Biocatalysis, 1987, Vol. 1, pp. 99–108
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REVIEW

ASPECTS OF BIOCATALYST STABILITY IN ORGANIC SOLVENTS

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(Received 26 January 1987; in final form 8 March 1987)

The stability of biocatalysis in systems containing organic solvents is reviewed. Among the examples presented are homogeneous mixtures of water and water-miscible organic solvents, aqueous/organic two-phase systems, solid biocatalysts suspended in organic solvents, enzymes in reverse micelles and modified enzymes soluble in water immiscible solvents. The stability of biocatalysts in organic solvents depends very much on the conditions. The hydrophobicity or the polarity of the solvent is clearly of great importance. More hydrophobic solvents (higher log P values) are less harmful to enzymes than less hydrophobic solvents. The water content of the system is a very important parameter. Some water is essential for enzymatic activity; however, the stability of enzymes decreases with increasing water content. Mechanisms of enzyme inactivation are discussed.

KEY WORDS Enzyme stability, biocatalyst stability, enzymes in organic solvents, biocatalysts in organic solvents, bioorganic synthesis.

INTRODUCTION

The use of enzymes in organic solvents has been reviewed several times (Butler, 1979; Antonini, Carrea and Cremonesi, 1981; Lilly, 1982; Carrea, 1984; Fukui and Tanaka, 1985; Lilly and Woodley, 1985). Information on enzyme stability in organic solvents has appeared in many articles, although it has seldom been the main issue. We present in this minireview a compilation and comparison of different systems used in bioorganic synthesis and we also discuss some of the aspects of stabilization in organic media. A characteristic feature of these systems is the amount of water present in the system. The purity of the enzyme preparations used may be essential for the stability properties registered and also for the catalytic performance. The examples presented will be for purified enzymes as well as for crude preparations and for more complex biocatalysts such as whole cell catalysts.

The different systems are schematically presented in Figure 1. Figure 1a illustrates enzyme catalysis in homogeneous solutions, where a water-miscible organic solvent is added to the mixture. When water-immiscible solvents are added, two-phase systems are formed (Figure 1b). A system offering better protection of the biocatalysts is shown in Figure 1c. Here the catalyst is immobilized in solid particles, offering an aqueous microenvironment, while the bulk solution is water-immiscible organic solvent. A further reduction in water content is achieved by precipitating the enzyme on a solid support; after evaporating off the water, the catalyst is added to an organic solvent. Here only

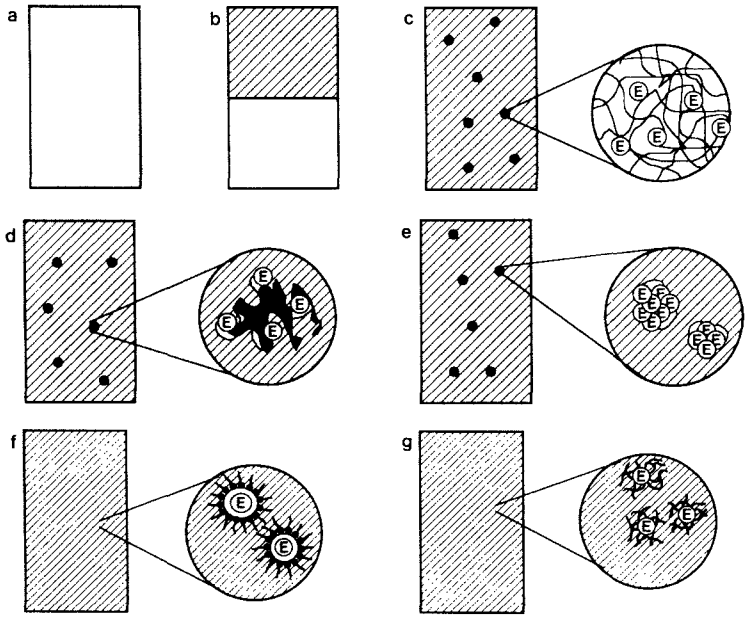

Figure 1 Schematic presentation of different ways to use enzymes together with organic solvents. Water is drawn as white areas and the organic solvent as shaded areas. In the magnified pictures individual enzyme molecules (circles marked with E) are shown. (a) Homogeneous system containing water and water-miscible organic solvent. (b) Two-phase system containing one aqueous phase and one organic phase. The enzyme is located in the aqueous phase. (c) Enzyme immobilized in water-containing, porous particles surrounded by organic solvent. (d) Enzyme adsorbed on porous particles surrounded by organic solvent. Small amounts of water are associated with the enzyme molecules and the support material. (e) Solid enzyme particles suspended in organic solvent. Small amounts of water are associated with the particles. (f) Enzyme in microemulsion (reverse micelles). Inside the reverse micelles the enzyme molecules are surrounded by thin water shells. (g) Covalently modified enzyme soluble in organic solvents. Small amounts of water are associated with the enzyme molecules and in some cases with the modifying residues.

