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Activity and Stability of Enzymes Incorporated into Acrylic Polymers

Zhen Yang,[†] Anita J. Mesiano,[†] Srikanth Venkatasubramanian,[†] Susan H. Gross,[‡] J. Milton Harris,[‡] and Alan J. Russell*,[†]

Contribution from the Department of Chemical Engineering & Center for Biotechnology and Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and Department of Chemistry, University of Alabama, Huntsville, Alabama 35801

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Abstract: Two enzymes (subtilisin and thermolysin) have been modified with a polyethylene glycol (PEG, MW 3400) having an acrylate group at one terminus and an active ester at the other terminus and then incorporated into polyacrylates during free-radical initiated polymerization in a variety of organic solvents. Despite the high activity of the radical intermediates, both enzymes remain active and stable after incorporation. The activity and stability of the resulting biopolymer produced by subtilisin have been compared, in both aqueous and organic media, to those for the native, PEG-modified, and traditionally-immobilized subtilisins. In aqueous solution, the biopolymer had a reduced K_m and k_{cat} relative to the native subtilisin, but the specificity constant (k_{cat}/K_m) was only reduced to one-ninth by incorporation into the polymer. In a flow-cell reactor, the biopolymer achieved the longest half-life (>100 days) relative to the other forms of the enzyme. The enzyme's tolerance toward both heat and a miscible organic solvent was also enhanced by incorporation into the polymer.

Introduction

Enzyme immobilization methods are continuously being developed for uses in a variety of areas such as the food, chemical, pharmaceutical, and agricultural industries. Most immobilization strategies are designed for enzyme function in mainly aqueous environments. However, the need for use of enzymes in organic media has dramatically increased in recent years,[1] and it is now necessary to develop new methodologies to enable the synthesis of enzyme preparations which are stable in nonconventional environments, including nonaqueous solvents. One such approach is to form a protein-containing polymer.[2] Incorporation of proteins into polymeric substances is generally performed in aqueous solution due to the limited solubility of enzymes in organic solvents.

Our recent work focuses on the synthesis of protein-containing polymers in an organic solvent,[3] and we have hypothesized that such an approach could produce solvent-stable enzymes. Briefly, an enzyme is modified with a heterofunctional polyethylene glycol acrylate, which has an active ester terminus to react with the ϵ-amino groups of the lysine residues in the enzyme molecule to enable enzyme solubility and retention of activity in organic solvents, and an acrylate group at the other terminus to participate in the classical acrylate polymerization reaction in organic media (see Figure 1). Incorporation of active enzymes into polymers which can be used in either aqueous, gas, supercritical fluid, or organic phases is of obvious significance. Monomeric acrylates are the building blocks for a variety of polymers[4] ranging from poly(methyl methacrylate) and controlled drug release matrices (hydroxymethacrylates) to contact lenses (hydroxyacrylates). Therefore, this approach is not limited to a particular protein or polymer. Ito and colleagues[5] have recently developed a method of grafting enzymes with various hydrophobic vinyl polymers (for example, polystyrene) in organic solvents with the aid of azobis(4-cyanovaleric acid). They have demonstrated that the modified enzyme dissolves in organic solvents while still retaining catalytic activity. The approach we are taking is distinct from the work of Ito in that we are attempting to design immobilization strategies which result in protein-containing polymers with high activity in both organic solvents and organic/aqueous mixtures.

In the study reported herein, subtilisin Carlsberg (a serine protease, EC 3.4.4.16) has been selected as a model enzyme to demonstrate the maintainence of enzyme's activity and stability after incorporation into acrylic polymers. To demonstrate the general feasibility of this approach, another enzyme, thermolysin (a metalloendopeptidase, EC 3.4.24.4), has also been modified with PEG and co-polymerized into a polyacrylate. Both enzymes are particularly useful in catalyzing reactions such as transesterifications[6,7] and peptide syntheses[8,9] in organic media.

Results and Discussion

Protein-Polymer Synthesis. Subtilisin was first modified with PEG-A (shown in Figure 1). The attachment of PEG to

* Address correspondence to Alan J. Russell, 300 Technology Drive, Center for Biotechnology & Bioengineering, University of Pittsburgh, Pittsburgh, PA 15219. Telephone number: 412-383-9740. Fax number: 412-383-9710.

[†] University of Pittsburgh.

[‡] University of Alabama, Huntsville.

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1995.

(1) Dordick, J. S. *Enzyme Microb. Technol.* **1989**; *11*, 194−211. Klibanov, A. M. *Trends Biochem. Sci.* 1989, *14*, 141−144.

(2) Fulcrand, V.; Jacquier, R.; Lazaro, R.; Viallefont, P. *Tetrahedron* **1990**; *46*, 3909−3920.

(3) Yang, Z.; Williams, D.; Russell, A. J. *Biotechnol. Bioeng.* **1995**, *43*, 10−17.

(4) Odian, G. *Principles of polymerization*, 3rd ed.; John Wiley & Sons: New York, 1991.

(5) Ito, Y.; Fujii, H.; Imanashi, Y. *Biotechnol. Prog.* **1993**, *9*, 128−130.

(6) Chatterjee, S.; Russell, A. J. *Biotechnol. Bioeng.* **1992**, *40*, 1069−1077.

(7) Yang, Z.; Zacherl, D.; Russell, A. J. *J. Am. Chem. Soc.* **1993**, *115*, 12251−12257.

(8) Cassells, J. M.; Halling, P. J. *Biotechnol. Bioeng.* **1989**, *33*, 1489−1494. Chen, K.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5618−5622. Oyama, K.; Nishimura, S.; Nonaka, Y.; Kihara, K.; Hashimoto, T. *J. Org. Chem.* **1981**, *46*, 5242−5244.

(9) Oyama, K.; Kihara, K. CHEMTECH **1984**, (February) 100−105. Wong, C.-H.; Wang, K.-T. *Experientia* **1991**, *47*, 1123−1129.

