Siloxane-Based Biocatalytic Films and Paints for Use as Reactive Coatings

Young Duk Kim,[1] Jonathan S. Dordick,[2] Douglas S. Clark[1]

[1]Department of Chemical Engineering, University of California, Berkeley, California 94720, USA; telephone: 510-642-2408; fax: 510-643-1228; e-mail: clark@cchem.berkeley.edu
[2]Department of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA

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Abstract: We have developed a new methodology for preparing films and paints suitable for use as biocatalytic coatings. The hydrolytic enzymes pronase and α-chymotrypsin were immobilized by either sol-gel entrapment or by covalent attachment into a polydimethylsiloxane (PDMS) matrix and cast into thin films or incorporated into an oil-based paint formulation. All of the coatings retained enzymatic activity and adhered to several different materials. The enzymatic films and paints also exhibited higher thermostability than enzyme free in solution or covalently attached to the outer surface of PDMS. A porous membrane based on a PDMS-immobilized enzyme was also prepared by an immersion precipitation process. Protein adsorption measurements showed that the enzyme-containing films and paints adsorbed less protein than enzyme-free controls, and that protein adsorption decreased with increasing proteolytic activity of the coating. These coatings thus provide the means to apply a stable enzymatic surface to a wide range of materials, and may be generally useful as biocatalytic paints and films. *Biotechnol Bioeng* **72**: 475–482, 2001
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INTRODUCTION

Silicone materials have mechanical and physicochemical properties intermediate to those of glass and organic resins. Distinctive characteristics of silicone materials include good thermal and oxidative stability, low-temperature flexibility, inertness, and "nonstick" surfaces. The latter property is particularly important for fouling-resistant coatings. In this connection, polydimethylsiloxane (PDMS), a commercially important linear silicone polymer with excellent nonstick properties, is being considered as a nontoxic antifouling coating for ship hulls to replace current antifouling paints containing toxic biocides (Vincent and Bausch, 1997; Wattermann et al., 1997). The unique structure of PDMS imparts a low surface energy due to tightly packed methyl groups, providing a low glass transition temperature (T_g) (Owen, 1988), which may be related to the ability to deter fouling by proteins and microorganisms.

The inertness and high stability of silicone materials have also been exploited for enzyme immobilization (Gill and Ballesteros, 2000; Gill et al., 1999; Shtelzer and Braun, 1994; Venon and Gudipati, 1995; Wu et al., 1994). Enzyme immobilization generally relies on the sol-gel approach to generate silica matrices by acid- or base-catalyzed hydrolysis of hydrolyzable silane compounds such as tetramethylorthosilicate (TMOS) (Avnir et al., 1994; Dave et al., 1994). Whereas high temperatures are required to prepare glass by the traditional melting of silica, sol-gel processes involve low-temperature hydrolysis of monomeric precursors, and are thus highly suitable for the microencapsulation of fragile biomolecules. The application of PDMS in enzyme immobilization has been limited, however, by the extremely hydrophobic nature of PDMS. To date, relatively low-molecular-weight PDMS (MW <4200) has been used as a minor component for sol-gel encapsulation in aqueous media, with the final product being immobilized-enzyme powders and hydrogels (Gill and Ballesteros, 1998; Reetz et al., 1996). If, on the other hand, PDMS could be incorporated as a major component and polymerized, a flexible immobilized enzyme film should result, stemming from the low glass transition temperature of the polymer.

In the present work, we describe a new method for incorporating enzymes into PDMS films under nonaqueous conditions, and report the formulation of a siloxane-based biocatalytic paint. Specifically, α-chymotrypsin (α-CT) and pronase were used to prepare highly stable enzyme-containing PDMS films and paints suitable for coating a variety of surfaces (e.g., metals and plastics). Moreover, the hydrolytic activity of the film had the effect of reducing protein adsorption below the relatively low level observed with unmodified PDMS. Biocatalytic PDMS films could also be cast into porous membranes at room temperature, demonstrating their potential for the preparation of biocatalytic filters. Thus, these formulations warrant further consideration as candidates for environmentally benign coat-

Correspondence to: D. S. Clark
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ings, resins, and paints with biocatalytic and antifouling properties.

