Site-Directed and Random Immobilization of Subtilisin on Functionalized Membranes: Activity Determination in Aqueous and Organic Media

S. Viswanath,[1,3] J. Wang,[2,3] L. G. Bachas,[2,3] D. A. Butterfield,[2,3] D. Bhattacharyya[1,3]

[1]Department of Chemical & Materials Engineering, University of Kentucky, Lexington, Kentucky 40506
[2]Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506,
[3]Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky 40506; telephone: (606) 257-2794; fax: (606) 323-1929; e-mail: db@engr.uky.edu

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Abstract: Kinetic comparisons have been made between a randomly immobilized and a site-specifically immobilized subtilisin BPN′ on microfiltration membranes of varying hydrophilicities in both aqueous and organic media. Site-directed mutagenesis was employed to introduce a single cysteine into the amino acid sequence of subtilisin at a location away from the active site. Immobilization of this mutant enzyme was then carried out using the single cysteine residue to orient the active site of the enzyme away from the membrane surface. Kinetic comparison of the immobilized mutant enzyme with the randomly immobilized wild-type enzyme in aqueous media showed an activity enhancement on both hydrophilic silica-containing and hydrophobic poly(ether)sulfone membranes. Higher loading efficiencies were observed for the site-directed enzyme on immobilization. Optimal enzyme loading values were calculated for the randomly immobilized enzyme. An enhancement of activity was also observed for the site-directed immobilized systems using nearly anhydrous hexane as the solvent. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **60**: 608–616, 1998.
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INTRODUCTION

Subtilisin is an alkaline protease with well-established solution kinetics. Its amino acid sequence (Boyer, 1971), gene (Strausberg et al., 1993), and X-ray structure (Bott et al., 1988) are known, thus allowing rational mutations of the active site and other regions of the enzyme. Amino acid substitutions on the enzyme can be carried out to generate enzyme variants with altered substrate specificities (Bott et al., 1991). For example, protein engineering was used to form a mutated subtilisin that would ligate peptides effectively in solution (Abrahmsen et al., 1991). Genetic modifications have also been used to enhance other enzyme properties such as thermostability, chemical oxidation, enhanced organic solvent stability, and pH dependence of activity (Arnold, 1990; Estell et al., 1985; Takagi et al., 1990; Thomas et al., 1985).

There have been a number of kinetic studies carried out on subtilisin BPN′ in aqueous solution to determine the extent of activity retained by the enzyme on immobilization to a synthetic support. Most of these studies were hampered by the random nature of the immobilization, which resulted in lower activity of the immobilized enzyme. Subtilisin was immobilized on porous glass via isothiocyanate coupling (Chapman and Hultin, 1975; Trzmiel et al., 1995), which resulted in the pH optimum of the enzyme to be shifted to the alkaline side upon immobilization. This shift was presumed to be due to the negative charge on the surface of the glass. However, the activity of the immobilized enzyme was much lower than that of the homogeneous enzyme in solution.

Soluble and immobilized enzyme systems are generally used as water based catalysts (Butterfield, 1996). However, there are many industrially important reactions that occur best in the absence of water. For example, esterification reactions that are equilibrium-limited are driven to completion in the absence of water or when the product water is partitioned away from the reaction mixture. Another motivating factor for considering enzyme catalysis in organic media is for the case of low reactant solubility in water. However, enzymes suffer from poor catalytic efficiency in organic media. Enzyme catalysis in organic media has been the subject of active research for the last decade (Griebenow and Klibanov, 1996; Wangikar et al., 1997; Zaks and Klib-

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anov, 1988). Hydrophobic water-immiscible solvents like hexyl acetate, octanol, and methylene chloride, as well as hydrophilic polar solvents like acetonitrile and tetrahydrofuran have been tested and the enzyme activity was found to be a function of the water content of the system.

Support hydrophilicity plays an important role in nonaqueous immobilized enzyme catalysis. Reslow et al. (1988) defined the term ''aquaphilicity'' of a support as being the ratio of the amount of water partitioned on the support to the amount of water in the solvent. They found that the immobilized enzyme activity decreases with increasing hydrophilicity of the support. The same concept of aquaphilicity was used by Orsat et al. (1994) when they covalently attached subtilisin Carlsberg to five membranes of varying aquaphilicity; however, they could not find a definitive trend in activity. Valivety et al. (1994) included the importance of enzyme loading when they found the activity of the enzyme to be nearly independent of the water activity at high enzyme loading indicating reduced surface area of the support available to compete with the enzyme for water.

Different site-directed immobilization strategies using gene fusion have been employed by our group on the enzymes β-galactosidase and alkaline phosphatase (Vishwanath et al., 1995, 1997). These strategies utilized avidin–biotin and antibody–antigen interactions for the immobilization of fusion proteins of β-galactosidase and alkaline phosphatase, respectively. It was demonstrated that site-directed immobilization resulted in higher catalytic efficiencies compared to the corresponding randomly immobilized enzyme.

The aim of this research is to improve the enzyme activity of immobilized subtilisin on membranes by ordered immobilization, and to understand the role of the membrane support and its hydrophilicity by studying the kinetics of the immobilized enzyme in organic solvents. Because wild-type subtilisin BPN′ has no cysteines, site-directed mutagenesis instead of gene fusion was used for site-specific immobilization. This was accomplished by introducing a single cysteine residue into the amino acid sequence of the enzyme.