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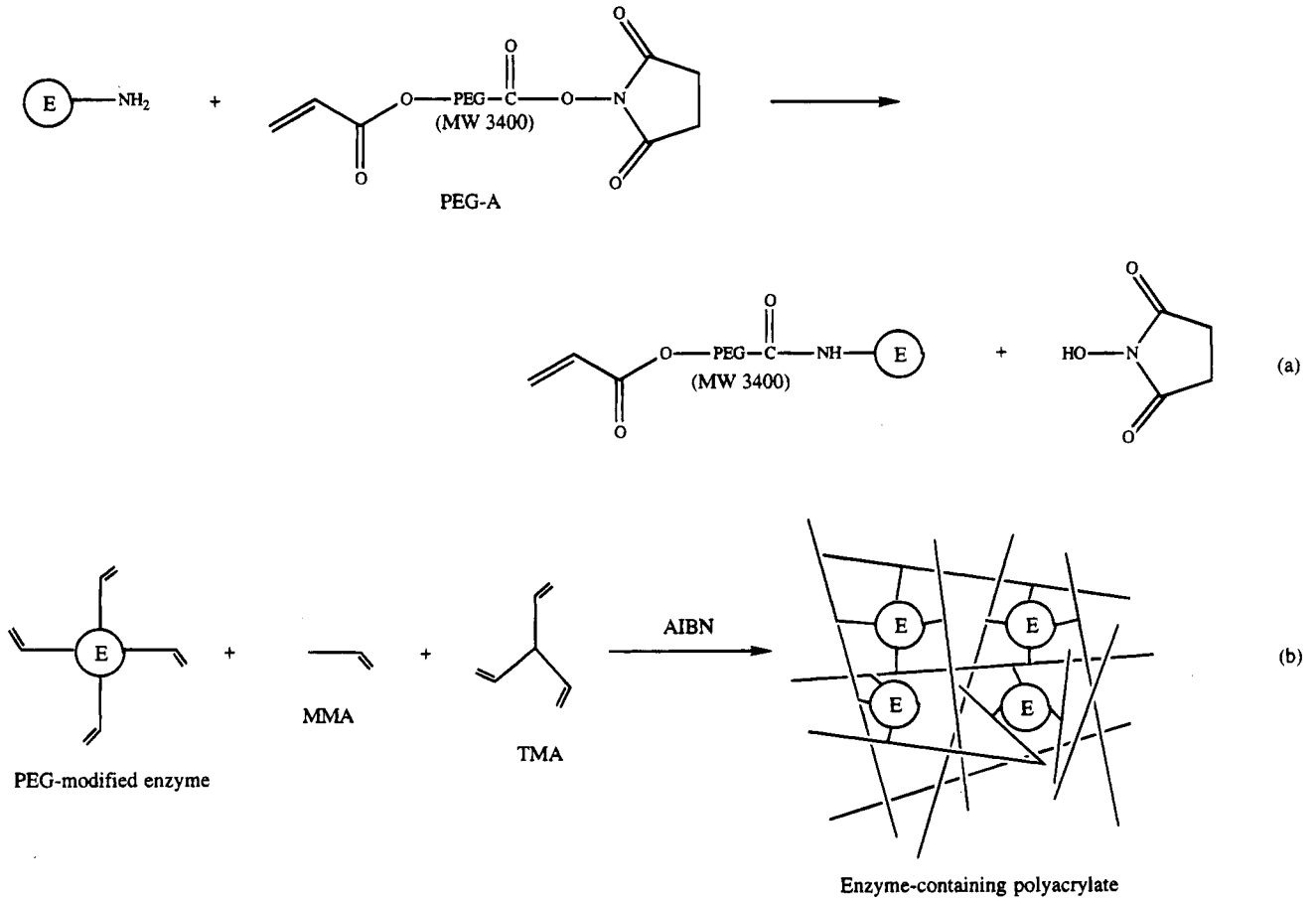


Figure 1. PEG-modification of subtilisin in aqueous solution (a) and its incorporation into polyacrylates during free-radical initiated polymerization in organic solvents (b).

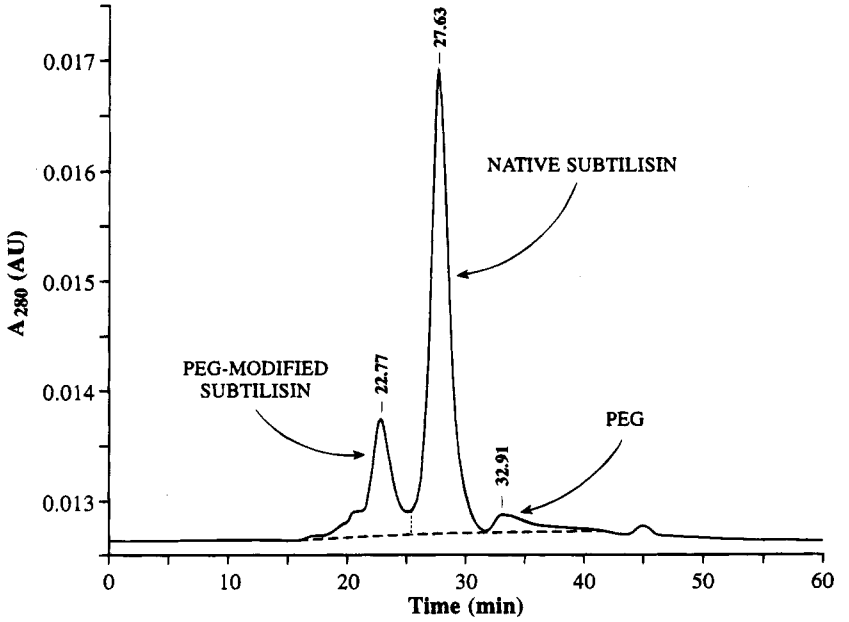


Figure 2. HPLC analysis of PEG-modified subtilisin. For experimental details see text.

the enzyme has been confirmed by HPLC analysis (Figure 2) with a modification degree of 18% (that is 18% of the subtilisin present was modified to some degree by the polyethylene glycol). The modified sample contained approximately equal concentrations of singly, doubly, triply, and quadruply modified proteins, as determined by mass spectroscopy (similar experiments are described in ref 3). The protein content of the PEG-modified sample was 22%, as determined by using the Bradford method.[10]

The PEG-modified subtilisin was then incorporated into polyacrylates by free-radical copolymerization with methyl methacrylate (MMA) and trimethylolpropane trimethacrylate (TMA).[3] After extensive rinsing, none of the aqueous supernatants showed enzyme activity. This was also the case when the polymer was crushed prior to rinsing. Thus, it appears that immobilization was close to 100% efficient, and the enzyme was covalently attached rather than adsorbed to the polymer

(10) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

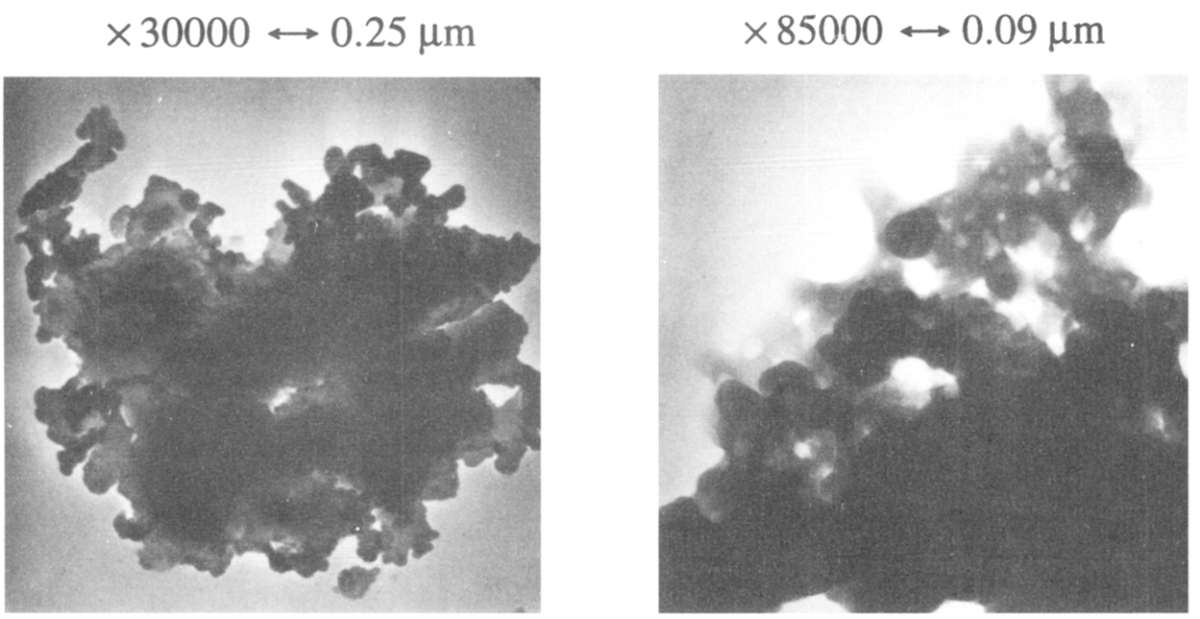


Figure 3. Transmission electron micrographs of the subtilisin-containing polyacrylate synthesized as described herein. This work was performed at R. J. Lee Inc, a company with expertise in the characterization of biomaterials.

