate buffer, pH 8.00) to remove the unreacted protein. The resulting membrane was cut into four 1×4 cm pieces. For activity measurements, the protein content of the papain stock solution was estimated by the method of Lowry et al. [20] and adjusted to 8 mg/ml. One piece of membrane was incubated with 2 ml of papain solution for 24–36 h. Deionized water (2 ml) was added to the



Scheme 1. A schematic illustration of the reaction between the MTS spin label and the single cysteine group of papain located at the active site.

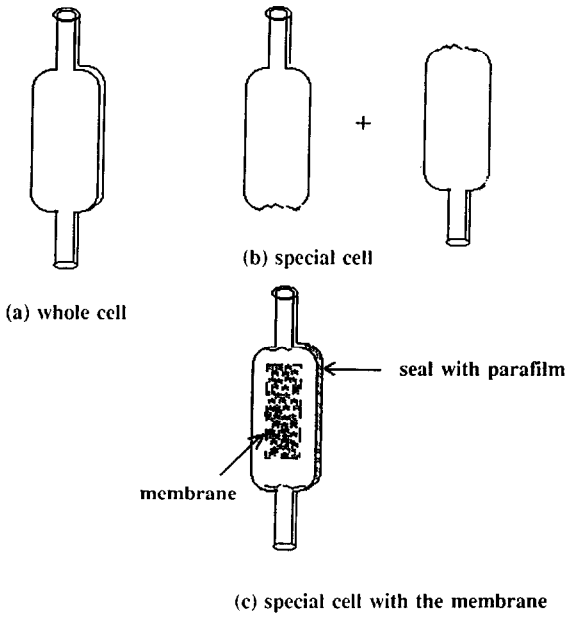
membrane system and the solution was homogenized by shaking. The protein concentration of the incubating solution was again estimated as above and the difference of the protein contents between the papain solution prior to incubation and that after incubation is the amount of papain immobilized on the polysulfone membrane. The membrane was then cut into four 2×2 cm pieces for enzymatic analysis.

EPR spectra acquisition

All the EPR spectra of the polysulfone membranes with immobilized MTS-labeled papain were recorded on a Bruker ESP-300 spectrometer with a TM rectangular cavity at room temperature, using a special quartz EPR cell. The cell is composed of two pieces, the front piece and the back piece. The membrane was taken directly from the buffer or solution in which it was stored or incubated, and laid between the front and back pieces of the cell. The edges of the cell were sealed with parafilm and buffer or solution was added into the cell if necessary to make sure the membrane was immersed in the buffer or solution (see Scheme 2). The use of this cell not only ensured that the membrane and the membrane-bound enzyme were fully hydrated, but also provides a mean of rapidly tuning the EPR instrument, i.e., it minimized microwave dielectric loss. Typical parameters for the acquisition of the EPR signals were the following: microwave frequency 9.78 GHz; microwave power 24.2 mW; modulation frequency 100 kHz; modulation amplitude 0.32 G; and scan width 130 G.

Assay for the amidase activity of papain immobilized on polysulfone membrane

A BAPNA (substrate) stock solution (2 m*M*) in 50 m*M* Tris (Tris buffer, pH 7.5), was prepared by dissolving 43.5 mg BAPNA in 1 ml DMSO, which was then dispersed into 49 ml of Tris. Thus the final DMSO content in the substrate stock solution was 1% (v/v). Papain exists in an inactive form until the active-site cysteine is activated [18]. Accordingly, an activating solution, 20 m*M* cysteine and 4 m*M* EDTA in 50 m*M* Tris, was also prepared. In order to determine the appropriate activation time,



Scheme 2. A schematic illustration of the EPR quartz cell: (a) intact cell, (b) special cell used in this study, (c) special cell with the membrane enclosed.

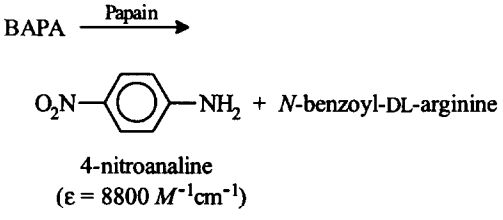
each 2×2 cm piece of polysulfone membrane with immobilized papain was activated in 1 ml of 0.1 *M* Tris containing 10 m*M* cysteine and 2 m*M* EDTA for different time periods at 37°C. Then 1 ml of substrate (final substrate concentration: 1 m*M*) was added and reacted for 15 min in a 37°C water bath with agitation. The amount of 4-nitroaniline (Scheme 3) released was estimated spectrophotometrically at 410 nm ($\epsilon = 8800\ M^{-1}\ cm^{-1}$). The final volume of the assay reagents is 2 ml instead of 1 ml used in the assay for free papain in solution [19]; thus, for the convenience of later comparison, all the calculated activities of membrane-immobilized papain are multiplied by a factor of 2. The appropriate time period to activate papain was found by activating the immobilized papain at 37°C for different time periods and then measuring the activity. The results are shown in Table 1. Ten minutes was found to be sufficient to fully activate the enzyme since the activities for longer activation time periods remained constant. Therefore, a 10 min activation time was chosen for all

Table 1
Effect of the activation time period on the measurement of the amidase activity of immobilized papain on polysulfone membranes

Activation time (min)	Activity (M/mg min)
10	27.3±0.3[a]
60	27.1±0.2
110	27.8±0.2
135	27.2±0.4

[a]Means ±S.D. are presented (N=3).

the activity measurements of immobilized papain on polysulfone membranes.



Scheme 3.

In the assay procedure, all the solutions used in this assay were prewarmed to 37°C in the water bath. Each 2×2 cm piece of polysulfone membrane with immobilized papain was activated in 1 ml of the activating solution containing 10 mM cysteine and 2 mM EDTA for 10 min at 37°C with agitation. Then different amounts of substrate (final substrate concentration: 0.01–1 mM) were added and the final volume of the reaction mixture was adjusted to 2 ml with 50 mM Tris. The reaction mixtures were incubated for 15 min at 37°C with agitation. The amount of 4-nitroaniline released was estimated spectrophotometrically as above. The kinetic parameters, K_m(app) and V_{max}(app), were obtained from the Lineweaver–Burk plot of the experimental data.