MATERIALS AND METHODS

Materials

α-Chymotrypsin (α-CT; from bovine pancreas), pronase (from *Streptomyces griseus*), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (suc-AAPF-*p*Na), L-leucine-*p*-nitroanilide (L*p*Na), *N*-acetyl-L-phenylalanine ethyl ester (APEE), 4-methylumbelliferyl-*p*-tri-methylammonium cinnamate chloride (MUTMAC), bovine serum albumin, polyvinylpyrrolidone (PVP; MW = 40,000), and azocasein were obtained from Sigma Chemical Co. (St. Louis, MO). Dimethyldimethoxysilane was obtained from Fluka (Milwaukee, WI) and poly(dimethylsiloxane-*block*-ethylene oxide) was obtained from Gelest, Inc. (Tullytown, PA). Tetramethyl orthosilicate (TMOS), methytrimethoxysilane (MTrMOS), tetraethyl orthosilicate (TEOS), 3-aminopropyltriethoxysilane (APTS), polydimethylsiloxane (PDMS), polysulfone, glutaraldehyde, chlorinated rubber, iron acetylacetonate, rosin, and dibutyltin dilaureate were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification.

Preparation of Biocatalytic PDMS Films

Sol-Gel Entrapment (Method 1)

Homogeneous sol was typically prepared by sonicating 1.46 g of TMOS, 1.52 g of MTrMOS, 0.4 g of distilled water, and 30 μL of 40 m*M* HCl for 10 min in a sonication bath containing a mixture of ice and water. To the freshly prepared sol was added 6.5 mL of enzyme solution containing 300 mg of enzyme in 100 m*M* phosphate buffer (pH 7.5). The resulting hydrogel was dried under vacuum for 24 h and crushed in a mortar to a fine powder of immobilized enzyme. The typical procedure for film formation was as follows: 1 mL of the aforementioned sol mixture containing enzyme was emulsified with 3 mL of 45% (w/w) PDMS solution in tetrahydrofuran (THF) by adding poly(dimethylsiloxane-*block*-ethylene oxide). The resulting mixture was stirred with 2 mg of dibutyltin dilaureate for 3 min and spread over the inner surface of a polypropylene plate, after which it was incubated at room temperature for at least 24 h to allow film formation by polymerization of the PDMS. The final film thickness was ca. 50 μm for all samples, as determined by the use of calipers.

Covalent Attachment (Method 2)

Ten milliliters of 1.25 wt% glutaraldehyde solution in 0.4 *M* phosphate buffer (pH 7.5) was added dropwise over a period of 15 min to 20 mL phosphate buffer containing 312 mg of APTS and 300 mg of enzyme. The resultant mixture was dialyzed for 24 h and freeze-dried for 48 h to obtain a lyophilized powder. To increase the activity of the immobilized CT PDMS film, polyvinylpyrrolidone (PVP) was added to the aqueous mixture before freeze-drying, yielding a final dry preparation of 79% (w/w) PVP. The film-formation procedure was the same as that of the sol-gel entrapment method.

Surface Attachment (Method 3)

PDMS films having no immobilized enzyme were prepared first from a mixture of 0.21 g of TMOS, 2.47 g of PDMS, and 12 mg of dibutyltin dilaureate. The PDMS film was then partially hydrolyzed in 3 *M* HCl for 30 min to provide silanol functional groups on the film surface, rinsed with deionized water, and dried under vacuum for 12 h. The PDMS films were then treated with 2% 3-aminopropyltriethoxysilane in acetone followed by reaction with 5% glutaraldehyde in 0.4 *M* phosphate buffer (pH 7.5) for 30 min. Enzyme solution (25 U/mL for CT or 4.4 U/mL for pronase) was added to the glutaraldehyde-treated films and incubated for 1 h at 4°C.

Membrane Formation

A homogeneous reaction mixture was prepared by sonicating and vortexing 12 mg of immobilized pronase powder (method 2), 0.02 g of TMOS, 0.2 g of PDMS, 4 mL of THF, and 20 mg of dibutyltin dilaureate. The mixture was stirred for 30 min to promote polymerization of PDMS, after which 0.5 g of polysulfone and 1 mL of *N*-methylpyrrolidone were added to prepare a casting solution. The casting solution was spread onto a glass plate and immersed into a water bath containing 10% (v/v) THF. This process induced a liquid–liquid phase separation of the casting solution, resulting in the formation of a porous membrane.