surface or trapped inside pores of the polymers. When subtilisin was modified with methoxy-PEG (with a terminal ether instead of a terminal acrylate at one end of the PEG), the polymer formed during the free-radical polymerization between MMA, TMA, and methoxy-PEG-modified enzyme did not exhibit any activity, supporting the above result and indicating again that the active biopolymer was formed via covalent incorporation of enzyme into the acrylic polymer via copolymerization of methacrylate groups.

A transmission electron micrograph of the biopolymer product, shown in Figure 3, confirmed that the biopolymer was macroporous. The protein content of the biopolymer was approximately 0.02% (by weight), based on the PEG-modified enzyme used, its protein content and modification degree, and the final weight of the polymer formed (this assumes 100% incorporated). This extremely low protein content was due to the limited solubility of the PEG-modified enzyme in the organic solvent (~2 mg/mL in toluene). We are currently attempting to improve the protein content by varying the solvent and the molar ratios of monomers. It is worth noting, however, that even with this low protein loading, the biopolymer we synthesized still exhibited a relatively high activity (its k_{cat}/K_m in aqueous solution was only eight times lower than for the native enzyme) and stability in both aqueous and organic media, as will be reported in the following sections.

Activity. After incorporation into polyacrylates, subtilisin was still active in catalyzing the transesterification of *N*-acetyl-L-phenylalanine ethyl ester by methanol in organic solvents. The initial reaction rate for the biopolymer in dry toluene (<0.01% water in the solvent) was 3.5 nmol/min/$mg_{protein}$, while in the presence of 0.05% (v/v) distilled water or 0.5 g $Na_2HPO_4 \cdot 12H_2O$ (to give a water activity of 0.85 at 30 °C[7]), the biopolymer promoted an initial reaction rate of 11.0 or 41.0 nmol/min/$mg_{protein}$, respectively. This indicates that the specific activity of the biopolymer in organic media was comparable to native subtilisin (0, 1.4, and 120 nmol/min/$mg_{protein}$ under similar conditions, but in acetonitrile[7]), and, like native subtilisin, after incorporation into polymers the enzyme also required water to become activated.

As expected, the biopolymer was also active in aqueous solution. We compared the activity of native and traditionally immobilized subtilisin (attached to oxirane-activated macroporous acrylic beads) to that of the biopolymer. At 20 °C and in the presence of 0.1 mM substrate (succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide), the initial rate of the hydrolysis reaction with the biopolymer was 6.7 μmol/min/$mg_{protein}$, similar to that for the traditionally immobilized enzyme (7.0 μmol/min/$mg_{protein}$), but much lower than the rate when the native subtilisin was used (146 μmol/min/$mg_{protein}$). It is of course very common for immobilization to decrease enzyme activity. To investigate these differences we determined the apparent K_m and V_{max} for each enzyme form (Table 1). The failure to obtain accurate values of the K_m and V_{max} for the traditionally immobilized enzyme suggests that the immobilized enzyme had a much higher K_m than the soluble one. Immobilization can often increase the apparent K_m of an enzyme due to the reduced accessibility of the enzyme active site to the substrates. However, the K_m for the biopolymer was dramatically reduced. This may be attributed to the high hydrophobicity of the acrylate polymers. The substrate used was hydrophobic, and as such could partition into the porous hydrophobic polyacrylate where the enzyme is incorporated. Therefore, the local concentration of the substrate in the environment surrounding the enzyme should be much higher than its bulk concentration, thus accounting for a lower apparent K_m.

Table 1. Kinetic Constants for Subtilisin-Catalyzed Hydrolysis of *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-Nitroanilide in Aqueous Solution

	K_m (mM)	$k_{cat(min)}$[a] (s^{-1})	$k_{cat(min)}/K_m$ (s^{-1} mM^{-1})
biopolymer	0.019 ± 0.005	>2.1	111
native subtilisin	0.19 ± 0.01	181 ± 5	953

[a] The turnover number is calculated by assuming that the polymerization of enzyme is 100% efficient. Reduced efficiency would result in an increase in this minimum value of k_{cat}.

In Table 1 the turnover number for each enzyme is reported as $k_{cat(min)}$. Since it is not possible to accurately ascertain the enzyme concentration in experiments with the immobilized enzyme, we have calculated the maximum enzyme content assuming that all the enzyme added to the polymerization mixture has been incorporated into the final polymer. If the incorporation was less than 100% efficient, then the turnover number would increase proportionally.

It was interesting to assess whether incorporation of subtilisin into acrylic polymers resulted in activity in aqeous/organic mixtures, since previous experiments have demonstrated that

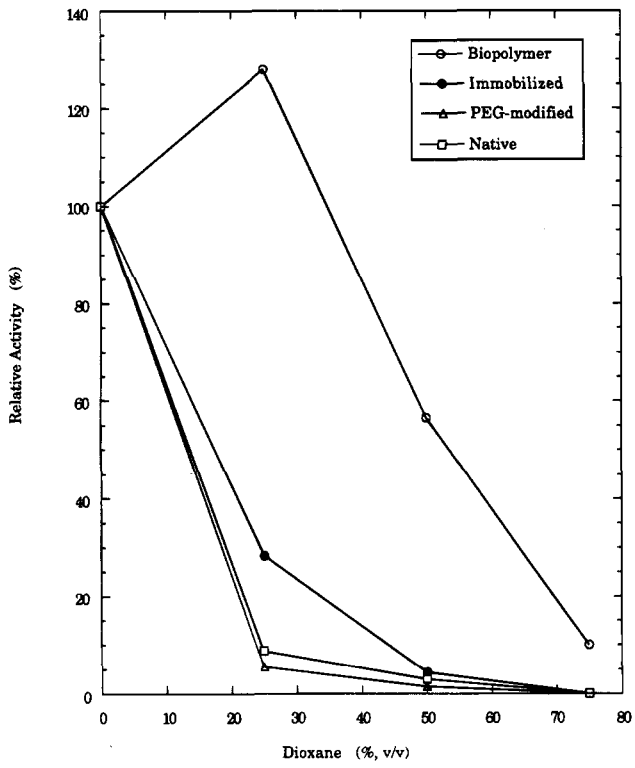


Figure 4. Activities of native, PEG-modified, polymerized, and immobilized subtilisins in various buffer/dioxane mixtures at 20 °C with *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide as the substrate. For experimental details see text.

apolar organic solvents such as dioxane strongly inhibited α-chymotrypsin-catalyzed hydrolyses in aqueous solution.[11] The initial rates for the hydrolysis reactions catalyzed by native, PEG-modified, polymerized, and traditionally immobilized subtilisin in various dioxane/buffer solutions were compared as shown in Figure 4. The biopolymer remained highly active in the presence of high concentrations of dioxane, while the activities of both the native and PEG-modified enzymes dropped precipitously as the dioxane concentration increased. The traditionally immobilized subtilisin exhibited limited activity in dioxane/buffer mixtures.

