

FINAL REPORT

Immobilized Enzyme System for Lignocellulosic Biomass Saccharification

Investigators

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Abstract

The overall objective of this research is to enhance the bioactivity of cellulase enzymes as well as the stability of the enzymes to increase its reusability through immobilization of the enzymes in the microenvironment of porous inorganic-organic hybrid sol-gel polymers. Recently, we have discovered that the microenvironment of these sol-gel polymers markedly increases the bioactivity of immobilized enzymes. Using these polymers for the confinement of the enzymes or as supports to covalently attach them will allow us to explore the effects of these two different approaches to immobilization on the bioactivity and reusability of cellulase enzymes. We have prepared polymers in which β -glucosidase, a cellulase enzyme, has been covalently immobilized onto sol-gel polymers and integrated with capillary electrochromatography as a separation scheme downstream from the immobilized enzyme. At room temperature, the immobilized enzyme in a sol-gel polymer with high porosity was able to partially hydrolyze D-(-)-salicin, our model substrate, to saligenin in a 5-minute period, while a room-temperature hydrolysis of salicin with the enzyme did not produce saligenin.

Background

The most critical step in the cellulose-to-ethanol bioconversion appears to be saccharification, the enzyme hydrolysis of cellulose to produce fermentable sugars that can yield ethanol. The high cost of cellulases makes it desirable to be able to reuse the enzymes and increase their bioactivity. Recently, we have discovered that confined enzymes that have been immobilized in microenvironments have markedly increased bioactivity. Specifically, we have reported that pepsin immobilized in a porous inorganic-organic hybrid sol-gel monolith exhibited a 700-fold increase in bioactivity¹ and similarly encapsulated trypsin was found to have enhanced stability and bioactivity.^{2,3} Covalent attachment of trypsin on a similar hybrid sol-gel polymer resulted in a 2000-fold increase in the bioactivity of the enzyme for the hydrolysis of benzoyl-L-arginine ethyl ester, a substrate commonly used to determine the bioactivity of trypsin.⁴ These porous hybrid sol-gel polymer monoliths indeed appear to be good candidates for the confinement of enzymes, resulting in increased enzymatic hydrolysis at room temperature.

Fundamental understanding of how confined, immobilized enzymes are able to increase their activity is presently lacking, although several hypotheses exist. The purpose of this study will be to elucidate this enzymatic enhancement mechanism and to apply it to the pressing need of converting biomass to biofuel.

Results

Our study of confined enzymes starts with β -glucosidase, one of the cellulase enzymes. We have prepared immobilized β -glucosidase in a porous sol-gel polymer monolith following a previously developed protocol for enzyme immobilization. The immobilized enzyme-sol-gel

polymer system is prepared inside a small-bore fused-silica capillary to allow for on-line chromatographic separation and detection of the hydrolysis products. Figure 1 shows how the polymer is prepared and how the enzyme is immobilized onto the polymer. Figure 2 is an SEM image of one of these sol-gel polymers, showing its porous nature.

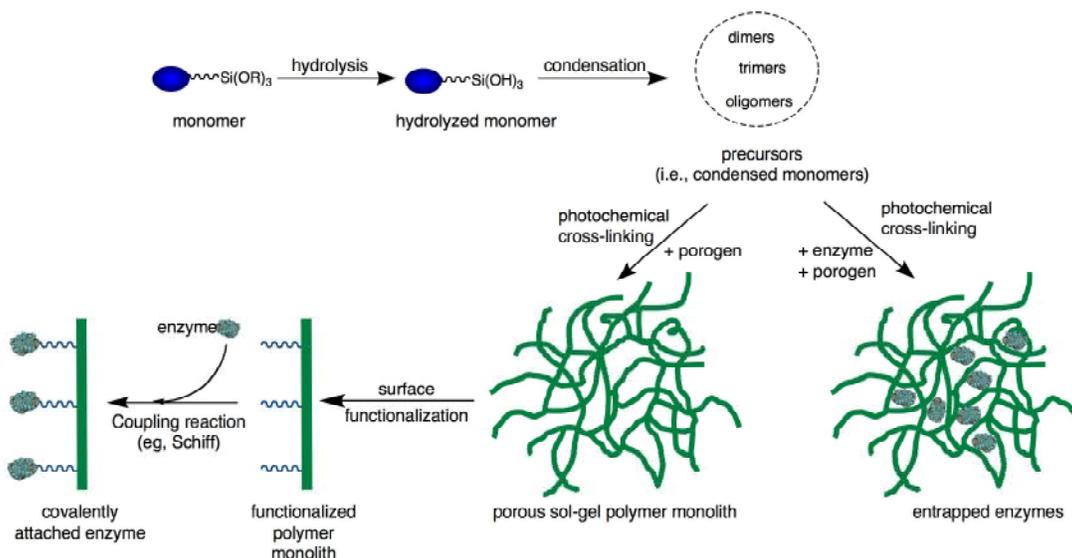


Figure 1. Schematic diagram of porous inorganic-organic sol-gel polymer preparation and immobilization of enzyme by covalent attachment and entrapment. ● is a photoactive group and R is an alkane chain.