small amounts of water are present in the proximity of the enzyme and in the support (Figure 1d). A similar picture is achieved if solid enzyme is added directly to the organic solvent. Since the protein is not soluble in organic solvents, insoluble aggregates will be formed (Figure 1e). Still another possibility is to solubilize the enzyme molecules in microemulsions and thus create an aqueous microenvironment for the enzyme in the bulk organic phase (Figure 1f). Finally, it is possible to modify the enzyme to make it soluble directly in the organic solvent (Figure 1g).

ENZYME CATALYSIS IN SYSTEMS CONTAINING WATER-MISCIBLE ORGANIC SOLVENTS

Modest concentrations of some water-miscible compounds enhance the stability of enzymes (Butler, 1979; Freeman, 1984; Asakura, Adachi and Schwartz, 1978). A well-known example of stabilization is the effect that glycerol or other poly-alcohols exert on many enzymes. Enzyme catalysis in the presence of high concentrations of polyols has recently become increasingly popular, since the

addition of polyols decreases the water activity and thereby may change the equilibrium for hydrolytic reactions. In this case, the enzyme is stabilized by the polyol and the equilibrium is shifted. Enzymes added to aqueous solutions containing high concentrations of water-miscible solvents unfold and lose their activity. Klyosov, van Viet and Berezin (1975) showed that α-chymotrypsin lost its typical catalytic activity when dissolved in dimethylsulphoxide (DMSO).

The sensitivity of enzymes to inactivation by solvents varies. In the literature there are examples where enzymes have been used in homogeneous mixtures of water and organic solvents without considerable loss of activity. A column containing immobilized glucose oxidase was operated continuously for 14 days in a 20% (v/v) acetone solution without any considerable decline in enzyme activity (Alberti and Klibanov, 1982). Yokozeki *et al.* (1982) carried out a transglycosylation reaction using immobilized whole cells (*Enterobacter aerogenes*). This biocatalyst retained its original activity after 35 days of operation in 40% (v/v) DMSO.

ENZYME CATALYSIS IN TWO-PHASE SYSTEMS

When water-immiscible solvents are added to aqueous solutions, two-phase systems are formed. Several bioconversions have been performed in two-phase systems, consisting of one organic phase and one aqueous phase. Antonini, Carrea and Cremonesi (1981) pointed out that the sensitivity of enzymes to organic solvents in two-phase systems varies greatly. Generally, solvents of lower polarity are less harmful to enzymes than are the more polar solvents. Carrea *et al.* (1974) studied the stability of β-hydroxysteroid dehydrogenase in two-phase systems containing water and different solvents. Of the solvents tested, n-hexane and isooctane produced the lowest amount of deactivation.

Khmelnitski *et al.* (1984) carried out peptide synthesis using α-chymotrypsin in a water–ethyl acetate two-phase system. They found that the free enzyme was inactivated rather quickly, while enzyme immobilized on Sorsilen, a terephthalate polymer, could be re-used successfully. It retained 50% of its initial activity after seven days of operation. Schutt *et al.* (1985) studied the hydrolysis of arylglycine derivatives by subtilisin in two-phase systems with the aim of preparing optically pure D-arylglycines. In the hydrolysis of DL-2-acetamidophenylacetic acid methyl ester they achieved almost quantitative yields when the immobilized subtilisin was used repeatedly 75 times (>150 h) in a two-phase system consisting of water and methyl isobutyl ketone.

As long as the enzyme is kept in the water phase and is not exposed to the interface, good operational stability can be foreseen. To achieve this goal, the enzymes often must be immobilized in or on a hydrophilic carrier. The larger the difference in polarity between the support and the organic solvent, the lower the risk of exposure of the enzyme at the interface. Some enzymes, lipases for example, exert their physiological function at interfaces and should therefore tolerate two-phase systems well.