Clement and Bender[11] showed that dipolar aprotic solvents such as dioxane, acetonitrile, and acetone affected the kinetics of α-chymotrypsin-catalyzed hydrolyses by increasing K_m but keeping k_{cat} nearly constant. Increasing the dioxane concentration in aqueous buffer from 0 to 22% resulted in a 100-fold reduction of the k_2/K_m for the α-chymotrypsin-catalyzed acylation of *N-trans*-cinnamoyl imidazole. It is thus not surprising that a remarkably low activity was observed in our experiments when the soluble subtilisin, whether native or PEG-modified, was placed in 25% dioxane in aqueous buffer. Clement and Bender concluded that solvents like dioxane were not only strong competitive inhibitors but also had effects on the enzyme via electrostatic interactions. The solvent apparently reduced the dielectric constant of the medium, thus increasing the electrostatic repulsion between the enzyme and the substrate, and in turn decreasing the binding. Since the local dielectric constant within the hydrophobic polyacrylate would be lower than in bulk water, it is reasonable to suggest that the addition of dioxane would not dramatically alter electrostatic interactions within the polymers. Therefore, since the enzyme in the polymer is already active, the addition of solvent has a smaller effect than on enzyme solubilized in buffer.

Another explanation for the relatively high activity of subtilisin incorporated into polyacrylates in the presence of high concentrations of dioxane can be related to the mobility of the enyzme. The addition of organic solvents to buffer results in conformational changes in enzyme molecules.[12] Immobilization of an enzyme on a solid support may exert a significant stabilizing effect on the enzyme, particularly when attached by multiple covalent linkages, and these more rigid enzyme molecules are expected to be more resistant to the conformational alterations induced by the solvents.[13]

(11) Clement, G.; Bender, M. *Biochemistry* **1963**, *2*, 836–843.

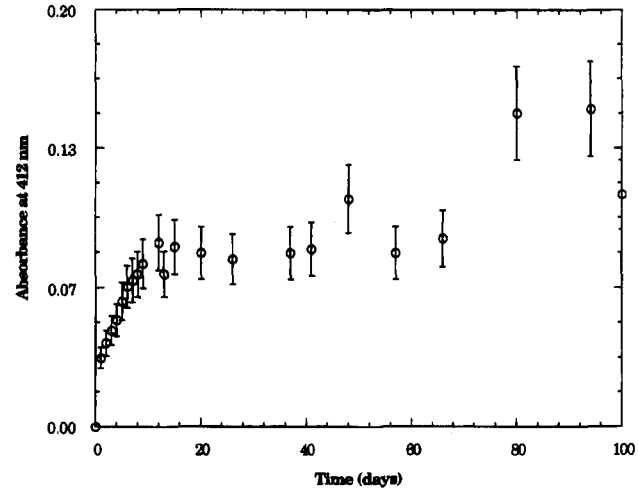


Figure 5. Activity of polymerized subtilisin in a continuous flow-cell reactor at 20 °C. For experimental details see text.

Stability in Aqueous Solution. In order to test the applicability of the biopolymer, it is important to determine subtilisin stability in continuous biocatalytic use. For this purpose, a flow-cell reactor was constructed and utilized to follow a subtilisin-catalyzed hydrolysis in aqueous solution.[3]

When the biopolymer was placed in the reactor, enzyme activity continued for over 100 days (Figure 5) after which time the experiment was terminated. The catalytic stability of the biopolymer is particularly interesting when one considers that native subtilisin begins to lose activity after only 5 days. Given the activity of the biopolymer in the presence of solvent denaturants, it was not surprising that the biopolymer was such a stable form of the enzyme. Because of cross-linking, the enzyme molecules in the biopolymer are probably more rigid, and the binding between the protein molecules and polyacrylate was probably much stronger than via the oxirane immobilization strategy (single point attachment). By using aldehyde-agarose gels as activated supports for enzyme immobilization, Guisán[13] has demonstrated that single-point attachment of enzyme to the support is fast and reversible but binding of the enzyme on the supports is dramatically stabilized by multipoint enzyme-support attachments.

The enhanced stability of the polymer-incorporated subtilisin can also be related to the decreased autolysis of the enzyme resulting from PEG-modification and immobilization, given the steric hindrance that the large PEG chain and the rigid polymer structure create. Our previous work[3] has shown that after PEG-modification, the half-life of subtilisin at 30 °C (3 h) was increased to 700 h for the singly modified enzyme and 1500 h for the doubly modified enzyme. Incorporation of subtilisin into rigid polyacrylates should also protect the enzyme from auto-hydrolysis. It is interesting that the half-life of native subtilisin in the flow-cell reactor (<40 days, data not shown) was much longer than that for the free enzyme incubated in aqueous buffer (3 h). This increase in half-life can be attributed to the presence of the substrate during the continuous reaction,

(12) Herskovits, T.; Gadegbeku, B.; Jaillet, H. *J. Biol. Chem.* **1970**, *245*, 2588–2598.

(13) Guisán, J. M. *Enzyme Microb. Technol.* **1988**, *10*, 375–382.

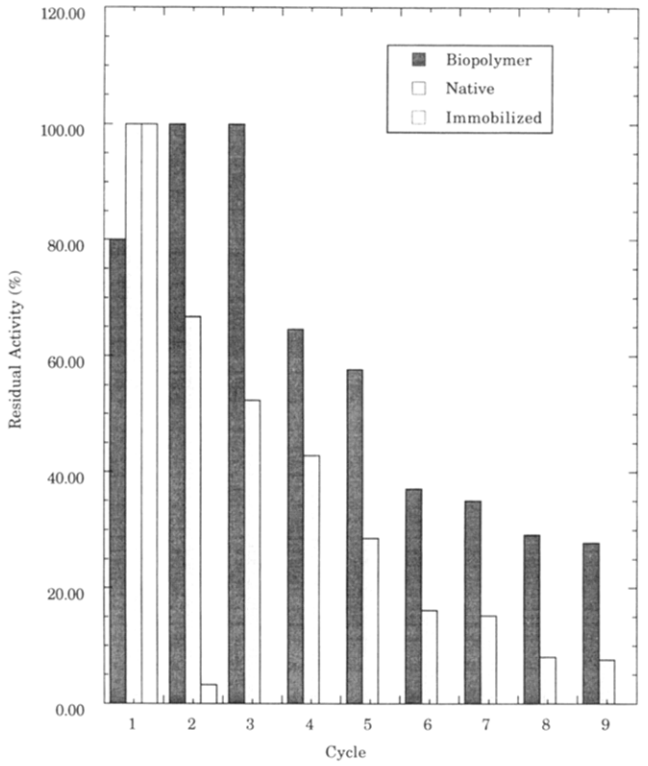


Figure 6. Recyclability of native, polymerized, and immobilized subtilisin in hexane at 30 °C. For experimental details see text.

since the active site of the enzyme was always occupied by the substrate molecules autolysis was probably minimized.

