

Flat-Sheet and Hollow Fiber Membrane Bioreactors: A Study of the Kinetics and Active Site Conformational Changes of Immobilized Papain Including Sorption Studies of Reaction Constituents

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Abstract: Papain, a sulfhydryl protease has been immobilized on flat-sheet modified polysulfone membranes and hydroxyethyl cellulose coated polyethersulfone hollow fibers. Amidase activity of the enzyme in solution and on the membranes has been assayed. Immobilized papain on the modified polysulfone membrane and the hollow fibers retains 12% and 25% of its activity (with 1 mmol dm⁻³ substrate) in solution, respectively. Loading experiments revealed decreased activity on the modified polysulfone membrane with increased enzyme loading. Adsorption experiments for the reaction product, *p*-nitroaniline, have been performed and an attempt has been made to correct for this in activity calculations. Apparent Michaelis–Menten parameters were determined for the modified polysulfone and hollow fibers, with both K_m and V_{max} being lower in the immobilized cases. Electron paramagnetic resonance study of the changes in active site conformation of an enzyme on a hollow fiber membrane are reported for the first time. Experiments using the sulfhydryl-specific (1-oxy-2,2,5,5-tetramethyl- Δ^3 -pyrroline-3-methyl)methanethiolsulfonate spin label depicted the presence of two subpopulations of immobilized papain on the hollow fibers, one of them active and one denatured.

Key words: immobilized enzymes, bifunctional membranes, hollow fibers, electron paramagnetic resonance.

INTRODUCTION

A bifunctional membrane is an entity in which a biological molecule is incorporated into a polymeric matrix cast in the form of a porous membrane, thereby imparting a biological function to the membrane.¹ Enzymes have been immobilized on different types of supports like microporous polymeric membranes, beads and gels. Membranes possess several advantages over other

support matrices and are the subject of this investigation.

Methods of immobilization fall into two broad categories of covalent binding and non-covalent or physical immobilization.² To attach the enzyme covalently onto polymer surfaces, the membrane polymer must be first modified to facilitate enzyme attachment. Polymer membranes with the hydroxyl group in their backbone are modified by reaction with glutaraldehyde, cyanogen bromide or FMP (2-fluoro-1-methyl pyridinium tosylate).^{3–6} Carboxyl group-containing polymers are

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typically activated using EDC (1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide).^{7,8} In all cases the enzyme is coupled to the activated membrane via the amine group of lysine or sometimes arginine.

In the current investigation, papain, a thiol protease was used as a model enzyme. This enzyme has been studied extensively in the solution state and its structure is fully defined.⁹⁻¹³ Immobilized papain on different supports has been researched by several investigators. The enzyme has been covalently attached to a porous silica support and also on porous (poly(γ -methyl L-glutamate) beads.^{14,15} Papain has been immobilized on porous chitosan beads,¹⁶ and the effects of varying surface concentrations, spacer length and pH on activities have been reported. Polyvinyl alcohol and polyvinyl butryl (PVA/PVB) copolymer membranes were used to attach papain covalently.^{6,17} Jayakumari and Pillai¹⁸ have investigated papain immobilization by utilizing supports of varying cross-linking density. Papain immobilization on modified alumina supports has been investigated by Hyndman and colleagues.^{19,20}

The enzyme undergoes conformational changes as a result of immobilization which directly influence its catalytic activity. To better understand the properties and functional response of the immobilized protein it is necessary to study these changes. Electron paramagnetic resonance (EPR) is a spectroscopic technique which is used to study changes at the active-site of the enzyme. EPR detects unpaired electrons and characterizes the interaction between nuclear and electron spins when the sample is placed in a magnetic field and irradiated with microwave radiation. Application of this technique necessitates the use of nitroxide 'spin labels' as probes since most biological molecules are not paramagnetic in their natural state.