Active-site accessibility of the immobilized papain

Two methods have been employed to study the solvent accessibility to the active site of the immobilized enzyme. In the first approach papain was first immobilized onto the membrane, as described above, and activated in 2 mM sodium cyanide and 0.8 mM EDTA for 2 h at 4°C. Excess MTS spin label in 0.1 ml of acetone was then added to the membrane system and reacted for 1 h at 4°C. Excess spin label was removed by washing extensively in 5P8 with stirring (the washing lasted two days and more than 15 changes of 5P8).

The second approach, paramagnetic broadening of the EPR signals by $K_3Fe(CN)_6$, is an equilibrium method. Each 1×4 cm piece of polysulfone membrane with immobilized MTS-labeled papain was incubated in $K_3Fe(CN)_6$ solution of different concentrations (0–500 mM) for 30 min at room temperature; the EPR spectrum was acquired subsequently.

pH studies

The buffers (0.1 and 0.2 M) used in the pH study were: acetate buffer for pH 3–5, phosphate buffer for pH 5–8, and Tris buffer for pH 9–10. In the study of the effect of pH on the active-site conformation of immobilized papain on polysulfone membranes, each 1×4 cm piece of polysulfone membrane was incubated in 0.1 M buffer of different pH (pH 3.03–9.71) for 15 min at room temperature before the EPR spectrum was acquired. The distance between the high-field and low-field resonance peaks, $2A'_{zz}$, was used as the parameter for the motion of the spin label at the papain active site [21,22]. The larger (smaller) this splitting parameter, the slower (faster) the motion of the MTS spin label in the active site of papain.

When measuring the papain activity under conditions of different pH, the activating solution and the substrate solution were prepared as described above except in deionized water. Each 2×2 cm piece of polysulfone membrane with immobilized papain was incubated in 0.5 ml 0.2 M buffer of different pH (pH 3.03–9.81) at 37°C for ~5 min. Then 0.5 ml of activating solution was added and papain was activated in 10 mM cysteine and 2 mM EDTA for 10 min. Substrate solution (1 ml) was then added and reacted at 37°C for 15 min with agitation (final substrate concentration was 1 mM BAPNA). The amount of 4-nitroaniline released was estimated spectroscopically as noted above.

Denaturant studies

The effects of denaturants, urea and guanidine hydrochloride, on the active-site conformation and the amidase activity of immobilized papain on polysulfone membrane were studied. Urea (10 *M*) guanidine-HCl (8 *M*) stock solutions in deionized water were prepared, respectively. In the conformational studies, each 1×4 cm piece of polysulfone membrane with immobilized MTS-labeled papain was incubated in urea (0–8 *M*) or guanidine-HCl (0–6 *M*) solutions of different concentrations for 30 min at room temperature before the EPR spectrum was acquired. When measuring the papain activity under different concentrations of urea or guanidine-HCl, the activating solution and the substrate solution were prepared as described above. Each 2×2 cm piece of polysulfone membrane with immobilized papain was incubated in 0.5 ml of different concentrations of urea (0–8 *M*) or guanidine-HCl (0–6 *M*) solutions at 37°C for ~5 min with agitation. Then 0.5 ml of activating solution was added and the immobilized papain was activated in 10 m*M* cysteine and 2 m*M* EDTA for 10 min. Substrate solution (1 ml) was then added and reacted at 37°C for 15 min with agitation (final substrate concentration was 1 m*M* BAPNA). The amount of 4-nitroaniline released was estimated as above.

Thermal stability studies

In the EPR spin-labeling study, each 1×4 cm piece of polysulfone membrane with immobilized MTS-labeled papain was incubated in 0.1 *M* phosphate buffer, pH 7.00 at 70°C in a water bath for different time periods (0–25 min); the membrane was taken out and cooled to room temperature before the spectrum was acquired.

In the study of thermal stability of the amidase activity of immobilized papain, each 2×2 cm piece of polysulfone membrane with immobilized papain was incubated in 50 m*M* Tris at 70°C in a water bath for different time periods (0–86 min); the membrane was taken out and cooled to room temperature in 50 m*M* Tris. The treated membranes were prewarmed to 37°C. Tris (0.5 ml) and 0.5 ml activating solution were then added and agitated for 10 min. Substrate solution (1 ml) was then added and reacted at 37°C for 15 min with agitation (final substrate concentration was 1 m*M* BAPNA). The amount of 4-nitroaniline released was estimated spectrophotometrically as above.

Reusability study

One piece of polysulfone membrane with immobilized papain was activated in 1 ml of 50 m*M* Tris containing 10 m*M* cysteine and 2 m*M* EDTA at 37°C for 10 min with agitation. Then 1 ml of substrate solution was added and reacted at 37°C for 15 min with agitation. The amount of 4-nitroaniline released was estimated as noted above. The same piece of membrane was transferred to another 2 ml of reaction mixture containing all the reagents for the assay (50 m*M* Tris containing 5 m*M* cysteine, 1 m*M* EDTA and 1 m*M* BAPNA) immediately after the first reaction. Again, the amount of 4-nitroaniline released was estimated as above after 15 min reaction at 37°C with agitation. The process was repeated 18 times.

Storage stability

Pieces (1×4 cm) of polysulfone membrane with immobilized, MTS-labeled papain were stored at 4°C in 0.1 *M* phosphate buffer, pH 7.00, for different time periods (0–25 days), and the EPR spectra of the membranes were then acquired.