After 100 days in the flow-cell reactor, 12% of the total supplied substrate had been hydrolyzed by the biopolymer. By suitably optimizing the flow rates, pH (the pH used in this test was 7.0 versus the optimal pH for native subtilisin of 8.0), and substrate concentration, it may be possible to obtain even better yields for this continuous process.

Stability in Organic Solvents. The recyclability of the biopolymer in organic media has also been compared to that for the native and traditionally immobilized enzyme. The enzyme was allowed to catalyze a transesterification reaction in hexane for 1 h (as described in the Methods section). The reaction mixture was then centrifuged and the supernatant decanted. The enzyme powder was then washed three times with methanol to remove the remaining reactants and products, dried thoroughly by air blowing, and then recycled into a fresh reaction mixture. As shown in Figure 6, the biopolymer was again the most stable, retaining 34% of its activity even after nine cycles. Native subtilisin possessed only 7.6% activity after nine cycles, while only 3.3% of the original activity was retained by the traditionally immobilized enzyme in the second cycle.

The cycling experiment described above was designed to be particularly harsh. Indeed, methanol is known to inactivate many proteins[12] and even solubilize others. We felt that if the biopolymer could withstand extensive rinsing in methanol, it would indicate the synthesis of a material with improved properties for use in organic solvents. Our results demonstrate that the material does indeed have these desirable properties. The particularly low stability of the traditionally immobilized subtilisin could result from the effect of methanol on the attachment chemistry. While it seems unlikely, since the enzyme was attached to oxirane-activated acrylic beads, methanol could act as a nucleophile, and break bonds between the oxirane and the enzyme.

Effect of Polymerization Conditions on Activity and Stability. In order to confirm the generality of the approach of enzyme incorporation into polyacrylates, we have studied the effect of polymerization conditions on activity and stability of the subtilisin-containing acrylic polymers. It is well-known that the morphology of the polymer can be significantly altered by changing the solvent during polymerization.[14] The effect of solvent on morphology is demonstrated in the scanning electron micrographs of the polymers in Figure 7. All the polymers are macroporous, but the pore sizes vary between 0.1 and 1 μm. Most of the biopolymer samples have a basic structure made of small spheres (nodules) which have agglomerated to form large unshaped entities (Figure 7a,b), while a few samples appear fluffy and granular, with no discernible regular shape (Figure 7c).

The specific surface areas of the biopolymer samples (Table 3), determined by the BET method,[15] are also dependent on the solvent used for polymerization. A large surface area was observed in the polymer synthesized in either toluene (185 m^2/g) or carbon tetrachloride (193 m^2/g), but the surface area dropped when the proportion of chloroform in the above two solvents increased, with the smallest surface area obtained in the presence of pure chloroform (only 6.4 m^2/g).

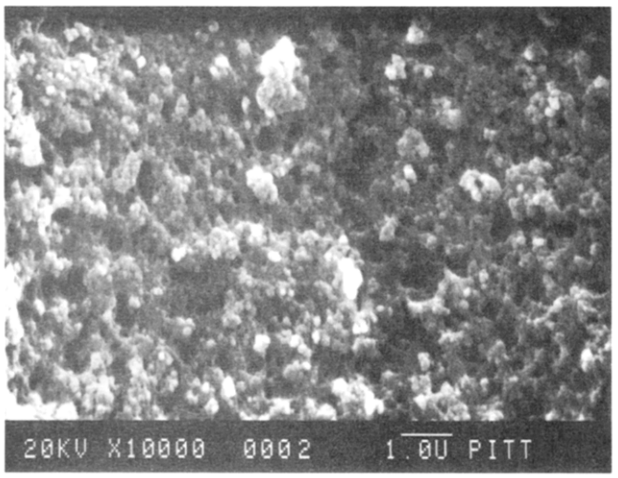
In spite of the different morphologies, however, the biopolymers promoted similar activities in aqueous buffer (Figure 8, open bars), with a reasonably high tolerance to heat. After the treatment for BET surface area measurements (heating at 130 °C for 2 h under vacuum), all the biopolymers retained 16–30% of their original activities (Figure 8, filled bars). The native subtilisin was totally inactivated after such a harsh treatment. Additionally, the activities of the biopolymers in the buffer/dioxane mixtures varied with dioxane content, irrespective of the solvent used in polymerization. They were all more tolerant toward dioxane than the native subtilisin, as indicated in Figure 4.

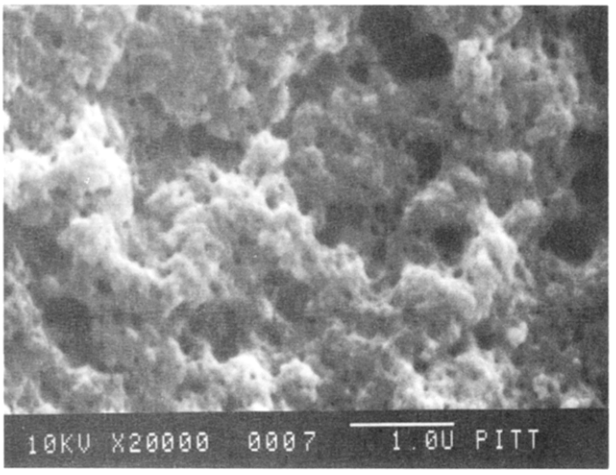
All the biopolymers have also been shown to be catalytically active in catalyzing transesterification reactions in organic solvents. The polymers showed variant activities in toluene depending on the solvent used for polymerization (Table 3). Comparatively speaking, the activities varied in the order of toluene > carbon tetrachloride > chloroform, and the biopolymers synthesized from toluene/chloroform mixtures were slightly more active than those produced from carbon tetrachloride/chloroform mixtures.

The above experiments have therefore demonstrated that the biopolymers synthesized in different organic solvents result in different pore sizes and specific surface areas, but they remained catalytically active in both aqueous and nonaqueous media. It appears that their activities lie in the same order of magnitude, irrespective of the morphology of the polymer, although the latter is of significant importance in controlling the diffusion and mass transfer of substrates and products. Interestingly, while previous experiments have shown that native subtilisin exhibits low activity in chloroform compared to solvents such as toluene,[7] the subtilisin-containing biopolymers synthesized during copolymerization in all solvent mixtures containing chloroform remained as active in either aqueous or organic media as those produced from toluene. Presumably, subtilisin has not been denatured or deactivated by exposure to chloroform during polymerization, but the rather low activity of the enzyme in chloroform might result from some other indirect effects, such as lowering the polarity of the enzyme's active site, and partitioning of substrates and products between enzyme active site and the bulk solvent phase.

(14) Guyot, A.; Bartholin, M. *Prog. Polym. Sci.* **1982**, *8*, 277–332. Rosenberg, J.-E.; Flodin, P. *Macromolecules* **1986**, *19*, 1543–1546. Rosenberg, J.-E.; Flodin, P. *Macromolecules* **1993**, *20*, 1518–1522. Rosenberg, J.-E.; Flodin, P. *Macromolecules* **1987**, *20*, 1522–1526.

(15) Brunauer, S.; Emmett, P. H.; Teller, E. *J. Am. Chem. Soc.* **1938**, *60*, 309–319.





