Preparation of Enzyme-In-Polymer Composites with High Activity and Stability

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Flash devolatilization was applied to incorporate an enzyme into a polymer matrix, which created a novel and highly active biocatalytic composite suitable for use in both aqueous and organic media. Enzymes were codissolved in toluene with commercial, high-molecular-weight polymers, and the solvent was then rapidly removed by injecting the mixture into a vacuum chamber. Subsequent cross-linking of the enzyme with glutaraldehyde resulted in stable entrapped enzyme within the polymeric matrices. For example, a composite of α-chymotrypsin and low-density polyethylene showed no significant loss of enzymatic activity in aqueous buffer over a period of one month. The normalized activity of this biocatalytic material in organic solvents was 3–13 times higher than that of native α-chymotrypsin lyophilized from aqueous buffer. Washing the composite material with aqueous buffer increased the activity in isooctane an additional ten fold. The composites of α-chymotrypsin and polyethylene demonstrated the feasibility of obtaining active and stable biocatalytic materials via the application of compositional quenching to a biological system.

Introduction

Enzymes are highly specific catalysts appropriate for both biological and chemical transformations. Many large-scale biocatalytic processes in the pharmaceutical, chemical, and food industries require active and stable enzymes incorporated onto or within a suitable heterogeneous matrix (Blanch and Clark, 1995). A variety of methods are available to incorporate an enzyme into a support matrix, including entrapment, covalent attachment, and adsorption (Wiseman, 1985; Clark, 1994; Blanch and Clark, 1995).

Recently, it has become possible to modify enzymes, via formation of hydrophobic ion-pair complexes so that they become soluble in organic solvents, while maintaining biocatalytic activity (Paradkar and Dordick, 1994). This solubilization enables the incorporation of enzymes into complex material systems (Wang et al., 1997; Novick and Dordick, 1998; Dordick et al., 1998). The present article reports a physical process that begins with the codissolution of the enzyme and ordinary polymers in a relatively dilute (ca. 10%, w/w) and single-phase solution. This solution is then flash devolatilized, and an enzyme-in-polymer microdispersion is created by the process of compositional quenching (Nauman et al., 1986, 1988). The composite is subsequently stabilized by treatment with glutaraldehyde.

The physical entrapment of α-chymotrypsin (CT) into commercial, high molecular-weight polymers is described in the present work. Polyethylene and polystyrene were used to trap CT within a porous matrix, permitting diffusion of substrates and products to and from the enzymes.

Materials and Methods

Materials

α-Chymotrypsin, glutaraldehyde, aerosol OT (dioctyl sodium sulfosuccinate), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, *N*-acetyl-Phe ethyl ester (APEE), and *o*-phthaldialdehyde reagent (OPA) were purchased from Sigma Chemical (St. Louis, MO). Polyethylene and polystyrene were donated from BP Chemicals (Warrensville Heights, OH) and Novacor (Calgary, Alberta), respectively. All other reagents and solvents were purchased from Aldrich (Milwaukee, WI) and were of the highest grade commercially available. The solvents were dried over 3-Å molecular sieves for 24 h prior

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to use, and the water content was less than 0.005% (v/v), as determined by Karl-Fischer titration.

Solubilization of α-chymotrypsin in organic solvents

Ten milliliters of an aqueous enzyme solution [containing 1 mg/mL CT, 1% (v/v) isopropanol, and 2 mM $CaCl_2$ dissolved in 10 mM bis-tris buffer, pH 7.8] were contacted with an equal volume of hexane containing 2 mM AOT. The two-phase mixture was stirred vigorously at 22°C and 300 rpm for 5 min, and then centrifuged at 7000 G for 10 min. Upon separation of the organic phase from the aqueous solution, the resulting enzyme–surfactant ion-paired complex was dried by evaporating hexane under vacuum, and then reconstituted into the organic solvent of choice. The concentration of protein in the organic phase was determined by UV absorbance at 280 nm, and the yield of extraction was greater than 90% (Paradkar and Dordick, 1994).

