0273-2289/04/118/0361/$25.00

Entrapment of Enzyme in Water-Restricted Microenvironment for Enzyme-Mediated Catalysis Under Microemulsion-Based Organogels

Datta Madamwar* and Amit Thakar

Post Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar-388 120, Gujarat, India, E-mail: datta_madamwar@yahoo.com

Received April 28, 2003; Revised October 6, 2003; Accepted October 24, 2003

Abstract

Nonaqueous enzymology has emerged as a major area of biotechnology research and development. Enzymes in organic solvents offer great potential for the biocatalysis of a wide range of chemical processes that cannot occur in water. One of the most commonly used methods for carrying out enzymatic conversions in organic solvents is enzymes solubilized in water-in-oil (w/o) microemulsions or water containing reverse micelles. In reverse micelles, enzyme molecules are solubilized in discrete hydrated micelles formed by surfactants within a continuous phase, i.e., nonpolar organic solvent. Under appropriate conditions, these solutions are homogeneous, thermodynamically stable, and optically transparent. However, there are very few examples of preparative-scale enzyme-catalyzed synthesis in water-in-oil microemulsion. One reason is that despite the advantages offered by microemulsion media, product isolation and enzyme reuse from such single-phase liquid medium is more complex than in competing methodologies in which the catalyst is present as a separate solid phase. Therefore, the approach simplifying product isolation, and enzyme reuse from microemulsion-based media, has been the use of a gelled microemulsion system, especially gelatin silica nanocomposite.

Index Entries: Reverse micelles; microemulsion organogels; lipase; organic solvents; esterification; enzymes.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Enzymes have been employed in catalytic processes for many years, but although they have found extensive application in aqueous solution, their use has been somewhat restricted by the fact that water-insoluble substrate required a large reaction volume and more complicated separation procedure *(1)*. This problem has been largely overcome in the last three decades by the development of methods that allow the use of enzymes in organic solvent *(2–5)*.

Hence, biocatalysis in organic media has become a field of increasing interest *(6–15)*. The main advantages of nonaqueous enzymology are increased solubility of the substrates, favorable thermodynamic equilibrium shift for many reactions, enhanced thermostability, and ease of recovery of the insoluble enzyme. However, many aspects of the application of these systems, including scale-up, cost-effectiveness relative to an alternative process, product separation, and enzyme recovery, have not been investigated in detail.

Various methodologies have been developed to carry out enzymatic conversions in organic solvents. These include stirred biphasic water/organic solvent mixture *(16)*, immobilization of enzymes on solid supports *(17)*, chemically modified biocatalyst *(18)*, enzyme slurries (addition of enzyme in organic solvent on an insoluble solid) *(19,20)*, and enzymes solubilized in water-in-oil (w/o) microemulsions or water containing reverse micelles *(9–11,21–24)*.

One of the most intensively studied methods has been the technique of solubilizing enzymes in hydrated reverse micelles or w/o microemulsion. Reverse micelles are spheroidal aggregates formed by surfactants in apolar media. In contrast to normal micelles in water, the polar groups of the surfactant molecules are directed toward the interior of the aggregate and form a polar core that can solubilize the water (the "water pool"), and lipophilic chains are exposed to the solvent. Under appropriate conditions, these are optically transparent and thermodynamically stable. These spheroidal droplets of 10–200 nm in diameter are spontaneously generated and remain dispersed in continuous organic phase as a result of molecular self-assembly *(25–27)*. The advantage of this procedure is that the enzyme is dispersed at the molecular level, rather than as a solid aggregate, in a stable liquid solution that is capable of solubilizing polar, apolar, and interfacially active substrates. Enzymes in such a system are effectively protected from inactivation by the organic solvent by a layer of water and surfactant shell *(1,11)*. An impressive variety of enzymes have been solubilized and studied in organic media containing reverse micelles or microemulsion droplets *(9–11,17–20)*. However, there are very few examples of preparative-scale regioselective or stereoselective enzymatic synthesis in w/o microemulsion. One reason is that despite the advantages offered by microemulsion media, product isolation and enzyme reuse from

this type of single-phase liquid medium is more complex than in competing methodologies in which the catalyst is present as a separate solid phase.

