Kinetics and Active Fraction Determination of a Protease Enzyme Immobilized on Functionalized Membranes: Mathematical Modeling and Experimental Results

Sowmya Ganapathi-Desai,[†] D. Allan Butterfield,[‡,§] and Dibakar Bhattacharyya*[,†,§]

Department of Chemical and Materials Engineering, Department of Chemistry, and Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky 40506-0046

A detailed study on the performance of a membrane bioreactor is presented, considering diffusion reaction models with product adsorption and structure−function correlations. The enzyme papain was utilized for experimental investigation both in the homogeneous state and on a modified polysulfone (MPS) membrane. Variation of enzyme loading on the membrane and enzyme concentration in the solution state depicted decreasing activity with increasing loading. The performance of the bioreactor was simulated using a diffusion reaction model within a recirculation loop. Electron paramagnetic resonance (EPR) spectroscopy was utilized to study the conformational changes of the active site of papain immobilized on the MPS membrane. Two models were applied to correlate the structure and function of the biocatalyst, based on loading (kinetics) and EPR (structure). The active fractions, λ, determined from the two models were 0.29 and in the range 0.25−0.3, respectively. The intrinsic kinetics (V_{max}) for the immobilized enzyme as determined by the correlations were in the range 101−121 μmol/(g·min), compared to 111 μmol/(g·min) for the homogeneous enzyme. This proves that the immobilized enzyme kinetics do approach homogeneous kinetics for papain on the MPS membrane, when corrected for adsorption and conformational changes.

1. Introduction

The immense potential of enzymes to catalyze reactions external to their biological environment was recognized early, and this potential has been harnessed in a variety of ways (Butterfield, 1996). Enzymes have been attached to various supports, including membranes, packed beds, and gels (Diaz and Balkus, 1996); there are certain benefits to utilizing membranes over other support matrices. The advantages of convective flow versus mainly diffusive transport in packed columns are some of the important differences that make membranes more amenable to affinity separations and immobilized enzyme reactions (Klein, 1990; Butterfield, 1996). Membranes thus possess several advantages over other support matrices and are the subject of this investigation.

The enzyme undergoes conformational changes as a result of immobilization which directly influence its catalytic activity (Butterfield et al., 1994; Bhardwaj et al., 1996). It could also deactivate with time and by leaching and eluting out of the support matrix (Shyam et al., 1975; Houng et al., 1993; Rialdi and Battistel, 1996). Leaching of the protein could arise especially when it is held in place by weaker forces, as in cases of physical adsorption. To better understand the properties and functional response of the immobilized protein it is necessary to study these changes. Electron paramagnetic spectroscopy (EPR) is a powerful tool that is widely used in several areas of study including biological systems, polymer chemistry, and free radical reactions and also to study fouling in ultrafiltration membranes (Butterfield et al., 1994a; Friedrich et al., 1980; Oppenheim et al., 1994). EPR requires the presence of a paramagnetic molecule (Butterfield, 1982). However, in using EPR spectroscopy to study biological molecules such as proteins, it is often the case that the protein in consideration is not paramagnetic in the natural state. This necessitates the use of an external probe called the "spin label" to impart paramagnetism. Molecules containing the nitroxide moiety are widely used as spin labels, due to the high stability of the nitroxide radical and its amenability to synthesis.

The information about the polarity and the local environment of the paramagnetic center of the spin label that can be determined and the relatively simple spectra of nitroxides that need to be analyzed [i.e., usually only electron−nuclear hyperfine coupling of the nitrogen nucleus ($I = 1$) and the unpaired electron are resolved in aqueous solution (Butterfield, 1982)] have resulted in wide application of these probes. The first detailed EPR analysis of an enzyme both in the solution and immobilized states was performed on α-chymotrypsin attached to a sepharose support (Berliner and Wong, 1974; Clark and Bailey, 1983; Clark and Bailey, 1984). Two subpopulations of the immobilized enzyme were observed for the first time, each differing in their motion. In this case the population with the higher activity was discovered to be the one with faster motion. The percentages of the two subpopulations in different samples of the

[†] Department of Chemical and Materials Engineering.
[‡] Department of Chemistry.
[§] Center of Membrane Sciences.
* Corresponding author. Telephone: (606) 257-2794. Fax: (606) 323-1929. E-mail: db@engr.uky.edu.

10.1021/bp980096p CCC: $15.00
Published on Web 11/14/1998

immobilized enzyme were delineated, assuming that the enzyme existed in the same two states in all samples, only differing in relative amounts of the two populations. Since this EPR investigation, the technique has been used to study changes in conformation of different kinds of immobilized enzymes, including alcohol dehydrogenase, β-galactosidase, subtilisin, papain, and glutamate dehydrogenase (Marg et al., 1986; Skerker and Clark, 1987; Clark et al., 1987, 1988; Telo et al., 1990; Zhuang and Butterfield, 1992; Butterfield et al., 1994; Ganapathi et al., 1995; Bhardwaj et al., 1996; O'Connor and Bailey, 1989; Viswanath et al., 1998). In most EPR reports of enzymes on supports, two subpopulations have been discerned, but just one population and as many as three subpopulations have also been observed (Zhuang and Butterfield, 1992; Song et al., 1992).

A mathematical model for a membrane bioreactor containing an immobilized enzyme involves the consideration of (a) substrate transport to and from the biomolecule, convective or diffusive mode, or both; (b) enzymatic reaction; (c) state of the biomolecule—partial denaturation; and (d) enzyme distribution on the support. Several models exist in the literature which describe the performance of membrane bioreactors considering some or most of the above-mentioned factors. Detailed modeling of packed bed reactors and stirred tank reactors where the enzymes are attached to beads, gels, and suspensions has also been performed (Lortie and Pelletier, 1992; Grulke et al., 1977; Santoyo et al., 1993).

Mathematical models of immobilized enzyme systems have considered different membrane configurations and transport conditions, but most of these models have assumed the existence of the protein on the support as a single population. This is not true in most cases, and consideration of the presence of more than one population and its effect on the parameters of the biocatalyst is vital. Biofunctional supports, polymeric or inorganic, could interact not only with the enzyme but also with the product and substrate. We have demonstrated in earlier work that this interaction could affect measured activity significantly (Ganapathi et al., 1995; Bhattacharyya et al., 1996). This model will consider the effect of adsorption of reaction constituents in addition to taking into account conformational changes at the enzyme active site.

