

## Imprinting Enzymes for Use in Organic Media

Joseph O. Rich and Jonathan S. Dordick

### 1. Introduction

Enzymes suspended in nonaqueous media are more rigid than in aqueous media (*1,2*). This increased rigidity is thought to be the result of increased electrostatic and hydrogen-bonding interactions among the surface residues of the protein in organic solvents (*3*). Despite this rigidity, enzymes remain active and selective in organic solvents, and this has led to a large number of applications that have impacted the chemical, pharmaceutical, and polymer industries (*4–6*). Interestingly, because of the rigid structure of biocatalysts when placed in organic media, it has been possible to alter selectivity (e.g., enantioselectivity and regioselectivity) and increase activity through pretreating the enzyme with an inhibitor/substrate analog (*7,8*). This process, known as “molecular imprinting,” locks the enzyme into a conformation that is favorable for catalysis during lyophilization through the addition of the desired substrate or a substrate analog to the enzyme solution prior to freezing (**Fig. 1**). Furthermore, the ligand may prevent the formation of inactive “microconformations” in the active site created during the lyophilization process (*9*). These hypotheses have been supported by experiments involving the addition of water to the imprinted enzymes. The addition of water to the organic solvent reaction mixture has been shown to strongly depress the activation phenomenon of imprinting because of an increase in enzyme flexibility upon rehydration (*8,10*).

Enzyme inhibitors (*8*), substrate analogs (*11*), and nucleophilic substrates (*10*) have all been successfully used as molecular imprints and have been shown to increase enzyme activity and control enzyme selectivity (both enantioselectivity and substrate selectivity) in organic media (*10,12,13*). The development of compounds with predetermined molecular recognition

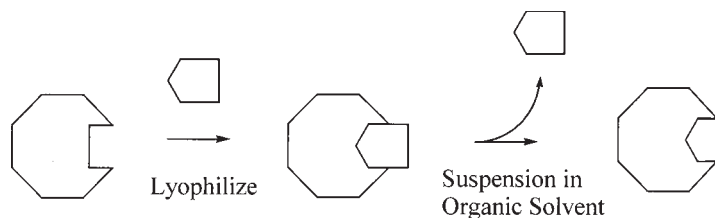


Fig. 1. Representation of the mechanism of enzyme alteration as a result of the addition of an imprinting compound to the lyophilizate. In the lyophilization step, the imprinting compound forces the enzyme active site into a conformation that is more favorable for catalysis. Upon suspension in a solvent in which the imprinting compound is soluble, or rinsing with such a solvent in order to remove the imprinter, the imprinted enzyme is able to accept the substrate of choice. If the imprinting compound is not removed from the active site, the imprinted enzyme is unable to function as a biocatalyst (adapted from **refs. 8 and 10**).

properties has included the imprinting of synthetic polymers (**14,15**), hydrogels (**16**), and proteins (**4**) with a template molecule. Furthermore, the induction of enzymic activity in proteins by lyophilization in the presence of a transition state analog has been recently reported (**17,18**).

Some important limitations of “molecular imprinting” should be considered when employing this technique. First, the imprinter must be sufficiently soluble in water to obtain the necessary concentration for imprinting, where the effectiveness of a nucleophilic substrate as an imprint is known to be dependent on the concentration of the imprint in the aqueous solution prior to lyophilization (**10**). Second, the use of imprinted enzymes is limited to nearly anhydrous media where enzymes are sufficiently rigid to maintain the imprint-induced activated conformation. Third, the application of imprinting of enzymes has not been extended beyond hydrolytic enzymes.

The use of additives to the aqueous solution prior to freezing has been shown to prevent the reversible denaturation of proteins during the drying process (**19,20**). These lyoprotectants, including sucrose and trehalose (and other carbohydrates) and polyethylene glycol, have been shown to increase the activity of many enzymes suspended in anhydrous organic solvents (**11**). This lyoprotection phenomenon occurs concomitantly with the imprinting, especially when imprinting with nucleophilic substrates such as sugars or nucleosides (**10**).