3. Results

Spin-labeling is an EPR technique in which a paramagnetic spin label is incorporated into the system of interest. Spin labels are generally of the nitroxide type [22–24]. The usefulness of nitroxide spin-labeling methods derives from the extreme sensitivity of EPR, the fact that opaque samples (such as immobilized enzymes on polymeric membranes) can be used, the molecular information on motion, polarity, etc., of the local environment near the paramagnetic center of the spin label that can be obtained, the relative simplicity of the resulting EPR spectra that must be analyzed, the nondestructiveness of the

method, and the fact that, generally, except for the spin label, the system is diamagnetic [22–24]. Several reviews of the use of spin-labeling in biological and synthetic membranes are available [22–24].

The EPR spectra of nitroxides are especially sensitive to molecular motion of the spin label. Under fast, isotropic motion, as normally occurs with nitroxides in solution, a sharp, three-line EPR spectrum results. However, if the motion becomes hindered, as for example occurs if the spin label is bound to the active site of an enzyme, then asymmetric line broadening occurs. In particular, due to the relaxation phenomena involved with nitroxides, the high-field ($M_I=-1$) resonance line becomes broader (sometimes much broader) than the low-field ($M_I=+1$) and center ($M_I=0$) resonance lines [23]. Further, as the motion slows, the resonance positions of the low- and high-field lines spread out. The distance (splitting) between the low- and high-field lines of the EPR spectrum of motionally-slowed nitroxide spin labels is given by the parameter, $2A'_{zz}$, (Fig. 1).

The unique advantages of this highly-sensitive magnetic resonance method enumerated above make EPR suitable for examining the structure of opaque biofunctional membranes such as biosensors, affinity membranes, and biocatalytic membranes [22–24]. We report here the first EPR study of a fully-hydrated biofunctional membrane.

The EPR spin-labeling technique is a very sensitive method for detecting active-site conformational changes of papain *in solution* under a variety of conditions, and the activity measurements corroborate the EPR results [19]. For example, MTS-labeled papain in solution gives a spectrum like that in Fig. 1A. However, it is difficult to obtain EPR spectra of fully-hydrated immobilized enzymes on polymeric membranes, perhaps because of the difficulty of inserting the membrane into the intact quartz EPR flat cell. As far as we could determine, no extensive study using EPR spin-labeling techniques has been performed on enzymes immobilized on polymeric membranes in which the membrane was kept bathed in buffer solution. These conditions

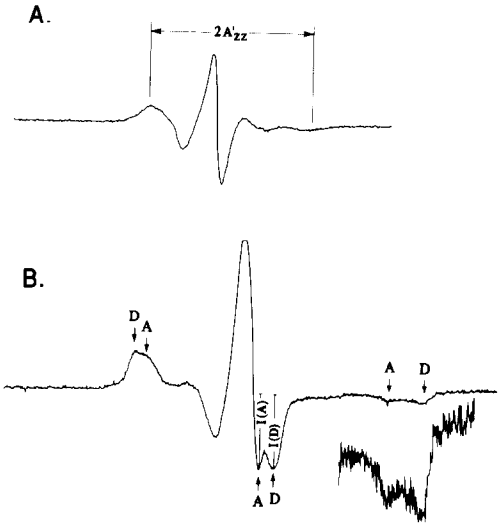


Fig. 1. (A) A typical EPR spectrum of MTS-labeled papain in solution. The splitting parameter, $2A'_{zz}$, is indicated. (B) A typical EPR spectrum of MTS-labeled papain immobilized on modified polysulfone membranes. The signals corresponding to subpopulation A and D are indicated. The EPR signal intensities below the baseline of $M_I=0$ center lines [$I(D)$ and $I(A)$] used for calculating the conversion parameter $I(D)/I(A)$ are also marked. Amplification of the instrumental gain demonstrates the existence of the two resonance peaks at the high-field lines (inset).

maintain the enzyme in its most natural state and approximate the conditions a biofunctional membrane would encounter in conventional engineering applications. As noted above, EPR studies of papain immobilized on a rather dry and dense membrane have been reported [16–18]. In the current study, using a special quartz EPR cell, the EPR spectra of immobilized papain on a modified polysulfone membrane have been successfully acquired, and more importantly, the immobilized enzyme was in full contact with the buffer or selected solutions (such as urea, guanidine-HCl) of interest when the spectrum was being taken. Therefore, the comparison between the EPR studies and the activity measurements and comparison of membrane-immobilized and free papain are more meaningful.

A typical EPR spectrum of immobilized papain on modified polysulfone membranes is shown in Fig. 1B. In contrast to the spectrum of

MTS-labeled papain in solution (Fig. 1A), the EPR spectrum of immobilized MTS-labeled papain on modified polysulfone membranes is a somewhat complicated. There are two subpopulations of immobilized enzymes, which are revealed by the EPR spin-labeling technique (Fig. 1B); that is, the spectrum is an overlap of two three-line nitroxide spin-label spectra. This is most obviously seen in the center of the spectrum where two minima exist in the $M_I=0$ central line. However, these two environments are also easily seen in the low-field $M_I=+1$ line, and upon computer amplification of the signal, in the high-field $M_I=-1$ line as well (Fig. 1B). The splitting between the high- and low-field lines, $2A'_{zz}$, which has been used very successfully in studying free papain in solution [19], was also employed to analyze the spectra of the immobilized papain on modified polysulfone membranes. For one subpopulation (called subpopulation *A*) $2A'_{zz}$ is about 60 G, while $2A'_{zz}$ is about 70 G for the other subpopulation (called subpopulation *D*). Thus the spin label has less motion at the active sites of both the immobilized enzyme subpopulations than that of papain in solution, which has a $2A'_{zz}$ of ~53 G [19]. It may be that the active-site cleft, at least the portion the spin label detected, of the immobilized papain has a more closed structure than that of the free papain in solution, which might be due to changes in the active-site structure upon immobilization and/or interactions between the enzyme and the polymeric support. Within the enzyme on the same polysulfone membrane, the active-site cleft of one subpopulation (with a $2A'_{zz}$ about 60 G) may be more open, and therefore provide for greater motional freedom, than that of the other subpopulation (with a $2A'_{zz}$ of 70 G).

