NON-COVALENT IMMOBILIZATION OF PAPAIN ONTO MODIFIED POLYSULFONE MEMBRANE USING AVIDIN-BIOTIN COUPLING: EPR AND KINETIC STUDIES

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ABSTRACT

Membrane-based bioreactors or immobilized enzymes are one example of biofunctional membranes, defined as entities composed of biological molecules attached to a polymeric matrix cast in the form of porous polymeric membranes. A detailed understanding of the structure and function of enzymes upon immobilization is essential to develop a membrane bioreactor with optimal properties involving minimal conformational changes at the active site of the enzyme. In this study, a sulfhydryl protease, papain (EC 3.4.22.2), was noncovalently immobilized onto the modified polysulfone membrane through the avidin-biotin complex. Various kinetic parameters for the amidase activity of noncovalently bound papain, using the substrate benzoyl arginine p-nitroanilide hydrochloride (BAPNA) were determined and the results were compared with those obtained from studies of papain in solution and papain directly immobilized onto the modified polysulfone membrane. As expected, there was a decrease in the enzymatic activity upon direct immobilization. However, insertion of the avidin-biotin complex as a non-covalent spacer resulted in an enzyme bioreactor which was more stable and reusable than the free enzyme.

Electron paramagnetic resonance (EPR) spin labeling techniques were used to characterize the active site conformational changes of papain immobilized on the modified polysulfone membranes through the avidin-biotin complex. This paper reports the pH dependence, reusability and storage stability studies of biofunctional membranes using avidin-biotin complex as a non-covalent spacer. A good correlation was found between the active site conformational changes and the amidase activity of papain upon noncovalent immobilization onto the modified polysulfone membrane.

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INTRODUCTION

Over the past few decades, electron paramagnetic resonance (EPR) spin labeling techniques have been used successfully to provide information on the active site environment of immobilized proteins[1-3]. EPR measurements do not require optically transparent samples, which makes it an excellent technique for studying active site conformational changes of enzymes immobilized on opaque support matrices. Clark and Bailey,[4-6] showed that in the case of spin- labeled α-chymotrypsin covalently immobilized on CNBr-activated sepharose, two different subpopulations of the immobilized enzymes exist in the resultant EPR spectra. The active site conformations of the immobilized enzyme corresponding to the two different subpopulations were found to be different.

Our group has also reported the presence of two EPR spectral subpopulations on studies done on papain directly immobilized on polymeric membranes.[7] We also reported the comparison of the effects of direct immobilization of papain on flat sheet modified polysulfone membrane (MPS) and hydroxyethyl cellulose (HEC) coated polyethersulfone hollow fibers.[8] As reported earlier, EPR studies showed two subpopulations of the immobilized papain on the hollow fibers. We have found that immobilization of spin-labeled enzymes, in general, have resulted in two conformationally different EPR spectral subcomponents. In addition to these findings, our past experience has shown that immobilizing an enzyme through a 6-carbon spacer enhances the enzymatic activity and minimizes the conformational changes at the active site of the enzyme.[9-11]

Most of the immobilization reactions reported earlier by our group[7] involved the direct formation of covalent bonds between the lysine residues on papain molecules and the aldehyde functional groups on modified polysulfone membranes. In all these studies, a short, sulfhydryl-specific spin label sulfhydryl specific spin label, (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrrolidine-3-methyl) methanethiosulfonate (MTS) was employed in the characterization of directly immobilized papain.

In the current study, the high binding affinity between avidin and biotin has been utilized to create a noncovalent spacer between the enzyme and the modified polymeric membrane. The bicyclic ring of biotin is necessary to bind to avidin. The biotinylation of papain was accomplished using NHS-LC-Biotin (Figure 1).