Various researchers have studied the active site structural features of immobilized enzymes by EPR spectroscopy. Investigations have dealt with immobilized horse liver dehydrogenase on sepharose and CNBr-sepharose supports.^{21,22} Thermal inactivation of immobilized α -amylase, chymotrypsin and trypsin has also been studied by EPR.²³ Activity measurements of papain, in conjunction with EPR, on PVB membranes showed a good relationship between the properties and structure of the immobilized enzyme.⁶

In most cases of immobilized enzyme systems studied by EPR spectroscopy, two subpopulations of the bound protein have been shown to exist; the more restricted subpopulation has been reported as being the less active.²⁴

Until now, EPR has been used extensively to characterize immobilized enzymes only on beads, gels, and occasionally on flat-sheet microporous membranes.²⁴ In this report, we present the first EPR study of immobilized enzymes on hollow fiber membranes and compare the findings with those of immobilized papain on flat-sheet membranes. We have also addressed the issues of

adsorption of reaction constituents on polymer membranes and the effects of this adsorption on the activity of the immobilized enzyme. Conformation changes at the active site of papain and measurements of its enzymatic activity are reported.

2 MATERIALS

Hydroxyethyl cellulose (HEC) coated polyether sulfone (PES) hollow fibers (1 μ m average pore size) were acquired from Sepracor. Flat-sheets of modified polysulfone blend, microporous (0.45 μ m average pore size, 152.4 μ m thickness) membranes (MPS), UltraBind US 450, were purchased from Gelman Sciences. Papain (lyophilized powder), *N*-benzoyl-DL-arginine-4-nitroanilide (BAPNA), DL-cysteine, and ethylenediaminetetraacetic acid (EDTA) [disodium salt, dihydrate, 99+ % purity] were purchased from the Sigma Chemical Co. Dimethyl sulfoxide (DMSO) [HPLC grade] was obtained from Aldrich. The spin label, (1-oxyl-2,2,5,5-tetramethyl- Δ^3 -pyrroline-3-methyl) methanethiolsulfonate (MTS), was purchased from Reanal, Budapest, Hungary.

3 EXPERIMENTAL METHODS

3.1 Membrane characterization

The commercial membranes that were used in various studies were characterized for surface area and pore size by nitrogen gas adsorption measurements, utilizing an Accelerated Surface Area and Porosimetry System from Micromeritics, model ASAP 2000. Prior to membrane runs, the instrument was calibrated using standard silica and alumina particles. Approximately 1 g of cut membrane pieces was loaded into the analyzer cell and degassed under 4 μ Torr vacuum for at least 24 h. The analysis was performed at liquid nitrogen temperature (77 K).

3.2 Product and substrate adsorption

Batch adsorption of the substrate, BAPNA, and product, *p*-nitroaniline (PNA), on the membranes was studied. The flat-sheet modified polysulfone membranes were loaded into a flow cell (4.7 cm in diameter) and the experiments were performed in a flow set-up (Fig. 1). *p*-Nitroaniline and BAPNA solutions of different concentrations were perfused through the membrane to acquire kinetic and equilibrium adsorption data. The HEC-coated hollow fibers were cut into pieces (0.4 cm length) and adsorption experiments were performed on loose fibers.

3.3 Enzyme immobilization

The HEC-coated hollow fibers were potted into a module using epoxy (6 fibers of 6.4 cm length each). They were first activated by reaction with glutaraldehyde at 25°C for 48 h, following which the fibers were treated with a papain solution at 4°C for 48 h to yield biofunctional hollow fibers. The flat-sheet MPS membranes were cut into discs (each disc weighing 0.08 g) and reacted with a solution of papain at 4°C for 24 h. In both cases, the membranes were washed extensively to remove any non-specifically adsorbed papain and then reacted with ethanolamine to cap unreacted aldehyde groups. The amount of papain immobilized was determined by difference in protein concentration before and after the immobilization reaction, utilizing Biorad's Bradford protein assay.