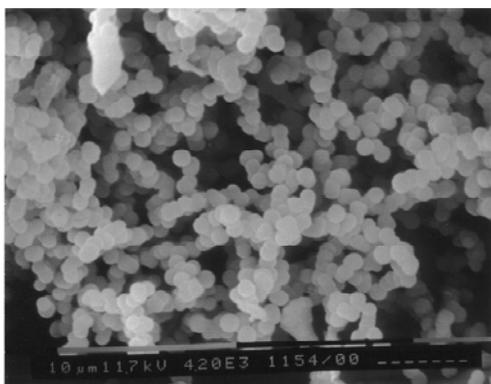


Figure 2. Scanning electron micrograph of a porous sol-gel polymer monolith (without enzyme).

The first generation immobilized- β -glucosidase-sol-gel polymer was evaluated. At room temperature, hydrolysis of salicin was observed by monitoring the formation of saligenin by UV absorbance (Figure 3). An increase in the residence time of salicin in the enzyme-sol-gel polymer by 5 minutes led to a 38% increase in the absorbance of saligenin. This result is remarkable given that saligenin was not observed when the hydrolysis was conducted in free

solution at room temperature with a higher concentration of the enzyme. (β -Glucosidase is typically used at 37°C.)

Three separately prepared first generation immobilized enzyme systems were evaluated. It was found that all three first generation systems had similar bioactivities up to 30 uses.

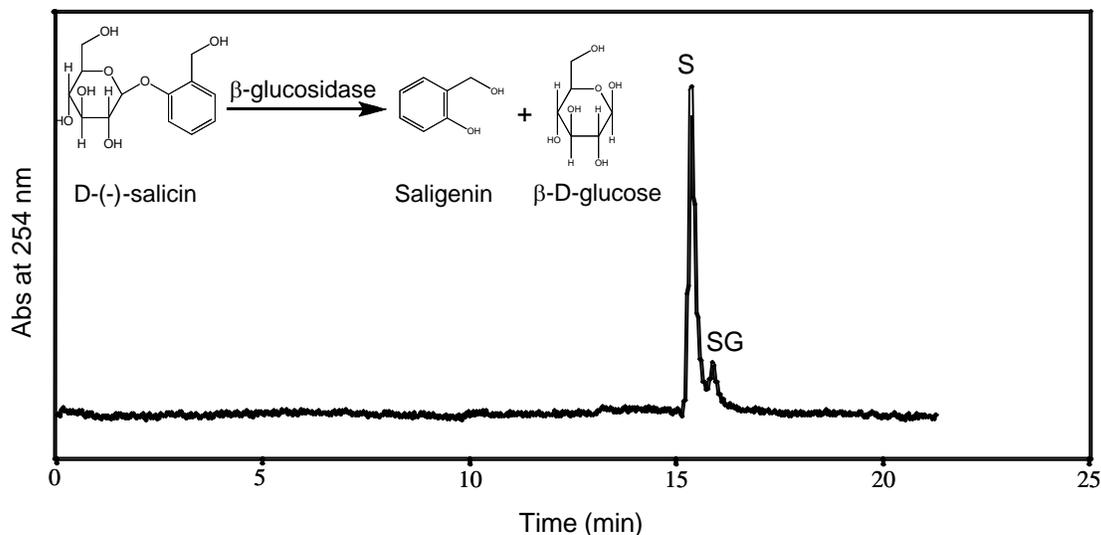


Figure 3. Separation of salicin (S) and saligenin (SG) after hydrolysis of salicin on a β -glucosidase enzyme immobilized in a sol-gel polymer monolith. Saligenin was formed after a 5-minute reaction with the immobilized enzyme.

When the residence time of salicin on the β -glucosidase-modified sol-gel was increased at room temperature, thereby increasing the reaction time, there was an increase in the saligenin peak height as shown in Figure 4.