The amidase activity follows Michaelis–Menten kinetics; however, the kinetic parameters, V_{max} and K_m, obtained from the Lineweaver–Burk plot of the experimental data, may not be the true values equivalent to those obtained in homogeneous reactions. Papain immobilized on modified polysulfone membranes has an apparent mean maximum reaction velocity of 27.9 *M*/mg min and an apparent K_m of 0.612 m*M* (Table 2).

3.1. pH studies

EPR spin-labeling is a sensitive method to study the active-site conformational change of enzymes [21] including free papain in solution [19] under different pH conditions. The results of a similar EPR study on immobilized papain are given in Fig. 2. For free papain in solution, the active site of papain is homogeneous. In the case of immobilized papain on polysulfone membranes, two subpopulations of immobilized

Table 2
Kinetic parameters V_{max}(app) and K_m(app), obtained from Lineweaver–Burk plots

Trial number	V_{max} (*M*/mg min)	K_m(app) (m*M*)	r[a]
1	29.3	0.643	0.9992
2	25.7	0.564	0.9991
3	28.6	0.628	0.9996
Mean	27.9	0.612	
S.D.	1.89	0.042	
Papain in solution[b]	68.6±1.49	0.377±0.0095	

[a] r is the correlation factor of linear regression.
[b] Mean ±S.D. [19].

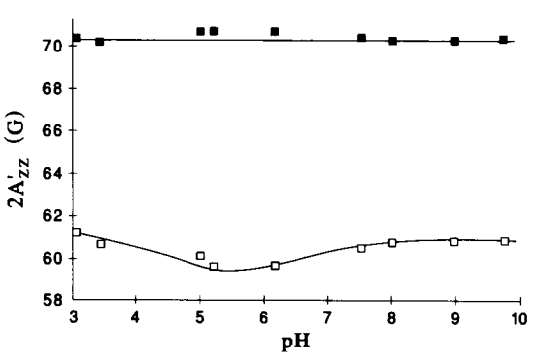


Fig. 2. Effect of pH on the active-site conformation of immobilized papain on modified polysulfone blend membranes. The splittings of the high- and low-field lines of subpopulation *A* (□) and subpopulation *D* (■) are plotted against the pH of the solutions. Each point is the mean of three trials and the standard deviation is less than 5% of the mean.

enzymes exist as noted above. For subpopulation A, $2A'_{zz}$ has a minimum around pH 5.22 and the $2A'_{zz}$ values increase on both sides of this pH. The response of the active-site conformation of the subpopulation A to pH is very similar to that of free papain in solution, though the magnitude of change is smaller [19]. Therefore, the relative positions of the amino acid residues necessary for the enzyme's activity may not change significantly compared to those of free papain in solution, although the active-site cleft of the subpopulation A might have a more closed structure than that of the free papain in solution since $2A'_{zz}$ is considerably larger. However, no detectable change on the motion of the spin label at the active site of subpopulation D as a function of pH was observed (Fig. 2). Indeed, the motion of the spin label at the active site of subpopulation D is so slow that it is close to the rigid limit value ($\approx 10^{-7}$ s rotational correlation time), which suggests that the free space in the active-site cleft of this subpopulation may be very small.

The pH dependence of the amidase activity of the immobilized papain is also very similar to that of free papain in solution (Fig. 3): in the pH range from 5 to 7, the relative activity remains fairly constant for both papain in solution and immobilized on modified polysulfone blend membranes. This result is consistent with the EPR results of the subpopulation A of the immobilized papain but not those of subpopulation D (Fig. 2), which suggests that subpopulation A may be the active form of the immobilized papain, while subpopulation D may have already been denatured upon immobilization.

3.2. Denaturant studies

EPR studies of denaturants such as urea and guanidine-HCl have shown that the active-site cleft of MTS-labeled free papain in solution might have a more closed structure as the protein became denatured [19]. The EPR spectra of immobilized papain under different concentrations (0–6 M) of urea solution are shown in Fig. 4. As the urea concentration increases, subpopulation A converts into subpopulation D, no matter upon which portion (low-field, mid-field, or high-field lines) of the spectra the observation is focused. Consider the low-field line. On the spectrum of the control (without urea), the low-field line is an overlap of two EPR signals, the one on the right is the low-field line of subpopulation A and the one on the left is that corresponding to the subpopulation D (Fig. 4a). As the urea concentration increases, the low-field line becomes sharper because of the conversion of the low-field line of the subpopulation A to the low-field line of the subpopulation D. At 6 M urea, the low-field resonance line is almost symmetric though a small shoulder still appears on the right-hand side of this line. This conversion is also seen in the high-field lines. There are two low-amplitude dips at the high-field end of the spectra (Fig. 4a), which correspond to the high-field lines of the subpopulation A (the one on the

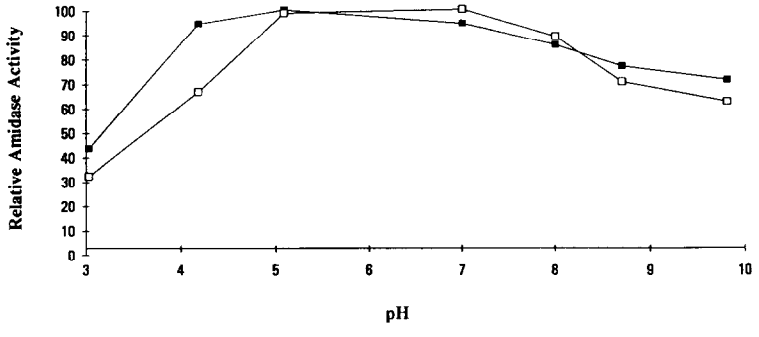


Fig. 3. Effect of pH on the amidase activity of immobilized papain on modified polysulfone blend membranes. The enzyme activities of the immobilized (■) and free papain (□) are plotted against the pH of the solutions. Each point is the mean of three trials and the standard deviation is less than 5% of the mean.