3.4 Activity measurements

The amidase activity of papain was assayed as described by Arnon,²⁵ with some modifications. A 2 mmol dm⁻³ solution of the substrate BAPNA was prepared in Tris buffer containing 1% (w/v) DMSO. The sulfhydryl group on cysteine-25 at papain's active-site remains blocked in the natural state and the enzyme has to be activated prior to the assay. This was accomplished by activating papain for 10 min in a solution containing cysteine and EDTA. An equal volume of substrate was added after activation, and the reaction mixture was circulated through a flow-through cell in the spectrophotometer where the absorbance of the solution was recorded at 410 nm, to determine the concentration of *p*-nitroaniline formed in the reaction (Fig. 1). All reactions were carried out at 37°C, and at a pH of 7.5.

The biofunctional MPS flat-sheet membranes were placed in the flow cell and initial rate measurements were made by operating the flow cell in a recirculation mode (Fig. 1). Immobilized enzyme was also activated with a cysteine and EDTA solution as described above, prior to substrate addition. The hollow fibers in the module were assayed for activity of papain covalently bound to them. Substrate solution were made to flow through the lumen of the fibers and the shell side where permeate was collected and recirculated. Apparent

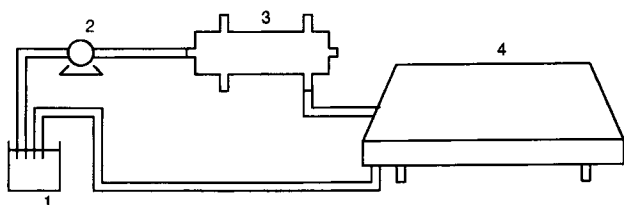


Fig. 1. Schematic of flow set-up used for activity measurements. 1, Reaction vessel; 2, peristaltic pump; 3, membrane module; 4, UV-Vis spectrometer.

Michaelis-Menten parameters were calculated from kinetic measurements. For both flat-sheet and hollow fibers, rate data were acquired for 20 min, when product concentration increased linearly with time.

3.5 EPR studies

3.5.1 Immobilization of spin-labeled papain on membranes

Papain was first spin-labeled using MTS, a sulfhydryl specific nitroxide label. The enzyme was activated in a solution containing NaCN and EDTA at room temperature for 2 h, after which it was reacted with MTS spin label. Spin-labeled protein was separated from excess unreacted spin label by gel filtration on a Sephadex G-25 (medium) column and used further. Spin-labeled papain was coupled to both flat-sheet and hollow fiber membranes. The hollow fiber membranes were first activated by reaction with glutaraldehyde as described above. The MPS membranes were cut into discs (4.7 cm diameter) and reacted with spin-labeled papain for 24–36 h at 4°C. Reaction with spin-labeled papain was also performed on activated hollow fibers, 6 cm in length for 36–48 h at 4°C. Both membranes were then washed extensively to remove non-specifically adsorbed protein.

3.5.2 EPR spectra acquisition

All EPR spectra were recorded on a Bruker 300 EPR spectrometer with a TM 8988 rectangular cavity at room temperature. The parameters used to acquire spectra were: microwave frequency 9.78 GHz; microwave power 24.2 mW; modulation frequency 100 kHz; modulation amplitude 0.32 G and scan width 130 G. A special quartz cell²⁴ was used to obtain spectra of flat-sheet membranes and the hollow fibers were placed in a 'round cell' to acquire spectra, where each fiber was inserted individually into the cell. In all cases, the biofunctional membranes were fully hydrated and the spectra thus obtained reflected true conditions to which the membrane-bound enzyme is exposed. The effect of urea, a denaturant, on the active site conformation of papain was also investigated. The experiments were performed by incubating pieces of MTS-labeled hollow fiber membranes in various concentrations of urea for 30 min at 25°C, after which EPR spectra were acquired.

4 RESULTS AND DISCUSSION

The two membranes, flat-sheet MPS and HEC-coated hollow fibers, were subjected to a set of experiments to fulfill our goal of characterizing and comparing the biofunctional membranes.

4.1 Pore volume analysis

The first set of experiments involved pore volume analysis by nitrogen gas adsorption which yielded pore

sizes and BET surface area measurements where appropriate. Protein immobilization is influenced by several factors, the nature and morphology of the membrane support being one of them. Surface area and pore size of the support affect protein loading, which in turn affects bioreactor performance.