When flow rates are relatively small it can be assumed that transport through the membrane takes place chiefly by diffusion and convective flow is neglected. For flat-sheet biofunctional membranes and affinity membranes, it has been found in various cases that radial gradients are negligible (Thommes and Kula, 1995; Vishwanath et al., 1995). This means that substrate is now diffusing through the membrane pore and diffusion of substrate in the pore is equal to the rate of enzymatic reaction. The model outlined below will consider diffusive flow of substrate in the axial direction.

2. Development of Mathematical Model for Bioreactor Including Adsorption Effects and Enzyme Conformational Changes

The model applied to the flat-sheet biofunctional membrane in this research incorporates a parameter to account for alterations in enzyme conformation, leading to the two subpopulations of the enzyme. The following are the assumptions of this model:

1. Uniform enzyme distribution.
2. Diffusion transport across the membrane in the axial direction.

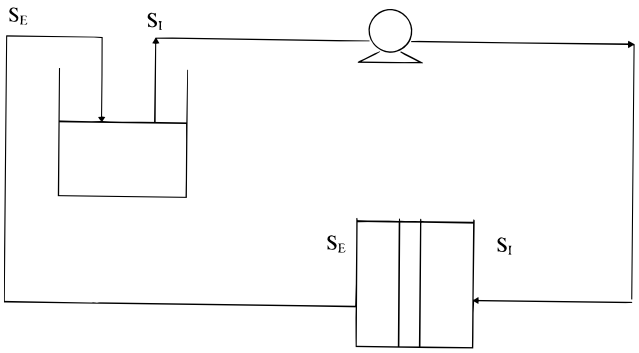

Figure 1. Schematic of recirculation setup through the membrane cell, where S_I represents substrate flowing into the membrane and S_E represents solution leaving the membrane after reaction and flowing back into the beaker.

3. Complete mixing in the bulk of the solution.
4. Michealis−Menten kinetics for the enzymatic reaction.
5. Enzyme on the membrane exists in more than one population.

$$D_{\text{eff}} \frac{\mathrm{d}^2 S}{\mathrm{d}x^2} = \frac{V_{\max} S}{K_{\text{m}} + \text{S}} \tag{1}$$

where D_{eff} is the effective diffusivity of the substrate in the solute and it is assumed that this does not vary with distance along the membrane. K_{m} and $V_{\max}$ are Michaelis−Menten reaction parameters.

The boundary conditions for this system are

$$x = 0, \quad S = S_0 \tag{2}$$

$$x = L, \quad \frac{\mathrm{d}S}{\mathrm{d}x} = 0 \tag{3}$$

Using nondimensional parameters

$$\bar{S} = S/K_{\text{m}}; \quad z = x/L; \quad R = r/a \tag{4}$$

$$\frac{\mathrm{d}^2 \bar{S}}{\mathrm{d}z^2} = \frac{L^2 V_{\max}}{D_{\text{eff}} K_{\text{m}}} \frac{\bar{S}}{1 + \bar{S}} \tag{5}$$

where $L^2 V_{\max}/D_{\text{eff}} K_{\text{m}}$ is the square of the Thiele modulus, represented by ϕ.

When the membrane bioreactor is operated by recirculating the reaction constituents through the membrane, the above equation must be solved in conjunction with the expression for substrate recirculation. The schematic of the recirculation setup is represented in Figure 1. A balance is taken around the feed tank in the schematic to give the following equation:

$$Q_{\text{f}}(S_{\text{e}} - S) = V \frac{\mathrm{d}S}{\mathrm{d}t} \tag{6}$$

where V is the volume of the feed tank, Q_{f} the flow rate through the membrane, S the substrate concentration in the feed tank, and S_{e} the concentration of substrate at the exit of the membrane. The initial condition for this system is

$$t = 0, \quad S = S_0 \tag{7}$$

By introducing a nondimensional variable for time and using the same representation for dimensionless S as before

$$\tau = \frac{t}{V/Q_f} \tag{8}$$

Substituting into eq 6

$$\frac{d\bar{S}}{\bar{S}_e - \bar{S}} = d\tau \tag{9}$$

By integrating eq 9 analytically and using the initial condition to determine the integration constant

$$\ln\left(\frac{\bar{S}_e - \bar{S}_0}{\bar{S}_e - \bar{S}}\right) = \tau \tag{10}$$

The dimensionless concentration profile within the membrane reactor can thus be obtained for each pass in the recirculation reactor configuration and substrate depletion with time is determined.

2.1. Adsorption of Product. In cases where either substrate or product adsorb on the membrane, the extent of this interaction and its effect on the measured reaction kinetics must be considered. Control tests must be performed on the membrane to determine the adsorption isotherm for the product. Considering an instance where product adsorption is significant, the following analysis is used:

Considering a linear isotherm

$$Q = k_a P \tag{11}$$

This isotherm describes a linear increase in amount adsorbed with increasing product concentration, where k_a is the adsorption coefficient. Q represents the adsorption capacity of the adsorbent in moles per gram. Assuming that by stoichiometry of the enzyme-catalyzed reaction one mole of adsorbing product is formed per mole substrate, the equation for substrate balance is

$$S = S_0 - P \tag{12}$$

For product that adsorbs on the membrane, total product is expressed as a sum of product adsorbed (ads) and not adsorbed (nads):

$$P = P_t = P_{nads} + P_{ads} \tag{13}$$

Therefore

$$S = S_0 - (P_{nads} + P_{ads}) \tag{14}$$

$$P_{ads} = f(Q,W) \tag{15}$$

The total moles of product formed is

$$(P_t)_{moles} = P_{nads}V_1 + QW \tag{16}$$

By substituting into eq 14

$$P_{nads} = \frac{(S_0 - S)V_1}{V_1 + k_a W} \tag{17}$$

for linear adsorption.

