Preparation of Active and Stable Biocatalytic Hydrogels for Use in Selective Transformations

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Enzymes can be entrapped or covalently incorporated into polymeric gels for use as selective catalysts for biotransformations,[1] functional components of biosensors,[2] and in therapeutic bioreactors (e.g., through hollow-fiber geometries).[3] In many cases, water-soluble vinyl monomers are employed including (meth)acrylic acid, acrylamide, and hydroxyethyl methacrylate, and polymerization is initiated chemically or by irradiation.[4] The resulting polymer chains that comprise the gel are either charged or amphiphilic and, therefore, have the potential to bind proteins or other biological macromolecules nonselectively.[5] As opposed to synthetic materials, natural neutral polysaccharides are highly hydrophilic and less sensitive to nonselective protein adsorption.[6] In particular, cellulose, dextran, and agarose have been used as matrixes for enzyme immobilization.[7−10] Nevertheless, these matrixes invariably require preactivation (e.g., with CNBr[7]) to allow covalent enzyme immobilization, and immobilization can be highly nonuniform.[7]

In the present study, we have developed a synthetic, nonpolysaccharide, sugar-based hydrogel for enzyme incorporation that has properties similar to that of vinyl polymers, yet with the inertness available from polysaccharides. This material, poly(sucrose acrylate) (PSA), is synthesized in two steps. First, regioselective acylation of sucrose with vinyl acrylate yields sucrose 1′-acrylate, which acts as a highly water-soluble monomer. This is followed by the rapid copolymerization with a suitably modified enzyme to give a homogeneous enzyme−hydrogel composite. PSA has large and controllable pore sizes (up to 1000 Å)[11] together with a high equilibrium water content that mimics a native aqueous environment for incorporated enzymes. Thus, highly active and stable enzymes are expected.

Three enzymes were chosen for this preliminary study: α-chymotrypsin (CT), subtilisin Carlsberg (SC), and horseradish peroxidase (HRP), which, taken in aggregate, represent a wide variety of enzyme mechanisms and substrate specificities. Enzyme was incorporated into the hydrogel according to the scheme depicted in Figure 1A. The biocatalyst was chemically derivatized with acryloyl chloride following a procedure of Martinek et al.[12] which resulted in little loss of native activity.[13] Sucrose 1′-acrylate was synthesized using Optimase M-440 (a commercial subtilisin Carlsberg preparation) suspended in pyridine as described by Patil et al.[11] Enzyme incorporation into the sucrose-based hydrogel was performed by copolymerizing the modified enzyme with sucrose 1′-acrylate in the presence of 1,2-dihydroxyethylene-bis-acrylamide as cross-linker.[14] The enzyme-containing hydrogels were packed into a plastic syringe (0.2 mm tip diameter) and ejected to break the gel into smaller particles with sizes ranging from 0.2 to 0.02 mm, as determined by microscopy (Figure 1B). The hydrogel was washed extensively with deionized water to remove any unbound materials.

The activity of all three enzymes (at a loading of ~0.5%, w/w) in the hydrogel ranged from 9 to 20% of the native enzyme activities in aqueous buffer. This is similar to activities observed with other enzymes incorporated into (or onto) hydrophilic matrixes.[15] Loss of native activity does not appear to be due to limitations of intraparticle diffusion as calculations of the observable modulus indicated that the internal effectiveness factor at this enzyme loading was nearly unity.[16] This

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(13) After modification with acryloyl chloride, unreacted reagents were removed from the enzyme solution via ultrafiltration (Amicon YM-10 filter, 10 000 MWCO, running buffer consisted of 50 mM, pH 7.8 Tris buffer), and the enzyme was then lyophilized. The activity of the modified enzyme was measured in 50 mM pH 7.8 Tris buffer via the hydrolysis of the chromogenic synthetic tetrapeptide, *N*-succ-AAPF-*p*-nitroanilide. The initial rate of formation of free *p*-nitroaniline was measured at 410 nm. Modified CT retained at least 80% of the native enzyme activity.