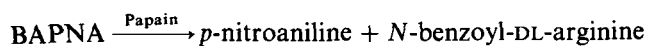
From the results of the pore volume analysis experiments, surface area measurements were made using the BET method. The BET surface areas of the MPS flat-sheet and HEC-coated hollow fibers were determined to be 9.22 and $7.26 \text{ m}^2 \text{ g}^{-1}$, respectively.

A theoretical estimate of the area available for a monolayer of papain immobilized on the membranes was made. The calculations were performed based on a uniform papain loading of 1 mg immobilized on the membranes. The area occupied by one molecule of papain¹⁴ is 18 nm^2 and therefore 1 mg of papain (MW = 23 000 daltons) requires an area of $4.71 \times 10^{17} \text{ nm}^2$. The area available on the MPS and hollow fiber membranes used in the experiments is 7.38×10^{17} and $1.38 \times 10^{18} \text{ nm}^2$, respectively. From this calculation it was determined that for a 1 mg immobilization of the enzyme adequate surface area was available on both membranes. But it should be noted that for single site covalent enzyme attachment, the area requirement will be considerably lower. This may explain the observed decreased activity with increased loading on the MPS membrane (see below).

4.2 Activity assay results

The extent of papain immobilized on HEC-coated hollow fibers was 5.7 mg g^{-1} or 0.78 mg m^{-2} (based on total membrane surface area determined by the BET method). In the case of the flat-sheet membranes, the amount of papain immobilized was 10.8 mg g^{-1} or 1.17 mg m^{-2} .

Papain hydrolyzes BAPNA, as shown in the reaction



The effect of enzyme loading on the activity of the bio-functional membrane was also studied. Four different enzyme loadings were considered, immobilizing papain on four discs of the MPS membrane, with a different initial enzyme concentration in each case. The same immobilization technique of a 24 h soak was applied as described above. The result reflects a clear reduction (Fig. 2) in activity with increased loading, which is consistent with the surface area calculations above; higher enzyme loading can lead to crowding of the enzyme on the surface, resulting in spatial restrictions, blocking the active site and/or denaturing the protein. Alternatively, multiple point attachments of the enzyme would decrease its conformational flexibility at the active site, thereby inhibiting the ability of the enzyme to adapt to binding of the substrate. The Lineweaver–Burk plot for

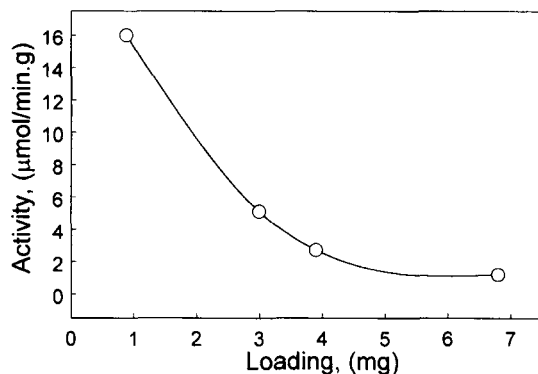


Fig. 2. Effect of enzyme loading on activity of papain immobilized on MPS membrane.

papain on the MPS flat-sheet membrane and HEC-coated hollow fibers is depicted in Fig. 3, and the apparent Michaelis–Menten parameters of papain on both membranes are reported in Table 1. All activity calculations are normalized with respect to enzyme loading. The MPS membrane with 3 mg bound papain retains only 12% of the activity (at 1 mmol dm^{-3} BAPNA) of papain in the homogenous state. The HEC-coated hollow fiber retains 25% of homogeneous results as shown in the plot of relative activity, for papain loading of 1 mg (Fig. 4). The apparent Michaelis–Menten constant, K_m^{app} , for both membranes is significantly lower than that for papain in solution. This unusual result implies increased affinity of the immobilized enzyme for the substrate. These results are of course apparent kinetic parameters, without consideration of diffusional resistance inside the pores, sorption of reaction constituents and changes in active site conformation of covalently bound papain.