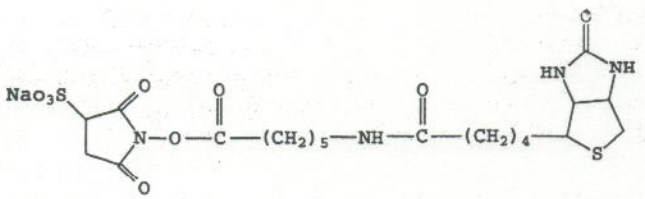

Figure 1. The structure of NHS-LC-biotin.

EPR spin labeling techniques were used to study the effects of biotinylation on the active site conformation and properties of papain in solution. No changes in the EPR parameters of active-site labeled biotinylated papain were found, consistent with the notion that biotin does not induce conformational changes in this enzyme.[12] The use of the avidin-biotin complex as a noncovalent spacer provided an alternative strategy for enzyme attachment. This paper reports the effect of noncovalent immobilization of papain onto the modified polysulfone membrane through the avidin-biotin complex on the structural and performance characteristics of papain.

MATERIALS AND METHODS

Materials

The modified polysulfone blend membrane, Ultrabind™ US450, was purchased from Gelman Sciences. According to the manufacturers, the average pore size of the membrane is 0.45 μm, and the thickness is 152.4 μm. The membrane has aldehyde functional groups on the surface available for covalent attachment. Papain, avidin (from egg white), DL-cysteine, N-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) were obtained from Sigma Chemical Company. Disodium ethylenediaminetetraacetic acid (EDTA) was obtained from Aldrich. The sulfhydryl specific spin label, (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrrolidine-3-methyl) methanethiosulfonate (MTS) was purchased from Reanal, Budapest, Hungary. The ultrafiltration membrane (10,000 MW cutoff) used for concentrating MTS-labeled papain, was obtained from Millipore Company. NHS-LC-Biotin was purchased from Pierce. All other chemicals and solvents were reagent grade.

Methods

A stock solution of papain was (4 mg/mL) was spin labeled, concentrated and analyzed as previously described.[13]

Biotinylation of MTS-labeled Papain The concentration of MTS-labeled papain was adjusted to 3 mg/mL using $NaHCO_3$ buffer, pH 8.0. Then NHS-LC-Biotin was added to 5 mL of MTS-papain solution (the mole ratio of NHS-LC-Biotin to papain was 5:1). The reaction was carried out at room temperature for 1 hour with stirring. The biotinylated MTS-labeled papain was then dialyzed extensively in 3L of 50 mM $NaHCO_3$ buffer, pH 8.0, at 0°C. The buffer was changed after every eight hours for two days. A 10,000 MW cutoff membrane was employed for dialysis.

NonCovalent Immobilization of Papain through the Avidin-biotin Complex A 3mL solution of avidin in 50 mM $NaHCO_3$ buffer (2mg/mL avidin) was prepared. Avidin was covalently attached to the polysulfone membrane and the system was incubated for one day at 4°C. Unreacted avidin was washed away with the 50 mM $NaHCO_3$ buffer pH 8.0. Biotinylated MTS-labeled papain solution was then added to the membrane and the system was again incubated for 24 hours at 4°C. Excess papain was washed away with 50 mM $NaHCO_3$ buffer. The resulting membrane was then cut into four 1X4 cm^2 pieces for EPR studies.

EPR Spectra Acquisition All the EPR spectra of MTS-labeled papain noncovalently immobilized on modified polysulfone membranes were recorded on a Bruker ESP-300 spectrometer with a TM rectangular cavity at room temperature using a special quartz EPR cell.[7] The membrane was taken directly from the buffer in which it was stored. Typical parameters for the EPR spectra were mw frequency, 9.78 GHz ; mw power, 24.2 mW ; modulation amplitude, 0.32 G; modulation frequency, 100 kHz ; scan width, 130G.