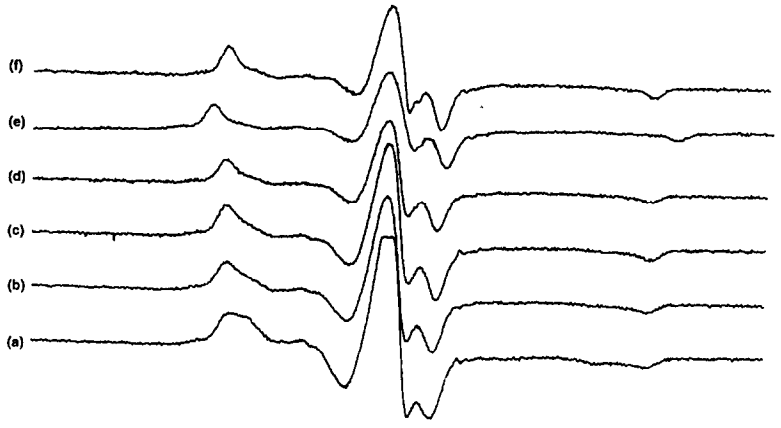


Fig. 4. EPR spectra of the immobilized papain on polysulfone membrane exposed to urea solutions of 0 (a), 1 (b), 2(c), 3 (d), 4 (e), and 6 *M* (f).

left) and the subpopulation *D* (the one on the right), respectively. The conversion can also be observed clearly at this portion of the spectrum; that is, the high-field line of the subpopulation *A* disappears while the intensity of the high-field line of the subpopulation *D* increases. Almost no sign of the high-field line of the subpopulation *A* can be observed at 6 *M* urea (Fig. 4f). It is both difficult and tedious to resolve these two subpopulations and doubly integrate them separately in order to monitor the conversion between these two subpopulations. Instead, if attention is focused on the negative portion (that portion of the spectrum below the baseline) of the $M_I=0$ mid-field lines (Fig. 1B), it can be seen that the intensity of the line (the one on the left) corresponding to the subpopulation *A* decreases while the line (the one on the right) corresponding to the subpopulation *D* increases as a function of that urea the concentration. The relative intensity of these two lines should be a good parameter to monitor the conversion between these two subpopulations and the parameter is defined in the following equation.

$I(D)/I(A)$ = (the intensity of the negative mid-field peak of the subpopulation *D*)/(the intensity of the negative mid-field peak of the subpopulation *A*)

The plot of the parameter, $I(D)/I(A)$, versus the urea concentration is given in Fig. 5. The conversion of subpopulation *A* into subpopulation *D* is clearly evidenced by an increased value as the urea concentration increases to ~5 *M* after which this parameter is approximately constant.

The activity measurements correlate with the EPR results (Fig. 6). The amidase activity of the immobilized papain decreases slowly at urea concentrations below 5 *M*. When the urea concentration is higher than 5 *M*, the decrease in activity becomes much more dramatic and just

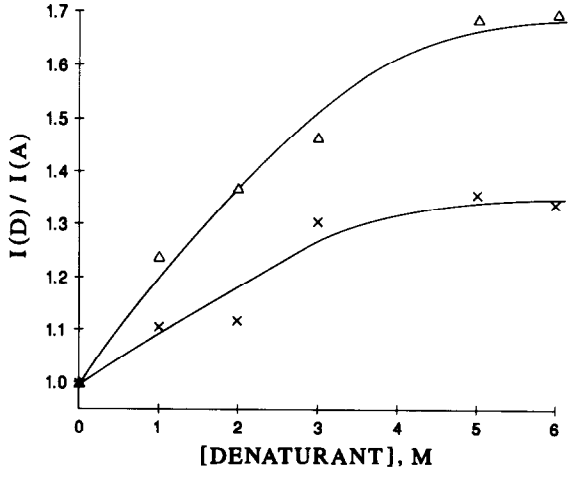


Fig. 5. The plot of the conversion (or denaturation) parameter, $I(D)/I(A)$, versus the urea (△) or guanadine hydrochloride (X) concentration.

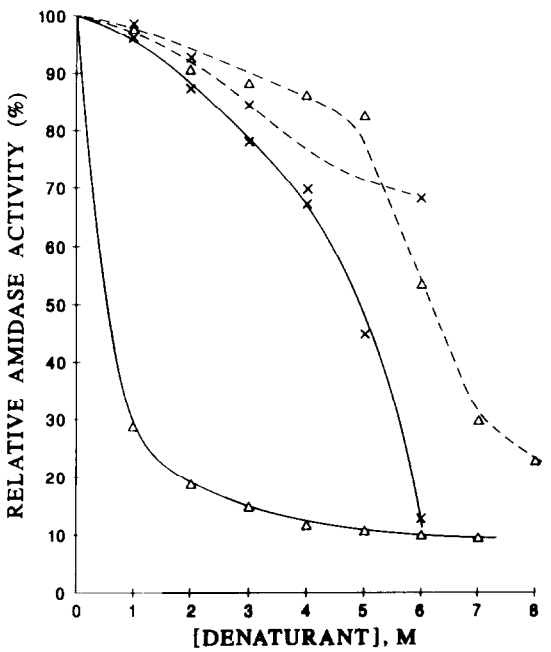


Fig. 6. Effect of urea (△) or guanadine hydrochloride (X) on the amidase activity of papain immobilized on modified polysulfone blend membranes. The enzyme activities of immobilized (——) and free papain (———) are plotted against the respective denaturant concentrations. Each point is the mean of three trials and the standard deviation is less than 5% of the mean.