ENZYMES IN SYSTEMS CONTAINING LITTLE WATER

It has been known for some time that enzymes can be stored in organic solvents with little loss of activity. Dastoli, Musto and Price (1966) showed that crystals of

α-chymotrypsin could be incubated in methylene chloride containing 0.25% water for 90 h with only 5% inactivation. Dastoli and Price (1967a) found that milk xanthine oxidase catalyzed the oxidation of crotonaldehyde when the enzyme was suspended in different solvents. It was shown that the effect of solvents on V_{max} for the enzyme probably reflect the general suitability of the solvent as a medium for redox reactions (Dastoli and Price, 1967b).

Recently, interest has focused increasingly on the catalytic activity of enzymes in the presence of organic solvents, and when practical applications are of interest the operational stability is of vital importance. When exposing an enzyme preparation to an organic solvent it has turned out to be very important to define the purity of the enzyme preparation and the water content in the final incubation mixture. An impure preparation may offer some protection for the catalytically active molecules both by a "protein dilution" effect and by creating a favourable microenvironment, e.g. by offering a favourable polarity in the vicinity of the catalytic site.

One example of such a crude preparation is *Nocardia* cells that have been used to oxidize cholesterol to cholestenone. The solubility of both substrates, cholesterol and oxygen, is higher in organic solvents than in water. Buckland, Dunnill and Lilly (1975) performed the bioconversion in several solvents and found carbon tetrachloride the most suitable. After seven runs (69 h) the reaction rate had fallen to half the value of the first run. Even better operational stability was achieved with immobilized cells. After 300 h of operation in 1,1,1-trichloroethane, cells entrapped in polyacrylamide and in alginate retained 50% and 68% of their initial activity, respectively (Duarte and Lilly, 1980). *Nocardia* cells can oxidize not only cholesterol but also other 3-β-hydroxy-Δ^5-steroids to the corresponding 3-keto-Δ^4-steroids. For the oxidation of dehydroepiandrosterone, polymer-entrapped cells in water-saturated benzene/heptane (1:1) showed an operational half-life of 20–24 h (Omata *et al.*, 1979). In the oxidation of pregnenolone in water-saturated chloroform/heptane (1:1) the operational half life of polymer-entrapped cells was extrapolated to be around 50 h (Omata, Tanaka and Fukui, 1980). It should be stressed in this context that at the present state of development only dead cells can be used, even if there are some indications that living cells can survive in, for example, microemulsions (Häring, Luisi and Meussdoerffer, 1985).

It has been shown by several researchers that lipases are quite stable in organic solvents. Bell *et al.* (1981) used *Rhizopus arrhizus* mycelia to hydrolyze triglycerides in olive oil. The hydrolysis was performed in a diisopropyl ether solution containing 0.06–0.38% (w/v) water. The loss of activity was 0.6–1 %/h at 30°C. Macrae (1983) reported that *Rhizopus niveus* lipase adsorbed on kieselguhr had been used for interesterification for 400 h with almost no loss of activity. The substrates, palm oil midfraction and myristic acid, were dissolved in petroleum ether (b.p. 100–120°C) saturated with water. In a similar example *Mucor miehei* lipase on Hyflo Supercel was used to catalyze the interesterification of palm oil midfraction and stearic acid in a packed bed reactor at 50°C (Macrae, 1985). The enzymatic activity decayed exponentially with a half-life of about 60 days.

Zaks and Klibanov (1984) studied the transesterification reaction between tributyrin and various alcohols catalyzed by porcine pancreatic lipase. The alcohols were dissolved in tributyrin. No extra solvent was added. The enzyme

was used as a crude powder suspension. It was shown that the heat stability of the enzyme was markedly dependent on the water content of the system. At a water content of 0.015% the activity half-life was more than 12 h at 100°C. The enzyme was also highly active under these conditions. If the water content was increased to 0.8% the stability decreased markedly, and in a water solution the enzyme lost its activity almost instantaneously at 100°C.

The stereoselective hydrolysis of DL-menthyl succinate by a whole cell (*Rhodotorula minuta* var *texensis*) catalyst was studied by Omata *et al.* (1981). Good operational stability was obtained when the cells were entrapped in polyurethane. The half-life was about 60 days when used in water-saturated n-heptane.

ENZYME CATALYSIS IN REVERSE MICELLES

A reverse micellar medium consists of tiny water droplets stabilized by surfactants in a bulk, water-immiscible organic solvent. Enzyme solubilization in such media has recently been reviewed (Luisi, 1985; Luisi and Laane, 1986). It has been reported that the storage stability of enzymes is sometimes better in reverse micelles than in water solutions (Barbaric and Luisi, 1981; Hilhorst, Laane and Veeger, 1982).