Assay for the Amidase Activity of Papain Noncovalently Immobilized on Modified Polysulfone Membrane The amidase activity assay for papain was performed as described previously.[7,8,13]

pH dependence Studies The buffers (0.2M) used in pH study were: acetate buffer for pH 3-5, phosphate buffer 5-8, and Tris buffer for pH 9-10. For studying the effect of pH on the active site conformation of noncovalently immobilized papain on polysulfone

membrane, each 1X4 cm^2 piece was incubated in buffers of different pH for 30 min. at room temperature before the EPR spectrum was acquired. The distance between the high field and low field resonance peaks, 2 A'_{zz}, was used as a parameter for characterizing the motion of spin label at the papain active site.[7,13]

While measuring the papain activity under different pH conditions, the activating solutions were prepared in deionized water as described above. Each 2X2 cm^2 piece of polysulfone membrane with noncovalently immobilized papain was incubated in 0.5 mL buffer of different pH. The solution was incubated for about 5 minutes at 37°C. Then 9.5 mL of the activating solution was added and papain was activated for 10 minutes. Ten mL of substrate solution was then added and reacted for 20 minutes with Continuous stirring (final substrate concentration was 1mM BAPNA). The amount of 4-nitroaniline released was estimated spectrophotometrically as described above. The relative amidase activity of papain under different pH was plotted.

Storage Stability and Reusability Studies These studies were performed in a manner previously described.[7,13]

RESULTS

EPR studies

Figure 2(a) shows a typical EPR spectrum generated by free MTS spin label in solution. The three equally spaced sharp lines arise due to free and isotropic tumbling of the nitroxide moiety of the spin label. The line widths are highly sensitive to the changes in the microenvironment of the spin label. Figure 2(b) represents the effect of the microenvironment, i.e., the active site of papain, upon the spin label motion. The characteristic asymmetric broadening of the high (M_I = -1) and low field (M_I = +1) resonance lines with a consequent loss in amplitude is due to the influence of active site structure on the rotational motion of the attached spin label. Thus, the motion of the spin label may be characterized by the splitting between the low and high field resonance lines. A hyperfine splitting parameter, $2A'_{zz}$, can be used to measure the splitting between the high and low field lines in the EPR spectrum. This parameter is characteristic of the spin label motion; a lower value of $2A'_{zz}$ implies less restricted motion of the spin label and vice-versa. This parameter has been successfully used in characterizing the active site conformations of free and immobilized enzymes.[7,13]

The comparison of the active site conformation of MTS-labeled papain in aqueous solution with that of immobilized papain is shown in Fig 2(c). The spectra of MTS-labeled papain in solution showed $2A'_{zz}$ value of 53G, which suggests that the motion of nitroxide moiety of spin label located in the active site of papain is restricted when compared with the motion of free spin label.

The EPR spectrum (Figure 2 (c)) of papain immobilized directly onto the modified polysulfone membrane showed the presence of two subpopulations whose $2A'_{zz}$ values are 60G and 70G, respectively.

Various structural and kinetic properties of biotinylated papain were compared with those of the free papain previously studied extensively by our research group. It was seen that there was no significant change in the active site conformation and enzymatic activity of papain upon botinylation.[12] In Figure 2(d), a typical EPR spectrum of MTS-labeled biotinylated papain in solution is presented. The typical value of $2A'_{zz}$ for MTS-labeled papain in solution is about 53 G. In the case of MTS-labeled biotinylated papain in solution, the $2A'_{zz}$ value was found to be 54G. Thus, it may be be concluded that addition of biotin on the surface of papain molecule does not affect significantly the motion of the spin label

at the active sites. Therefore, the conformation and structure of papain's active site remain essentially unaltered upon biotinylation.