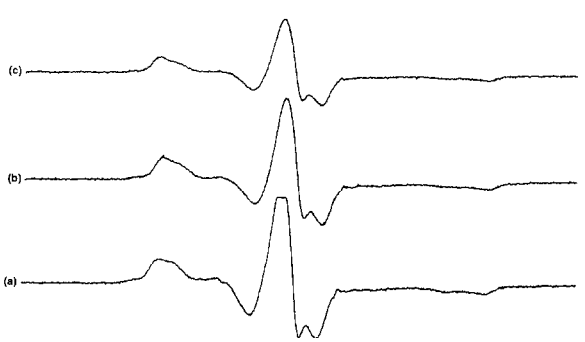


Fig. 7. EPR spectra of the immobilized papain after (a) 0, (b) 10 and (c) 25 min incubation at 70°C.

23.2% of the activity left at 8 *M* urea. Since $I(D)/I(A)$ is relatively constant between 5 and 6 *M* urea, the results suggest that the spin-label motion in the active site is maximally hindered at 5 *M* urea and that subsequent loss of activity at urea concentrations > 5 *M* may be due to global denaturation of the protein. Comparing the effects of urea for soluble and immobilized papain, it is obvious that immobilization of the enzyme enhances its stability toward denaturation by urea (Fig. 6).

Similar results were obtained in the studies of denaturation by guanidine-HCl. The measurements of the denaturation (or conversion) parameter, $I(D)/I(A)$, and the amidase activity also are summarized in Fig. 5 and Fig. 6, respectively. Again, the EPR results are consistent with the activity measurements and the denatured form (subpopulation *D*) of the immobilized enzyme has a more restricted active-site cleft than that of the active form (subpopulation *A*). There is also conversion from subpopulation *A* into subpopulation *D* upon addition of guanidine-HCl. The resistance to denaturation by guanidine-HCl after immobilization is even more significant than that of urea. At guanidine-HCl concentration as high as 6 *M*, the immobilized papain still has 69.1% of its activity remaining compared to only 12.9% for the enzyme in solution (Fig. 6).

3.3. Thermal stability studies

Immobilization of papain greatly increases the thermal stability of the enzyme. Immobilization on modified polysulfone membranes even protects the mixed disulfide bond between the MTS spin label and the active site of papain. This bond is not stable for free papain in solution under high temperature [19], but in contrast, there is no detectable release of the spin label from the active site of the immobilized papain after incubation at 70°C for 10 and 25 min (Fig. 7). However, some of the active enzyme (subpopulation *A*) did denature (was converted into subpopulation *D*) after 10 and 25 min of incubation at 70°C. The denaturation (or conversion) parameter, $I(D)/I(A)$, increases from 1.014 of the control to 1.179 and 1.175 after 10 and 25 min incubation at 70°C, respectively. Further denaturation after 10 min at 70°C may be very slow: there is no observable change between the EPR spectrum of the immobilized papain after 10 min incubation at 70°C and that after 25 min incubation.

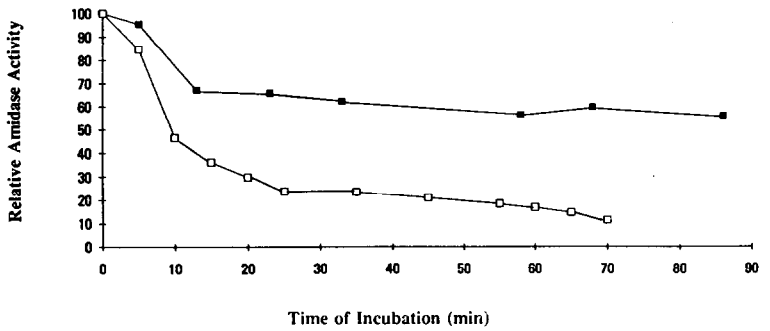


Fig. 8. Thermal stability of immobilized papain on modified polysulfone blend membranes. The enzyme activities of immobilized (■) and free papain (□) are plotted against the incubation time at 70°C. Each point is the mean of three trials and the standard deviation is less than 5% of the mean.

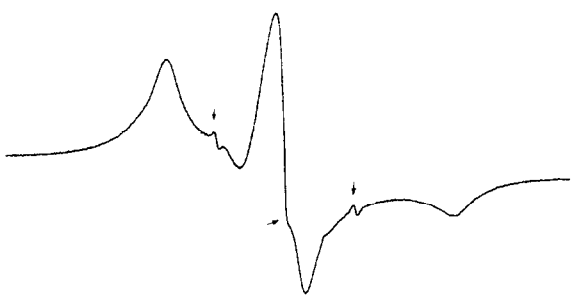


Fig. 9. An EPR spectrum obtained by first immobilizing papain on modified polysulfone blend membranes, then reacting with MTS spin label. The signals marked by arrows are from excess free spin label.

The enhanced resistance to thermal inactivation upon immobilization can also be observed in the amidase activity measurements (Fig. 8). The immobilized papain has 55.6% of its activity after incubation for 86 min at 70°C while only 55 min are needed to decrease the activity of free papain below 20% [19]. Furthermore, free papain loses almost all its activity after a 70 min incubation at 70°C with a bit more than 10% of the activity left (Fig. 8). Consistent with the EPR studies, the amidase activity of the immobilized papain stays around 60% of the control after 13 min of incubation at 70°C (Fig. 8). Further denaturation did occur, but at a very slow rate (Fig. 8).

3.4. Reusability studies

One of the most significant advantages of immobilized enzymes over the native enzymes in solution is their reusability. The reusability of the immobilized papain on modified polysulfone blend membranes was studied and the results suggested a constant activity for at least 18 cycles after an initial equilibrium period (data not shown).