Hilhorst, Laane and Veeger (1982) studied hydrogen production by hydrogenase in reverse micelles with cetyltrimethylammonium bromide as surfactant and a chloroform/octane mixture as solvent. They found that the enzymatic reaction stopped after 18 h of operation. In a combined enzyme system containing hydrogenase, lipoamide dehydrogenase and 20 β-hydroxysteroid dehydrogenase in reverse micelles, the reduction of progesterone continued for at least 9 h (Hilhorst, Laane and Veeger, 1983). Doddema *et al.* (1985) found that the operational stability of cholesterol oxidase was higher in whole cells ($t_{1/2} > 10$ days) than in reverse micelles ($t_{1/2} \sim 20$ h).

Lee and Biellmann (1986) solubilized *Streptomyces* cholesterol oxidase and catalase in reverse micelles with cyclohexane as solvent, tetradecyltrimethylammonium bromide as surfactant and a water content of 10%. When oxidizing cholesterol under these conditions cholesterol oxidase lost 5% of its activity in 5 h.

MODIFIED ENZYMES SOLUBLE IN WATER-IMMISCIBLE ORGANIC SOLVENTS

Enzymes are not normally soluble in organic solvents. However, by modifying them with polyethylene glycol (PEG), for example, it is possible to dissolve enzymes in organic solvents. Several different enzymes have been modified in this way (Inada *et al.*, 1986). Activity of these modified enzymes has been studied in benzene and chlorinated hydrocarbons. PEG-modified lipase exhibited good storage stability in benzene (Yoshimoto *et al.*, 1984). Little data on the operational stability of the PEG-modified enzymes has been reported, although in some cases the enzymes have shown catalytic activity for a few days (Matsushima *et al.*, 1986).

The PEG-chains seem to play an important role in this system. They make it possible to dissolve the proteins in strongly apolar solvents. At the same time it is assumed that the PEG-chains bind water molecules, thereby creating a microenvironment that is favourable for enzyme catalysis. The true mechanism behind the function of PEG-modified enzymes in organic solvents remains, however, to be elucidated.

ON OPTIMIZING ORGANIC SOLVENTS IN BIOCATALYSIS

Some investigators have tried to elucidate those properties of the solvents which are important for their use together with biocatalysts. It has been observed that apolar solvents are often less harmful to enzymes than solvents with higher polarity (Cremonesi *et al.*, 1975). Physical properties of the solvent, such as water solubility (Duarte and Lilly, 1980) and dielectric constant (Omata, Tanaka and Fukui, 1980; Harbron, Smith and Lilly, 1986), have been used to correlate the activity of a biocatalyst in different solvents. However, the usefulness of these parameters seems to be limited by the lack of sufficient experimental data. Brink and Tramper (1985) found that the Hildebrand solubility parameter and the molecular weight were useful characteristics for predicting the effect of organic solvents on immobilized *Mycobacterium* cells catalyzing epoxidation of propene and 1-butene. Solvents with high molecular weight and low Hildebrand solubility parameter (low polarity) gave the highest retention of catalytic activity. In such solvents the highest activity of anaerobic bacteria has been retained (Playne and Smith, 1983).

Laane, Boeren and Vos (1985) proposed that log P was a more useful parameter than the Hildebrand solubility parameter. Log P is defined as the logarithm of the partition coefficient of a given component in the octanol-water two-phase system. The authors used the same set of experimental data as Brink and Tramper (1985), and found a better correlation when log P was used than when the Hildebrand solubility parameter was used. From the log P value alone it was possible to predict the usefulness of a solvent in systems containing whole-cell catalysts. Log P values can be determined experimentally or calculated from the hydrophobic fragmental constants of Rekker (1977). Log P values have also been used to find suitable conditions for enzymatic conversions in reverse micelles (Hilhorst *et al.*, 1984). Reslow, Adlercreutz and Mattiasson (1987a) studied the esterification of N-acetyl-L-phenylalanine with ethanol by α-chymotrypsin adsorbed on porous glass beads in various organic solvents. It was shown that solvents with higher hydrophobicity (higher log P values) gave higher enzyme activities and also better thermostability and operational stability than less hydrophobic solvents. The amount of water added to the enzyme preparations greatly influenced the initial reaction rates. When compensating for the water solubility in the organic solvent an even better correlation was achieved (Reslow, Adlercreutz and Mattiasson, 1987b). For some solvents optimal water contents were determined. The thermostability decreased with increasing water content.