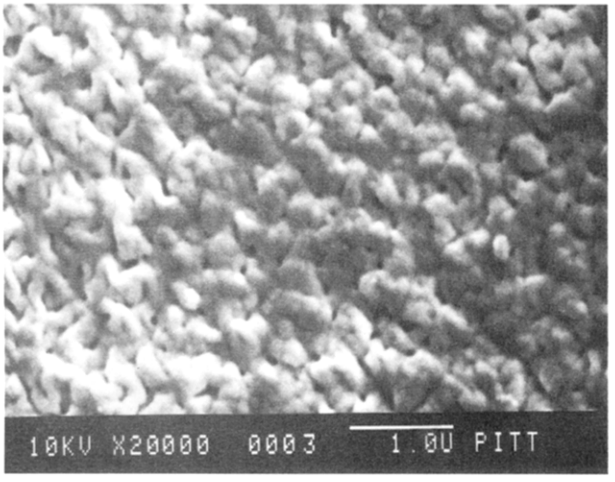


Figure 7. Scanning electron micrographs of the subtilisin-containing polyacrylates synthesized from different organic solvents: (a, top) for biopolymer A2, (b, middle) for biopolymer B2, and (c, bottom) for biopolymer A3.

Thermolysin-Containing Polyacrylates. The immobilization method described above should have general utility to other enzymes, since almost all enzymes contain lysine residues. Thermolysin, a metalloendopeptidase which is thermostable and particularly useful in peptide hydrolysis and synthesis,[9,16,17] has also been modified with PEG and incorporated into acrylic polymers in the same way as described for subtilisin.

HPLC analysis has confirmed that 79% of the native thermolysin can be modified with PEG-A. The activity of thermolysin in aqueous solution was determined by monitoring the hydrolysis of a dipeptide substrate, 3-(2-furylacryloyl)-glycyl-leucine amide (FAGLA), spectrophotometrically at 345 nm.[16] The K_m for both native and PEG-modified thermolysins were 2.9 and 4.0 mM respectively, and the k_{cat} values were 90 and 118 s^{-1}, respectively. Hence, PEG-modification did not lead to the alteration of the enzyme's catalytic efficiency (k_{cat}/K_m was 29.5 s^{-1} mM^{-1} for the PEG-modified enzyme compared to 31.0 s^{-1} mM^{-1} for the native enzyme).

To demonstrate the activity of thermolysin after incorporation into polyacrylates, the thermolysin-containing biopolymer (1 mg) was added to 10 mL of pH 7, 20 mM Tris-HCl buffer containing 5 mM $CaCl_2$ and 1 mM FAGLA (as substrate), and the enzyme activity was determined while varying the reaction temperature from 20 to 90 °C (Figure 9). No reaction occurred in the absence of biopolymer. The same optimum reaction

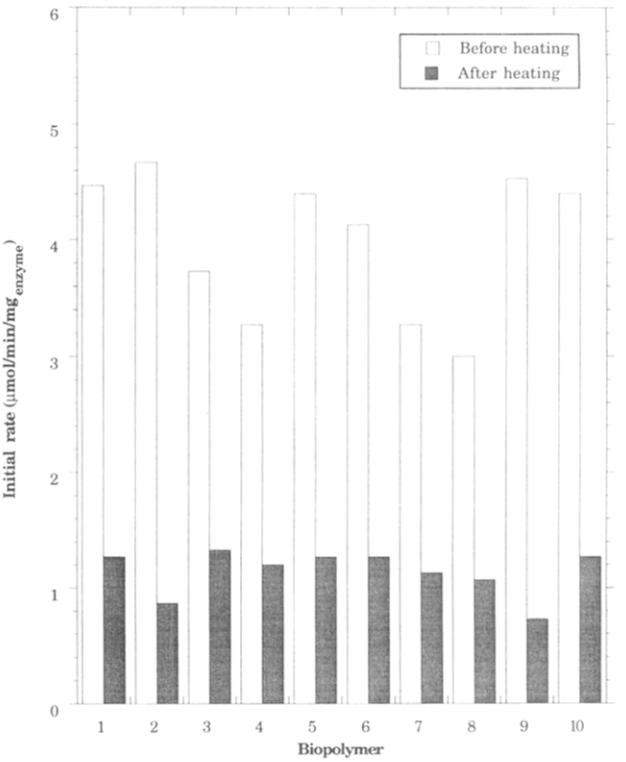


Figure 8. Activities of subtilisin-biopolymers in aqueous buffer (20 mM Tris-HCl, pH 8.0) before and after heating at 130 °C under vacuum for 2 h.

Table 2. Synthesis of Subtilisin-Containing Biopolymers in Different Organic Solvents

In Toluene/Chloroform Mixtures

biopolymer	A1	A2	A3	A4	A5
toluene (mL)	4	3	2	1	0
chloroform (mL)	0	1	2	3	4
PEG-subtilisin (mg)	6	6	6	6	6
MMA (mL, distilled)	0.2	0.2	0.2	0.2	0.2
TMA (mL, 50% in $CHCl_3$)	1	1	1	1	1
AIBN (mL, 0.5% in toluene)	1	1	1	1	1

In Carbon Tetrachloride/Chloroform Mixtures

biopolymer	B1	B2	B3	B4	B5
carbon tetrachloride (mL)	4	3	2	1	0
chloroform (mL)	0	1	2	3	4
PEG-subtilisin (mg)	6	6	6	6	6
MMA (mL, distilled)	0.2	0.2	0.2	0.2	0.2
TMA (mL, 50% in $CHCl_3$)	1	1	1	1	1
AIBN (mL, 0.5% in toluene)	1	1	1	1	1

(16) Feder, J. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 326−332.
(17) Heinrikson, R. L. *Methods Enzymol.* **1977**, *47* (Part E), 175−189.
(18) Harris, J. M.; Yalpani, M.; Van Alstine, J. M.; Struck, E. C.; Case, M. G.; Paley, M. S.; Brooks, D. E. *J. Poly. Sci., Poly. Chem. Ed.* **1984**, *22*, 341.

Table 3. Surface Areas of Subtilisin-Biopolymers and Their Enzyme Activities in Toluene

solvent mixture	toluene/chloroform					carbon tetrachloride/chloroform				
biopolymer	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5
chloroform % (v/v)	0	25	50	75	100	0	25	50	75	100
surface area (m^2/g)	185	141	7.46	7.08	6.39	193	109	42.9	9.92	6.39
activity in toluene (μmol/min/mg_{enzyme})	0.44	0.63	0.97	0.26	0.27	0.32	0.17	0.08	0.03	0.27

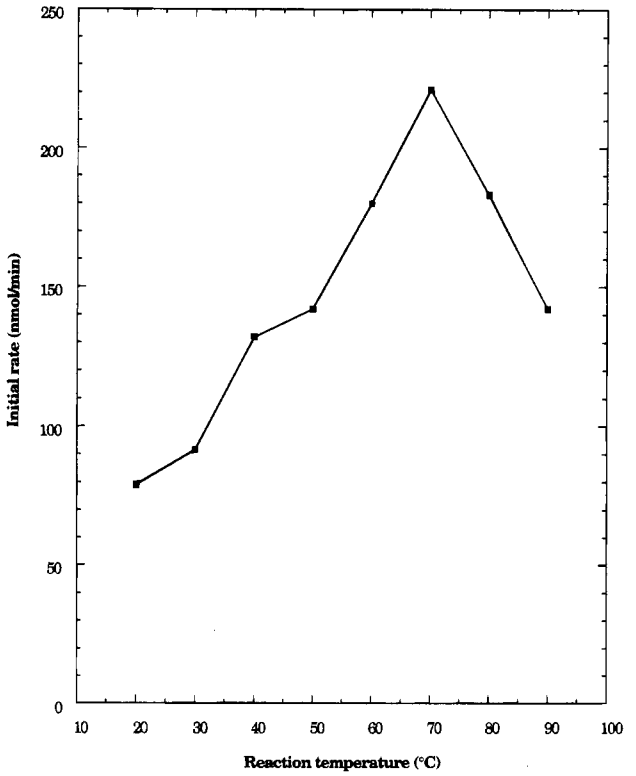


Figure 9. Activities of thermolysin-biopolymer in aqueous solution at different reaction temperatures.