3.5. Active-site accessibility of the immobilized papain

The EPR data above suggest that both subpopulations of immobilized papain have active-site clefts with less motional freedom for the MTS spin label than that of the free papain in solution. One factor which affects the activity of the immobilized enzyme is the accessibility of the substrate and solvent to the active site of the immobilized enzyme. The difference in accessibility of the two subpopulations may result in differences in specific activity. Two methods have been employed to study the active-site accessibility of the immobilized papain. In the first approach, it was found that the MTS spin label could not bind to the active site of subpopulation *D* if the enzyme were first immobilized and then spin-labeled (Fig. 9). Only the active subpopulation *A* was labeled by the MTS spin label as evidenced by equivalent values of $2A'_{zz}$ of Fig. 9 and subpopulation *A* in Fig. 1B. This may be

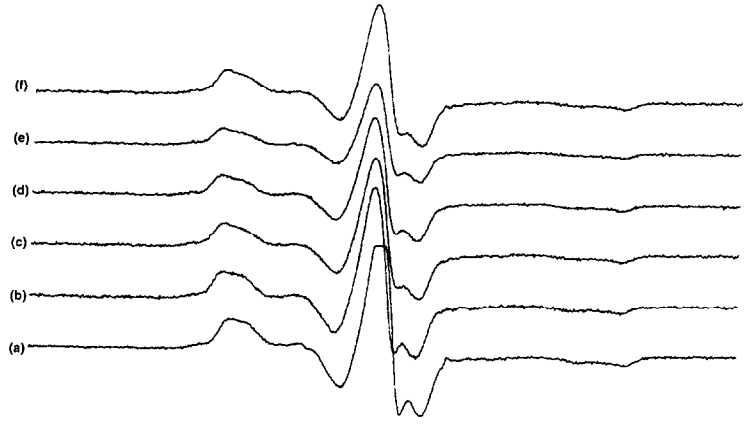


Fig. 10. EPR spectra of immobilized papain on modified polysulfone blend membranes exposed to $K_3Fe(CN)_6$ solutions of 0 (a), 100 (b), 200 (c), 300 (d), 400 (e), and 500 mM (f).

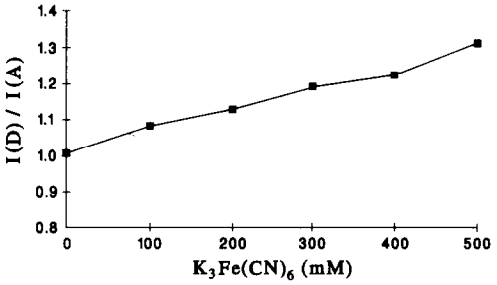


Fig. 11. Line-broadening effect of $K_3Fe(CN)_6$ monitored by the parameter $I(D)/I(A)$.

Table 3
Storage stability of immobilized papain on polysulfone membrane monitored by $I(D)/I(A)$

Day	$I(D)/I(A)$
3	0.966
5	0.920
9	0.946
14	0.950
20	0.990
25	0.925

due to the fact that the active-site cleft of subpopulation D is so closed that the MTS spin label and/or activating agents are not able to get into it. Therefore, only the active subpopulation A is easily accessible by solvent while the inactive subpopulation D is not.

In the second approach, $K_3Fe(CN)_6$ was employed to broaden the EPR signals of the MTS spin label. If the $K_3Fe(CN)_6$ molecule is small enough to get into the active site of the immobilized papain and get close enough to the spin label, the EPR signal of the spin label will be broadened due to the paramagnetic relaxation between the spin label and the paramagnetic molecule $K_3Fe(CN)_6$ [23,24]. A series of EPR spectra of the immobilized papain under different $K_3Fe(CN)_6$ concentrations are given in Fig. 10. No broadening effect was observed for subpopulation D, but there is indeed line broadening in the EPR signals of subpopulation A, indicating that $K_3Fe(CN)_6$ is accessible to the nitroxide spin label in subpopulation A but not subpopulation D. As above, the parameter $I(D)/I(A)$ is also a good parameter to monitor solvent accessibility to the active sites. Since the total integrated intensity of the spectrum is fixed, line broadening has the effect of reducing the peak amplitude. If $K_3Fe(CN)_6$ causes paramagnetic relaxation of subpopulation A, then $I(A)$ should decrease and $I(D)/I(A)$ should increase. The results are plotted in Fig. 11. As the $K_3Fe(CN)_6$ increases, the value of $I(D)/I(A)$ increases providing additional evidence that the active subpopulation A has good substrate accessibility to

its active site while the denatured subpopulation *D* has not.

3.6. Storage stability

Papain immobilized on modified polysulfone blend membranes has high storage stability: the EPR spectrum of the immobilized papain has no observable change after 25 days of storage in 0.1 *M* phosphate buffer, pH 7.00, at 4°C. $I(D)/I(A)$ values for the spectra are given in Table 3, and suggest that the relative population monitored by $I(D)/I(A)$ does not vary during the storage over this time period. This result is also consistent with the observation that no protein was liberated from the membrane into solution during this period (data not shown).

4. Discussion

The EPR spin-labeling technique using MTS spin label has been shown to be very sensitive for studying the conformation of native papain in solution [19], and this current study suggests that it is also very sensitive to the conformational change of immobilized papain on modified polysulfone membranes. From the literature ([4,5–8,11,14], see Introduction) and this study, it appears that a general trend exists that immobilized enzymes have two major subpopulations, no matter what enzyme was immobilized and what kind of support was used. More than two subpopulations of the immobilized enzymes were also observed in a few cases [12,13]. All the immobilization matrices mentioned above, polymeric or inorganic, are in the form of beads. Although Zhuang and Butterfield [16–18] were the first to use EPR to characterize the structure of enzymes immobilized onto polymeric membranes, these systems were not ideal since the membranes were not fully hydrated and were dense. Moreover, the spin label used, though specifically bound to the single cysteine in the active site, was quite lengthy, causing the nitroxide group to protrude to the outside of the active site. This current research, using MTS, a small spin label bound to the cysteine group but confined to the active-site cleft, and a special quartz EPR cell, is the first to characterize immobilized enzymes on a fully-hydrated membrane. In this study, two major subpopulations were also found and characterized for fully-hydrated papain immobilized on modified polysulfone membranes.

