Functional Fibers for Immobilization of Biomolecules M02-CD05

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GOAL

This project aims to generate novel fibrous supports for encapsulation/immobolization of biomolecules, specifically enzymes. These fibrous materials offer versatile porosity and ultra-high specific surface for superior enzyme binding and substrate accessibility. Several synthesis and processing concepts have been explored to create specific surface chemistries targeted to bind enzyme proteins. This research pioneers the concept development of fibrous support for the conservation, recovery and reuse of specialty enzymes.

ABSTRACT

A hydrolyzing enzyme, lipase (triacylclglycerol ester hydrolyses, EC 3.1.1.3), has been used as the model biomolecule thus far in this study. Methods to assay the activities of this enzyme in soluble as well as in immobolized forms have been developed. Several chemical approaches have been exploited to incorporate enzyme molecules either on fiber surfaces or inside the fibers. The effectiveness of surface activation mechanisms, efficiency of enzyme incorporation and the activities of the bound enzymes have been accessed, optimized and compared with free enzymes as well as physically encapsulated enzymes.

Concept

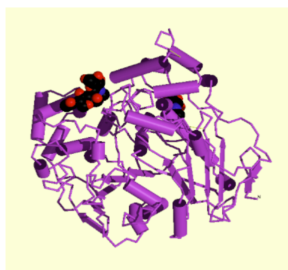
Enzymes are versatile biocatalysts, capable of catalyzing diverse and unique reactions that are highly specific, often stereo specific, in their catalytic mechanisms, enabling simplified steps toward structurally specific product formation and making them highly desirable for targeted reactions. Advances in biotechnology, in recent years, have made more efficient generation of specific enzymes available, expanding their potential, practical use in large-scale conversion of chemicals and materials.

In this research, two approaches have been investigated: (1) incorporating enzyme proteins as part of interpenetrating network and (2) chemically bonding enzyme molecules to fiber surfaces. One of the goals has been to preserve and enhance enzymatic activities, especially under environment that typically render enzymes inactive. Both approaches were explored during the first half year of this project whereas emphasis has shifted toward the second in the second half of the first and through the first half of the second year.

The primary emphasis has been to introduce functional groups on fiber surfaces with specific focus on synthetic approaches to regulate surface architecture, the chemical characteristics of the spacers (length and structure) and functional groups. These primary functional groups are then extended to include spacers or converted to specific active functional groups. To react with ε-aminolysine, potential functional groups have been identified and include those containing primary functional groups of hydroxyl -OH, carboxylic -COOH, and aldehyde –C(O)H. Since few polymers contain these primary functional groups, most require introduction of these reactive functional groups to fibers. We have selected compounds with reactive acylated end groups to react with the highly reactive ε-aminolysine to form piptide bonds.

Enzymes can be covalently bonded to solids via various chemical bonding techniques, such as crosslinking, multi-functional reagents, or surface reactive functional groups. Enzymes immobilized by water-insoluble supports can serve as reusable and removable catalysts, which can potentially possess improved storage and operational stability. Among these methods, chemical covalent bonds offer the strongest links, and thus the most stable enzyme-solid complexes. To chemically bond enzymes to a solid, the structures and functions of both the enzymes and the solids should be considered. Work on modification of enzymes has mostly involved reactions of ε-aminolysine residues due to the more reactive nature of the primary amine. The findings that physico-chemical properties of selective modification of ε-aminolysine residues in enzymes are only slightly affected, even in markedly modified proteins, also suggests that ε-aminolysine is non-essential for catalytic activity. Therefore, this project targets chemical strategies on fibrous materials for immobilization of enzymes involving reactions with ε-aminolysine.

The specific enzyme used is triacylclglycerol ester hydrolyses (EC 3.1.1.3) from fungus (candida rugosa) [534 protein residues; active residue(s): -SER (CH_2OH) 209; -GLU ($-CH_2CH_2COOH$) 341; and HIS 449]. Typically, lipases catalyze hydrolysis of triacylclglycerol to glycerol and fatty acid, thus their activities can be assayed by the quantity of fatty acid produced.