An algebraic equation results, and total product and product adsorbed can then be determined. Therefore, after solving eq 5 for S and determining the substrate profile within the membrane pore, eqs 13−17 are utilized to obtain total and measured product profiles in the membrane. The effect of product adsorption on actual activity and on product profiles within the membrane pore can be assessed using this treatment.

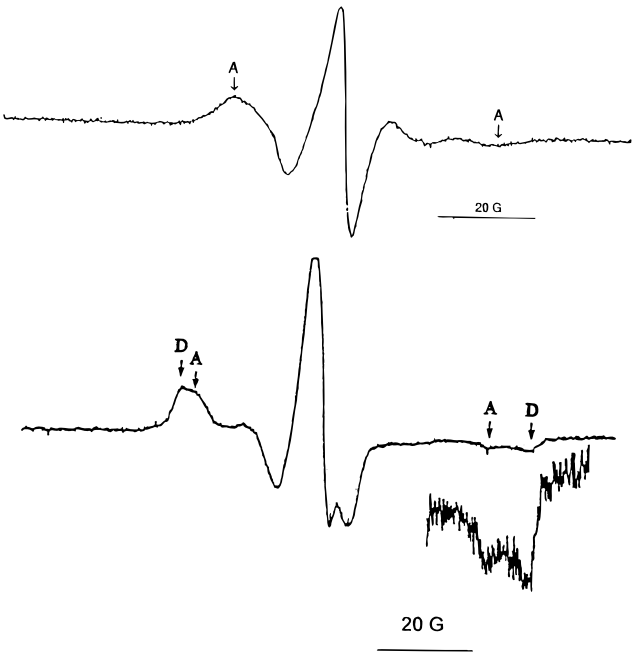

Figure 2. (a) Typical EPR spectrum of MTS-labeled papain in the homogeneous state, the distance between the arrows indicates the hyperfine splitting parameter, $2A_{zz}'$ for the single population (A). (b) Typical EPR spectrum of papain on the MPS membrane, with two subpopulations. The hyperfine splitting parameter, $2A_{zz}'$ for the subpopulations, A and D, are indicated.

2.2. Determination of Active Fraction in Immobilized Enzyme. It is essential to recognize the extent of reduction in enzyme activity due to variation at the active site and also quantify this reduction. To achieve this goal, parameters obtained from EPR spectroscopy are utilized. If the spectra of the immobilized enzyme are composed of more than one population, the spectra are deconvoluted to determine the percentage composition of each of the populations. Nitroxide spin labels in solution generate EPR spectra of three equally spaced lines of about the same intensity; when motional restrictions are placed on the spin label asymmetric line broadening occurs. This is evident in Figure 2a, the spectrum of spin-labeled papain in the homogeneous state. When the spin label is immobilized on the single sulfhydryl group of papain, which is then attached to the membrane, greater motional restrictions are placed on the label (Figure 2b). Slower motion is also characterized by the hyperfine splitting between the low-field and high-field lines designated as $2A'_{zz}$. The larger the $2A'_{zz}$, the slower the motion of the spin label. A typical two-component spectrum is defined by a broad hump at the low-field line, and as expected, two resonance positions versus one for the $M_I = -1$ line (Figure 2b). The two subpopulations are characterized as A (with higher activity) and D (the more "denatured" subpopulation) with hyperfine splitting parameters ($2A'_{zz}$) of 60 and 70 G, respectively (Butterfield et al., 1994b). A specific method for deconvolution of the spectra and determining the fractions of A and D is outlined below.

The technique utilized to ascertain the fraction of active enzyme considers immobilized enzyme activity at different loadings and then utilizes EPR spectroscopy to compare and validate results obtained from the loading model. We can assume that the activity at a given loading can be represented as a sum of two activities A_A

and A_D by the following equation:

$$A_M = \lambda A_A + (1 - \lambda)A_D \quad (18)$$

where A_A represents the activity of homogeneous enzyme and A_D the activity of immobilized enzyme at very high loading. This portrayal of activity is valid when normalized activity of the biofunctional membrane decreases with increasing loading, and A_D therefore represents activity of the more denatured population. Then, λ is the fraction of more active enzyme and $(1 - \lambda)$ the fraction of enzyme with lower activity. Thus utilizing λ obtained from eq 18 the active fraction can be determined; this value can then be applied to the deconvolution model for the EPR spectra, and the nature of the spectra thus generated can be examined.

Deconvolution of EPR Spectrum.

Assumptions:

1. Spectra are composed of two populations, A and D, defined by their respective motions.
2. G_A is the spectrum of the active subpopulation.

The equation for the composite spectrum can be written in terms of the two spectra

$$G_M = \lambda G_A + (1 - \lambda)G_D \quad (19)$$

Thus, λ is calculated from eq 18 and this value of λ is inserted into the deconvolution model for the EPR spectra. The resulting subtracted spectrum for the more denatured subpopulation is then analyzed for characteristic features. Further, the deconvolution model can be solved for λ over the entire mole fraction range and the subtracted spectra examined. A quantitative analysis of the immobilized enzyme subpopulations can thus be achieved by utilizing the best fit for the active fraction.

2.3. Evaluation of Model Parameters and Method of Solution. To solve the system of equations presented above, a number of parameters have to be determined. Parameters necessary to solve differential eq 5 are D_{eff}, L, K_m, and V_{max}. The substrate diffusivity, D_{eff}, is assumed to be the diffusivity of the solute in water in the pores of the membrane. This is estimated using the Wilke−Chang equation. The length of the membrane pore or membrane thickness is a parameter specified by the supplier. This would vary if the pores were tortuous, and the tortuosity factor would have to be considered. Kinetic parameters K_m and V_{max} have to be obtained by initial rate experiments. Adsorption experiments, involving product adsorption on the membrane at various concentrations, will yield the constants of the adsorption isotherm. The membrane weight, W, is attained by a simple weight measurement. The initial substrate concentration, S_0, is a known parameter specified by the experimenter. To correct for conformational changes occurring at the active site of the enzyme, EPR experiments have to be performed. The obtained spectra have to be analyzed and deconvoluted to determine the fraction of active enzyme, λ.