Previous studies of imprinting have led to suggestions that the mechanism of the imprinting-induced rate enhancement involves the conservation/alteration of the enzyme active site (**11**). Similarly, alteration of the substrate specificity via imprinting must also involve the alteration of the enzyme structure, particularly in the vicinity of the transition

state. Kinetic observations of altered substrate selectivity show that there must be a structural component to imprinting enzymes (**10**), and this is consistent with Fourier transform infrared (FTIR) studies probing the secondary structure (**21**) and molecular dynamics simulations (**10**) of imprinted proteins.

In conclusion, molecular imprinting of enzymes for use in organic solvents represents a rapid, simple, and often effective methodology to increase enzyme activity and alter enzyme selectivity. Such an approach, in combination with genetic manipulation of enzyme structure (e.g., directed evolution and DNA shuffling) can be used complementarily for the design of biocatalysts with desired properties, particularly for use in extreme environments.

## 2. Materials

### 2.1. Imprinting

1. Subtilisin Carlsberg (E.C.3.4.21.62) solution containing 7–15 U/mg solid using casein as the substrate (Sigma): Measure 20 mg of enzyme into a freeze-drying flask.
2. Prepare a solution of 20 mg/mL of water-soluble substrate (e.g., sucrose or thymidine) in 10 mM sodium phosphate buffer, pH 7.8.

### 2.2. Measurement of Enzyme Activity

1. Imprinted enzyme prepared as detailed in **Subheading 3.1**.
2. Organic solvent dried over 4-Å molecular sieves for 24 h.
3. Prepare a solution of 0.1 M *n*-butyric acid vinyl ester (TCI America) and 10 mM nucleophile in a suitable organic solvent.
4. High-performance liquid chromatographic (HPLC) grade acetonitrile and water, both containing 0.1% (v/v) trifluoroacetic acid (*see* **Note 1**).

## 3. Methods

### 3.1. Imprinting

1. Add 2 mL of substrate solution to 20 mg subtilisin Carlsberg in a glass freeze-drying flask (native enzyme may be prepared by excluding the substrate from the buffer solution).
2. Gently mix the solution for 15 s.
3. Flash-freeze the enzyme–substrate solution in liquid N<sub>2</sub>.
4. Lyophilize the mixture for 24 h (*see* **Note 2**).
5. Store the imprinted enzyme in a desiccated environment at –20°C.
6. If necessary, the imprinting compound may be removed from the solid enzyme preparation by washing with a suitable anhydrous organic solvent (*see* **Note 3**).

### 3.2. Measurement of Enzyme Activity

1. Suspend 1 mg of the imprinted enzyme in 1 mL of anhydrous solvent containing nucleophile and acyl donor in a screw-cap vial.
2. At suitable time intervals (e.g., every 2 h), withdraw samples for analysis by a suitable method (see **Note 1**).

## 4. Notes

1. The analysis of enzyme-catalyzed transesterifications in anhydrous organic solvents typically involves the use of HPLC or GC. For sucrose, GC analysis using a flame ionization detector (FID) and a HP-1 capillary column was employed following precolumn derivitization of the carbohydrates using 1,1,1,3,3,3-hexamethyldisilazane (Sigma Sil-A). The progress of thymidine acylation reactions was followed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a ODS-AQ (C18) column (YMC) using ultra-violet detection at 254 nm and an isocratic mobile phase of 1 mL/min acetonitrile/water (60:40).
2. Lyophilizations were performed using either a Labconco 4.5 or 12 freeze-dryer. The freeze-dryer normally obtained a vacuum of  $(10-20) \times 10^{-3}$  torr with a condenser temperature of approx  $-50^{\circ}\text{C}$ .
3. The removal of a competitive substrate or enzyme inhibitor from the imprinted enzyme preparation may be accomplished by rinsing the enzyme preparation several times (at least three times) with a dry organic solvent in which the imprinting compound is soluble. The nature of the rinsing organic solvent, however, can also affect enzymic activity. If the imprinting molecule is the substrate of interest, it is not necessary to perform this added step.

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