It must, however, be stressed in this context that evaluation of the effects of organic solvents on both isolated enzymes and viable cells can never give a perfect correlation with a parameter such as log P. The mechanisms of interaction are probably very different. In the latter case, solubilization of membrane

components with a subsequent disruption of ion balances etc. is a major problem, whereas, in the case of enzyme denaturation, direct interaction between the solvent and the protein molecule is essential for the effects observed.

MECHANISMS OF ENZYME INACTIVATION

When a protein is heated above a certain temperature the protein unfolds and thereby loses its native conformation. This process has been studied extensively (Tanford, 1968, 1970). When enzymes unfold they lose their catalytic activity. If an enzyme solution is heated above the transition temperature and then cooled down, enzymatic activity can be regained. However, often only a fraction of the activity can be restored because of irreversible inactivation mechanisms. These mechanisms were investigated for lysozyme solution at 100°C by Ahern and Klibanov (1985). At pH 4 and 6 deamidation of asparagine residues was the main irreversible inactivation mechanism, while at pH 8 it was the formation of incorrect (scrambled) structures due to disulfide interchange. Other irreversible inactivation mechanisms were hydrolysis of peptide bonds and destruction of cystine residues. Ribonuclease was irreversibly inactivated at high temperatures by the same mechanisms as lysozyme (Zale and Klibanov, 1986). In all the irreversible inactivation mechanisms studied, water participates directly or indirectly.

The transition temperatures of protein unfolding can be measured by differential scanning calorimetry (Donovan, 1984). The addition of water-miscible organic solvents such as lower alcohols (Fujita, Miyanaga and Noda, 1979) or DMSO (Fujita, Izumiguchi and Noda, 1982) destabilizes the proteins so that the transition temperatures decrease.

Some investigations have shown that enzymes are quite thermostable in nonpolar organic media (Zaks and Klibanov, 1984; Ayala *et al.*, 1986; Reslow, Adlercreutz and Mattiasson, 1987a). In these investigations the thermostability decreased with increasing water content. However, at very low water contents the initial enzyme activity increased with increasing water content. After reaching a maximum the initial reaction rate then decreased with increasing water content (Zaks and Klibanov, 1984; Reslow, Adlercreutz and Mattiasson, 1987a). Solid, dry enzymes have also been shown to be quite thermostable (Mullaney, 1966). A probable explanation is that the irreversible inactivation mechanisms mentioned occur slowly at low water content.

A popular way of describing the stabilizing effect obtained by the reduction of the water content of an enzyme preparation in an apolar solvent might be that eventually there is not enough water for larger conformational changes to take place. This effect can then be similar to that achieved when enzymes are immobilized by multipoint attachment to a support, thereby freezing them into one conformation.

A further factor of importance when evaluating the operational stability of enzymes is the effect of diffusion-controlled processes. The study of immobilized enzymes in an aqueous environment has shown that much of the stabilizing effect ascribed to immobilization is a result of diffusion limitations due to the use of an excess of enzyme molecules (Cheetham, 1983). In the case of enzyme catalysis in organic media these potential effects have not yet been fully investigated.

Under operational conditions, inactivation caused by reactants and products may play an important role in the inactivation of enzymes. Introduction of an organic phase as substrate reservoir and/or product extractant, however, may help to overcome these problems.

CONCLUSION

The examples presented in this review show that it is sometimes possible to obtain very good stability of biocatalysts in systems containing organic solvents. Some enzymes are more sensitive than others. The purity of the enzyme is of importance. Often enzymes in whole cell catalysts are more stable than purified enzymes. Sometimes, the stability can be increased by immobilization.

The stability of biocatalysts in organic solvents depends very much on the conditions. The hydrophobicity or the polarity of the solvent is clearly of great importance. More hydrophobic solvents (higher log P values) are less harmful to enzymes than less hydrophobic solvents. The water content of the system is a very important parameter. Some water is essential for enzymatic activity. However, the stability of enzymes decreases with increasing water content.

Acknowledgements

The authors wish to thank Dr Rajni Kaul for valuable discussions and Mr Scott Bloomer for linguistic advice. The support of the National Swedish Board for Technical Development (STU) and the Biotechnology Research Foundation (SBF) is gratefully acknowledged.

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