The nonlinear ordinary differential eq 5 is solved numerically using a subroutine from the IMSL Fortran library, BVPFD. This routine solves the equation using a variable order, variable step size finite-difference method. The linear ordinary differential equation describing recirculation of substrate (9) is solved analytically, and eq 5 is then placed inside the recirculation loop and solved for the requisite number of passes.

3. Experimental Methods

Papain, a sulfhydryl protease and amidase, was used as a model enzyme to conduct activity experiments. Data from kinetics experiments on homogeneous and immobilized papain were utilized to determine model parameters. Flat-sheet, microporous, polymeric membranes (0.45 μm average pore size, 152.4 μm thickness) from Gelman Sciences were used for covalent attachment of the enzyme. The commercial membranes were available in the activated form, with functional aldehyde groups for protein attachment. Enzyme immobilization was performed by a flow-through method where the membrane was placed in a flow cell and enzyme solution was recirculated through the membrane at a flow rate of 3 mL/min for 90 min at a temperature of 25 °C. The same flow cell was utilized for kinetic measurements and adsorption experiments. After immobilization the membrane was washed extensively to remove any nonspecifically 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 Biorads' Bradford protein assay. The concentration of the enzyme solution for immobilization was altered as required for studies involving variation in enzyme loading.

Comprehensive measurements of the kinetics, using amidase activity, and conformational changes, using EPR spectroscopy, were conducted on the modified polysulfone (MPS) membrane. The amidase activity of papain, using *N*-benzoyl-DL-arginine-4-nitroanilide (BAPNA) as a substrate and using an activating solution of 10 mM cysteine and 2 mM EDTA, was assayed in all cases. Adsorption measurements of the product, *p*-nitroaniline, on the MPS membrane were conducted to ascertain its effect on the measured activity (Ganapathi et al., 1995). The adsorption experiments were conducted in the membrane cell described above where *p*-nitroaniline solutions of different concentrations were perfused through the membrane to acquire kinetic and equilibrium adsorption data. The effect of enzyme loading on the activity of the homogeneous and immobilized enzyme was studied. Kinetic experiments were carried out in the recirculation mode, as outlined in Figure 1. The reaction mixture was circulated through a cell in the spectrophotometer where the absorbance of the product formed was recorded at 410 nm. The UV−visible spectrophotometer used in all experiments was the Bausch & Laumb Spectronic 1001.

EPR experiments were performed at the active site of papain, using the nitroxide spin label (1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiolsulfonate (MTS) (Butterfield et al., 1994). Papain was first spin labeled with MTS and then attached to the membrane. 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 the following: microwave frequency, 9.78 GHz; microwave power, 24.2 mW; modulation frequency, 100 kHz; modulation amplitude, 0.32 G; scan width, 130 G. A special quartz flat cell (Butterfield et al., 1994) was used to obtain spectra of the MPS membrane.

4. Results and Discussion

4.1. Enzyme Kinetics and Effect of Enzyme Loading. The results of the kinetics and adsorption experiments have been reported in an earlier work (Ganapathi et al., 1995). Table 1 depicts the kinetic parameters for papain in the homogeneous state and on the MPS membrane; the Michaelis−Menten parameter and the maximum reaction rate are presented. It was found that *p*-nitroaniline adsorbed significantly on the MPS membrane, with measured activity only 25% of the adsorption

Table 1. Michaelis−Menten Parameters for Papain in Solution and on the MPS Membrane

membrane	V_{max}, μmol/(g·min)	K_m, mM	correl coeff, r^2
MPS[a]	7.6	0.5	0.99
homogeneous papain	111.1	1.4	0.98

[a] Apparent values for membrane.

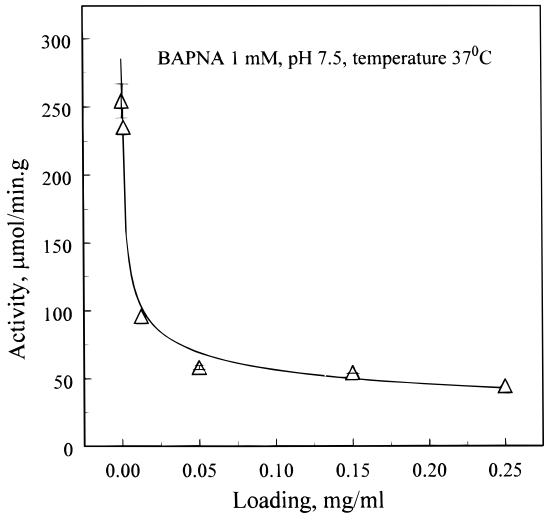

Figure 3. Effect of papain concentration on activity in the homogeneous state. All activities measured at 1 mM initial substrate concentration.

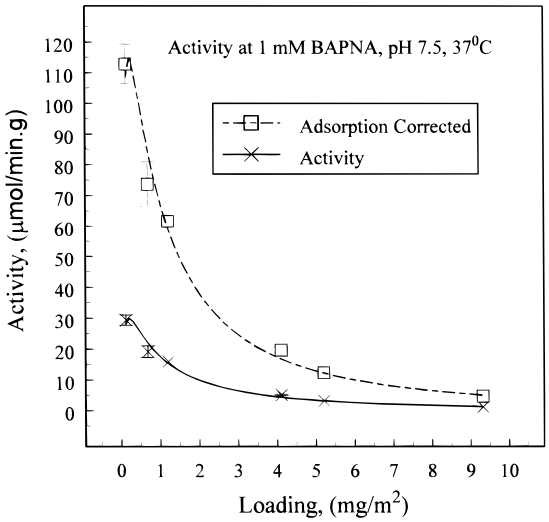

Figure 4. Effect of papain loading on activity on the MPS membrane. The two plots represent measured activity and activity corrected for adsorption of *p*-nitroaniline. All activities measured at 1 mM initial substrate concentration.

corrected activity of immobilized papain. Enzyme loading was determined to be a factor in the activity of the enzyme, both in the homogeneous and immobilized states, and is depicted in Figures 3 and 4. In both cases the amidase activity of papain decreased with increased loading, with activity reported per gram of enzyme. At higher loadings between 1 and 5 mg, the activity of homogeneous papain is almost constant; but at very low loadings, the activity measured is far higher. This anomalous result could be explained by two scenarios: (a) papain being a protease could self-digest and increasing concentrations of the enzyme could lead to this phenomenon and (b) papain self-associating at high concentrations (Coates and Swann, 1970; Pandit and Rao, 1974) forming dimers. Also, being a cysteine protease, this enzyme could dimerize via the sulfhydryl group (Kelly and Zydney, 1994), and in both cases, dimerization leads to reduced activity.