When papain was immobilized onto the modified polysulfone membrane through the avidin-biotin complex, the two spectral subpopulations of the enzymes were again observed (Figure 2(e)). This spectrum resulted because of the overlap of two three-line nitroxide spin label spectra. One subpopulation had $2A'_{zz}$ value of 58.2 G and the other about 68.4 G. Thus, the complex formation does restrict the motion of the active site spin label for both subpopulations as expected. Also, the D subpopulation has a $2A'_{zz}$ value close to that of the denatured population of directly immobilized papain,[7] while the other subpopulation has a $2A'_{zz}$ value that is close to that of biotinylated papain (see above). Hence, subpopulation A represents the active form of immobilized papain. Comparison of $2A'_{zz}$ values for the active subpopulation A of immobilized enzymes reveals that the motion of the active site spin label is slightly less restricted in the case of noncovalently immobilized papain ($2A'_{zz}$ =58.2G) as compared to directly immobilized papain ($2A'_{zz}$ = 60G). This shows that a relatively more open active site structure results upon insertion of avidin-biotin complex as a noncovalent spacer between the enzyme and the polymeric membrane.

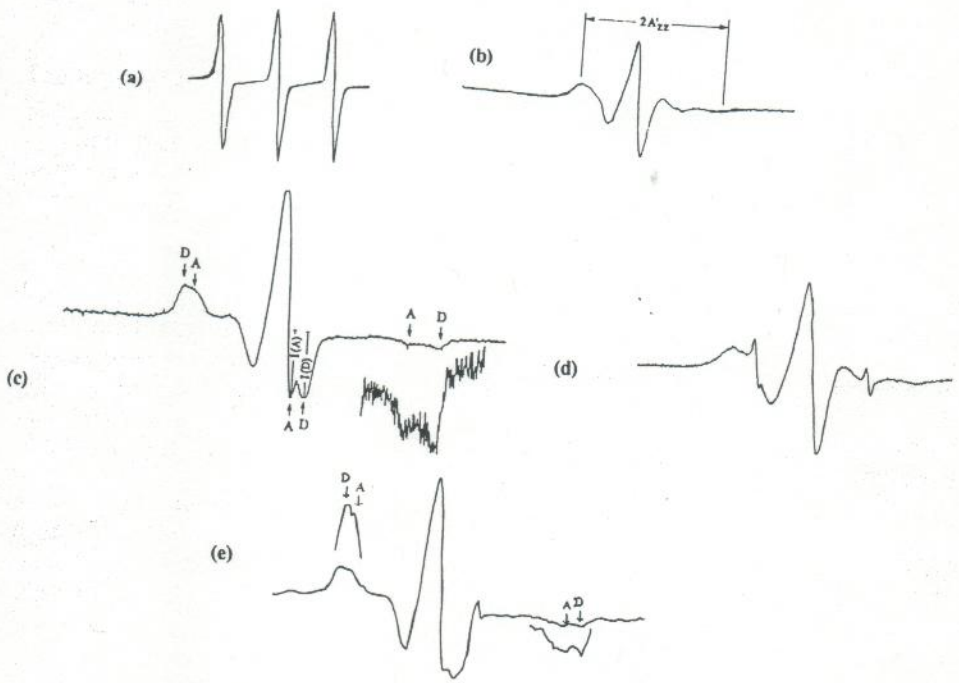

Figure 2. Typical EPR spectra of MTS label (a) in solution. (b) showing the effect of active site conformation on its motion. The splitting parameter, $2A'_{zz}$ is 53G. (c) bound to papain directly immobilized onto the polysulfone membrane. Two subpopulations A and D are observed. (d) bound to biotinylated papain in solution (e) bound to papain noncovalently immobilized on the polysulfone membrane via avidin-biotin complex.

Kinetic Studies

Table I summarizes the kinetic parameters of free and immobilized papain obtained from Lineweaver-Burk plots of the data. The amidase activity of the enzyme follows Michaelis-Menten kinetics. The apparent values of the various parameters are reported. It can be seen quite clearly from the values of the various parameters that there was an

increase in the value of K_m(app) and a decrease in the enzyme activity represented by V_{max}(app). It has been reported previously that the activity of an enzyme is usually reduced upon immobilization.[7] The higher values of K_m (app.) observed for immobilized enzymes may be due to alterations in the active site conformation, consistent with the EPR findings.