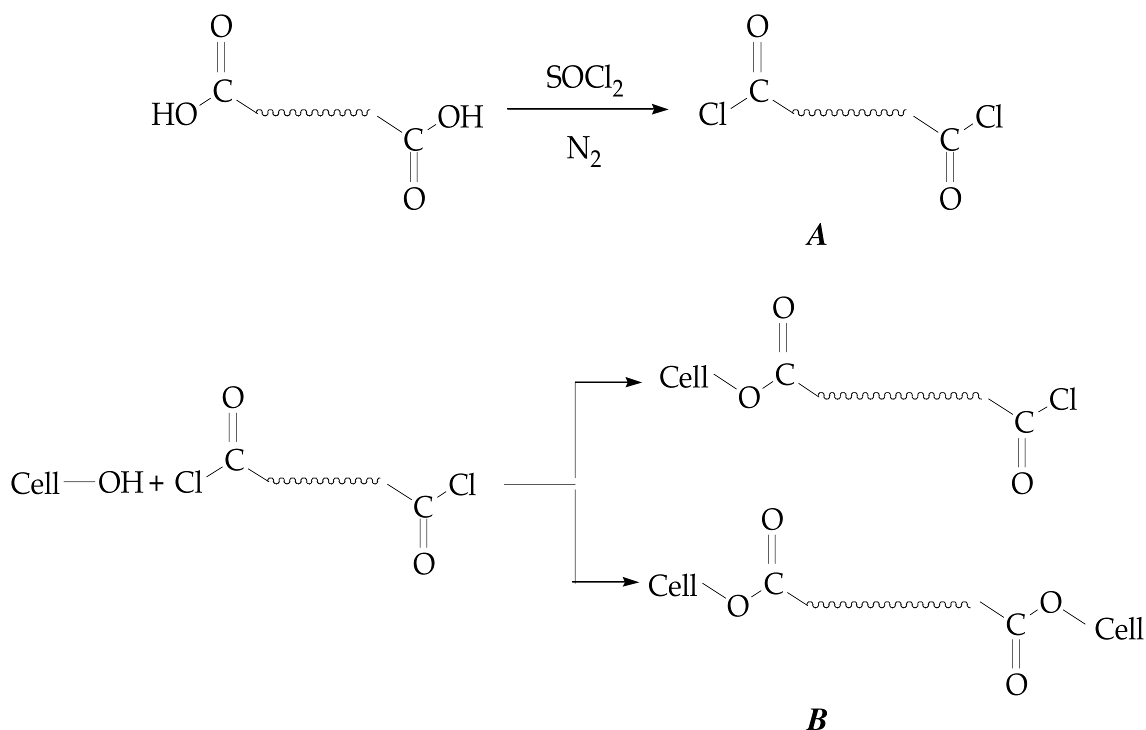


Triacylclglycerol ester hydrolyses (EC 3.1.1.3)

Progress

This project has specifically targeted reactive fiber chemistry that has an affinity toward the polar side groups of the enzyme protein, specifically amines of the ε-aminolysine, as binding sites. Consideration of the fibrous supports includes such desirable characteristics as ultra-high specific surface area, good chemical, mechanical and thermal stability, hydrophilicity and insolubility.

An amphiphilic polyethylene glycol (PEG) linker is used to activate fiber surfaces. The linker containing two carboxylic groups was first converted to the more reactive acyl chloride (***A***) then reacted with the hydroxyle groups on the surface of cellulose fibers (***B***):



Esterification of cellulose by PEG-diacylchloride results in acidic half-esters of cellulose or cellulose diesters, and was confirmed by the strong ester carbonyl stretching around 1750 cm^{-1} in FTIR (***Figure 1***). The level of esterification is positively related to and controlled by the molar amount of the PEG-diacylchloride.