Figure 4 depicts the decrease in activity with increased loading for papain immobilized on the membrane. The pronounced increase in activity upon correction for adsorption is evident for immobilized papain. The trend on the membrane could be a direct manifestation of the enzyme's behavior in solution. Higher enzyme loading can lead to crowding of the enzyme on the surface, resulting in spatial restrictions, blocking of the active site, and/or denaturation of 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. Papain possesses 10 lysine amino acid residues which could react with the aldehyde group on the membrane. These could clearly lead to multipoint attachments of the enzyme which could block the active site, leading to a reduction in measured activity.

4.2. Enzyme Reaction Model with Adsorption. In the investigation of the performance of a biofunctional membrane, it has been hypothesized that there are three chief factors that influence immobilized enzyme activity: (a) diffusion resistance, (b) product or substrate adsorption, and (c) alterations in immobilized enzyme conformation. The effect of diffusion resistance is considered by applying the diffusion reaction model presented in eq 1.

The diffusion reaction equation was applied to the specific case of the MPS membrane. The value of D_{eff} is 5×10^{-6} cm^2/s, determined by the Wilke−Chang equation. The value of V_{max} [7.6 μmol/(g·min) (Table 1)] was converted into units of millimoles per liter per second by considering the volume of the support containing the catalyst, the membrane in this case. The membrane volume, based on water intake, was supplied by the manufacturer (Gelman Sci.) to be 0.163 mL. This yielded a V_{max} of 2.3×10^{-3} mmol/(L·s). The values for K_m and L, the length of the pore, were 0.5 mM (Table 1) and 0.015 24 cm, respectively. Therefore, the Thiele modulus, $\phi = L(V_{max}/D_{eff}K_m)^{1/2}$, for this system was found to be 0.5.

The diffusion reaction equation was solved within the recirculation loop as described earlier in the section on model development for the specific case of the MPS membrane. The values of feed-tank volume, V, and flow rate through the membrane, Q_f, used to solve the equation were 0.024 L and 2 mL/min (3×10^{-5} L/s), respectively. Substrate profiles were generated at different times; these were plotted in terms of product formed using material balance for substrate and product. The calculated product profiles were then compared with experimentally obtained plots of P versus t. All experimentally obtained product values were corrected for adsorption, using the linear isotherm. The plots of calculated and measured product formation with time, are depicted in Figures 5 and 6, for substrate profiles of 1 and 0.5 mM. The calculated plots from the recirculation model represent a good fit at 1 mM, with measured (and adsorption corrected) values being slightly higher than model calculations. At a concentration of 0.5 mM, the calculated values are in excellent agreement with the measured (and adsorption corrected) values. It must be mentioned that the assumption of equilibrium adsorption has been made in correcting for measured product concentrations. Thus it can be concluded that the operation of the MPS membrane bioreactor is in the reaction-controlled regime, with the calculated values from the

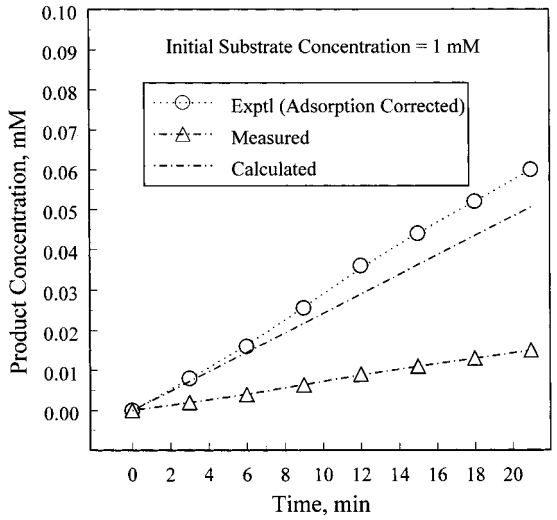

Figure 5. Calculated, measured, and measured + adsorption corrected product formation with time for papain on the MPS membrane, for substrate concentration of 1 mM.

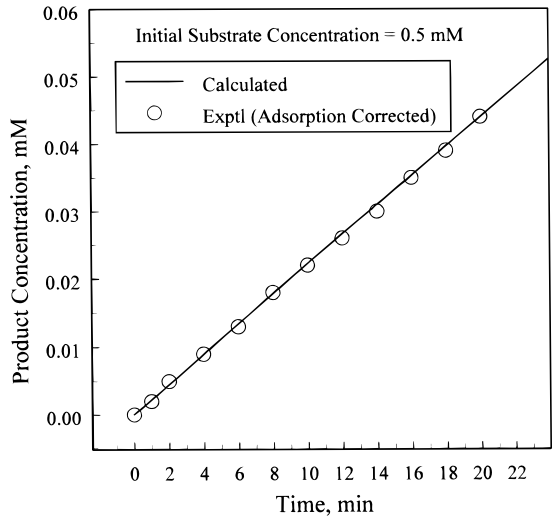

Figure 6. Calculated, measured, and measured + adsorption corrected product formation with time for papain on the MPS membrane, for substrate concentration of 0.5 mM.

model in good agreement with experimental data at a Thiele modulus of 0.5.

Adsorption of product is an important factor that can have pronounced impact on enzyme activity measurements. The specific instance of *p*-nitroaniline adsorption on the MPS membrane showed that experimentally measured activities were only 25% of the corrected activity. The effect of adsorption is demonstrated using the linear adsorption isotherm (eq 17) for product adsorption on a flat-sheet membrane. Simulations for the system at a constant substrate concentration (1 mM) at a Thiele modulus of 0.5 are presented in Figure 7. The large difference associated with the measured and total product concentrations is obvious. This model incorporating product adsorption is a clear illustration of the profound effect that adsorption has on actual enzyme activity and kinetics.