Comparison of the kinetic parameters of papain immobilized directly and noncovalently shows that the insertion of a noncovalent spacer introduces a degree of flexibility in the enzyme which is absent in the case of direct immobilization. This result is consistent with the values $2A'_{zz}$ indicated above.

Table I. Apparent values of kinetic parameters obtained from the Lineweaver-Burk plots. Each value indicates the mean and standard deviations of three measurements

	V max (μmol/g.min) (Apparent)	Km (mM) (Apparent)
Papain (in solution)	110 ± 0. 15	1.2 ± 1.32
Papain (in solution) after biotinylation	100 ± 0.09	2.0 ± 1.24
Papain immobilized directly on polysulfone membrane	13.2± 1.41	12.3 ± 2.88
Papain immobilized on polysulfone membrane via avidin-biotin complex	32.3 ± 0.54	10.7± 2.45

pH Studies

The pH of the system had almost negligible affect on the conformation of the immobilized papain (pH 5-9). The $2A'_{zz}$ values for the subpopulation D showed no observable changes. The $2A'_{zz}$ values for the active subpopulation A also remained almost unchanged in this pH range. These results suggest that the conformation of the papain active site is unaffected in this pH range.

Reusability Studies

Figure 3 indicates that papain immobilized on the modified polysulfone membrane through avidin-biotin complex does not loose its activity for at least 20 cycles after an initial period of equilibrium. This is one of the most important advantages of a membrane-based enzyme bioreactor.

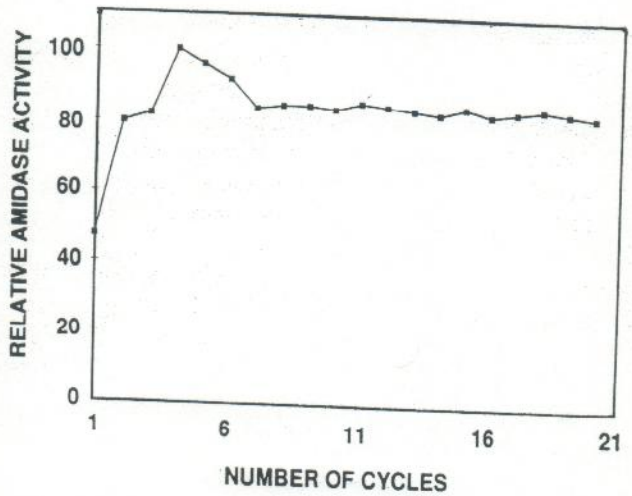

Figure 3. Reusability studies done on noncovalently immobilized papain

The Effect of Storage on the Amidase Activity

The storage stability of noncovalently immobilized papain was studied by storing the system in 5mM NH_4HCO_3 buffer, pH 8.0 at 4°C for different time periods. There was no significant alteration in the activity and the active site conformation of the enzyme on storage.

In the EPR studies, a parameter previously employed by our group[7] to characterize immobilized enzymes, I (D) / I (A), was used to assess the change in the spectra over a period of storage. Focusing on the portion below the baseline of the spectrum, it was shown previously that the intensity of the M_I=0 mid field line corresponding to the active and denatured subpopulations changes with denaturing of papain. Hence, the relative intensity of these two lines, I (D)/ I(A), is a good parameter to investigate the denaturation of the enzyme upon immobilization. This parameter can be defined as

$$I(D)/I(A) = \frac{\text{the intensity of the negative mid-field peak line of the subpopulation D}}{\text{the intensity of the negative mid-field peak line of the subpopulation A}}$$

A decrease in the value of I (D)/ I(A) would correspond to a decrease in the enzymatic activity. It was observed that the enzymatic activity is retained even after 25 days of storage and that I (D)/ I(A) remains relatively constant over this time period.