MATERIALS AND METHODS

All aqueous kinetic assays were carried out using the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and the kinetic assays in the nonaqueous solvent were carried out using the substrate *N*-CBZ-L-alanine-*p*-nitrophenyl ester (Sigma, St. Louis, MO). The cross-linkers 4-(4-*N*-maleimidomethyl)cyclohexane 1-carboxyl hydrazide (M_2C_2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), and *N*-(γ-maleimidobutyryloxy)sulfosuccinimide ester (Sulfo-GMBS) were obtained from Pierce (Rockford, IL). The reducing column was procured as part of the ReduceImm reducing kit from Pierce.

Genetic Modification of Subtilisin

Serine 145 in subtilisin (obtained from plasmid pSbt) (Strausberg et al., 1993) was mutated to cysteine by overlap extension site-directed mutagenesis (Horton and Pierce, 1991). The mutagenesis also introduced a nonspecific glycine-98-to-serine mutation (Huang et al., 1997). The mutated subtilisin was reinserted into pSbt to form pLGB105. The expression vector pLGB105 was transformed into a protease-deficient strain of *Bacillus subtilis* to express the mutant subtilisin. The enzyme was purified from the cell culture supernatant by ammonium sulfate precipitation, followed by cation exchange chromatography on a BioCAD Sprint perfusion chromatography system (PerSeptive Biosystems, Framingham, MA). The wild-type subtilisin was similarly expressed and purified from a protease-deficient strain of *B. subtilis* harboring pSbt.

Immobilization Supports

Flat sheets of poly(ether)sulfone blend (MPS) membranes (Ultrabind US 450) were purchased from Gelman Sciences. The membrane matrix was composed of poly(ether)sulfone blended with polyacrolein to provide aldehyde functionality. The average pore size of the membrane was 0.45 μm, and the thickness of the membrane was 152 μm. The surface area of the membrane was determined by nitrogen gas adsorption studies using the Brunauer–Emmett–Teller pore (BET) isotherm, to be 9 m^2/g (Ganapathi et al., 1995). The other membrane used for enzyme immobilization was purchased from Whatman (product # 6872-1703, Actidisk), and was composed of poly(vinyl chloride) (PVC) sheets with embedded aldehyde-functionalized silica (approximately 65% silica) particles to provide functionality for enzyme attachment. These membranes were preactivated with aldehyde functionality and ready for use. The average membrane pore size was 1 μm, and the membrane thickness was 100 μm. The surface area of the membrane was reported to be 80 m^2/g. The saturation transfer electron paramagnetic resonance (STEPR) measurements of the immobilized subtilisin were also carried out on a cellulose acetate (Schleicher & Schuell) membrane, which was first converted to cellulose by hydrolysis followed by oxidation of the hydroxyl groups to aldehyde functionality to enable covalent attachment. The average pore size of this membrane was 0.2 μm and the thickness was 126 μm.

Immobilization of Wild-Type Subtilisin

The attachment of the wild-type enzyme on the membranes was achieved by traditional Schiff-base chemistry. The enzyme solution was convected through the membrane for 1.5 h at room temperature using 100 m*M* phosphate buffer at pH 7.5. This enabled coupling of lysine residues of the enzyme to aldehyde groups on the membrane. The membrane was washed with 0.5 *M* NaCl solution after the immobilization step to remove noncovalently bound protein. This was followed by a washing step using deionized water. The unreacted aldehyde groups on the membrane were blocked by using a 50 m*M* ethanolamine solution.

Immobilization of the Mutant Enzyme

The mutant enzyme had a single cysteine residue, which could bind with the cysteine residue of another enzyme molecule to form a disulfide bond. Just prior to immobilization and in order to reduce any intermolecular disulfide bond, the enzyme was passed through a Reduce-Imm column as suggested by the manufacturer. The mutant enzyme was either directly immobilized on the MPS and Actidisk membranes or bovine serum albumin (BSA) was used as a spacer molecule. In the case of direct immobilization of the mutant enzyme, the cross-linkers M_2C_2H and PDPH were used. Both of these are heterobifunctional cross-linkers with hydrazide as one of the end groups. The immobilization was carried out by recirculating the cross-linker solution through the membrane for 2 h. The membrane with the bound cross-linker was washed with deionized water and buffer repeatedly to remove the unbound cross-linker. It was ensured that the pH of the wash solutions was kept at neutral pH as hydrolysis of the maleimide group can readily occur above pH 8.0. Next, the membrane's unreacted aldehyde groups were capped with Tris. The freshly reduced enzyme was convected through the M_2C_2H-containing membrane for 2 h to enable attachment of the sulfhydryl groups to the maleimide group of the cross-linker. The pH at this step was adjusted to 7.0, where the reactivity of the maleimide group with sulfhydryls is 1000-fold faster than with amines. When PDPH was used as the cross-linker, the hydrazide attachment step was the same as for M_2C_2H. When the mutant enzyme was convected through the PDPH-containing membrane, a pH of 7.5 was used to ensure optimal attachment.