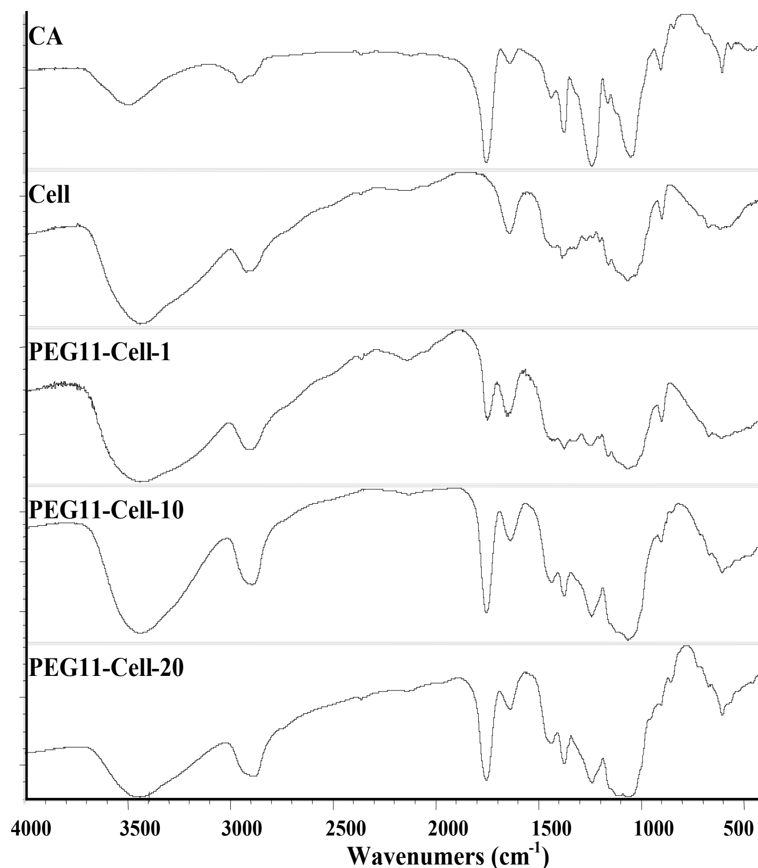


Figure 1. FTIR of PEG-modified cellulose

While the cellulose diesters can serve as intramolecular or intermolecular crosslinks between cellulose chains, only the cellulose half-esters bear reactive acylchloride toward the amines of the enzyme molecules. The highest amount of free acid as well as total acid/ester was found at a COCl/OH ratio of 10 (Figure 2).

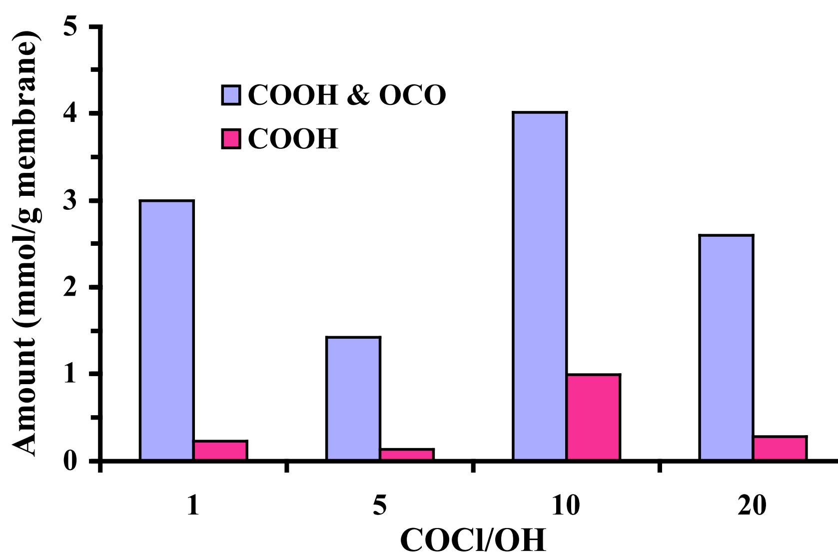


Figure 2. Free carboxylic and total ester and carboxylic on modified cellulose

The surface modified fibers with either acylchloride or acid groups can then react with the amine of the ε-aminolysine to form amide bonds aided by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The coupling reaction was found to be most efficient at a 0.2 EDC/COOH molar ratio, pH 4 and 26ºC for 7 h.

The catalytic activities of the lipase enzymer were measured by photometric determinations of the monoacylglyceride produced. A triglyceride emulsion was prepared by emulsifying 5g olive oil in 95 ml 0.89% NaCl using gum arabic as the emulsion reagent for 10 min. The incubation mixture was prepared by mixing olive suspension, 10 mM deoxycholate and 1M triethanolamine buffer (pH 8.5) at the volume ratio of 50:5:45, final concentration being 30 mM, 0.5 mM and 0.5 M, respectively. Lipase activity was measured at 30 C and pH 8.5. Immobilized lipase was added to 1.0 ml incubation mixture, incubated in a 30 C bath equipped with shaker and denatured by heating for 10 min at 90 C. 5.0 ml chloroform and 2.5 ml copper reagent were added and mixed in a shaker. The mixture was centrifuged for 5 min to separate the phases and the aqueous phase was removed. To assay the enzyme, 2.0ml chloroform layer was mixed with 0.25 ml 11 mM diethyldithiocarbamate. Photometric determination is performed at 440 or Hg 436 nm at ambient temperature against the corresponding sample blank that can be prepared in the same procedure except that the enzyme is not activated before the assay. The catalytic activities of the bound lipase enzyme were found to be positively related to the amount of free acid on the modified cellulose (***Figure 3***).