Compositional quenching

Polyethylene was dissolved in toluene at 85°C, while polystyrene could be dissolved at room temperature. When dissolution was complete, soluble CT was added and mixed only long enough to ensure homogeneity. For sample sizes of approximately 3 mL, the total mixing time was about 45 s. The mixture was then flashed adiabatically into a vacuum chamber. The product was a 3-mm-diam. porous string of polyethylene (or polystyrene) with an expected enzyme loading of 4% (w/w) CT. The strings were dried in a vacuum at room temperature for 24 h (Nauman et al., 1986).

Glutaraldehyde treatment

To cross-link the entrapped enzyme and prevent its diffusion out of the polymer matrix, the compositionally quenched composites were treated with glutaraldehyde (30 mM and 50 mM) for different lengths of time (20, 60 and 120 min). After glutaraldehyde treatment, the materials were washed excessively with buffer (10 mM phosphate, pH 7.8) and distilled water, and dried under vacuum at room temperature for 24 h.

Activity and stability of polymer-entrapped α-chymotrypsin

The activity and stability of the CT–polymer composites were determined in aqueous buffer (10 mM phosphate, pH 7.8) and several organic solvents [isooctane, *tert*-amyl alcohol, acetone, and acetone supplemented with 1% (v/v) water]. The activity was measured via the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in aqueous solution (Kim and Dordick, 1997), and via the transesterification of 10 mM APEE with 1 M *n*-propanol in organic solvents (Kim et al., 2000). The activity of the biocatalytic composites was measured as a function of time to determine stability.

Determination of α-chymotrypsin loading

The amount of entrapped CT was determined using the OPA (*o*-phthaldialdehyde) reagent after complete acid-hydrolysis of CT (Sears and Clark, 1993). CT–polymer composite (1 mg) was incubated in 1 mL of 6 N HCl solution at 105°C for three days. To determine the amino group concentration, the solution was neutralized by adding 1 mL of 6 N NaOH solution. Then, 0.75 mL of neutralized sample was mixed with 1.5 mL of 0.1 mg/mL OPA in 50 mM sodium borate buffer (pH 9.5). After 30 s, the fluorescence emission at 445 nm (ex 347 nm) was measured, and the concentration of CT was calculated by using a standard curve prepared with free CT. All the experiments were performed together with blank samples (polymers without CT), and the results were obtained in triplicate after subtraction of the fluorescence emission of the blanks. The amount of CT that leached out from the polymeric matrices was measured in a similar manner.

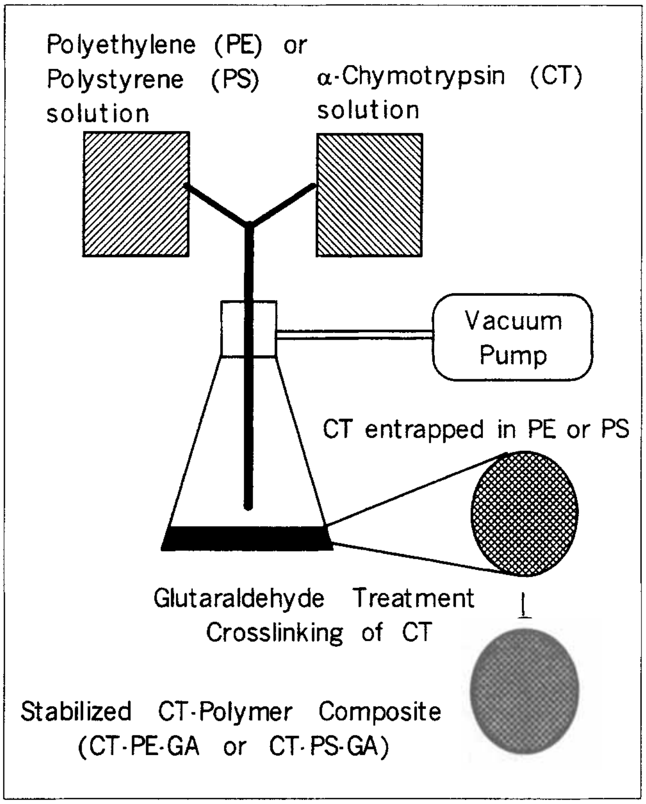

Figure 1. Preparation of enzyme-polymer composites.