The attachment of the mutant enzyme to the membrane via BSA involved first covalently linking the amine residues of BSA to the aldehyde groups on the membrane by Schiff-base chemistry. This was achieved by convecting the BSA solution through the membrane for 1.5 h at room temperature using phosphate buffer. The unreacted aldehyde groups on the membrane were capped with Tris. The heterobifunctional cross-linker Sulfo-GMBS was first attached to BSA by recirculating for 1 h. Then, a freshly reduced enzyme preparation was recirculated through the membrane at pH 7.0 for 1 h to enable attachment of the cysteine residue of the enzyme to the maleimide group on the cross-linker.

Protein Assay

The amount of unbound subtilisin in the supernatant was determined by the Bradford protein assay (Bradford et al., 1976) using the protein assay kit from Bio-Rad and by an activity (kinetic) analysis. The bound protein was calculated by difference. Direct colorimetric determination of bound enzyme on the membrane was not used because of the adsorption of dye on the membrane.

Catalysis in Aqueous Media

The rate of subtilisin-catalyzed hydrolysis was measured by monitoring the increase in the absorbance of the product *p*-nitroaniline at 410 nm using a UV–vis spectrophotometer. The concentration of the enzyme in the homogeneous experiments was 3.9 mg/L. The substrate concentration range was 0.05–1 m*M*. In the case of the immobilized enzyme, a continuous flow set-up was used (Vishwanath et al., 1995). This enabled an on-line measurement of the rate of reaction. The product *p*-nitroaniline adsorbs strongly on the hydrophobic MPS membrane used for immobilization. Hence, a correction for the adsorbed product was made using the equilibrium isotherm for *p*-nitroaniline on the MPS membranes. We have previously reported the kinetics and equilibrium adsorption behavior of *p*-nitroaniline on the MPS membranes (Ganapathi et al., 1995).

Catalysis in Organic Media

The organic solvent hexane was saturated with water prior to use in experiments. Further, it was ensured that the substrate 1-butanol would not compete for the available water by first adding the 1-butanol to the hexane and then saturating the mixture with water. For the kinetic analysis, solutions were prepared in such a way that they were 1 *M* in 1-butanol and contained concentrations of the *N*-CBZ-alanine-*p*-nitrophenyl ester substrate in the range of 0.5–3 m*M*. All concentrations are with respect to the total volume of the solution. The reaction used for kinetic assays was a transesterification reaction:

N-CBZ-alanine-*p*-nitrophenyl ester + 1-butanol
$\rightarrow$ *N*-CBZ-alanine-butyl ester + *p*-nitrophenol

The kinetic assays were conducted in the recirculation setup (Vishwanath et al., 1995) with the membrane-bound enzyme housed in a dead-end filtration mode using either MPS or PVC–silica membranes. The product *p*-nitrophenol (pK_a 7.2) was partitioned into a buffer solution at pH 7.8 to ensure complete product recovery. The absorbance of the partitioned product in the aqueous phase was measured at 410 nm using a UV–vis spectrophotometer.

Saturation Transfer Electron Paramagnetic Resonance Studies

The spin-label 4-(ethoxyfluorophosphinyloxy)-TEMPO (EFT) (Sigma) was attached specifically to the active site serine 221 of subtilisin (Subramaniam et al., 1996). The STEPR spectra were recorded on a Bruker ESP-300 spectrometer with a TM rectangular cavity at room temperature. STEPR spectra were obtained using the second harmonic of the modulation frequency detected 90° out-of-phase under microwave power saturating conditions. This phase-sensitive detection permits isolation of the signal due to saturation effects only, thus increasing the sensitivity of the measurement. The spectrum at low power is ''self-nulled''; that is, the phase angle necessary to eliminate any in-phase signal is determined. The microwave power was increased from the normal 10–20 mW for in-phase EPR to 100 mW for STEPR samples. Modulation at 50 kHz and detection of

the second harmonic at 100 kHz at 90° out-of-phase to the modulation frequency leads to the detection of the STEPR spectra.

RESULTS AND DISCUSSION

Experiments with the immobilized enzyme were carried out with wild-type and mutant subtilisin BPN′ and comparisons were made among the different immobilized enzyme–membrane configurations in aqueous media (Table I). The effect of enzyme loading on immobilized activity and the storage stability of the immobilized enzyme were studied to optimize the enzyme membrane systems. In all cases, the kinetic parameters K_M and V_{max} were determined initially from Lineweaver–Burk plots, and verified by nonlinear regression analysis. The values of relative activity (RA) were determined by dividing the activity of the immobilized enzyme by the homogeneous activity corresponding to the same amount of enzyme and multiplying by 100.

Wild-Type Subtilisin

When wild-type subtilisin was immobilized on the MPS membrane, the immobilized activity dropped to 11% of the homogeneous activity. This large drop in activity is primarily due to two reasons. First, the hydrophobic interaction that occurs between the enzyme and the membrane can result in denaturation of the enzyme due to changes in active site conformation. The second reason is the random immobilization between the enzyme molecules and the support. There are 11 lysine residues per molecule of subtilisin, and therefore, the enzyme can covalently bind to the support from different locations on its structure. Further, there exists a potential of forming more than one bond between the membrane and each enzyme molecule. This large-scale anchoring of the enzyme to the support can also cause a change in the conformation of the enzyme.