Formulation of Biocatalytic Paint

The following components were combined and mixed with a mechanical stirrer for 2 h at room temperature: 0.5 g of PDMS; 0.5 g of chlorinated rubber; 50 μL of dimethyldimethoxysilane; 20 mg of powdered silane-functionalized α-CT (by Method 2); 50 μL of poly(dimethylsiloxane-*block*-ethylene oxide); 38 mg of iron acetylacetonate dissolved in 0.3 mL of THF; 0.5 g of rosin; and 1.5 mL of xylene. Using iron acetylacetonate as the polymerization catalyst produced a red-orange paint; alternatively, replacing iron acetylacetonate with the more toxic dibutyltin dilaureate, with and without cuprous oxide, produced green and pale yellow paints, respectively. Protein adsorption was

measured using poly(ethylene terephthate) films (6 cm × 6 cm) painted on both sides with the biocatalytic paints.

Active-Site Titration

The concentration of active α-CT was determined by active-site titration with MUTMAC. Fluorescence measurements were carried out in 1-cm cuvettes in a FluoroMax-2 spectrofluorimeter (Jobin Yvon-Spex Instruments, Edison, NJ). The samples were excited at 356 nm, and emission was followed at 451 nm. A baseline was established by adding 250 μL of 0.2 m*M* MUTMAC solution in water to 3.0 mL of potassium phosphate buffer (pH 7.3, 1 *M* NaCl). For the α-CT-containing PDMS films and paint, 1-cm^2 sections were inserted into the cuvette and stirred for 30 s, at which point the fluorescence of liberated 4-methylumbelliferone was measured, corresponding to the concentration of functional active sites.

Enzyme Activity Assays

Assays of pronase and α-CT were performed using L*p*Na and suc-AAPF-*p*Na as substrates, respectively, in Tris-HCl buffer (0.1 *M*, pH 7.8) at 25°C. The substrate concentrations ranged from 0.25 to 4.0 m*M* for L*p*Na and from 0.125 to 2.0 m*M* for suc-AAPF-*p*Na. The product of each hydrolysis reaction, *p*-nitroanilide, was measured spectrophotometrically at 405 nm using a Beckman DU-6 UV-Vis spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). Film and paint samples were assayed by placing a small (ca. 0.5-cm^2) patch in the cuvette, which settled to the bottom, out of the light path. The kinetic parameters V_{max} and K_m were obtained by fitting initial rate data to the Michaelis–Menten equation through Eadie–Hofstee plots. All plots were linear, indicating the absence of diffusional limitations (Blanch and Clark, 1995), except for the case of pronase immobilized by method 1, which showed slight curvature. From the *y*-intercept of the Eadie–Hofstee plot, the Thiele modulus, ϕ, was determined to be ca. 1.5 (Blanch and Clark). However, the reported V_{max}/K_m value was determined from the linear portion of the plot and therefore reflects the intrinsic kinetics. The intrinsic catalytic efficiencies (k_{cat}/K_m) were obtained by normalizing V_{max}/K_m by the concentration of active enzyme determined in active-site titration measurements. The reproducibility of kinetic parameters was verified from two independent sets of rate data. Based on the uncertainty of the least-squares fitting, the individual kinetic parameters had relative errors ranging from 0.25% to 13.6%, with the majority being less than 10%.

Thermal Stability

Samples were immersed in Tris-HCl buffer (pH 7.8) and incubated in a shaker at 75°C (50°C for α-CT). Periodically, samples were removed from the incubation solution, rinsed with buffer at room temperature, and assayed for residual activity as described previously. Remaining activity was determined by collecting initial rate data at 25°C using a substrate concentration of 0.5 m*M*.

Protein Adsorption

Bovine serum albumin (BSA) was chosen as a model protein for protein adsorption studies. Experiments were carried out by immersing a sample film of 6 cm^2 in a propylene centrifuge tube containing 2 mL of 20 μg/mL protein solution in 50 m*M* phosphate buffer (pH 7.0). Tubes were shaken at 30°C for 24 h at 250 rpm. The film was then removed from the tubes, and the remaining protein solution was vortexed. The protein concentration remaining in solution was determined according to the amino acid analysis procedure described by Sears and Clark (1993).