Results and Discussion

The flash devolatilization process is shown in Figure 1. The first step was dissolution of the polymers in toluene to form 10% (w/w) solutions. The polyethylene (PE) was dissolved at 85°C, while the polystyrene (PS) was dissolved at room temperature. These polymer solutions were then mixed with solubilized CT and flash devolatilized. The mixing was done rapidly (approximately 45 s) by magnetic stirring to minimize exposure of the enzyme to the high temperature (85°C) needed to keep the polyethylene in solution.

The activity of the enzyme–polymer composites decreased very rapidly in buffer (10 mM phosphate, pH 7.8), especially at early incubation times (Figure 2), due to CT leaching from the polymer matrix. The protein content and activity in the

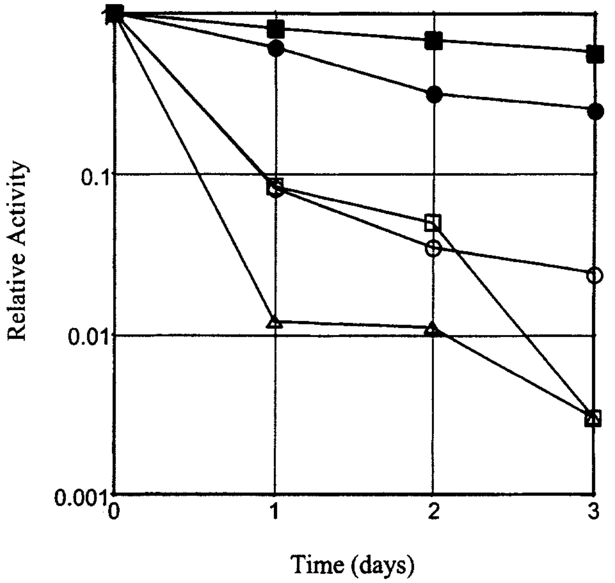

Figure 2. Stability of vacuum-sprayed α-chymotrypsin.

Samples (1–2 mg) were incubated in 1 mL of aqueous buffer solution (10 mM phosphate buffer, pH 7.8) at 30°C and 250 rpm. The residual activity at each time point was measured via the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, after washing samples with aqueous buffer solution, and normalized to the relative activity representing the ratio to the initial activity. Polyethylene (low density, ○; high density, △) and polystyrene (□) were used as polymers, and closed symbols represent samples treated with 30 mM glutaraldehyde for 20 min.

Table 1. Optimization of Glutaraldehyde Treatment

Polymer	Glutaraldehyde (mM)	Time for Treatment (min)	Residual Activity* After 7 d μmol/ $g_{\text{composite}} \cdot h$	Loss of Activity** after 7 d μmol/ $g_{\text{composite}} \cdot h$
Polyethylene (low density)	30	20	2.88	30.16
	50	20	10.68	5.53
	50	60	18.48	0.97
	50	120	16.08	0.50

*Residual activity was measured by the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in 10 mM phosphate buffer (pH 7.8) after excessive washing.

**Loss of activity is the difference between an initial activity ($t = 0$) and residual activity at $t = 7$ days.

washing buffer was consistent with the activity loss in the composite. The rapid CT leaching from the untreated composite was surprising. Although solubilized in organic solvents, the CT is almost certain to be incompatible with the polyethylene matrix. Compositional quenching is expected to produce a two-phase composite, with CT as the dispersed phase. Polystyrene is used below its glass transition temperature and polyethylene is semicrystalline. The low diffusivities expected in these matrices should prevent CT leaching. However, the flash devolatilization product is highly porous and exposes the CT phase domains to the washing buffer, resulting in rapid CT leaching.