temperature (70 °C) was also observed for both native and PEG-modified thermolysins. The activation energies, determined by Arrhenius plots, were 9.1 and 17.3 KJ/mol for the native thermolysin and the biopolymer, respectively. We believe that this almost 2-fold increase in activation energy can be attributed to the increased steric hindrance offered by the polymer network to the approach of the substrate to the active site of the enzyme. The activity and stability of thermolysin-containing polyacrylates in both aqueous and nonaqueous environments and their use in peptide synthesis are currently under investigation in our laboratory.

Conclusions

Our results have shown that it is generally feasible to incorporate enzymes (such as subtilisin and thermolysin), after modification with PEG-acrylate, into acrylic polymers during polymerization in organic solvents. This approach enables enzymes to remain active and stable in aqueous and organic media and mixtures of the two. The enzymes' tolerance against heat and organic solvents (such as methanol and dioxane) can be significantly enhanced. In spite of the decrease in subtilisin activity upon polymerization, the overall productivity of the catalyst can be enhanced because of the remarkable stability of the material. Kinetic investigation of subtilisin-catalyzed reactions suggests that polymer design can be used to modify the apparent binding between the enzyme and its substrate. Further applications of this approach are being assessed in our laboratory.

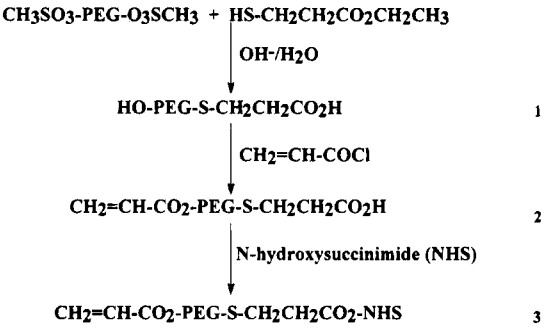


Figure 10. Reaction scheme for heterofunctional poly(ethylene glycol) synthesis. For details see the Experimental Section.

Experimental Procedures

Materials. The enzymes, native and immobilized subtilisin Carlsberg (attached to oxirane-activated macroporous acrylic beads) and thermolysin, were purchased from Sigma Chemical Co. The NHS activated polyethylene glycol acrylate (PEG-A) (MW 3400) was synthesized as described below. All other reagents were from either Sigma or Aldrich and were of the highest purity available. Methylene chloride was dried over molecular sieves. Chromatographic gels were supplied by Pharmacia. For the PEG syntheses, HPLC work was performed on a Bio Rad liquid chromatograph with an LDC Model 1107 refractive index detector and a Waters Ultrahydrogel 250 column. ^1H NMR spectra were determined on a Bruker 200 MHz NMR spectrometer, and IR work was performed on a Matteson Galaxy series FT-IR Model 3025 interfaced with a Pionex computer.

Heterofunctional Poly(ethylene glycol) Synthesis. The reaction scheme shown in Figure 10 was utilized to synthesize the heterofunctional PEG molecules necessary for the modification of subtilisin.

Synthesis of ω-Carboxyl α-Hydroxyl PEG, 1. In a round bottom flask equipped with a magnetic stir bar, reflux condenser and nitrogen line, PEG-3400-mesylate (**8**) (70.0 g; 11% substituted; 0.0206 mol) was added to a mixture of 280 mL of toluene and 420 mL absolute ethanol and stirred to dissolve. Sodium hydroxide (3 × excess; 2.32 g; 0.0581 mol) was dissolved in 56 mL of absolute ethanol and added to the PEG-mesylate. Ethyl-3-mercaptopropionoate (3 × excess; 7.84 mL; 8.15 g; 0.0607 mol) was added to the reaction via syringe, and the reaction was heated under a nitrogen atmosphere to 60 °C for 3 h. The reaction mixture was cooled to room temperature, filtered to remove insoluble salts, and concentrated to about 250 mL under reduced pressure. This was added to 1200 mL of cold diethyl ether, giving the desired ester as a pale yellow precipitate which was dried in vacuo overnight.

In an Erlenmeyer flask equipped with a magnetic stir bar, PEG ester from the preceding step (70.0 g; 0.0206 mol) was added to 840 mL of distilled deionized water and stirred to dissolve. In a separate beaker, sodium hydroxide (1.4 g) was added to 35 mL of distilled deionized water and stirred to dissolve. The sodium hydroxide solution was added to the PEG solution until the pH was 12−13. The solution was then stirred at room temperature for 1 h. Oxalic acid was added to adjust the pH to 3. The solution was extracted with CH_2Cl_2 three times (200/200/200 mL). The extract was dried over $Na_2SO_4/MgSO_4$, filtered, concentrated under reduced pressure to about 150 mL, and added to 1000 mL of cold diethyl ether to precipitate the product. The product **1** was dried in vacuo overnight: yield 61.6 g, 88%. The mixture was purified by ion exchange chromatography on Sepharose FF (Pharmacia): ^1H NMR (DMSO-d_6) 2.63 ppm (m, $-SCH_2CH_2-$, 4H), 3.50 ppm (s, PEG backbone, 304 H), 4.57 ppm (t, O*H*, 1H).

Synthesis of ω-Carboxyl α-Acryloyl PEG, 2. In a round bottom flask equipped with a mechanical stir bar and a rubber septum attached to a nitrogen line and a bubbler, PEG acid from the preceding step

(1.00 g; 0.000294 mol) was dissolved in 30.0 mL of dry CH_2Cl_2. Triethylamine (2.5 × excess; 0.0744 g; 0.00103 mol) was weighed in a small test tube, diluted with 1 mL of CH_2Cl_2 and added via syringe. Acryloyl chloride, 98% (2.5 × excess; 0.0665 g; 0.00103 mol), was weighed in a small test tube, diluted with 1 mL of CH_2Cl_2; and added via syringe. The reaction was stirred under nitrogen overnight. The mixture was concentrated under reduced pressure until slightly viscous and dripped into 80 mL of cold diethyl ether to precipitate the product. After drying in vacuo 3 h, the product was dissolved in distilled deionized water (100 mL) and stirred for 30 min. The aqueous solution was extracted in CH_2Cl_2 three times (30/30/20 mL). The extract was dried over $Na_2SO_4/MgSO_4$, concentrated under reduced pressure until slightly viscous, and dripped into 100 mL of cold diethyl ether to precipitate the product **2**: yield 0.946 g, 95%; ^{1}H NMR (DMSO-d_6) 2.625 ppm (m, SCH_2CH_2, 4H), 3.50 ppm (s, PEG backbone, 304 H), 5.96 ppm (dd, C*Ha*HbCHcCOO, 1H), 6.20 ppm (dd, CHaHbC*Hc*COO, 1H), 6.35 ppm (dd, CHa*Hb*CHcCOO, 1H).

