PREFACE

Enzymatic catalysis has gained considerable attention in recent years as an efficient tool in the preparation of natural products, pharmaceuticals, fine chemicals, and food ingredients. The high selectivity and mild reaction conditions associated with enzymatic transformations have made this approach an attractive alternative in the synthesis of complex bioactive compounds, which are often difficult to obtain by standard chemical routes. However, the majority of organic compounds are not very soluble in water, which was traditionally perceived as the only suitable reaction medium for the application of biocatalysts. The realization that most enzymes can function perfectly well under nearly anhydrous conditions and, in addition, display a number of useful properties, e.g., highly enhanced stability and different selectivity, has dramatically widened the scope of their application to the organic synthesis.

Another great attraction of using organic solvents rather than water as a reaction solvent is the ability to perform synthetic transformations with relatively inexpensive hydrolytic enzymes. It is worth reminding the reader that in vivo, the synthetic and hydrolytic pathways are catalyzed by different enzymes. However, elimination of water from the reaction mixture enables the "reversal" of hydrolytic enzymes and thus avoids the use of the expensive cofactors or activated substrates that are required for their synthetic counterparts. Also, one should bear in mind that water is by no means an ideal solvent for synthesis; it is relatively expensive to remove on a large scale and it often participates in unwanted side reactions. Thus, the use of enzymes in conventional industrial solvents generally makes it easier and cheaper to incorporate a biotransformation step into the overall synthetic sequence.

Indeed, there are numerous examples of the successful application of enzymes in low water media to industrial-scale production of pharmaceuticals, food ingredients, and fine chemicals.

Methods are very important in any area of research, even more so in a field like nonaqueous biocatalysis, where many methods have been developed relatively recently and have not yet been standardized completely in all laboratories. All too often, the format of standard research papers does not allow methods to be fully described. The importance of key details may be known in the originating laboratory, but may not be appreciated in another, because they cannot be stressed enough, nor reasons explained. The prime objective of *Enzymes in Nonaqueous Solvents* is to address this issue because it was com-

piled to communicate such details. There will also be critical features of methods that are at present not appreciated by anyone, but that may be causing different results in different laboratories. Here again, the fuller presentations in this book should be a basis for the identification of such differences.

For the convenience of the reader, the editors decided to split the submitted material into three parts; broadly, these deal with the biocatalysts, synthetic chemistry, and systems other than just neat organic solvents or solvent mixtures. Those familiar with the subject will no doubt appreciate that such a separation is to a large extent arbitrary and is bound to result in some overlaps. The editors felt, however, that this would provide the book with a certain structure and make it easier for the reader to find specific pieces of relevant information. In addition, each part has a short introduction that surveys the contributions included.

Authors of standard research papers are understandably keen to emphasise their interesting results. Some signs of this can perhaps be detected in contributions to this volume too. As editors, we have tried to encourage authors to include as much detail as possible in describing their methods, and not to dismiss this as rather boring or unnecessary. We hope the result of the authors' efforts will prove valuable to all who are interested in studying or using enzymes in nonaqueous media.

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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

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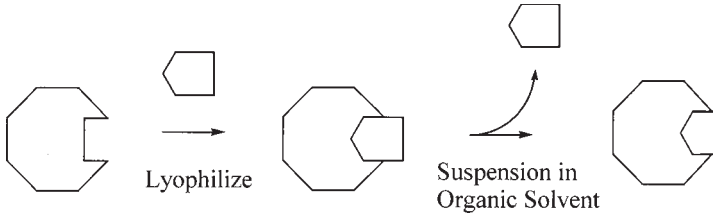

Fig. 1. Representation of the mechanism of enzyme alteration as a result of the addition of an imprinting compound to the lyophilizate. In the lyophilization step, the imprinting compound forces the enzyme active site into a conformation that is more favorable for catalysis. Upon suspension in a solvent 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 infared (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 m*M* 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 m*M* 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_2.
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 ultraviolet 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) × 10^{-3} torr with a condenser temperature of approx –50°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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