To prevent the loss of enzyme, the flash devolatilized samples were treated with 30 mM glutaraldehyde for 20 min. Glutaraldehyde reacts with amino groups on proteins, leading to intermolecular cross-linking and larger protein aggregates (Solomon and Levin, 1974). These larger protein aggregates would be less likely to diffuse out of the polymer matrix. As shown in Figure 2, the glutaraldehyde treatment stabilized the CT–polymer composites, yielding 18- and 51-fold higher activity than nontreated samples for PE and PS, respectively, after incubation in aqueous buffer for 3 days.

The glutaraldehyde treatment was optimized to achieve even better stability. The amount of glutaraldehyde was varied from 30 mM to 50 mM, and reaction times were varied from 20 min to 120 min. As shown in Table 1, optimal treatment was 60-min reaction with 50 mM glutaraldehyde. Indeed, after 7 days, less than 5% of the CT that was loaded initially had leached out of the CT-polyethylene composite. The different initial activities (sum of the two righthand values in Table 1) were due to loss of enzyme activity during the glutaraldehyde treatment and subsequent wash. Clearly, the first entry, with substandard glutaraldehyde cross-linking conditions, led to substantial loss of the enzyme from the composite, yet with increased glutaraldehyde concentration and incubation times, less enzyme is leached from the composite.

The actual loading of CT in the CT–polyethylene composite was determined by the OPA method after acid-hydrolysis of CT. Just after flash devolatilization, and with no glutaraldehyde treatment, the CT loading was 3.96 ± 0.04% (w/w), which is consistent with the theoretical loading of 4% (w/w) CT to polyethylene. After the glutaraldehyde treatment (50 mM GA for 60 min) and excessive washing, about 50% of CT remained, and the measured CT loading decreased to 1.96 ± 0.11% (w/w). This value was used to calculate the normalized initial rate per enzyme loading. After incubation in aqueous buffer for 7 days, the measured CT loading was 1.88 ± 0.04% (w/w), consistent with the observed loss of about 5% in enzyme activity after 7 days (Table 1). This suggests that glutaraldehyde treatment stabilizes the composite very efficiently.

Figure 3 shows the operational stability of free CT, CT–PE composite, and the GA-treated sample (50 mM GA for 60 min) in aqueous buffer (10 mM phosphate, pH 7.8). The activity of free CT decreased rapidly, presumably due to autolysis (Kawamura et al., 1981); the CT–PE composite also showed a decrease of activity due to the leading of CT from PE into the aqueous solution. On the other hand, the GA-treated composite was stabilized so that no significant loss of enzyme activity occurred over a period of one month.

Table 2 shows the reactivity of the treated composite in organic solvents. The activity of the GA-treated composite (CT–PE–GA) was measured by the transesterification of 10 mM APEE with 1 M *n*-propanol in isooctane, *tert*-amyl alcohol, acetone, and acetone supplemented with 1% (v/v) water. For comparison, the activity of lyophilized α-chymotrypsin was measured in the more conventional lyophilized suspension (Kim et al., 2000). The rate of transesterification was normalized based on the measured loading of CT, and the normalized rate of CT–PE–GA showed 5.0-, 3.3-, and 13-fold higher activity than lyophilized CT in isooctane, acetone, and acetone supplemented with 1% (v/v) water, respectively. One striking discovery is that the activity of CT–PE–GA was increased by about 10 times in isooctane via washing with buffer