Microemulsion-Based Organogels

One approach to simplify product isolation and enzyme reuse from microemulsion-based media has been the use of a gelled microemulsion system. Haering and Luisi *(28)* first described the formation of gelatin-based organogel in 1986. In 1991, Atkinson et al. *(29)* determined its physical and structural characteristics and proposed that the microemulsion-based organogels (MBGs) comprise an extensive, rigid, interconnected network of gelatin/water rods stabilized by a monolayer of surfactants, in coexistence with a population of conventional w/o microemulsion droplets.

Enzymes immobilized in MBGs offer considerable potential for use in organic synthesis. Apart from providing reproducibility of structure and the ability to disperse the biocatalyst at a molecular level, MBG provides a semisolid matrix, where the large hydrophobic/hydrophilic surface permits synthesis using water-insoluble substrates in organic solvents. MBGs, to a great extent, overcome the major problems associated with enzyme-catalyzed organic synthesis in w/o microemulsion, most notably, the efficient reuse of the enzyme and its suitability for large-scale synthesis *(5,28)*. However, organogels are not adequate for biological applications, because they have poor mechanical and thermal stability and offer little resistance to an aqueous environment. The gels increase in volume, become opaque and glutinous, and lose rigidity *(30)*. This may be owing to osmotic swelling as a result of the high effective ionic strength of the water contained within the gel, compared with the external water phase. Soni and Madamwar *(31)* made similar observations when butyric acid was used in a reaction system in which MBG rapidly degenerated and ultimately solubilized. Rees et al. *(1)* also observed similar results when ethanoic acid was used in the reaction system.

One possible way to overcome this problem is to improve matrix stability by developing hardened gelatin-based organogel systems, while maintaining the advantages of the microemulsion-based system *(30)*. To do this gelatin organogels are hardened by *in situ* polymerization of metal alkoxides such as tetramethoxysilane *(30)*. During this process, oil-soluble inorganic precursor in the surfactant-stabilized aqueous gelatin network forms water-soluble monomer (e.g., silicic acid), which subsequently polymerizes. As a result of this inorganic polymerization, a highly porous material is formed, which can be considered as a gelatin-silica nanocomposite *(30)*, which significantly improved thermal, mechanical, and chemical stability, when compared with that of gelatin organogels. In 1995, Reetz et al. *(32)* successfully entrapped lipase in hydrophobic sol-gel materials prepared from silicium alkoxide to obtain high product yield. Another advantage of this system is transparency, which permits direct spectroscopic investigation.

Preparation of Organogel

Typically, organogel is prepared by heating gelatin powder with the required amount of water to form a clear solution at 50–55°C. A reverse micellar solution at the same temperature is added and the mixture shaken vigorously until all the components are fully dispersed. The mixture is allowed to cool with occasional shaking to ensure that the system remains homogeneous, until it gels below 25°C *(1,33)*. Organogels are thermally reversible provided that they are shaken during cooling to avoid phase separation, and gels always melt at similar temperature *(33)*.

Hardened gelatin organogels are prepared by the addition of tetraethoxysilane (TEOS) when the system containing aqueous gelatin in reverse micellar solution is at 50°C and gelatin has melted completely to clear solution. This transparent homogeneous dispersion is gently quenched to 20°C with shaking, and a transparent gelatin is obtained. This enzyme containing nanocomposite is allowed to stand for 3 wk in a closed vial and is air-dried and ground to a powder, which significantly increases the surface area, thereby exposing active enzyme to the substrate *(30)*.

The hydrolysis of TEOS uses most of the microemulsion water originally present in the water pool. The remaining water remains in the core of the reverse micelle to hydrate the enzyme *(30)*.