The rate of reaction was analyzed by measuring the amount of the product *p*-nitroaniline in the permeate. There is a significant amount of product sorbed to the membrane which, if not accounted for, can result in erroneous kinetic data (Ganapathi et al., 1995). Hence, the amount of product adsorbed on the membrane that is in equilibrium with the product in the reaction mixture was added up to give the total amount of product formed in the reaction. This is shown as the adsorption corrected relative activity (RA) in Table I. When the wild-type enzyme was immobilized on hydrophilic PVC–silica membranes, the relative activity increased from 11% to 48%. This increase in activity indicates the importance of reducing hydrophobic interactions between the enzyme and the membrane. It should be noted that the enzyme loading on the PVC–silica membrane was almost 4-fold higher than that of the MPS membrane due to the larger surface area of the former. In addition, this high activity was obtained in spite of random immobilization of the enzyme on the PVC–silica membrane.

Mutant Subtilisin

Mutant subtilisin, which has a cysteine away from the active site, was immobilized on the MPS membrane using M_2C_2H as a cross-linker. The adsorption corrected RA was 28%, almost 3-fold higher than the RA of the wild-type enzyme on the same membrane. This increase in activity can be attributed to the site-directed nature of the immobilization, whereby there is only one bond between a specific location on each enzyme molecule (i.e., amino acid residue 145) and the membrane. This bond is located away from the active

Table I. Kinetic results obtained with various site-directed and random subtilisin immobilization strategies in aqueous media. The kinetic data are reported as average ± standard deviation ($n = 3$).

Enzyme	Amount immobilized[a] (mg)	K_M (m*M*)	V_{max} (μmol mg^{-1} min^{-1})	RA (%)
Wild-type				
Homogeneous	—	0.21 ± 0.013	95 ± 5.7	100
Randomly immobilized on MPS membrane	0.12 ± 0.006	0.25 ± 0.011	10.4 ± 0.9	11[b]
Randomly immobilized on PVC–silica membrane	0.45 ± 0.005	0.58 ± 0.019	45.1 ± 2.7	48
Mutant				
Homogeneous	—	0.15 ± 0.008	89.6 ± 4.9	100
Site-directed immobilization on MPS membrane	0.16 ± 0.008	0.23 ± 0.009	25.3 ± 1.8	28[b]
Site-directed immobilization on MPS membrane with BSA as spacer	0.022 ± 0.004	0.3 ± 0.014	40.7 ± 3.2	45[b]
Site-directed immobilization on PVC–silica membrane	0.127 ± 0.007	0.46 ± 0.015	78.6 ± 4.16	83

[a]Refers to protein immobilized on 4.7 cm diameter membranes.
[b]Corrected for product p-nitroaniline adsorption on MPS membrane.

site of the enzyme, orienting the enzyme's active site away from the membrane surface. This resulted both in a reduction in denaturation of the enzyme by multiple binding and in enhanced substrate accessibility to the enzyme's active site. However, the RA of this immobilized enzyme did not approach that of the homogeneous enzyme. This can be attributed to the hydrophobic interactions between the enzyme and the membrane. Therefore, it was important to reduce this hydrophobic interaction by either using a hydrophilic membrane or by distancing the enzyme away from the support by using a spacer.

When BSA was used as a spacer, the adsorption-corrected RA increased to 45% of the homogeneous activity. However, it should be noted that the amount of enzyme immobilized on the membrane was an order of magnitude lower than the other immobilizations. This was due to the chemistry of the process, where BSA's amino groups were used for coupling to both the site-directed enzyme and the membrane. This low amount of membrane immobilized enzyme implies that the higher activity observed when using BSA as a spacer molecule could be due to the sparse loading on the membrane; i.e., to a reduction of protein–protein interaction among neighboring subtilisin molecules. Therefore, in order to properly evaluate the potential advantages of site-directed immobilization, the mutant enzyme was immobilized on a hydrophilic PVC–silica membrane without the BSA spacer. This immobilization approach gave a relative activity of 83%. In this system, there was minimal hydrophobic interaction between the enzyme and the membrane support and no multiple binding due to the site-directed nature of the immobilization through the single cysteine residue on the mutant subtilisin. Therefore, the immobilized activity approached the homogeneous value.

Effect of Loading on Enzyme Activity

Different amounts of the wild-type and mutant enzymes were immobilized on the MPS membranes and the immobilized activity (normalized as μmol mg^{-1} min^{-1}) was determined. The normalized activity of the wild-type subtilisin decreased with increasing enzyme loading (Fig. 1). The reproducibility of these data was verified by performing replicate experiments at different amounts of immobilized enzyme. However, higher enzyme loading implies a higher throughput. Therefore, optimization of the amount of enzyme loaded on the membrane is required. It is possible to determine the optimal enzyme loading by using these data. When this activity is not normalized, i.e., when the rate is expressed as μmol/min, the activity that corresponds to the randomly immobilized enzyme goes through a maximum (Fig. 2). This activity then corresponds to the maximum activity that can be achieved with the given enzyme–membrane system. The curve in Fig. 2 was fitted to the following decay function by using nonlinear regression analysis:

$$V_{max} = k_3 E_0 \exp(-k_d E_0),$$

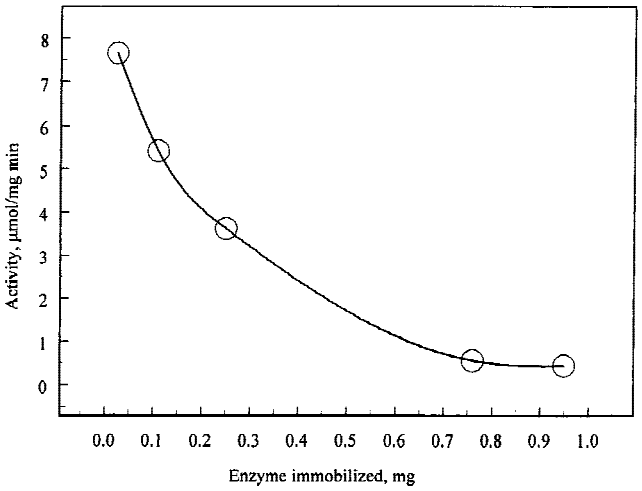

Figure 1. Effect of enzyme loading on the activity for subtilisin randomly immobilized on the MPS membrane.

where the normalized maximum rate was expressed as an exponential function of loading. The parameter k_d is a denaturation constant due to protein–protein interactions and is a measure of the optimal loading possible on the given support surface. This model fits the experimental curve to within 2% (sum of squares of the error) and allowed the determination of the denaturation constant caused by protein–protein interaction. The denaturation constant determined from regression analysis was 93 (μmol of enzyme)$^{-1}$ for wild-type subtilisin that was randomly immobilized on the MPS membrane. The reciprocal of this value gives the optimal enzyme loading possible on the membrane, which was 0.28 mg of enzyme. Since, the membrane internal surface area used is 0.73 m^2; this translates to an optimal loading of 0.38 mg/m^2 of membrane internal surface area.

Figure 2 also shows the effect of loading on the activity for the mutant enzyme immobilized in a site-directed fashion on the MPS membrane. The activity increases linearly

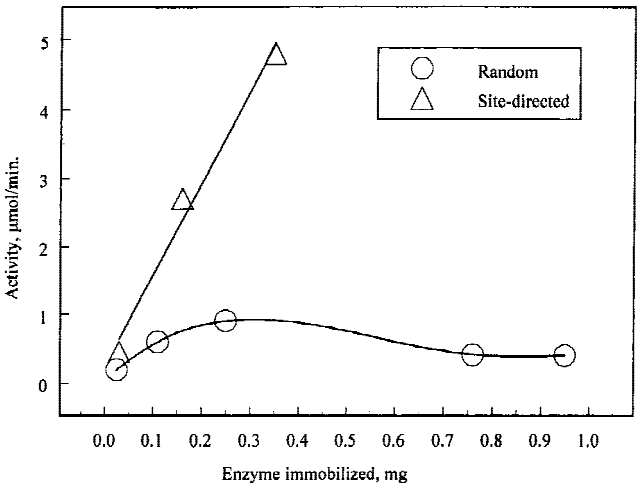

Figure 2. Effect of enzyme loading on the activity (in μmol/min) for subtilisin immobilized on the MPS membrane.

with loading, which indicates that the optimal enzyme loading for the site-directed immobilized enzyme on the same MPS membrane was higher than the range of amount of immobilized enzyme tested. In addition, the activity of the randomly immobilized enzyme was always lower than the activity corresponding to the same amount of site-directed immobilized enzyme. Replicate experiments showed that relative standard deviation was <7%. The increased activity obtained with the site-directed immobilized subtilisin can be attributed to the proper orientation of the active site of the enzyme molecules away from the membrane pore surface. This orientation reduces the possibility of steric hindrance of the active site caused by adjacent enzyme molecules and by the membrane surface, which is reflected in higher V_{max} values. This is an advantageous feature of site-directed immobilized systems as more enzyme can be loaded to enable higher throughput while retaining the same activity. Similar advantages of oriented immobilization have been reported previously for IgG molecules (Rao et al., 1998). Finally, it should be noted that the use of convective flow in conjunction with macroporous membranes facilitated substrate accessibility.

Another aspect of enzyme immobilization is loading efficiency, which is the ratio of the amount of enzyme bound to the membrane to the amount of the feed enzyme initially taken. It is important that this efficiency is at its highest so that loss of valuable enzyme in the supernatant is minimized. In the site-directed immobilized mutant enzyme system, there is a one-to-one binding between each enzyme molecule and a functional group on the membrane. This implies that a higher loading efficiency would be possible for the site-directed system as compared to the random immobilization. This is because of the possibility of multiple bonding between each randomly immobilized enzyme molecule and the membrane, thus resulting in an improper usage of the membrane surface area. Figure 3 shows a comparison of loading efficiencies between the site-directed and the randomly immobilized systems. Much higher loading efficiencies are observed for the site-directed system. To further prove this, the loading efficiency data corresponding to 0.7 mg of randomly immobilized enzyme were duplicated and the loading efficiencies obtained were significantly lower than that of the site-directed immobilized enzyme. The maximum efficiency is around 50%, and this can be improved further by increasing the number of macropores in the membrane. An increase in the number of macropores would enable higher enzyme accessibility to the entire surface area provided by the support.