4.3 Adsorption experiments

The amidase activity of papain involves hydrolysis of BAPNA to yield PNA as one of the products. Kinetic measurements on biofunctional polymer membranes like the flat-sheet MPS and HEC-coated hollow fibers, where both substrate and product are transported

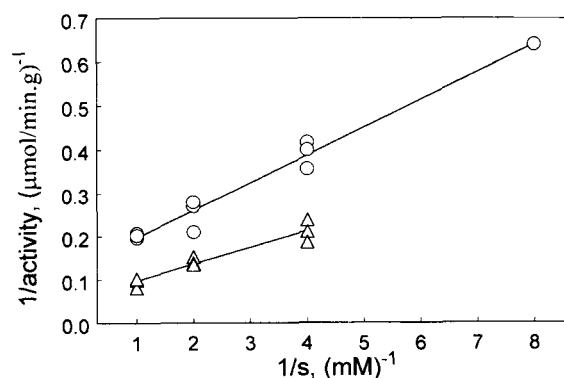


Fig. 3. Lineweaver–Burk plot for papain on MPS membrane and hollow fiber, with enzyme loadings of 3 mg and 1 mg respectively.

TABLE 1
Michaelis–Menten Parameters

Membrane	V_{max} ($\mu\text{mol min}^{-1} \text{g}^{-1}$)	K_m (mmol dm^{-3})	Correlation coefficient (r^2)
MPS ^a	7.46	0.48	0.99
Hollow fiber ^a	17.5	0.69	0.96
Papain in solution (no membrane)	111	1.44	0.98

^a Apparent values for membranes.

through the membranes, results in their adsorption. If significant adsorption takes place, a correction of activity is required to present a more accurate picture of immobilized enzyme kinetics.

Adsorption experiments of both *p*-nitroaniline and BAPNA indicated that PNA had a higher capacity and preliminary tests of its adsorption have been considered. Figures 5 and 6 depict PNA adsorption on the MPS membrane. The kinetics of the adsorption process displayed an exponential decrease in the residual concen-

tration of *p*-nitroaniline at all initial concentrations at which the experiment was carried out. Data from all experiments were fitted to an exponential curve (Fig. 5). Adsorption experiments at various concentrations of the product were carried out to determine the equilibrium adsorption curve. The isotherm displays a linear increase in adsorption capacity (*Q*) up to a concentration of 0.24 mmol dm⁻³. A correction for product adsorption (using Fig. 5 data) in the activity of the bifunctional MPS membrane with a papain loading of 3 mg reflected an increase in activity to 18% of that in solution. Calculations of corrected activity assuming equilibrium adsorption were also performed considering the adsorption isotherm in Fig. 6. For the MPS membrane, the equilibrium corrected activity was determined to be 47% of homogeneous papain activity. Both the adsorption corrections on activities are indicated in Fig. 4.

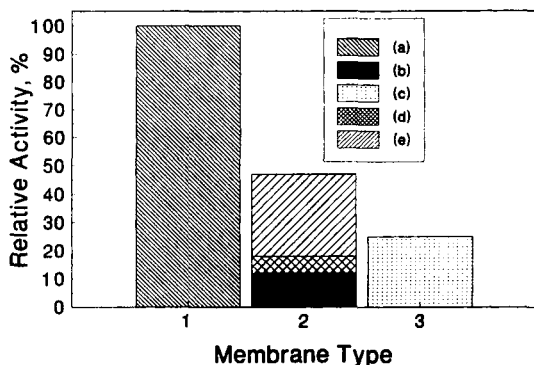


Fig. 4. Activity (1 = homogeneous, 2 = flat-sheet membrane, 3 = hollow fiber membrane) of papain on flat-sheet and hollow fiber membranes, relative to homogeneous activity of papain in solution. (a)—Homogeneous activity, (b)—MPS membrane, (c)—HEC hollow fibers, (d)—MPS membrane, activity correction using adsorption vs time data, (e)—MPS membrane, activity correction assuming equilibrium adsorption.