Composition of MBG Preparation

Gelatin

Gelatin is a naturally occurring gelling polymer. It is the generic term given to denatured collagen, a structural protein. The collagen is a triple helical rod with a length of about 3000 Å, a radius of 7 Å, and a molecular weight of about 300,000. The collagen structure is stabilized by hydrogen bonds, enhanced by the presence of water molecules, which bridge the strand of the helix. Gelatin forms the individual strand of collagen helix derived by acid or alkaline hydrolysis. Gelatin dissolves in water above 30°C, but on cooling below this temperature an elastic gel is formed. This is because protein partially regains the collagen helical conformation with neighbor strands crosslinking to form an infinite network *(30,34)*.

The strength of aqueous gelatin gel depends on the molecular weight of the biopolymer. The strength is also reflected by the so-called bloom number, which is roughly proportional to the protein's molecular weight *(29)*.

The primary function of gelatin is to organize cavities in a percolating network, which is then hardened by TEOS polymerization *(30)*.

Microemulsion

Microemulsions are self-emulsifying stable dispersions of oil and water stabilized by interfacial film of surfactant molecules. In contrast to normal micelles in water, the polar group of the surfactant molecules is directed toward the interior of the aggregate and forms a polar core that can

solubilize the water (the water pool), and the lipophilic chain is exposed to the solvent *(29)*.

These systems are also characterized by their small particle size (generally <100 nm), making them stable at a wide range of temperatures, and they are thermodynamically stable. In contrast to the conventional emulsion, these systems are formed spontaneously without the need for any mechanical work *(30)*.

Microemulsion consists of the following:

1. Surfactant:
   a. Anionic: These surfactants form w/o microemulsion, and the most widely used is Aerosol OT ([AOT], sodium di-2-ethylhexasulfosuccinate). Other anionic surfactants include sodium dodecyl sulfate and cetyl pyridinium chloride.
   b. Cationic: One of the most studied cationic surfactants is cetyltrimethylammonium bromide. Generally, in this case a cosurfactant is required to obtain reverse micellar solution. The hydration number is low for cationic surfactant.
   c. Nonionic: Various nonionic surfactants such as Tween, Triton, and Brij series have been employed. An important feature of Tween is that it is nontoxic. The hydrophobic lipophilic balance of nonionic surfactant shifts toward a lipophilic nature when temperature increases and a hydrophilic nature when temperature decreases.
   d. Zwitterionic: The first preparation of reverse micelles was done with a zwitterionic surfactant, phospholipid phosphatidylcholine *(35)*.
2. Nonpolar solvents: High-grade, nonpolar solvents such as *n*-hexane, *n*-heptane, dodecane, benzene, xylene, isooctane, cyclohexane, and toluene are used to dissolve amphiphiles. When the substrate of enzymatic reaction is water soluble, it will solubilize in the aqueous core of reverse micelles. Hence, no direct effect is observed on the reaction with a variation in solvents. However, in the case of reactions such as lipase-catalyzed hydrolysis of olive oil (when substrate is immiscible or poorly soluble), the effect of solvent on the apparent activity is probably related to the effect of solvent on the substrate partitioning between the aqueous and organic phases *(26)*.
3. Cosurfactant: Some surfactant needs cosurfactant in order to reside in the organic phase and form w/o microemulsions. Cosurfactants that are mainly long chain alcohols such as hexanol usually participate in the interfacial region of the w/o microemulsion *(36)*.
4. Buffer/water: A small amount of water/buffer is necessary to stabilize the reverse micelles. Water/surfactant molar ratio (W_0) is one of the important parameters in enzyme-catalyzed reactions. *Wo* controls the size of reverse micelles. The water molecules that are located in the core of the reverse micelles have different properties from that of bulk water *(37)*.