For the MPS membrane, diffusional transport inside the pores is not controlling and measured kinetics represent the intrinsic kinetics of the system. The effect of adsorption on the kinetics of the biofunctional membrane is significant (Figure 4). Therefore the next step, after elimination of diffusion resistance and correction

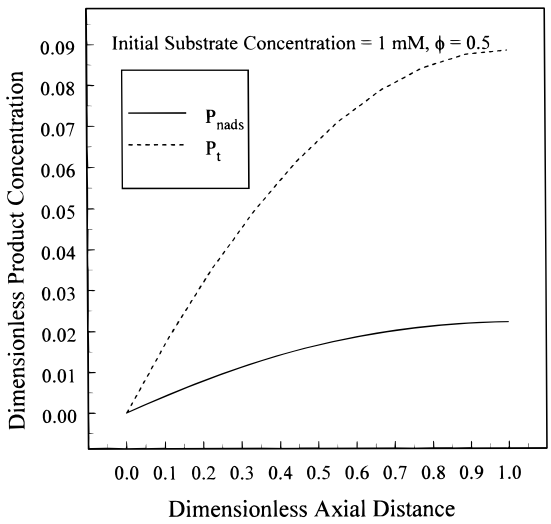

Figure 7. Effect of adsorption on product profiles inside membrane pore at an initial substrate concentration of 1 mM and a $\phi = 0.5$. Total product, P_t, and product not adsorbed, P_{ads}, are shown; their difference indicates adsorbed product.

for product adsorption, is evaluating changes in enzyme conformation at the active site of the enzyme and correlating this with enzyme function.

4.3. Determination of Active Fraction. Quite often immobilized enzymes on beads, gels, and polymer membranes exist in two environments. The loading and deconvolution models presented in eqs 18 and 19 have been employed to delineate these two subpopulations. It is obvious that the actual fraction of active sites, λ, is a function of the particular enzyme being studied. The loading model (eq 18) was applied to papain on the MPS membrane. All activities applied to eq 18 were those obtained for substrate concentration of 1 mM (pH 7.5, temperature 37 °C). The activity used for A_M was that obtained on a membrane with a typical loading of 3 mg and the corresponding activity in solution was used for A_A. In the case of A_D the activity of the membrane with the highest loading available was used. For both A_M and A_D, the activities measured were corrected for adsorption. The parameters used were the following: A_M, 19.5 μmol/(g·min) (Figure 4. Effect of Loading on MPS Membrane); A_A, 54.3 μmol/(g·min) (Figure 3. Effect of Loading on Homogeneous Papain Activity); A_D, 4.8 μmol/(g·min) (Figure 4. Effect of Loading on MPS Membrane). By substituting these values into eq 18, we get a λ value of 0.29. This suggests that the fraction of the active environment of papain on the MPS membrane is only 29%; (that fraction which has the activity of homogeneous papain at the same loading). Equation 18 has been applied to papain at other loadings on the MPS membrane (Figure 4) using the corresponding activity of homogeneous papain (Figure 3). The resulting plot of λ versus loading is depicted in Figure 8 (in each case the loading represents that on a membrane with a BET area of 0.7 m^2). It is interesting that at low loadings the active fraction of immobilized papain is almost constant between 0.85 and 0.9. But as loading increases, there is a significant drop in λ, to 0.15 for the high loading case.

For loadings of 1 and 3 mg, the amounts of active enzyme were 0.93 and 0.87 mg, respectively. This implies that there is a limit to the loading capacity of active enzyme on the membrane of about 0.9 mg. All λ determinations were made using the same value for A_D, that at the 7-mg loading.

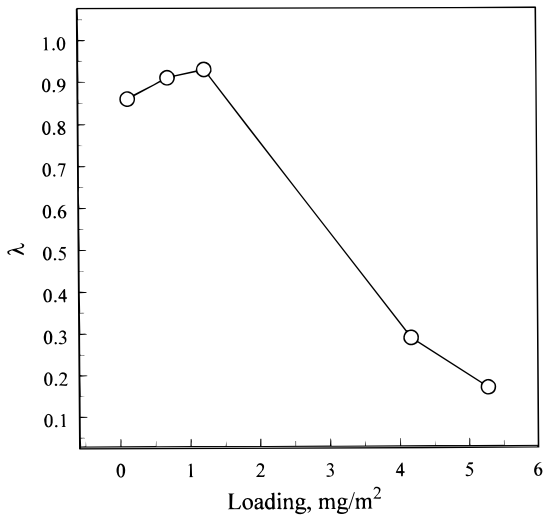

Figure 8. Variation of active fraction, λ, with increasing membrane loading, obtained from the loading model.

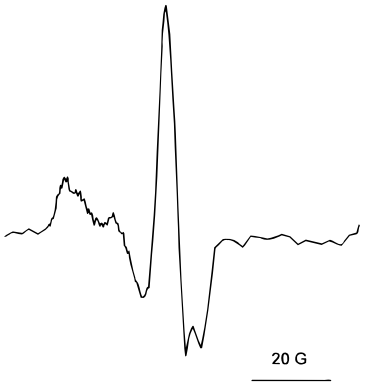

Figure 9. Digitized EPR spectrum of MTS papain on the modified polysulfone membrane, G_C, used for all subtractions.

The next step involves using EPR spectroscopy and applying the deconvolution model (eq 19) to the spectra obtained. Simulations of the deconvolution model were performed using the solution spectrum of papain as G_A and the membrane spectrum as G_C (Figure 9) at various λ. G_A and G_C denote digitized spectra of MTS papain in solution and on the membrane. The process of subtraction of the two spectra was conducted over the entire range of active fraction from 0.0 to 1.0. It was observed that at values of λ below 0.2 and above 0.6 the spectra lost the physical characteristics of a first-derivative EPR curve and were therefore discarded for this system.