The activities of the immobilized enzyme subpopulations are highly correlated with the mobility of the spin labels at the active sites of the immobilized enzymes. For all the immobilized enzymes mentioned above immobilized on beads [4,5–8,11,14], the subpopulation that has faster spin-label motion at the active site also has higher specific enzyme activity. Thus the rise in mobility at the active site may result in the increase of enzyme activity. An extensive study of the two subpopulations of chymotrypsin immobilized on Sepharose 4B by Clark and Bailey had found that the subpopulation with higher spin-label mobility had much higher ($\sim$16 times higher) specific activity than the subpopulation with slower spin-label motion [4]. It was also found that the overall activities of the immobilized chymotrypsin on different types of sepharose supports might depend on the distribution of the immobilized enzyme among these two subpopulations [4,7]. Deactivation studies by 50% *n*-propanol [5,7] had shown that the relative distribution of the more mobile and more immobilized forms had changed significantly as a result of alcohol exposure and the deactivated enzyme was primarily the latter, but clear characterization of these two subpopulations was not yet possible.

In the current study, both subpopulations of immobilized papain on polysulfone membranes have more restricted spin-label motion at the active sites than the corresponding native form in solution. Immobilization did change the conformation of the enzyme but not homogeneously; that is, subpopulation *A* may have a more open active-site cleft than subpopulation *D* since spin-label motion was faster in the former as assessed by the splitting parameter $2A'_{zz}$. The pH dependence of the immobilized papain suggests that subpopulation *A* may be the active form while subpopulation *D* may be the denatured form of the immobilized enzyme. Denaturant (urea or guanidine-HCl) and thermal inactivation of the

immobilized papain confirm this assumption. Subpopulation *A* can be converted into subpopulation *D* by addition of urea or guanidine-HCl, the denaturants which can completely denature the immobilized papain at high concentration (total loss of its amidase activity). The conversion also occurs under high temperature but at a slower rate. Therefore, higher spin-label mobility at the active site of the immobilized enzyme does correspond to higher activity in the case of papain.

The solvent and substrate accessibility to the active site is also one of the important factors affecting the activity of hydrolytic enzymes. $K_3Fe(CN)_6$ can broaden the EPR signal of subpopulation *A* but not subpopulation *D*. More direct evidence that the subpopulation *A* is the active form of the immobilized enzyme was obtained by immobilizing papain on polysulfone membrane first and then spin-labeling. In this way, only the active subpopulation *A* can be labeled by the MTS spin label. Therefore, loss of activity for the subpopulation *D* may be partly due to the inaccessibility of solvent (and/or substrate) to its active site due to its significant conformational change.

The apparent (uncorrected for possible diffusional resistance or substrate/product adsorption) Michaelis constant K_m(app) of the immobilized papain is about two times that of the free papain in solution, while the pH profile resembles that of papain in solution. This result may be due to not only the change in the conformation of the enzyme after immobilization, but also the partitioning effect introduced by the polymeric support. The substrate concentration at the microenvironment of the immobilized papain may be different from that of the bulk solution even at equilibrium because of the partition effect [25]. However, given the large pore size of the modified polysulfone blend membrane used in these studies, we favor enzyme conformational changes to account for an increased value of the apparent K_m. This interpretation is consistent with the EPR findings as well.

Papain immobilized on polysulfone membranes has much higher thermal stability and stronger resistance to the inactivation by denaturants such as urea and guanidine-HCl than papain in solution. The enhancement of stability by immobilization is also observed in many other enzyme immobilization studies [8,11,26]. Papain immobilized on polysulfone membranes is highly reusable as there was no significant decrease of amidase activity for 18 cycles of reactions.

In conclusion, the EPR spin-labeling technique is a powerful method for the study of immobilized enzyme systems, whose investigation by optical spectroscopies is ordinarily hindered by the opacity of the supports. EPR offers the possibility to correlate active-site structure to enzymatic activity in immobilized systems. In this study, insight into the conformation of a fully-hydrated,immobilized enzyme under a variety of conditions that could be encountered in engineering processes has been gained. This insight will help in the understanding of the rapidly growing field of biofunctional membranes in which applications of sensors, affinity membranes, and bioreactors show great promise.

Nomenclature

BAPNA	*N*-benzoyl-DL-arginine-4-nitroanilide
5P8	5 m*M* phosphate buffer, pH 8.0
MTS	(1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrrolidine-3-methyl)-methanethiosulfonate
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
$K_3Fe(CN)_6$	potassium ferricyanide
$2A'_{zz}$	EPR splitting parameter; a measure of rotational mobility of the spin label bound to the single SH group of papain located at the active site of the enzyme: the greater the value of this parameter, the slower the rotational motion of the spin label.

subpopulation *A*	fraction of immobilized papain in which the enzyme is still active. This fraction is characterized by a smaller value of $2A'_{zz}$ than subpopulation *D*.
subpopulation *D*	fraction of immobilized papain in which the enzyme is inactive.
K_m(app)	apparent Michaelis constant of immobilized papain.
V_{max}(app)	apparent maximum velocity of the enzymatic activity of immobilized papain.
LADH	liver alcohol dehydrogenase
CNBr	cyanogen bromide
Tris	Tris(hydroxymethyl)aminomethane buffer, pH 7.5
DMSO	dimethyl sulfoxide
ϵ	molar adsorbtivity
M_I	nuclear spin quantum number
G	Gauss, a measure of magnetic field strength
$I(D)$	amplitude of the negative mid-field peak of the EPR spectrum of MTS bound to subpopulation *D*.
$I(A)$	amplitude of the negative mid-field peak of the EPR spectrum of subpopulation *A*.
$I(D)/I(A)$	conversion or denaturation factor: the higher this value, the more of immobilized papain exists in denatured form.

Acknowledgements

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