(14) Sucrose acrylate monomer (940 mg), cross-linker (5 mg), and the modified enzyme (1 mg) were dissolved in 5 mL of 50 mM pH 7.8 Tris buffer. The redox initiators, sodium persulfate and TEMED, were then added at a concentration of 1 wt % each. Polymerization was initiated by aspirating the solution to remove oxygen. Polymerization was carried out over an ice bath to reduce potential inactivation of the enzyme. For CT and SC, *N*-glutaryl-L-phe-*p*-nitroanilide was added as a substrate during free radical polymerization to protect the active site from inactivation and to reduce potential autolysis during the process.

(15) The activities of PSA-containing enzymes relative to the native enzyme dissolved in aqueous buffer were 16, 20, and 8.9% for CT, SC, and HRP, respectively. This is similar to the values of CT incorporated onto poly(HEMA) (7.8%), carboxymethylcellulose (18%) (Brümmer, W.; Hennrich, N.; Klockow, M.; Lang, H.; Orth, H. D. *Eur. J. Biochem.* **1972**, *25*, 129), Sephadex G-200 (15%) (Mosbach, K. *Immobilized Enzymes*; Mosbach, K., Ed.; Academic Press: New York, 1976; Vol. XLIV, pp 31), and poly(acrylamide) (25%).[12]

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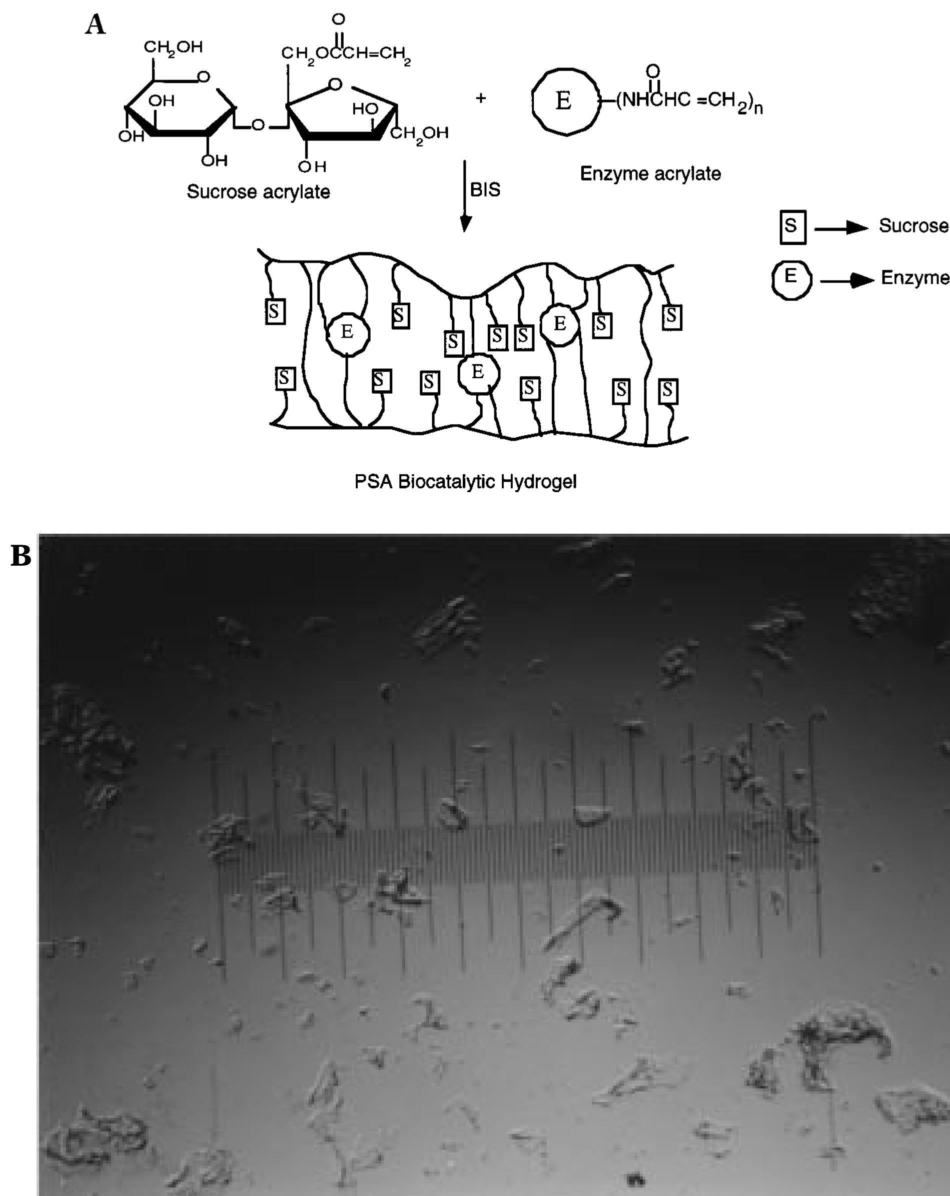