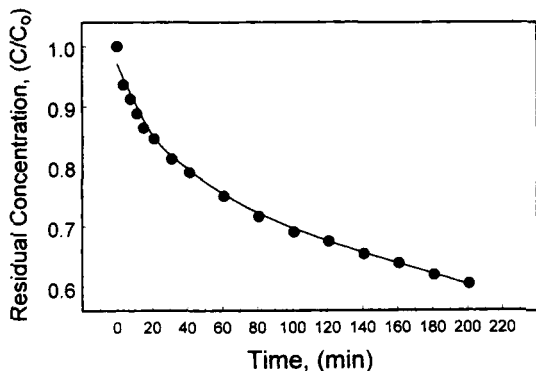


Fig. 5. Time profile of adsorption of product on MPS membrane. Residual concentration, *C/C*₀ at any time *t* is reported relative to the initial concentration.

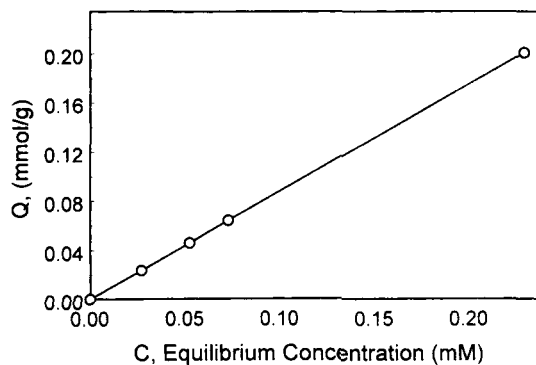


Fig. 6. Adsorption isotherm for *p*-nitroaniline on MPS membrane.

4.4 EPR results

Nitroxide spin labels in solution generate EPR spectra of three equally spaced lines of about the same intensity, arising from the interaction between electron and nuclear magnetic moments when placed in a magnetic field and irradiated with microwaves. However, when motional restrictions are placed on the spin label, asymmetric line broadening occurs. This is clearly depicted in Fig. 7, a spectrum of MTS-labeled papain in solution. The relaxation phenomena in nitroxides leads to greater

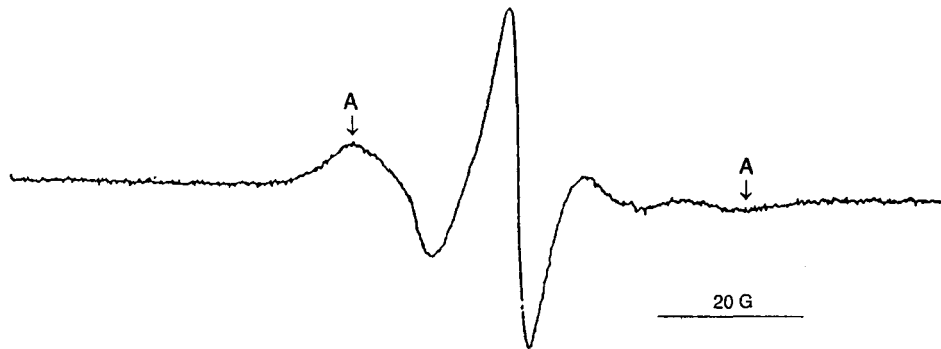


Fig. 7. EPR spectrum of MTS-labeled papain in solution. Distance between arrows indicates the hyperfine splitting parameter, $2A'_{zz}$.

broadening of the high field resonance line ($M_1 = -1$) than for the low field ($M_1 = +1$) and center ($M_1 = 0$) resonance lines.^{1,26,27} Immobilization of the spin label, MTS in this case, on the single SH group of papain, which is then attached to the membrane, leads to further motional restrictions manifested by line broadening and separation of the peaks of the EPR spectrum. Slower motion is also characterized by the hyperfine splitting between the low-field and high-field lines designated as $2A'_{zz}$, the hyperfine splitting parameter. The larger the $2A'_{zz}$, the slower the motion of the spin-label attached to the single SH group of papain located at the active site.²⁴ Figure 7 clearly shows this splitting of MTS-labeled papain in solution is characterized by one population with a $2A'_{zz}$ splitting of 53 G.

