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

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A detailed study on the performance of a membrane bioreactor is presented, considering

Substituting into eq 6

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

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

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

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

Therefore

The total moles of product formed is

By substituting into eq 14

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.

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 2 A¢zz. The larger the 2 A¢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 M

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and A_D by the following equation:

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, ì is the fraction of more active enzyme and (1 - ì) the fraction of enzyme with lower activity. Thus utilizing ì 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

Thus, ì is calculated from eq 18 and this value of ì 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 ì 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, ì.

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 í m average pore size, 152.4 í m thickness) from Gelman Sciences were used for covalent attachment of the enzyme. The commercial membranes were availcall8-236(f[uT]TJ T*ss))]confoimilized

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 ×Thl38 4177-,03(Thl (Thl (Ó8 * 085 (eff4.2ion)-5072ion)38 40con-)]T

model in good agreement with experimental data at a

ô	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
S_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

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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 â-galactosidase Adsorbed to a Phenol-formaldehyde Resin. J. Food Proc. Eng. 1977, 1,

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