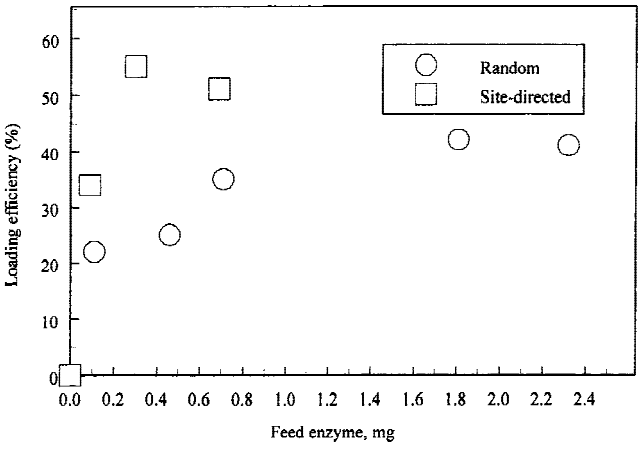

Figure 3. Loading efficiencies of random and site-directed immobilized subtilisin on the MPS membrane.

Activity Analysis in Organic Media

The transesterification activity of different membrane-bound subtilisin systems with 1-butanol and CBZ-alanine-*p*-nitrophenyl ester was measured in hexane saturated with water. It should be noted that the protease activity of subtilisin is much higher than its esterase activity. Moreover, because the immobilized enzyme is now in an organic phase, it would display even lower activity. Hence, organic phase enzymatic catalysis would be an excellent area to test the efficiency of site-directed immobilized enzyme systems.

The amount of water present plays a crucial role in enzymatic organic catalysis. There is a partitioning of the available water among the enzyme, the support and the organic phase. It has been reported in the literature (Reslow et al., 1988) that a hydrophilic support partitions water away from the immobilized enzyme and thus reduces the activity of the enzyme. However, a hydrophobic support can induce hydrophobic interactions between the enzyme and the support, which can reduce the activity by changing the active site conformation. Hence, there is an interplay between water partitioning and hydrophobic denaturation caused by the support in nonaqueous enzymatic catalysis. One more factor that plays a role is the enzyme loading on the support. A higher enzyme loading results in a larger surface coverage by the enzyme and, therefore, lesser water partitioning by the support (Valivety et al., 1994).

Kinetic Comparisons

Commercially available subtilisin Carlsberg was covalently immobilized on the MPS membrane using random immobilization via lysine residues. The kinetic results showed a 17-fold enhancement in activity as compared to Reimann et al. (1994) who carried out the same reaction and product analysis using subtilisin Carlsberg adsorbed on 6 nm pore diameter silica beads. Because of this activity enhancement observed when immobilizing the enzyme on a membrane rather than beads, we decided to explore site-directed enzyme–membrane systems in organic reactions. The PVC–silica membrane is more hydrophilic due to the presence of the silica beads and, as shown in Table II, gave a higher activity for the mutant subtilisin as compared to the MPS membrane. Reslow et al. (1988) had established that supports with higher hydrophilicity would give lower reaction rates due to the water competition phenomenon (i.e., they can partition water away from the enzyme). However the

Table II. Activity results obtained with the different subtilisin enzyme-membrane combinations used in hexane saturated with water. Data are averages ± standard deviation ($n = 3$).

Enzyme–membrane system	Amount immobilized (mg)	K_M (mM)	V_{max} (μmol mg^{-1} min^{-1})
Wild-type on PVC–silica membrane	0.45 ± 0.03	0.57 ± 0.021	1.11 ± 0.14
Mutant (site-directed) on PVC–silica membrane	0.13 ± 0.008	1.15 ± 0.029	2.6 ± 0.32
Mutant (site-directed) on MPS membrane	0.35 ± 0.012	2.21 ± 0.033	0.8 ± 0.11

results shown in the above table are in contradiction to this. This can be attributed primarily to three effects:

(A) The organic solvent hexane is hydrophobic. This rigidifies the enzyme, and the already rigid enzyme is not affected by the hydrophilicity of the support.

(B) The higher hydrophilicity of the PVC–silica membrane minimizes hydrophobic denaturation of the immobilized enzyme.

(C) A hydrophilic support would partition water away from the solvent, and, hence, a water concentration gradient would develop with higher water concentration near the membrane surface as compared to the bulk of the organic solvent. This implies a higher water availability in the enzyme vicinity and, hence, a higher activity.

The higher activity shown by the mutant subtilisin PVC–silica system compared to the wild-type subtilisin PVC–silica system can be directly attributed to the site-directed immobilization of the mutant subtilisin. The active sites of the mutant were properly oriented away from the membrane surface, leading to good accessibility for the substrate molecules. The importance of substrate accessibility is crucial in nonaqueous systems due to potential substrate solvation problems that already exist.

The most interesting comparison of activities is between the wild-type subtilisin PVC–silica system and the mutant subtilisin MPS system. The higher activity of the wild-type subtilisin PVC–silica system underlines the importance of reduction of hydrophobic denaturation in this system. Thus, even though the active sites of the wild-type enzyme molecules were not properly aligned away from the membrane surface, the reduction of hydrophobic denaturation led to higher activity being shown by the wild-type subtilisin PVC–silica system compared to the mutant subtilisin MPS system.