Figure 1. (A) Copolymerization of sucrose-1′-acrylate with acryloylated enzyme to form PSA biocatalytic hydrogel. (B) Micrograph of water-swollen PSA hydrogel particles swelled in aqueous buffer. The scale represents 1 mm total length with 10 μm divisions.

is not surprising given the large pore sizes of the hydrogel material.[11]

The thermal stability of CT-containing hydrogels was significantly higher than that of the native enzymes (Figure 2). The stabilization is particularly evident at 90 °C where the enzyme was found to have a half-life of 1.2 h compared to seconds for the native enzyme (Table 1). The sugar-based hydrogel is more stabilizing to CT than are other conventional hydrophilic matrixes (Table 1). In some cases, the degree of stabilization at 50–60 °C is up to 2 orders of magnitude, and may be a result of intimate entrapment of CT by the sugar-based hydrogel as compared to surface immobilization of CT on polysaccharide materials. Some of this stabilization may be the result of reduced propensity of a polymer-bound CT preparation from undergoing autolysis. However, the stabilizing influence of sucrose, a nonreducing sugar, may also play a role in stabilizing CT in the hydrogel network, and such interactions cannot be ignored, particularly if the sugar groups remain in close contact with the enzyme molecules.[17] In addition to CT, HRP was also stabilized by incorporating into PSA (Table 1), although not to as great an extent as for CT. The relatively low number of surface lysine residues on HRP[18] (6 versus 17 for CT)[11] which can be modified with an acrylic functionality and copolymerized with the sucrose acrylate may result in a lightly cross-linked

(16) Diffusional effects were determined by calculating the observable modulus (Blanch, H.; Clark, D. *Biochemical Engineering*; Marcel Dekker: New York, 1996): $\Phi \equiv$ intrinsic reaction rate/rate of diffusion $= (v_{\mathrm{observed}}/D_{\mathrm{effective}}S_{\mathrm{O}})(R/3)^2$ where $v_{\mathrm{observed}} = 1.98 \times 10^{-6}$ mmol product/(s cm^3 catalyst), $D_{\mathrm{effective}} = 10^{-6}$ cm^2/s (estimated), $S_{\mathrm{O}} = 0.2$ mM, and $R = 1.05 \times 10^{-2}$ cm (largest particle size). These values give a Φ of 0.12 which corresponds to an η_{I} of 1.0.

(17) Sugars are known to protect enzymes during the lyophilization process (Dabulis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **1993**, *41*, 566). Sugars have also been shown to stabilize enzymes in aqueous environments (Gray, C. J. *Biocatalysis* **1988**, *187*, 1).

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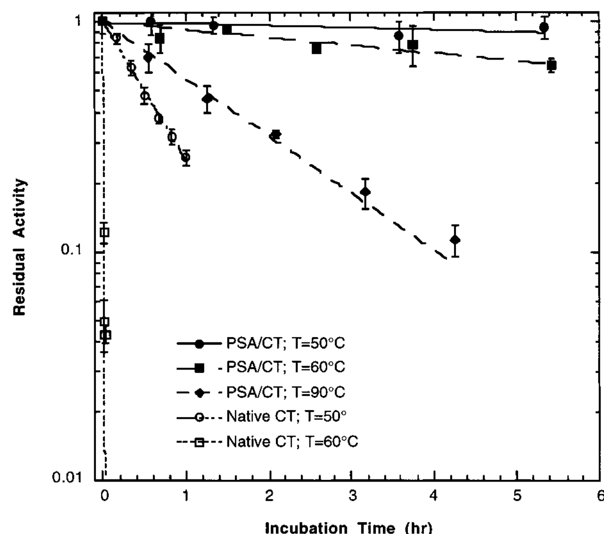