Structural Dynamics of MBG

Structural characterization of MBG has been attempted using a wide variety of techniques, including conductivity measurements and diffusion of radioactive traces in MBGs, in addition to a preliminary analysis of small-angle neutron-scattering measurement and X-ray-scattering data *(29,33)*. These results, together with information from nuclear magnetic resonance (NMR), suggest the existence of extensive ion-conducting channels and a mobile oil phase *(29,33,34)*. Jenta and colleagues *(29,34)* have proposed a schematic model based on neutron scattering and conductivity.

The proposed MBG comprises an extensive, rigid, interconnected network of gelatin/water rods stabilized by a monolayer of surfactant coexisting with a population of conventional w/o microemulsion droplets *(34)*.

In 1991, Rees et al. *(1)* first reported the use of MBG as a solid-phase immobilization matrix for lipase. Their studies noted some surprising properties of the matrix including the physical stability of pelleted MBG in apolar solvents. Under the most appropriate conditions, the matrix fully retained the surfactant, gelatin, water, and enzyme components.

Quellet et al. *(38)* reported on the basis of light and X-ray-scattering data that the spherical shape of the microemulsion droplet in gelatin-free dispersion is maintained in the gel. The polymer initially occupies the microdroplet until a critical concentration is reached above which the gelatin molecules link the droplets together through the oil to form a continuous aggregate of droplets where gel exhibits high electrical conductivity.

The organogel structure is sensitive to the surfactant and gelatin concentration, water content, added salts, and nature of the oil used as the continuous phase. Organogel structure is sensitive to the composition of both the dispersed and continuous phases *(34)*.

The change from gel to fluid or two-phase behavior occurs at the gelatin helix coil transition temperature. The phase separation is similar to the upper temperature phase transition of additive-free microemulsion, but the process is modified owing to the presence of gelatin, which fixes the upper temperature phase boundary of the gelatin melting temperature, essentially independent of W_0.

Factors Influencing Organogels

Polar solvents that can directly participate in noncovalent solvent-protein interaction and replace or remove water are more highly deactivating than low-polarity organic solvents such as hydrocarbon, which do not replace water and have a low capacity for stripping essential water *(29,39)*.

Oil influences the gelation process and nature of the finally formed MBG. The nature of hydrocarbon oil used in organogel formation has an important influence on gel stability. Oils with a lower molecular volume than heptane, such as cyclohexane, which has a better solvate ability and penetrates the surfactant tail groups, promote the discrete microemulsion droplet structure at the expense of gelation *(4,29)*.

As the alkane chain length of solvent increases, the initial rate of the reaction decreases. Higher activity can be obtained when branched and cyclic hydrocarbon, such as isooctane and cyclohexane, are used rather than the *n*-alkanes *(29)*.

The molar ratio of water to surfactant is known as the "R" value. It is a key parameter because it relates both the size of the microemulsion droplets and the activity of the water present in the core. Phase stability studies have shown that transparent, single-phase organogel can be prepared over a range of intermediate *R* value of 30–60 with 14% (w/v) gelatin and 0.4 mol/dm^3 of AOT *(29,34)*. This system offers relatively high condensation activity and good physical stability.

At low *R* values most of the water molecules in the microemulsion are tightly bound to the sulfonate groups and the sodium ions and, hence, are unavailable for the metal alkoxide hydrolysis reaction. Organogels with *R* values higher than 60 can also be formed at the cost of its optical transparency. However, this can be overcome by keeping the water concentration constant and decreasing the surfactant concentration *(31,34)*.

Phase stability studies indicate that the presence of a small amount of gelatin changes the shape and extent of the single-phase microemulsion region with respect to temperature *(34)*.

Optically transparent organogels are generally only obtained at gelatin concentrations higher than 10% (w/v). At low gelatin concentration (<7% [w/v]), the initial reaction rate is higher compared with that of high gelatin concentration (>10% [w/v]). However, the physical stability of the MBGs is affected by lower gelatin concentration, resulting in glutinous gel and finally disintegration, after prolonged contact with the external substrate/oil phase. By contrast, the composition of gel at higher gelatin content remains extremely stable and physically rigid and maintains structural integrity for longer periods in contact with the external reaction medium. A gelatin concentration of 15% (w/v) effectively denotes the upper gelatin limit in organogel formation of $R = 66.5$. The limiting factor in these gel systems is the solubility of gelatin in water *(34)*.