RESULTS AND DISCUSSION

Preparation of Immobilized Enzyme PDMS Films

Three different methods were used to immobilize CT and pronase in a PDMS matrix. Figure 1 summarizes the preparation of immobilized enzyme PDMS films by methods 1 and 2. Sol-gel entrapment, the first step of method 1, is a convenient way to synthesize a host matrix and entrap enzymes or other proteins while retaining their functional characteristics (Braun et al., 1990; Ellerby et al., 1992). To this end, enzymes were entrapped in sol-gel particles, the particles dispersed in a solution of PDMS in THF, and the PDMS cured via a room-temperature vulcanizing process that involved condensing silanol-terminated PDMS with the silane crosslinker TMOS, catalyzed by dibutyltin dilaureate.

The covalent attachment approach (method 2) produced a material in which the chemically modified enzyme is covalently bound to the PDMS matrix. This method requires the initial chemical treatment of enzyme with 3-aminopropyltriethoxysilane (APTS) to provide a chemical functionality reactive with PDMS. The modified enzyme was then incorporated into polydimethylsiloxane by a condensation reaction between silanol end groups in PDMS and ethoxy groups in APTS. Figure 1 shows that both methods 1 and 2 produced homogeneous, immobilized enzyme films. Both preparations were suitable for coating various materials following pretreatment of the surface with epoxy adhesive (e.g., steel, neoprene, and PVC), as shown in Figure 2. A third method involving direct surface attachment (method 3) is a more conventional way to immobilize enzymes onto a silica support, and was employed for the sake of comparison. In this case, the surface of a pre-made PDMS film was activated with 3 *M* HCl and derivatized with APTS, followed by covalent attachment of enzyme using glutaraldehyde. Thus, this method requires that film formation precedes enzyme attachment, and does not produce a biocatalytic composite suitable for coating different surfaces.

Methods 1 and 2 are also suitable for preparing porous immobilized enzyme membranes. To demonstrate this con-

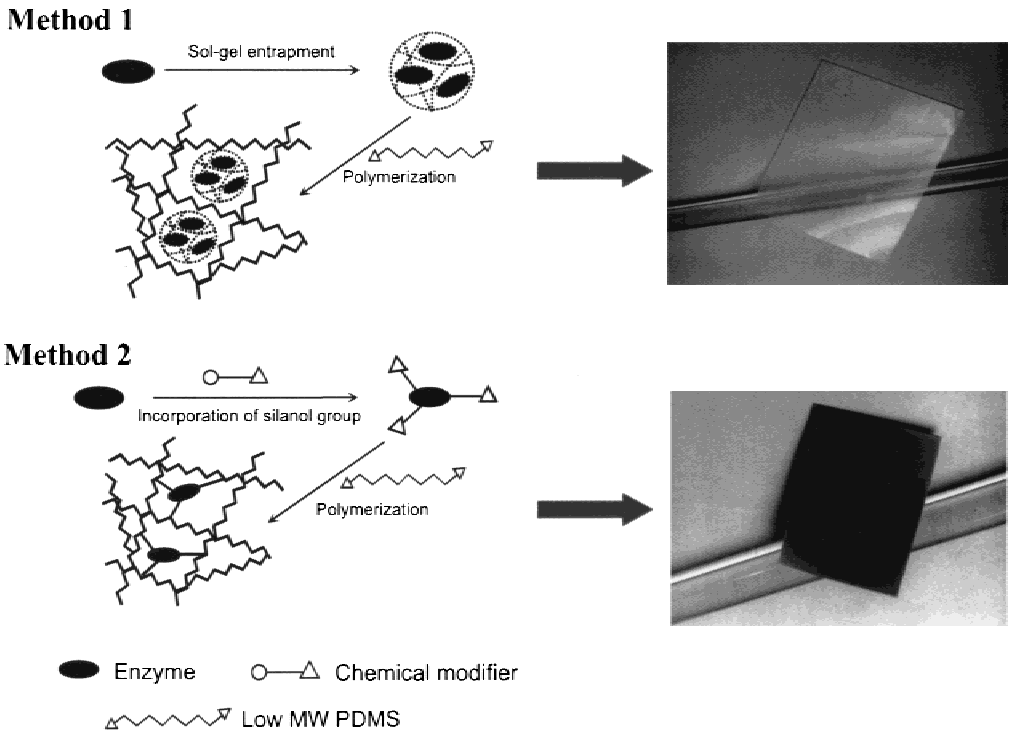

Figure 1. Incorporation of enzyme into polydimethylsiloxane polymers via sol-gel entrapment (method 1) and covalent attachment (method 2).