Initial STEPR Studies

Spin-labeling is an EPR technique in which a paramagnetic reporter group (usually a nitroxide) is attached to the system of interest (Butterfield, 1982, 1990). In contrast to the 10^{-11} to 10^{-7} s time scale of in-phase EPR spin-labeling, STEPR is sensitive to molecular motion on the 10^{-7} to 10^{-3} s time scale (Thomas et al., 1976). In STEPR, rotational diffusional transfer of saturation among the different manifolds of the resonance spectrum occurs; i.e., rotational modulation of nitroxide hyperfine and Zeeman anisotropies is the predominant mechanism for diffusional saturation transfer. The sensitivity of STEPR to slower motion than in-phase EPR makes this technique potentially useful for insight into slower molecular motion of spin-labels covalently attached to the active site of immobilized enzymes (Butterfield et al., 1997; Subramaniam et al., 1996). In preliminary studies, STEPR was used to examine the active site motion of spin-labeled immobilized subtilisin in hexane and water. The principal parameter extracted from these initial STEPR spectra is the rotational correlation time, τ, which may be considered the time necessary for the spin-label attached to Ser 221 of subtilisin to rotate through an angle of one radian. The larger the τ, the slower the motion of the spin-labels. Rotational correlation times are determined from STEPR spectra by analysis of the $M_I = 0$ central region of the resulting spectrum through reference to a published standard curve of similar measurements for spin-labeled hemoglobin in solvents of different viscosity (Thomas et al, 1976). This calibration curve has been used by others in STEPR studies of enzymes (Affleck et al., 1992). Initial studies of EFT-labeled immobilized subtilisin suggest that τ is about 3-fold shorter in the polar organic solvent acetonitrile than in the nonpolar organic solvent hexane, each containing identical amounts of water. These initial findings are consistent with the results of Guinn et al. (1991), who showed that τ was lower for spin-labeled enzymes in polar organic solvents relative to nonpolar organic solvents. We also compared τ of EFT-labeled subtilisin immobilized on MPS and cellulosic membranes using STEPR, and found that τ was smaller for the latter membranes, implying faster motion of the spin-label attached to subtilisin immobilized onto the more polar membrane support. (Although hydrophilic PVC–silica membranes were used for activity studies, these membranes interfere with the STEPR studies, and therefore cellulose membranes, which are also hydrophilic, were used in the STEPR studies to get insight of the motion of the active site of immobilized subtilisin.) Consequently, our initial STEPR studies in nonpolar solvents suggest that hydrophobic denaturation by the support may be more important than water partitioning to the support in terms of loss of membrane-bound enzyme activity. This suggestion is consistent with our findings (see Table II) showing wild-type subtilisin randomly immobilized on hydrophilic PVC–silica membranes has higher activity than mutant subtilisin

immobilized on MPS membranes in water-saturated hexane.

Storage Stability of Immobilized Mutant Subtilisin on Actidisk Membrane

The storage stability (at 4°C) of the immobilized mutant subtilisin on the PVC–silica membrane was tested over a period of time. The RA of the enzyme–membrane system after day 10 was 80% of the activity at day 1. The RA at day 3 was almost identical to the activity at day 1. This was after exposing the enzyme–membrane system to organic solvents like hexane and 1-butanol. Thus, the storage stability of the mutant enzyme on the PVC–silica membrane was appreciable for repeated use.

CONCLUSIONS

The site-directed enzyme immobilization method resulted in the attachment of the enzyme to the membrane by a single bond per enzyme molecule via the genetically introduced cysteine residue. This resulted in a large enhancement of activity as compared to the random immobilization strategies. Membrane–enzyme hydrophobic interactions became more important in this binding strategy compared to previous site-directed immobilization schemes due to the absence of spacer protein molecules (Vishwanath et al., 1995, 1997). This interaction was minimized by using hydrophilic silica membranes. The advantageous aspect of the absence of a spacer protein in this immobilization strategy is the higher loading efficiencies that could be obtained. A higher optimal loading was observed for the site-directed enzyme on the hydrophobic MPS membrane as compared with the randomly immobilized enzyme. Further, the stability of the immobilized system was enhanced since the immobilization procedure involved the attachment of the enzyme using a covalent linkage.

A high activity was retained by the site-directed immobilized enzyme on the PVC–silica membrane. The hydrophilic environment provided by the PVC–silica membrane reduced hydrophobic denaturation of the enzyme. Water competition effects were negligible compared to the effects of denaturation in a nonpolar solvent like hexane. Initial STEPR studies also confirmed this phenomenon. For the case of immobilized enzyme reactions in solvent systems, one can optimize various parameters (such as solvent partitioning, substrate and product solvations, etc.), and conceivably, higher enzyme activity compared to nonimmobilized systems may be obtained.

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References

Abrahmsen, L., Tom, J., Burnier, J., Butcher, K. A., Kossiakoff, A., Wells, J. A. 1991. Engineering subtilisin and its substrates for efficient ligation of peptide bonds in aqueous solution. Biochemistry **30**: 4151–4159.

Affleck, R., Haynes, C. A., Clark, D. S. 1992. Solvent dielectric effects on protein dynamics. Proc. Natl. Acad. Sci. U.S.A. **89**: 5167–5170.

Arnold, F. H. 1990. Engineering enzymes for non-aqueous solvents. Trends Biotechnol. **8**: 244–249.

Bott, R., Graycar, T., Estell, D. 1991. Engineering altered substrate specificity into subtilisin BPN′, pp. 429–440. In: D. Kamely, A. M. Chakrabarty, and S. E. Kornguth (eds.), Biotechnology. Kluwer Academic Publishers, Boston.

Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B., Power, S. 1988. The three-dimensional structure of *Bacillus amyloliquefaciens* subtilisin at 1.8 Å and an analysis of the structural consequences of peroxide inactivation. J. Biol. Chem. **263**: 7895–7906.

Boyer, P. D. 1971. The Enzymes, Vol. III. Academic Press, New York.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72**: 248–254.

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

Butterfield, D. A. 1990. Principles of biological membrane structure: Alterations in the physical state of one side of the membrane by modulation of the physical state of the opposite side. J. Membr. Sci. **53**: 3–17.

Butterfield, D. A. (ed.) 1996. Biofunctional Membranes. Plenum Press, New York.

Butterfield, D. A., Subramaniam, R., Bhattacharyya, D., Viswanath, S., Huang, W., Bachas, L. G. 1997. Biofunctional membranes: Electron paramagnetic resonance studies of the active site structure of enzymes site-specifically immobilized onto polymeric supports through molecular recognition. Polym. Mater. Sci. Eng. Preprints **76:** 602–603.

Chapman, J. D., Hultin, H. O. 1975. Some properties of a protease (subtilisin BPN′) immobilized to porous glass. Biotechnol. Bioeng. **17**: 1783–1795.

Estell, D. A., Graycar, T. P., Wells, J. A. 1985. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. J. Biol. Chem. **260**: 6518–6521.

Ganapathi, S., Butterfield, D. A., Bhattacharyya, D. 1995. 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. **64**: 157–164.

Griebenow, K., Klibanov, A. 1996. On protein denaturation in aqueous–organic mixtures but not in pure organic solvents. J. Am. Chem. Soc. **118**: 11695–11700.

Guinn, M., Skerker, P., Kavanaugh, P., Clark, D. 1991. Activity and flexibility of alcohol dehydrogenase in organic solvents. Biotechnol. Bioeng. **37**: 303–308.

Horton, R. M., Peare, L. R. 1990. Directed mutagenesis: A practical approach. Wiley, New York.

Huang, W., Wang, J., Bhattacharyya, D., Bachas, L. G. 1997. Improving the activity of immobilized subtilisin by site-specific attachment to surfaces. Anal. Chem. **69**: 4601–4607.

Orsat, B., Drtina, G., Williams, M., Klibanov, A. 1994. Effect of support material and enzyme pretreatment on enantioselectivity of immobilized subtilisin in organic solvents. Biotechnol. Bioeng. **44**: 1265–1269.

Rao, S. V., Anderson, K. W., Bachas, L. G. 1998. Oriented immobilization of proteins. Mikrochim. Acta **128**: 127–143.

Reimann, A., Robb, D. A., Halling, P. J. 1994. Solvation of CBZ-amino acid nitrophenyl esters in organic media and the kinetics of their transesterification by subtilisin. Biotech. Bioeng. **43**: 1081–1086.

Reslow, M., Adlercreutz, P., Mattiasson, B. 1988. On the importance of

support material for bioorganic synthesis. Eur. J. Biochem. **172**: 573–578.

Strausberg, S., Alexander, P., Wand, L., Gallagher, T., Gilliland, G., Bryan, P. 1993. An engineered disulfide cross-link accelerates the refolding rate of calcium-free subtilisin by 850-fold. Biochemistry **32**: 10371–10377.

Subramaniam, R., Butterfield, D. A. 1996. Structure–activity correlation of subtilisin immobilized on modified poly(ether)sulfone membrane by covalent and non-covalent linkages, pp. 201–208. In: D. A. Butterfield (ed.), Biofunctional Membranes. Plenum Press, New York.

Takagi, H., Takahashi, T., Momose, H., Inouye, M., Maeda, Y., Matsuzawa, H., Ohta, T. 1990. Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. J. Biol. Chem. **265**: 6874–6878.

Thomas, D. D., Dalton, L. R., Hyde, J. S. 1976. Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance. J. Chem. Phys. **65**: 3006–3024.

Thomas, P. G., Russell, A. J., Fersht, A. R. 1985. Tailoring the pH dependence of enzyme catalysis using protein engineering. Nature **318**: 375–376.

Trzmiel, T., Fortak, M., Galas, E. 1995. The properties of subtilisin, *Novo* type, immobilized on porous glass. Acta Biotechnol. **15**: 123–129.

Valivety, R. H., Halling, P. J., Peilow, A. D., Macrae, A. R. 1994. Relationship between water activity and catalytic activity of lipases in organic media. Eur. J. Biochem. **222**: 461–466.

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

Vishwanath, S., Watson, C. R., Huang, W., Bachas, L. G., Bhattacharyya, D. 1997. Kinetic studies of site-specifically and randomly immobilized alkaline phosphatase on functionalized membranes. J. Chem. Technol. Biotechnol. **68**: 294–302.

Wangikar, P. P., Michels, P. C., Clark, D. S., Dordick, J. S. 1997. Structure and function of subtilisin BPN′ solubilized in organic solvents. J. Am. Chem. Soc. **119**: 70–76.

Zaks, A., Klibanov, A. 1988. The effect of water on enzyme action in organic media. J. Biol. Chem. **263**: 8017–8021.