The synthetic activity of MBGs is adversely affected by the formation and retention of the water coproduct. This can be taken care of by selective removal of water using a concentrated solution of dry reverse micelles *(31)*.

The addition of salt to the system moves the upper temperature phase boundary away from the gelatin melting temperature. This prevents the polymer from crosslinking between droplets that aggregate near the phase boundary in the absence of salt. Added salt also electrostatically screens the charged group or the gelatin molecules, causing disruption of the network structure because it is difficult for intergelatin crosslinks to be formed *(34,40)*.

Silica-hardened organogels are examined by Si-NMR and scanning electron microscopy, which suggests that gelatin organizes the cavities in a percolating network, which is then hardened by *in situ* polymerization of metal alkoxides. This hardening with silica makes it impossible for the

immobilized enzyme to leak out after repeated use. Here the optical transparency of the matrix is a great advantage because it permits direct spectroscopic observations *(29,40,41)*.

Conclusion

Enzyme-containing microemulsion-based gels provide a novel method for performing chemical transformations in apolar organic solvents. Fairly good reports are available *(1,31,33,41–43)* demonstrating immobilization of lipases in these systems with retention of activity, but perhaps more important, it has been shown that they are of practical use in preparative-scale synthesis *(37)*. The system could be further improved by hardening the gel while maintaining the advantage of the microemulsion system.

References

1 Rees, G. D., Nascimento, M. da Groca, Jenta, T. R. J., and Robinson, B. H. (1991), *Biochim. Biophys. Acta* **1073,** 493–501.
2 Fletcher, P. D. I., Freedman, R. B., Rees, G. D., and Robinson, B. H. (1987), *Biochim. Biophys. Acta* **912,** 278–282.
3. Claudia, S., Sellapan, S., and Madamwar, D. (1997), *Appl. Biochem. Biotechnol.* **62,** 183–189.
4 Wu, J.-C., Son, B.-D., Xing, A.-H., Hayashi, Y., Talukder, M. M. R., and Wang, S.-C. (2002), *Process Biochem.* **37,** 1229–1233.
5 Nagayanna, K., Yamasaki, N., and Imai, M. (2002), *Biochem. Eng. J.* **12,** 231–236.
6 Luisi, P. L., Giomini, M., Pileni, M. P., and Robinson, B. H. (1988), *Biochem. Biophys. Acta* **947,** 209–246.
7 Borzeix, F., Monot, F., and Vandecastede, T. P. (1992), *Enzyme Microb. Technol.* **14,** 791–797.
8 Soni, K. and Madmawar, D. (2000), *Process Biochem.* **35,** 971–975.
9. Soni, K., Shah, C., and Madamwar, D. (2000), *Biocatalysis Biotransformation* **18,** 331–341.
10 Shah, C., Sellappan, S., and Madamwar, D. (2000), *Process Biochem.* **35,** 971–975.
11 He, Z.-M., Wu, J.-C., Yao, C.-Y., and Yu, K.-T. (2001), *Biotechnol. Lett.* **23,** 1257–1262.
12 Klibnov, A. M. (1989), *Trends Biochem. Sci.* **14,** 141–144.
13. Svensson, I., Wehtje, E., Adlercruetz, P., and Mattison, B. (1994), *Biotechnol. Bioeng.* **44,** 549–559.
14 Jaeger, K. E. and Reetz, M. T. (1998), *TIBTECH* **16,** 396–403.
15 Antonini, E., Carrea, G., and Cremonesti, P. C. (1981), *Enzyme Microb. Technol.* **3,** 291–296.
16. Carrea, G. (1984), *Trends Biotechnol.* **2,** 102–106.
17. Marlot, C., Langrand, G., Triantaphylides, C., and Baratti, J. (1985), *Biotechnol. Lett.* **7,** 647–650.
18. Inada, Y., Takahashi, K., Yoshimoto, T., Ajima, A., Matsushima, A., and Saito, Y. (1986), *Trends Biotechnol.* **7,** 190–194.
19. Dabulis, K. and Klibnov, A. M. (1993), *Biotechnol. Bioeng.* **41,** 566–571.
20. Kirchner, G., Scollar, M. P., and Klibanov, A. M. (1985), *J. Am. Chem. Soc.* **107,** 7072–7076.
21 Fletcher, P. D. I., Freedman, R. B., Rees, G. D., and Robinson, B. H. (1985), *Biochim. Biophys. Acta* **832,** 204–211.
22 Grandi, C., Smith, R. E., and Luisi, P. L. (1981), *J. Biol. Chem.* **256,** 837–843.
23 Hilhort, R., Laane, C., and Veeger, C. (1983), *FEBS Lett.* **159,** 225–228.