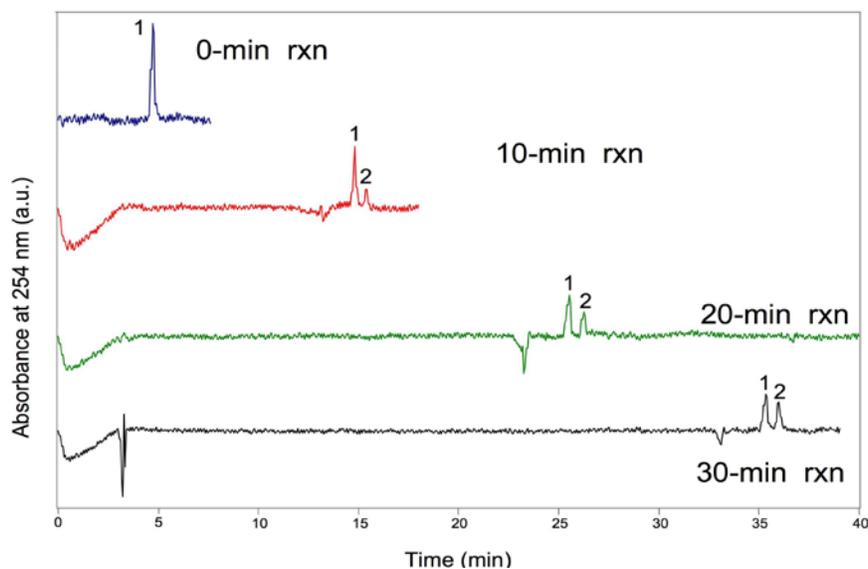


Figure 4. Room-temperature hydrolysis of salicin (**1**) with β -glucosidase immobilized on a sol-gel support at different reaction times, followed by separation of **1** from the product saligenin (**2**) by capillary electrochromatography. Injection of salicin solution at 3 kV for 3 sec; 10-kV separation voltage; 254 nm detection; 20°C. Mobile phase contained 5 mM ammonium acetate (pH 6.5) and 40% acetonitrile.

At zero-min reaction time, only the salicin peak is observed when the reaction solution is analyzed by capillary electrochromatography equipped with a UV-absorbance detector. At 10-min, a small saligenin peak is eluted after the salicin peak. At 20-min reaction time, the peak height of salicin and saligenin are about equal as shown in Figure 5. At 30-min reaction time, the peak height for saligenin is slightly increased compared to the salicin peak height.

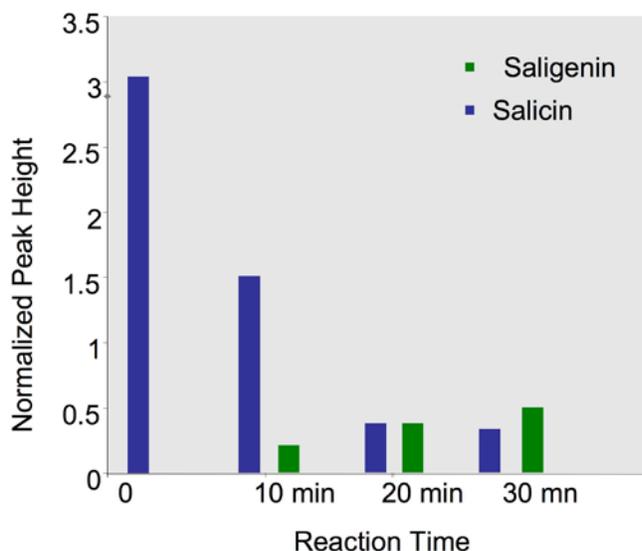


Figure 5. A plot of the disappearance of salicin (1) and the appearance of saligenin (2).

An increase in the acetonitrile content of the mobile phase by 10% did not significantly affect the enzyme's bioactivity, but did result in better peak separations (as shown in Figure 4).

Conclusions

A sol-gel polymer with immobilized glucosidase in a capillary was used about 30 times with no significant decrease in enzyme bioactivity for the hydrolysis of salicin to saligenin at room temperature. Optimization of the sol-gel structure may lead to increased hydrolysis of our model substrate. A better understanding the kinetic parameters of the enzyme-immobilized sol-gel material will lead to a better performing material.

References

1. Kato, M.; Sakai-Kato, K.; Jin, K.; Kubota, M.; Toyo'oka, T.; Dulay, M.T.; Zare, R.N. Integration of On-line Protein Digestion, Peptide Separation and Protein Identification Using Pepsin-Coated Photopolymerized Sol-gel Columns and Capillary Electrophoresis/Mass Spectrometry. *Anal. Chem.* **2004**, *76*(7), 1896-1902.
2. Sakai-Kato, K.; Kato, M.; Toyo'oka, T. On-line Trypsin Encapsulated Enzyme Reactor by the Sol-gel Method Integrated Into Capillary Electrophoresis. *Anal. Chem.* **2002**, *74*, 2943-2949.
3. Kato, M.; Inuzuka, K.; Sakai-Kato, K.; Toyo'oka, T. Monolithic Bioreactor Immobilizing Trypsin For High-Throughput Analysis. *Anal. Chem.* **2005**, *77*(6), 1813-1818.

4. Dulay, M.T.; Baca, Q.J.; Zare, R.N. Enhanced Proteolytic Activity of Covalently Bound Enzymes in Photopolymerized Sol-Gel. *Anal. Chem.* **2005**, *77*, 4604-4610.