CONCLUSIONS

Although several studies of enzymes immobilized on polymeric surfaces (beads) have been reported, our group was the first to use EPR to characterize membrane-based enzyme bioreactors,[9-11] to investigate the structure and function of enzyme immobilized onto the hollow fiber membranes,[8] and, in the current study, the first to use an avidin-biotin spacer for EPR studies of biofunctional membranes.

The avidin-biotin complex, because of its high noncovalent affinity, indeed provides a practical linkage between the enzymes and the polymeric membranes as shown in this

study. Since the enzyme bound with a spacer is further separated from the surface of the membrane, the enzyme, therefore, may have a higher mobility and the substrate-enzyme complex probably is more stable. However, this immobilized enzyme is not stable in the presence of denaturants because, avidin, being a protein, does not retain its conformation . Consequently, its biotin-binding capability is affected. Thus, the use of avidin-biotin complex as a noncovalent spacer is a useful method for enzyme immobilization, and one that offers an excellent alternative to direct immobilization.

ACKNOWLEDGMENTS

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REFERENCES

1. L.J. Berliner, S.T. Miller U.Y. Rosa and G.P. Royer, "An ESR Study of the Active Site Conformations of Free and Immobilized Trypsin". *Biochim. Biophys. Acta.* 315:195(1973)

2. G. A. Marg, G.L. Millhauser, P.S. Skerker and D.S.Clark, "Application of EPR Methods in Studies of Immobilized Enzyme Systems". *Ann. N. Y. Acad. Sci.*, 469:253(1986)

3. L.J. Berliner, "Spin Labeling in Enzymology: Spin Labeled Enzyme and Proteins". *Meth. Enzymol.*, 49:418(1978)

4. D.S. Clark and J.E. Bailey, "Structure-function Relationships in Immobilized Chymotrypsin Catalysis". *Biotechnol. Bioeng.*25:1027(1973)

5. D.S. Clark and J.E. Bailey, "Characterization of Heterogeneous Immobilized Enzyme Subpopulations using EPR Spectroscopy". *Biotechnol. Bioeng.*, 26:231(1984)

6. D.S. Clark and J.E. Bailey, "Kinetics and EPR Spectroscopy Studies of Immobilized Chymotrypsin Deactivation". *Ann. N. Y. Acad. Sci.*, 434:31(1984)

7. D. A. Butterfield, J. Lee, S. Ganapathi and D. Bhattacharyya, "Biofunctional Membranes IV. Active Site Structure and Stability of an Immobilized Enzyme, Papain, on Modified Polysulfone Membrane Studied by Electron Paramagnetic Resonance and Kinetics". *J. Membr. Sci.*, 91:47(1994)

8. S. Ganapathi , D. A. Butterfield and D. Bhattacharyya, "Flat Sheet and Hollow Fiber Membrane Bioreactors: A Study of Kinetics and Active Site Conformational Changes of Immobilized Papain Including Sorption Studies of Reaction Constituents". *J. Chem. Technol. Biotechnol.*(1995) (in press)

9. P. Zhuang and D.A. Butterfield, " Structural and Enzymatic Characteriztion of Papain Immobilized onto Vinyl Acohol/ Vinyl Butyral Copolymer Mmbrane. *J. membr. Sci.*66:247(1992)

10. P. Zhuang and D.A. Butterfield, "Spin Labeling and Kinetic Studies of a Membrane Immobilized Proteolytic Enzyme". *Biotech. Prog.*, 8:204(1992)

11. P. Zhuang and D.A. Butterfield, "Optimization of Covalently Coupling Enzymes to Polymeric Membranes". *J. Appl. Polym. Sci.*, 47:1329(1993)

12. J. Lee, "Electron Paramagnetic Resonance Studies of Membranes", M.S.Dissertation, University of Kentucky, 1993

13. D.A. Butterfield and J. Lee, "Active Site Structure and Stability of Thiol Protease, Papain, Studied by Electron Paramagnetic Resonance Employing a Methanethiosulfonate Spin Label". *Arch. Biochem. Biophys.*, Vol. 310, No.1:167 (1994)