In earlier EPR studies of papain spin-labeled at the active site with MTS and covalently attached to the MPS membrane,²⁴ we demonstrated the presence of two active-site subpopulations, A and D. These were characterized by two values of the hyperfine splitting parameter, 60 and 70 G, respectively. The active sub-

population A with a splitting of 60 G thus had more motion than D, which with a splitting of 70 G was more immobilized, and both active site environments displayed less motion than the active site of MTS papain in solution.

Denaturant studies using urea and guanidine hydrochloride were also reported for the MPS membrane,²⁴ and a conversion of the subpopulation A to D was discerned with increasing concentration of urea. This conversion of the active subpopulation to the denatured one was characterized by the conversion parameter, $I(D)/I(A)$ defined by:

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

In the current study EPR experiments of MTS-papain on HEC-coated hollow fibers were performed to determine the nature of changes at the active site of the

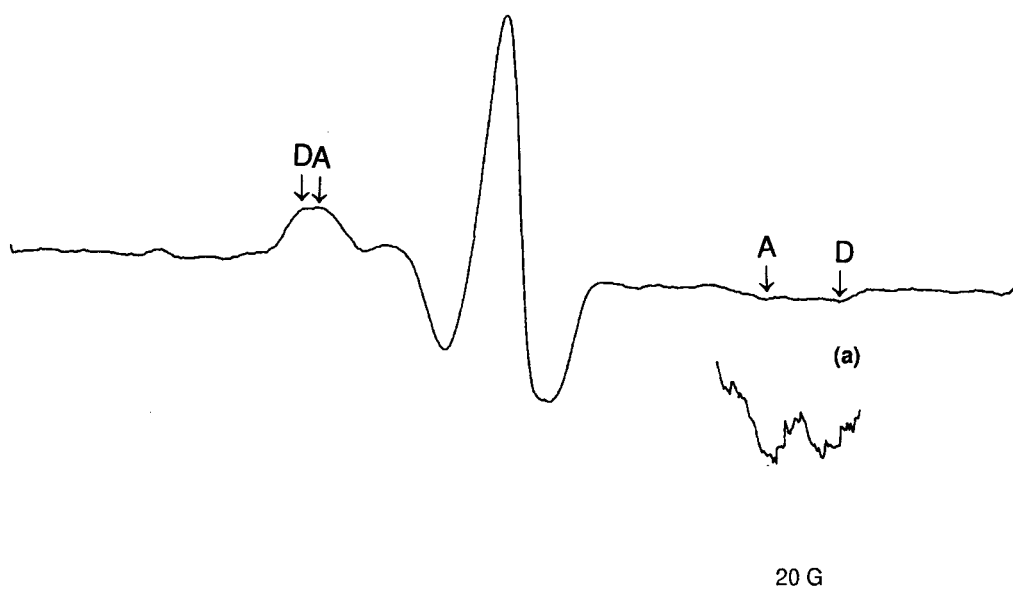


Fig. 8. EPR spectrum of MTS-labeled papain on hollow fiber, where A and D indicate the active and denatured subpopulations respectively. (a)—Signal enhancement of two subpopulations at the high-field line.