cept, an immersion precipitation process (Kim et al., 1999a, 1999b; Mulder, 1991; Vadalia et al., 1994) was employed to prepare membranes from immobilized enzyme PDMS. In this process, a homogeneous PDMS solution in THF is contacted with water (the nonsolvent), and subsequent exchange of THF and water across the interface effects liquid–liquid phase separation resulting in the porous membrane. To prevent the pore structure from collapsing during drying, polysulfone was added to the PDMS as a reinforcing agent. Figure 2a shows the porous structure of a membrane prepared with immobilized pronase PDMS and polysulfone. The pore size can be controlled by adjusting process parameters such as polymer concentration, molecular weight of the PDMS, and the choice of solvent/nonsolvent pair (Reuveres and Smolders, 1987; Reuvers et al., 1987).

Preparation of Biocatalytic Paint

A PDMS-based enzyme-containing paint was formulated to demonstrate the feasibility of a biocatalytic paint with potential for application as a fouling-resistant coating. Conventional antifouling paints contain toxic agents such as cuprous oxide or tributyltin, which are widely used as biocides. In lieu of such compounds, our formulation contained silanol-functionalized enzyme (method 2) for biocatalytic activity. The PDMS was polymerized with iron acetylacetonate, which acted as both a polymerization catalyst and red-orange pigment (Fig. 2d). Once air-dried, the painted surface was smooth and firm to the touch. Because the enzyme was covalently attached to the PDMS, there was no apparent release of enzyme from the final formulation (data not shown).

Active-Site Concentration and Catalytic Properties

Active-site titration in aqueous buffer, performed with the fluorogenic α-CT substrate MUTMAC, revealed that the percentage of active and accessible immobilized α-CT ranged from 1.8% of the immobilized enzyme for the paint to 13% for method 2 (Table I). The higher active enzyme content obtained in method 2 suggests that the former contains a more porous network than the latter. Indeed, it is expected that a paint would consist of a dense packing of polymer chains, thereby resulting in reduced enzyme accessibility. In both cases, samples were also prepared using polyvinylpyrrolidone (PVP) as a lyoprotectant during freeze drying, which resulted in much more active formulations. Polyethylene-glycol, KCl, and soybean trypsin inhibitor were also used as possible lyoprotectants but none of these produced significant activation (data not shown).

The α-CT preparations were assayed in aqueous buffer for the hydrolysis of the chromogenic substrate suc-AAPF-*p*Na. In general, K_m increased upon immobilization and k_{cat} decreased. Thus, the catalytic efficiency, k_{cat}/K_m, decreased upon immobilization, with method 1 yielding the highest value. The exact cause(s) of the substantial reduction in k_{cat}/K_m remain to be determined; however, possibilities include conformational changes to the enzyme induced by local crowding of enzyme molecules and/or unfavorable