Figure 2. Thermal inactivation of native CT and CT−PSA biocatalytic hydrogel. Enzyme (0.5%, w/w, loading in the PSA matrix) was suspended in aqueous buffer (50 mM Tris, 1 mM $CaCl_2$, pH 7.8), and the suspensions were shaken at 150 rpm for the indicated periods of time. Residual activity was measured as described in the text at 22 °C.[13]

Table 1. Summary of Results of CT and HRP Thermal Stability Experiments and Comparison of CT−PSA Thermal Stability to Other Hydrophilic Matrixes[a]

enzyme system	temp (°C)	k_i (h^{-1}) (half-life, h)	reference
	CT		
native	50	1.39 (0.50)	this work
PSA	50	0.02 (34.450	this work
sepharose CL-4B	50	0.27 (2.6)	22
dextran T-40 (soluble)	50	0.83 (0.83)	22
poly(acrylamide) (entrapped)	50	2.10 (0.33)	22
native	60	148 (2.8×10^{-3})	this work
PSA	60	0.078 (8.8)	this work
poly(methacrylate)	60	0.085 (8.2)	12
native	90	(0)	this work
PSA	90	0.58 (1.2)	this work
	HRP		
native	50	1.41 (0.49)	this work
PSA	50	0.63 (1.1)	this work
native	60	3.30 (0.21)	this work
PSA	60	1.07 (0.65)	this work

[a] The first-order inactivation rate constant (k_i) was determined by an exponential fit of the residual activity versus incubation time. The enzyme half-life is also shown (in parentheses) for completeness.

peroxidase-gel composite that lacks a high degree of sugar−protein interaction that may be necessary for stabilization.

This stabilizing influence of the sugar-based hydrogel was examined further in longer-term stability studies. To that end the CT incorporated within the PSA hydrogel was found be stable in continuous operation (in a continuous stirred tank reactor (CSTR) for nearly three weeks with no loss of activity (Figure 3). This not only indicates the enhanced stability of the enzyme but also shows that the enzyme is strongly attached to the hydrogel and that autolysis is eliminated.

The sugar-based biocatalytic hydrogels were more active in organic solvents than their native enzyme counterparts. For example, in the transesterification of Bz-L-Tyr-OEt with L-Leu-NH_2, the CT−PSA compos-

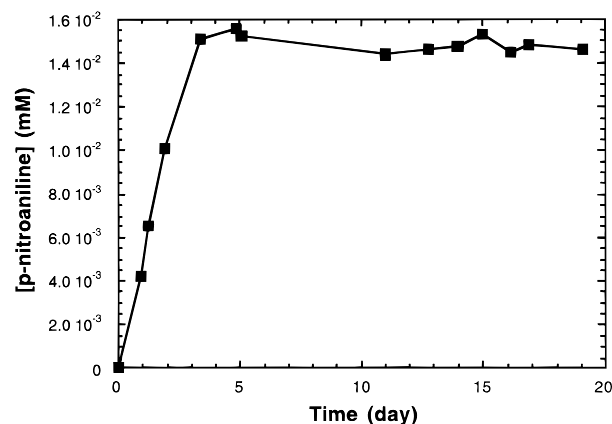

Figure 3. Long-term stability of PSA−CT hydrogel in a CSTR. A total hydrogel mass of 300 mg (containing 1.5 mg of CT) was suspended in 500 mL of aqueous buffer (50 mM Tris, 1 mM $CaCl_2$, pH 7.8) and the suspension shaken at 150 rpm at 22 °C. *N*-Glutaryl-Phe-*p*-nitroanilide was used as the chromogenic substrate, and the buffer solution containing 0.5 mM of the substrate was provided to the CSTR at a flowrate of 0.5 mL/min.