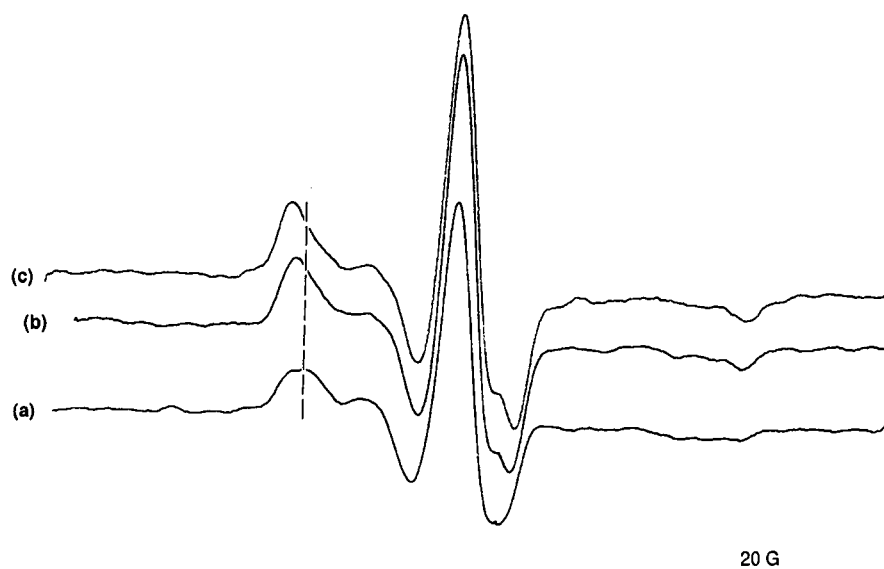


Fig. 9. Effect of denaturant on the EPR spectrum of hollow fiber. (a)—Fiber before urea treatment, (b) and (c)—fiber treated with 1 and 2 mol dm⁻³ urea, respectively. The dashed line indicates the position of the active subpopulation (A) associated with the low-field, $M_1 = +1$ resonance line. Note that with increasing urea, this peak is lost and only the denatured subpopulation is observed. See text.

immobilized enzyme. The spectrum of spin-labeled papain on the hollow fiber is depicted in Fig. 8. The spectrum displays similar characteristics of MTS-papain on the MPS membrane. Two subpopulations, A and D, are evident in the spectrum of the hollow fiber characterized by hyperfine splitting parameters of 57 and 70 G, respectively. This definite broadening of line-widths and the presence of two subpopulations at the active site of the enzyme, are clearly demonstrated in Fig. 8(a) under increased spectral amplification. Based on the similarity of peak heights of MTS in subpopulations at all three respective resonance lines, we estimate about 50% of the papain molecules are in the active form. The urea denaturation experiments with hollow fibers were performed to further substantiate the presence and nature of the subpopulations A and D. The effect of urea on the spectrum of the hollow fiber is shown in Fig. 9. For the hollow fiber, $I(D)/I(A)$ increases from 1 (with no urea) to 1.3 to 1.7 upon increasing urea concentration to 1 mol dm⁻³ and 2 mol dm⁻³, respectively, displaying a trend similar to that of the MPS membrane. We can therefore conclude that MTS-papain exists as two subpopulations on the hollow fibers, with the second subpopulation having a $2A'_{zz}$ splitting of 70 G being the denatured one.

Immobilization of the protein thus alters its inherent conformation and also its performance as a biocatalyst. These changes are manifested in reduced enzyme activity and the main factors contributing to this loss of activity are enzyme conformational changes, adsorption of reaction constituents on the support, and diffusional resistance to transport inside the support matrix. In this investigation we have established that in specific

instances adsorption can significantly affect measured activity, and demonstrated, utilizing EPR spectroscopy, that two subpopulations of immobilized papain can be discerned on different types of polymeric supports, with the denatured subpopulation contributing to about 50% of enzyme deactivation.

5 CONCLUSIONS

Immobilization of papain on flat-sheet and hollow fiber membranes revealed considerable loss in activity of the immobilized system compared with homogeneous enzyme activity. Biofunctional membrane activity decreased substantially with increased loading of papain on the MPS membrane, which can be explained by multi-point enzyme attachment and crowding, thus leading to insufficient surface area availability for the enzyme on the membrane. Significant adsorption of *p*-nitroaniline on the membranes was observed and a correction was performed based on kinetic and equilibrium adsorption data.

EPR investigation of the active-site conformational changes of papain on the hollow fiber membrane displayed the presence of two subpopulations as on the flat-sheet membrane. Denaturant studies also depicted that one of them was denatured and one active.

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