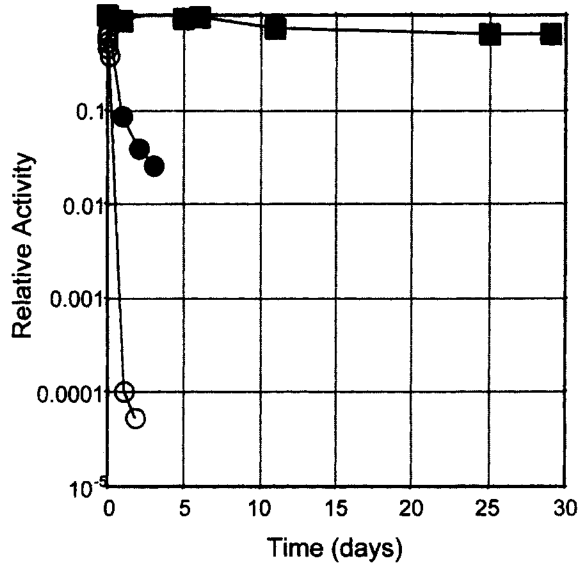

Figure 3. Stabilizing effect of glutaraldehyde treatment after vacuum spraying.

α-Chymotrypsin was vacuum-sprayed together with low-density polyethylene in toluene (●), and treated by 50 mM glutaraldehyde for 60 min. (■). Open circles represent the stability of free α-chymotrypsin in aqueous buffer (10 mM phosphate, pH 7.8).

solution just before initial-rate measurement. This increase of CT activity is most likely due to prehydration of CT within the composite material. It should be noted that such a prehydration is not feasible with lyophilized CT powder, as it would dissolve in the aqueous wash. The stability of CT–PE–GA, either unwashed or washed, was also evaluated. Only a small decrease in CT activity was observed in isooctane after a seven day incubation (Table 2).

Table 2. Activity of Vacuum-Sprayed α-Chymotrypsin and Polyethylene in Organic Solvents[*]

	Solvents	Activity[**] μmol/ $g_{\mathrm{composite}} \cdot h$	Rate/CT[†] μmol/ $g_{\mathrm{CT}} \cdot h$
Vacuum sprayed α-chymotrypsin (CT–PE–GA)	Isooctane	7.53	384
	tert-Amyl alcohol	0.26	13
	Acetone	0.02	0.93
	Acetone + 1% (v/v) water	6.59	336
	Isooctane (buffer washing)[‡‡]	74	3776
After 7-d incubation	Isooctane	6.42	341
	Isooctane (buffer washing)[††]	70	3723
Free α-chymotrypsin (suspended)[‡]	Isooctane	49	77
	Acetone	0.18	0.28
	Acetone + 1% (v/v) water	16	25

[*] Activity was measured by the transesterification of 10 mM APEE with 1 M *n*-PrOH. The vacuum sprayed α-chymotrypsin was treated by 50 mM glutaraldehyde for 60 min and excessively washed by buffer and distilled water, and dried under vacuum.

[**] Activity represents transesterification rate per gram of catalysts.

[†] Rate per CT was calculated based on the actual loading of CT that was determined by the OPA method after excessive acid hydrolysis. The values of CT loading were 1.99% (w/w) and 1.88% (w/w) at $t = 0$ and 7 days, respectively.

[††] The composite was washed with buffer solution several times just before initial rate measurement.

[‡] α-Chymotrypsin (5 mg/mL) was lyophilized from 20 mM phosphate buffer (pH 7.8), and the weight percentage of CT is 64% (w/w) in the presence of buffer salts.

Conclusions

We have made novel enzyme-in-polymer composites from ordinary, commercial polymers and enzymes that have been made soluble in organic solvents. These biocatalytic materials were stable in aqueous buffer, and had high activity and stability in organic solvents. Brief exposure of the enzyme to 85°C did not cause a significant loss in activity. Retention of CT in the entrapped form within the polymer matrix was optimized by glutaraldehyde cross-linking. It may, however, also be possible to avoid this step and to avoid the loss of approximately half of the starting enzyme by selecting a different matrix polymer. The compositional quenching process is simple and takes only minutes to perform. The key requirement is solubilization of the protein in a suitable organic solvent. To date, we and others have demonstrated that a wide variety of enzymes can be dissolved in suitable organic media (Wang et al., 1997; Novick and Dordick, 2000; Mori and Okahata, 1997). Therefore, the approach described herein should be applicable to a wide range of enzymes and polymers to yield diverse biocatalytic materials with synthetic utility.

Acknowledgment

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