Synthesis of NHS Ester of ω-Carboxyl α-Acryloyl PEG, 3. In a round bottom flask equipped with a magnetic stir bar and a rubber septum attached to a nitrogen line and a bubbler, intermediate **2** (0.946 g; 0.278 mmol), *N,N'*-dicyclohexyl carbodiimide (1.5 × excess; 0.0861 g; 0.417 mmol), and *N*-hydroxysuccinimide (1.5 × excess; 0.0480 g; 0.417 mmol) were dissolved in 30.0 mL of CH_2Cl_2. The flask was immersed in an ice bath and stirred overnight. The next day the reaction mixture was filtered, concentrated under reduced pressure, filtered, and precipitated into cold diethyl ether: yield 0.592 g, 63%; ^{1}H NMR (DMSO-d_6) 2.625 ppm (m, SCH_2CH_2, 4H), 2.81 ppm, (s, succinimide, 4H), 3.50 ppm (s, PEG backbone, 304 H), 5.96 ppm (dd, C*Ha*HbCHcCOO, 1H), 6.20 ppm (dd, CHaHbC*Hc*COO, 1H), 6.35 ppm (dd, CHa*Hb*CHcCOO, 1H). Hydrolysis, followed by titration of the resulting acid, indicated 2.49 × 10^{-4} moles of acid per g of PEG, or 85% of the theoretical value; FT-IR 1783 cm^{-1} C=O stretch (succinimidyl ester end groups), 1114 cm^{-1} C−O−C stretch.

Biopolymer Synthesis. Enzyme modification. Subtilisin (20 mg) and PEG-A (30 mg) (the molar ratio of enzyme to PEG is 10:1) were dissolved in 5 mL of borate buffer (0.1 M, pH 9.0). After magnetic stirring for 5 h at room temperature, the solution was dialyzed against phosphate buffer (20 mM, pH 6.0) for 24 h to remove the unreacted PEG-A, followed by lyophilization on a Labconco lyophilizer for 48 h. The protein content of the modified sample was determined by the Bradford method,[10] and the modification degree was measured by HPLC analysis and time of flight mass spectroscopy. For modification of thermolysin, the reaction temperature was set at 40 °C.

Typically, a Waters HPLC system, equipped with a Pharmacia Superdex 75 HR 10/30 column packed with dextran (75 μm), was used to separate PEG, PEG-modified subtilisin, and subtilisin. The reaction mixture after PEG-modification (100 μL of 2 mg/mL of lyophilized powder in buffer) was injected onto the column. The mobile phase used was pH 7.0 phosphate buffer (50 mM plus 10 mM NaCl) at a flow rate of 0.5 mL/min, and the different components were detected with a UV detector at 280 nm.

Polymerization. PEG-A modified subtilisin (6 mg) was dissolved in toluene (4 mL) in an 8-mL screw-capped Wheaton vial with the aid of sonication. Then methyl methacrylate (0.2 mL) (MMA), trimethylolpropane trimethacrylate (1 mL) (TMA, 50% v/v in chloroform, as cross-linker), and α,α'-azobisisobutyronitrile (1 mL) (AIBN, 0.5% w/v in toluene, as initiator) were added. After degassing with nitrogen for 1 min, the polymerization was initiated by UV light at 365 nm at room temperature. Twenty-four hours later, a turbid, stiff gel was formed occupying the entire reaction volume. The biopolymer product was then obtained by crushing, washing 3−5 times with distilled water (to remove any unreacted enzyme) and methanol (to remove any unreacted MMA, TMA, AIBN, and remaining toluene), and drying in a fume hood overnight.

Polymer Characterization. The specific surface area was determined from adsorption measurements with nitrogen according to the BET method.[15] Prior to measurements, the polymer samples were heated at 130 °C under vacuum for 2 h to remove the remaining water and solvent. Scanning electron micrographs were obtained by using JEOL 35 scanning electron microscope in the Department of Material Science and Engineering. Studies on transmission electron microscopy were performed at R. J. Lee Inc., a company with expertise in characterization of biomaterials.

Activity Assays. The activities of native, PEG-modified, polymerized, and traditionally immobilized subtilisin in aqueous solution were detected by following the enzyme-catalyzed hydrolysis of *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide with a Perkin-Elmer spectrophotometer, and their activities in organic media were measured by following the enzyme-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester by methanol with a Hewlett-Packard 5890 series II gas chromatograph, as described previously.[7] For the biopolymer activity in aqueous solution, biopolymer (5 mg) was added to a screw-capped reaction vial containing substrate (10 mL, 0.01−0.2 mM) in Tris-HCl buffer (20 mM, pH 8.0), then incubated in a water bath for temperature control, and stirred with a magnetic stirrer. Aliquots (2 mL) were removed at regular intervals (up to 70 min) and centrifuged for 1 minute, the supernatant was then used for absorbance determinations at 412 nm and then resuspended with the precipitate, and finally the entire mixture was then immediately transferred back to the reactor. The extinction coefficients for *p*-nitroaniline were determined in the aqueous−dioxane mixtures and were then used to convert observed absorbances to molar concentrations.

The activity of thermolysin in aqueous solution was determined by following the hydrolysis of a dipeptide substrate, 3-(2-furylacryloyl)-glycyl-leucine amide (FAGLA), spectrophotometrically at 345 nm.[16] For native and PEG-modified thermolysins, enzyme solution (20 μL) was added to 2 mL of 20 mM Tris-HCl buffer (pH 7, with 5 mM $CaCl_2$) containing substrate varying in concentration between 0.5 and 10 mM. To determine the activity of the thermolysin-containing biopolymer at different reaction temperatures, 1 mg of biopolymer was added to 10 mL of the same buffer containing 1 mM substrate at a fixed temperature changing between 20 and 90 °C.

Flow Cell Reactor. A flow cell apparatus[3] was designed and constructed for testing the activity and stability of the subtilisin-containing biopolymer as well as those of the native and immobilized subtilisin. *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide (0.1 mM) in 20 mM phosphate buffer (pH 7.0) was incubated in ice in order to minimize the auto-hydrolysis of the substrate. Biopolymer (50 mg) was enclosed inside a dialysis bag (approximate volume 0.5 mL), which was then placed within the 5 mL reactor, the flow rate of the substrate solution was set to 0.22 mL/min, and the absorbance at 412 nm was followed continuously with a spectrophotometer.

Stability in Organic Solvents. Native subtilisin (2 mg), biopolymer (50 mg), or immobilized subtilisin (250 mg) was added to 2 mL of hexane containing *N*-acetyl phenylalanyl ethyl ester (30 mM) and methanol (1.2 M). Enzyme activity was determined, during incubation of the reaction mixture at 30 °C and 300 rpm, using gas chromatography as described previously.[7] After incubation for 1 h (the reaction was still in the initial rate phase) the enzyme was recovered by centrifugation, and the supernatant was replaced with methanol. This procedure was repeated three times in order to remove any residual substrate, and after the final resuspension the enzyme was dried in air prior to the addition of fresh substrate in hexane. Complete cycles were performed nine times for each enzyme sample.

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