24 Samama, J. P., Lee, K. M., and Biellmann, J. F. (1987), *Eur. J. Biochem.* **163,** 609–617.
25 Fletcher, P. D. I. and Horsup, D. I. (1992), *J. Chem. Soc. Faraday Trans.* **88,** 855–864.
26. Yang, F. and Russell, A. J. (1994), *Biotechnol. Bioeng.* **47,** 60–70.
27. Luis, G. P., Valencia-Gonzalez, M. J., and Diaz-Garcia, M. E. (1996), **92,** 312–319.
28. Haering, G. and Luisi, R. L. (1986), *J. Phys. Chem.* **90,** 5892–5895.
29 Atkinson, P. J., Robinson, B. H., Howe, A. M., and Heenan, R. K. (1991), *J. Chem. Soc. Faraday Trans.* **87,** 3389–3397.
30 Schuleit, M. and Luisi, P. L. (2001), *Biotechnol. Bioeng.* **72,** 249–253.
31 Soni, K. and Madamwar, D. (2001), *Process Biochem.* **36,** 607–611.
32. Reetz, M. T., Zonta, A., and Simpelkamp, J. (1995), *Angew. Chem.* **107,** 373–385.
33 Atkinson, P. J., Grimson, M. J., Heenan, R. K., Hawe, A. M., and Robinson, B. H. (1989), *J. Chem. Soc. Chem. Commun.* **23,** 1807–1809.
34 Jenta, T. R. J., Batta, G., Reese, G. D., and Robinson, B. H. (1997), *Biotechnol. Bioeng.* **54,** 416–427.
35 Hanahan, D. J. (1952), *J. Biol. Chem.* **195,** 199–206.
36 Bansal, V. K., Shah, D. O., and O' Connell, J. P. (1980), *J. Coll. Interface Sci.* **75,** 462–469.
37. Douzou, P. (1980), in *Advances in Enzymology*, vol. 51, Meister, A., ed., Academic Press, New York, pp. 30–69.
38. Quellet, C., Eicke, H. F., and Sager, W. (1991), *J. Phys. Chem.* **95,** 5642–5655.
39. Farago, B., Huang, S. Richter, D., Safran, S. A., and Milner, S. T. (1990), *Prog. Coll. Polym. Sci.* **81,** 60–68.
40. Capitani, D., Sagre, A. L., Haering, G., and Luisi, P. L. (1988), *J. Phys. Chem.* **92,** 3500–3504.
41. Luisi, P. L., Scartazzini, R., Haering, G., and Schurtenberger, P. (1990), *Coll. Polym. Sci.* **268,** 356–374.
42 Rees, G. D., Robinson, B. H., and Stephenson, G. R. (1995), *Biochim. Biophys. Acta* **1259,** 73–81.
43. Nagishi, S., Shirasawa, S., Arai, Y., Suzuki, J., and Mukataka, S. (2003), *Enzyme Microb. Technol.* **32,** 66–70.