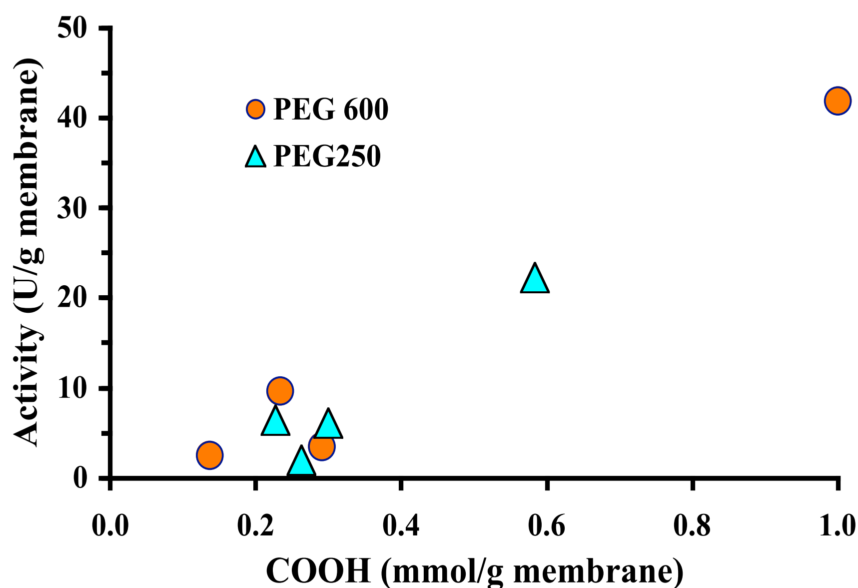


Figure 3. Enzyme activity relative to free carboxylic acid on modified cellulose

After exposure to organic solvents, the activities of the free lipase were reduced to 20 to 30% of its original level (***Figure 4***). The lipase bound to the cellulose fiber, on the other hand, shows much higher activities following exposures to the same organic solvents. For instance, the retention of bound enzyme activities is over 80% in cyclohexane and over 60% in toluene, more than double that found in the soluble form.

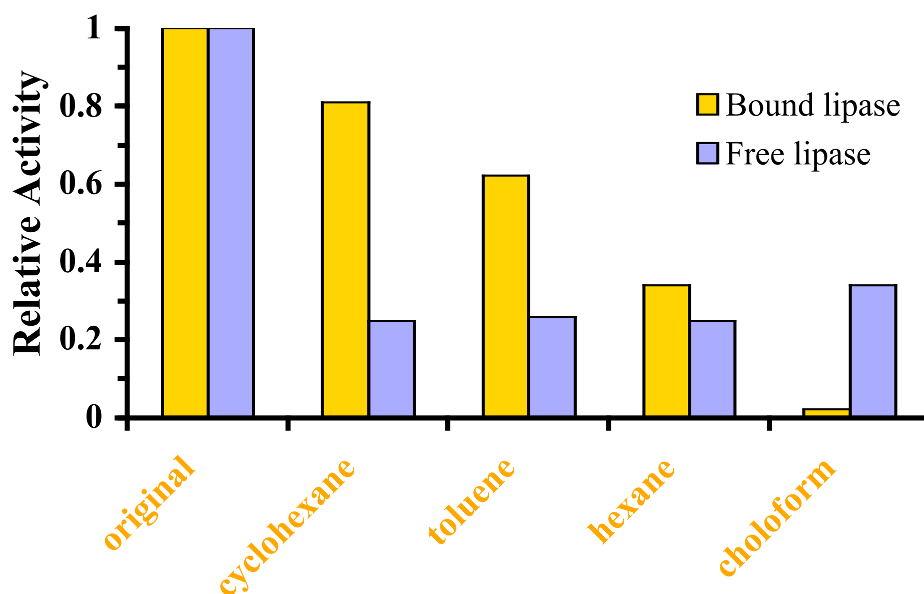


Figure 4. Enzyme activity following exposure to organic solvents for 24h

Although the standard assay temperature for this lipase is 30 C, comparisons of catalytic activities between the free and bound enzymes were investigated at elevated temperatures. The lipase, in its free form, maintains its activity up to 40 C before dropping to nearly half at 50 C (***Figure 5***). The bound lipase, however, has the same or higher activities at higher temperatures, i.e., up to 60 C. Most significantly, while free lipase lost all activity at 80 C, the bound lipase still retained 80% of its activity. The original fibrous and porous structures remain essentially unchanged throughout activation, immobilization, and activity assay.