On simulating spectra using different values of the active fraction, it was determined that for λ in the range of 0.25 and 0.3 the subtracted spectrum (Figure 10, $\lambda =$ 0.25) possesses the characteristics of an EPR spectrum with a single population. Thus it is clear that the deconvolution and loading models deliver values of λ which are close, within experimental error. From the deconvolution model, which assumed that two subpopulations of the immobilized enzyme were present on the membrane, the more active subpopulation was between 25 and 30% of the total enzyme on the membrane. Using the value of λ (0.29) obtained from experimental data (the loading model), the error in the calculation of λ from the deconvolution model was between 3% and 14% (for λ values between 0.25 and 0.3).

The assumption of two subpopulations is a reasonable one; all EPR spectra in this investigation display two

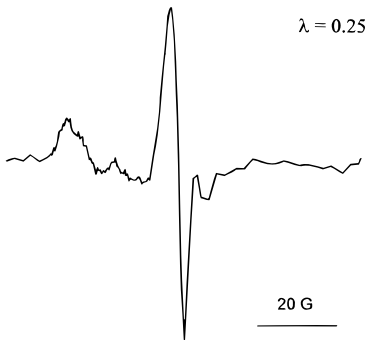

Figure 10. Subtracted spectrum, G_D, obtained at $\lambda = 0.25$.

environments on the membrane, and this phenomenon has been encountered on other membranes and on beads (Ganapathi et al., 1995; Clark and Bailey, 1984). The loading model supplied the value of λ to be 0.29. This model assumed that the fraction of the enzyme with more denaturation was the one with highest enzyme loading; in solving eq 18, A_D was taken at a 7-mg loading. It is possible that lower activities could be attained at higher loadings, leading to an exact match for λ from both models.

The determination of λ for papain on the MPS membrane leads to quantifying changes in enzyme conformation at the active site. From this, the actual amount of active enzyme is known; hence the normalized maximum activity can be corrected for this value of λ. Thus for the randomly immobilized enzyme system of papain on the MPS membrane, the kinetics representing the actual amount of active enzyme is V_{max}(intrinsic) $= V_{max}/\lambda$, where λ lies in the range 0.25−0.3. Taking into account adsorption correction, the intrinsic V_{max} for the MPS membrane with a loading of 3 mg lies between 101 and 121 μmol/(g·min), for λ values of 0.25 and 0.3, respectively. This is in excellent agreement with the normalized V_{max} for homogeneous papain, which is 111 μmol/(g·min).

5. Conclusions

The amidase activity of the enzyme varies with enzyme loading; the measured activity is significantly higher at low enzyme loadings for both homogeneous and immobilized enzymes. Mathematical modeling of the bioreactor by a diffusion reaction model depicted a reaction controlled system ($\phi = 0.5$). Calculated values of product formed with time; from the recirculation loop equations agreed reasonably with measured adsorption corrected values. Loading and spectral deconvolution models were applied to evaluate the active component in immobilized papain; λ values from the two models were both in the range 0.25−0.3. Thus both kinetic and structural information obtained from the two models of loading and deconvolution result in values of λ within experimental error. Finally, the structure and function of the immobilized protease, papain, have been correlated, demonstrating that, when corrected for adsorption and considering only the fraction of active enzyme, immobilized enzyme kinetics approach homogeneous kinetics.

Notation

Greek Symbols

η	effectiveness factor
λ	fraction of active enzyme
ϕ	Thiele Modulus

τ	dimensionless time in recirculation equation ($t/(V/Q_f)$)

Symbols

A	hyperfine tensor
$2A'_{zz}$	hyperfine splitting parameter
a	pore radius, L
BAPNA	*N*-benzoyl-DL-arginine-4-nitroanilide
D_{eff}	effective diffusivity, L^2/s
E	enzyme concentration, mol/L^3
EDTA	ethylenediaminetetraacetic acid
G	EPR spectrum
k_a	adsorption constant in linear isotherm, L^3/g
K_m	Michaelis−Menten parameter, mol/L^3
L	pore length, L
M_I	nuclear spin quantum number
MPS	modified polysulfone
MTS	(1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)methanethiolsulfonate
P	product concentration, mol/L^3
PES	polyethersulfone
PNA	*p*-nitroaniline
Q	adsorption capacity, mol/gram
Q_f	flow rate through the membrane, L^3/s
S	substrate concentration, mol/L^3
$\bar{S}$	dimensionless substrate concentration (S/K_m)
t	time, s
V	volume of feed tank, L^3
V_1	volume of reactor, L^3
V_2	volume adsorbed, L^3
V_{max}	maximum reaction rate, mol/L^3.sec
W	weight of membrane, gram
x	axial distance, L
z	dimensionless reactor length

Subscripts

0	initial condition
A	population with higher activity
ads	adsorbed
C	composite (spectrum parameter)
D	population with lower activity
e	exit
M	membrane
nads	not adsorbed
s	surface

Acknowledgment

This work was supported in part by a grant from the National Science Foundation (CTS-9307518).

References and Notes

Berliner, L. J.; Wong, S. S. Spin-labeled sulfonyl fluorides as active-site probes of protease structure *J. Biol. Chem*. **1974**, *249*, 1668−1677.

Bhardwaj, A.; Lee, J.; Glauner, K.; Ganapathi, S.; Bhattacharyya D. B.; Butterfield, D. A. Biofunctional Membranes: An EPR Study of Active Site Structure and Stability of Papain Non-covalently Immobilized on the Surface of Modified Poly(Ether)Sulfone Membranes through the Avidin−Biotin Linkage. *J. Membr. Sci.* **1996**, *119*, *241−252*.

Bhattacharyya, D.; Ganapathi, S.; Vishwanath, S.; Summers, M. In *Biofunctional Membranes*; Butterfield, D. A., Ed.; Plenum: New York, 1996; pp 117−129.

Butterfield, D. A. Spin labeling in disease. *Biol. Magn. Reson.* **1982**, *4*, 1−78.

Butterfield, D. A. *Biofunctional Membranes*; Plenum: New York, 1996.

Butterfield, D. A.; Lee, J. Active-site structure and stability of the thiol protease papain studied by electron paramagnetic resonance employing a methanethiosulfonate spin label. *Arch. Biochem. Biophys.* **1994**, *310*, 167−171.