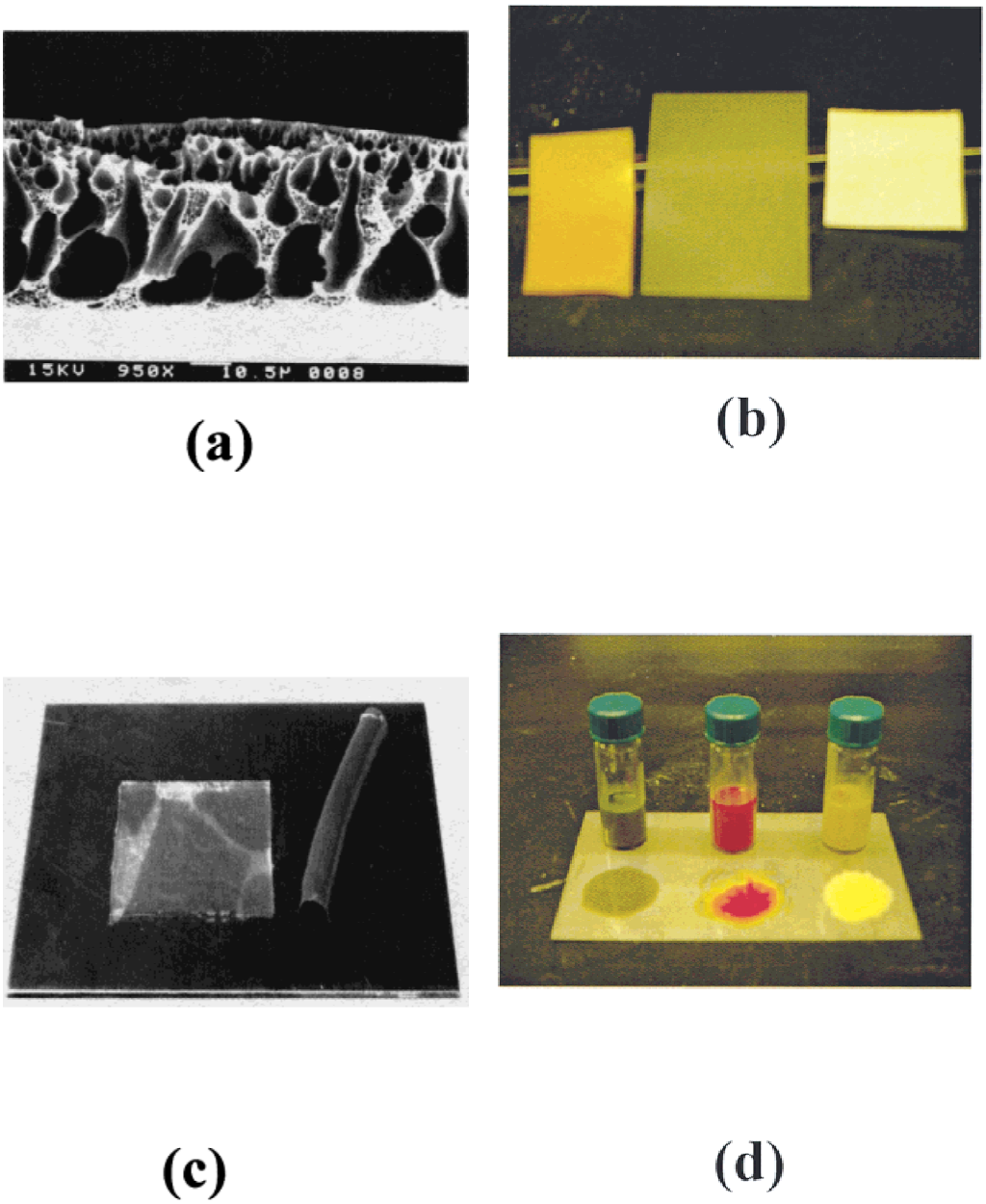

Figure 2. Various formulations of biocatalytic films and paints. (a) Scanning electron micrograph of pronase-containing PDMS membrane prepared using the immersion precipitation process (cross-section, 950-fold magnification). (b) Pronase-containing PDMS films (from left: method 2, method 1, and porous membrane). (c) α-CT-containing PDMS films coated onto a steel plate and a neoprene tube. (d) PDMS-based biocatalytic paints, prepared with (from left) cuprous oxide and dibutyltin dilaureate, iron acetylacetonate, and dibutyltin dilaureate.

interactions between the enzyme and the surrounding hydrophobic matrix, and, in the case of method 2, partial denaturation resulting from the formation of covalent bonds between the enzyme and PDMS during polymerization.

Pronase, an unusually nonspecific protease, was also formulated into paint and films. Pronase films and paint were all active for the hydrolysis of L*p*Na (Table II), as reflected by the values of V_{max}/K_m for the immobilized enzyme (k_{cat}/K_m could not be calculated because no active-site titrating agent is available for pronase). Interestingly, method 2 yielded the higher value of V_{max}/K_m for immobilized pronase, in strong contrast to the results for k_{cat}/K_m of immobilized α-CT. Moreover, including PVP during the freeze-drying step had no effect on the activity of pronase films prepared by method 2 (data not shown). The pronase-containing paint had activity comparable to that of the films. In addition, the membrane prepared by immersion precipitation showed considerable activity, illustrating that biocatalytic membranes can be prepared at room temperature using the appropriate solvent/nonsolvent system.

Thermal Stability of Films and Paints

Figure 3 shows the thermal stability of the biocatalytic PDMS films and paints determined by the initial reaction

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