Table 2. Initial Rate of Transesterification of *N*-Acetyl-Phe-Ethyl Ester with 1-Propanol (CT) and Vinyl Butyrate with (*S*)-*sec*-Phenethyl Alcohol (SC) in Different Organic Solvents[a]

solvent	CT−PSA activity ($v_i/[E]_0$) mM P/(h mM E_0)	native CT activity ($v_i/[E]_0$) mM P/(h mM E_0)	enhancement $v_{hydrogel}/v_{native}$
hexane	14.0	0.42	33.7
tert-amyl alcohol	0.39	0.036	10.7
diisopropyl ether	0.45	0.056	7.95
solvent	**SC−PSA activity ($v_i/[E]_0$) mM P/(h mM E_0)**	**native SC activity ($v_i/[E]_0$) mM P/(h mM E_0)**	**enhancement $v_{hydrogel}/v_{native}$**
S-isomer			
hexane	69.3	0.78	88.8
tert-amyl alcohol	5.5	8.1	0.68
R-isomer			
hexane	2.9	0.63	4.6
tert-amyl alcohol	6.4	0.35	18.3

[a] Data are normalized to the concentration of active site as determined via aqueous phase active site titration with the fluorogenic serine protease titrant, MUTMAC.[21] Values represent averages of three individual experiments with standard errors not greater than 15%.

ite was ca. 15-fold more reactive than the lyophilized, suspended enzyme.[19] To elucidate further this activation phenomenon in organic solvents, we examined both CT- and SC-catalyzed transesterification reactions in both polar and nonpolar media. In particular, CT−PSA showed nearly 34 times the activity of the lyophilized, suspended enzyme in hexane for the transesterification of N-Ac-L-Phe-OEt with 1-PrOH (Table 2). The high degree of activation in hexane may be due to the strong PSA−enzyme interactions that are likely to remain intact in such a nonpolar solvent. Thus, the enzyme may remain protected from direct interaction with the organic solvent. The sugars in the polymer may also enhance the activity of the incorporated enzymes by

(19) Solvent consisted of 7:3 isooctane:THF with 0.02% (v/v) added water. Initial rate of PSA−CT peptide synthesis was 0.22 mM product/(h mM E_o), while for the native enzyme the initial rate was 1.5×10^{-2} mM product/(h mM E_o).

partitioning any available water to the surface of the enzyme, which would be necessary for activity.[20] PSA–enzyme interactions are likely to be weakened in more polar organic solvents as solvent–sugar interactions may become stronger. This is consistent with lower degrees of activation of CT–PSA relative to the lyophilized, suspended enzyme in *tert*-amyl alcohol.

Even more striking results are obtained with SC for the transesterification of (*S*)-*sec*-phenethyl alcohol with vinyl butyrate (Table 2). The reaction of SC–PSA in hexane is nearly 2 orders of magnitude faster than the native enzyme. Interestingly, in *tert*-amyl alcohol, the native enzyme is *more* reactive than SC–PSA. These results are consistent with the aforementioned hypothesis that strong enzyme–PSA interactions are present and that these interactions are expected to be strongest in nonpolar solvents. Thus, the disruption of these interactions in a more polar solvent is expected to result in a smaller magnitude of this effect. Indeed, for SC, in *tert*-amyl alcohol, the activating influence is completely lost. Activation of the SC–PSA matrix for catalysis in hexane is also evident for the less reactive *R*-isomer; however, this activation is more significant in *tert*-amyl alcohol. Evidently, the mechanism of the interaction between SC and its microenvironment differs for catalysis with the *S*- and *R*-isomers. This is not surprising as the orientation of the *R*-isomer in the active site of SC must be opposite to that of the *S*-isomer if catalysis is to take place.

In summary, poly(sucrose acrylate) serves as an activating and stabilizing hydrogel matrix for the incorporation of enzymes. Such “biocatalytic hydrogels” may have a variety of uses ranging from catalysts for backed bed reactors, biocatalytic and nonfouling coatings, or as in vivo medical implants.

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