Butterfield, D. A.; Lee, J.; Ganapathi, S.; Bhattacharyya, D. 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. *J. Membr. Sci.* **1994**, *91*, 47−64.

Clark, D. S.; Bailey, J. E. Structure−Function Relationships in Immobilized Chymotrypsin Catalysis. *Biotechnol. Bioeng.* **1983**, *25*, 1027−1047.

Clark, D. S.; Bailey, J. E. Characterization of Heterogeneous Immobilized Enzyme Subpopulations Using EPR Spectroscopy. *Biotechnol. Bioeng.* **1984**, *26*, 231−238.

Clark, D. S.; Skerker, P. S.; Fernandez, E. J.; Jagoda, R. V. Spectroscopic Studies of Structure−Function Relationships in Free and Immobilized Alcohol Dehydrogenase. *Ann. N. Y. Acad. Sci.* **1987**, *506*, 117−128.

Clark, D. S.; Skerker, P. S.; Randolph, T. W.; Blanche, H. W.; Prausnitz, J. M. Conformational Analyses of Enzymes and Substrates in Processing Environments. *Ann. N. Y. Acad. Sci.* **1988**, *542*, 16−29.

Coates, J. H.; Swann, J. C. A reinvestigation of the association behavior of papain in solution. *Biochim. Biophys. Acta* **1970**, *214*, 545−547.

Diaz, J. F.; Balkus, K. J. Enzyme immobilization in MCM-41 molecular sieve. *J. Mol. Catal.* **1996**, *2*, 115−126.

Friedrich, C.; Noel, C.; Ramassuel, R.; Rassat, A. Preparation and Characterization of Spin-labeled Polystyrene. *Polymer* **1980**, *21*, 232−235.

Ganapathi, S.; Butterfield, D. A.; Bhattacharyya, D. 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. *J. Chem. Technol. Biotechnol.* **1995**, *64*, 157−164.

Grulke, E. A.; Okos, M. R.; Sykerson, A. Simulation of an Immobilized Enzyme Particle using β-galactosidase Adsorbed to a Phenol-formaldehyde Resin. *J. Food Proc. Eng.* **1977**, *1*, 351−372.

Houng, J. Y.; Yu, H. Y.; Chen, K. C.; Tiu, C. Analysis of Substrate Protection of an Immobilized Glucose Isomerase Reactor. *Biotechnol. Bioeng.* **1993,** *41*, 451−458.

Kelly, S. T.; Zydney, A. L. Effects of Intermolecular Thiol-Disulfide Interchange Reactions on BSA Fouling During Microfiltration. *Biotechnol. Bioeng.* **1994**, *44*, 972−982.

Klein, E. *Affinity Membranes*; John Wiley & Sons: New York, 1990; pp 129−148.

Lortie, R.; Pelletier, D. Comparison Between Dispersion and Plug-Flow Models for Fixed-Bed Enzyme Reactors. *AIChE J.* **1992**, *38*, 1477−1480.

Marg, G. A.; Milhauser, G. L.; Skerker, P. S.; Clark, D. S. Application of EPR Methods in Studies of Immobilized Enzymes Systems. *Ann. N. Y. Acad. Sci.* **1986**, *469*, 253−258.

O'Connor, K. C.; Bailey, J. E. ESR Investigations of Free and Immobilized Glutamate Dehydrogenase. *Biotechnol. Bioeng.* **1989**, *34*, 110−116.

Oppenheim, S. F.; Buettner, G. R.; Dordick, J. S.; Rodgers, V. G. J. Applying Electron Paramagnetic Resonance Spectroscopy to the Study of Fouling in Protein Ultrafiltration. *J. Membr. Sci.* **1994**, *96*, 289−297.

Pandit, M. W.; Rao, M. S. N. Self-associaiton of papain. *Biochim. Biophys. Acta* **1974**, *371*, 211−218.

Rialdi, G.; Battistel, E. Unfolding mechanism and stability of immobilized papain. *J. Thermal Anal.* **1996**, *47*, 17−25.

Santoyo, A. B.; Carrasco, J. L. G.; Gomez, E. G.; Rodriguez, J. B.; Morales, E. M. Transient Stirred-Tank Reactors Operating with Immobilized Enzyme Systems: Analysis and Simulation Models and Their Experimental Checking. *Biotechnol. Prog.* **1993**, *9*, 166−173.

Shyam., R.; Davidson, B.; Vieth, W. R. Mass Transfer and Biochemical Reaction in Enzyme Membrane Reactor Systems-II. *Chem. Eng. Sci.* **1975**, *30*, 669.

Skerker, P. S.; Clark, D. S. Catalytic properties and active-site structural features of immobilized horse liver dehydrogenase *Biotechnol. Bioeng.* **1988**, *32*, 148−158.

Song, Y.; Means, G. E.; Wan, X.; Berliner, L. J. A spin label study of immobilized enzyme spectral subpopulations. *Biotechnol. Bioeng.* **1992**, *40*, 306−312.

Telo, J. P.; Candeias, L. P.; Empis, J. A. A.; Cabral, J. M. S.; Kennedy, J. F. Immobilized enzymes—active, partially active or inactive? ESR studies of papain immobilization. *Chimmicaoggi Maggio* **1990**, 15−18.

Thommes, J.; Kula, M. R. Membrane Chromatography: An integrative concept in the downstream processing of proteins. *Biotechnol. Prog.* **1995**, *11*, 357.

Vishwanath, S.; Huang, W.; Bachas, L. G.; Bhattacharyya, D. Site-directed and random enzyme immobilization on functionalized membranes: kinetic studies and models. *J. Membr. Sci.* **1995**, *108*, 1−13.

Viswanath, S.; Wang, J.; Bachas, L. G.; Butterfield, D. A.; Bhattacharyya, D.; Site-directed and random immobilization of subtilisin on functionalized membranes: activity determination in aqueous and organic media. *Biotechnol. Bioeng.* **1998**, in press.

Zhuang, P.; Butterfield, D. A. Denaturation studies of active-site labeled papain using electron paramagnetic resonance and fluoroscence spectroscopy. *Biophys. J.* **1991**, *60*, 623−628.

Accepted October 5, 1998.

BP980096P