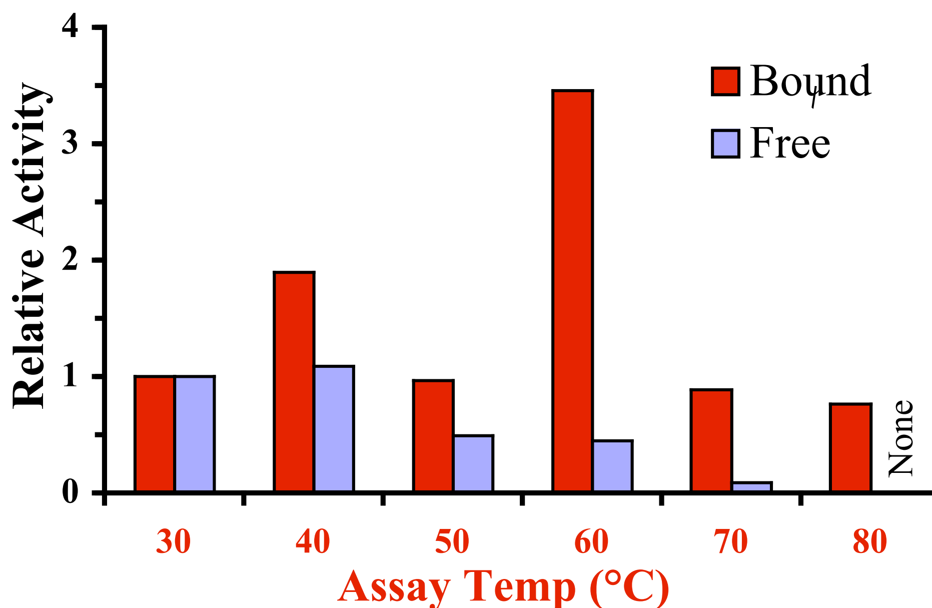


Figure 5. Enzyme activity assayed at elevated temperatures

Currently, considerable effort has been extended to investigate grafting of amphiphilic polymers on ultra-high specific surface cellulose fibers. The focus is on studying the reaction parameters to control the density and chain lengths of the polymers, the binding with enzyme, and the catalytic activities of the bound enzymes and their stability. Other methods to incorporate enzymes in the bulk of the fibers are also being re-examined.

Web site

http://trc.ucdavis.edu/textiles/ntc%20projects/ylhsieh_ntc.html

Acknowledgements

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Related Publications and Presentations

Liu, H. and Y.-L. Hsieh, Ultra-fine Fibrous Cellulose Membranes from Electrospinning of Cellulose Acetate, *Journal of Polymer Science, Polymer Physics*, 40:2119-2129 (**2002**).

Liu, H. and Y.-L. Hsieh, Surface methacrylation and graft-*co*-polymerization of Ultra-Fine Cellulose fibers, *Journal of Polymer Science, Polymer Physics,* (41):953-964 (**2003**).

Hsieh, Y.-L., Ultra-high specific surface fibers for incorporation of proteins and enzymes, Cellulose and Renewable Materials Division, 225th National Meetings American Chemical Society, New Orleans, March 23-27, **2003**.

Wang, Y. and Y.-L. Hsieh, Enzyme immobilization via electrospinning of polymer/enzyme blends, Division of Polymer Chemistry, 225th National Meetings American Chemical Society, New Orleans, March 23-27, 2003, *Polymer Preprint* **2003** 44(1):1212-3.

Hsieh, Y.-L., Ultra-high specific surface fibers for incorporation of proteins and enzymes, Cellulose and Renewable Materials Division, 225th National Meetings American Chemical Society, New Orleans, March 23-27, **2003**.

Hsieh, Y.-L., Y. Wang, and H. Chen, Amphilic links for bound enzymes on ultra-high specific surface fibers, 226th National Meetings American Chemical Society, New York, Sept. 7-11, 2003**.** *Polymer Preprint* **2003** 44(2):565-6.