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Synthetic Polymers as Solubilizing Vehicles for Enzymes in Water-Poor Media

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Abstract—A recent method for exposing enzymes to organic solvents is reviewed. By complex formation between the enzyme and polymers that *per se* are soluble in organic solvents it is possible to disperse the enzyme in the organic medium in such a way that an optically transparent (in the visible region) solution is obtained. After reaction, the separation of the enzyme from the organic medium can be obtained simply by addition of water. The enzyme can be recovered from the water phase. Physico-chemical studies have revealed that the enzyme is more stable in the complex-bound form.

Introduction

It is now well established that many enzymes can operate under almost anhydrous conditions.[1] Often a reaction medium consisting of an apolar organic solvent is used. Most enzymes are not soluble in the apolar organic solvents, and thus there is a need to create systems to improve the contact between the catalyst and its surroundings. A range of different alternative methods to create contact between the enzyme and the solvent are available today.

The microenvironment around the individual enzyme molecule has a great impact on the performance of the enzyme. Therefore, some efforts have been spent on developing different methods to expose the enzyme to the solvent. The easiest and one among the earliest was the use of dry enzyme that has been suspended in the solvent.[2] In this case a microenvironment which to a large extent was dominated by protein was created. However, since the enzyme also makes up the interior of the particles, diffusional hindrance can be expected. Chemical crosslinking of enzyme crystals has been used to increase the operational stability in organic media.[3] Another alternative that has become increasingly popular is to use the enzyme deposited on a solid support. The properties of the support strongly influence the behaviour of the enzyme and thus it has to be selected carefully.[4] The catalytic density of the supported enzyme is lower than with dry enzyme in the solution. Also diffusional limitations can be less pronounced in the immobilized preparations. Efforts have been made to achieve homogeneous catalysis by enzymes in organic media. The use of microemulsions to dissolve the enzyme creates systems that are optically transparent, in which the enzyme molecules are distributed within a large number of very small water droplets.[5] The water activity in these systems will be higher and more difficult to control as compared to the situation when the enzyme is immobilized. Furthermore, since the microemulsion systems are made up from an organic solvent, a detergent and water, the enzyme will be exposed to all the three types of reagents. Detergents may sometimes denature the enzyme. An alternative way to create a microdroplet around the individual enzyme molecule is to use PEG-modification,[6] in which polymer chains (PEG) are covalently coupled to the enzyme molecule. The preparations can then be dissolved in e.g. benzene and chloroform. Enzymatic activity is expressed upon addition of minute amounts of water to the system, probably because water partitions into the PEG-layer around the enzyme molecule and thus forms a water layer around it in the PEG-rich zone. Such preparations have been described for a range of enzymes. A problem is that upon PEG modification a lot of enzymatic activity is lost. The system behaves as if the enzyme is soluble in the bulk organic solvent and it is observed as optically transparent.

A further step along this path is to suspend the enzyme in a microemulsion where the detergent molecules are modified such that they can be polymerized to form very small solid particles in which the enzyme is entrapped.[7] Still the dimensions are so small that the preparations look optically clear to the naked eye or when analyzed in simple photometers.

The present paper reviews a new way to create a 'soluble' enzyme in organic solvents by forming non-covalent, reversible bonds between the enzyme molecule and polymer molecules.

Formation of Enzyme–Polymer Complexes

In studies with polymers as process aids for enzymatic reactions in organic media it has been observed that chymotrypsin in the presence of ethyl cellulose (apparent molecular weight: 54.5 kDa; degree of substitution: 2.42–2.53) can be dispersed in toluene. This could be a good alternative to covalent modification of enzymes with for example polyethylene glycol. Further studies have been carried out to investigate the dispersion process; they are reviewed in this paper.

Initially it was vital to investigate the size of the enzyme-containing species. A few studies were made using radioactively labelled albumin. The albumin was treated

with toluene containing ethyl cellulose. Samples were taken at intervals and filtered through a 5 μm filter. The amount of radioactivity in the filtrate increased with increasing incubation time during the 24 h of the experiment.[8] At the end of the experiment about 70 % of the radioactivity was detected in the filtrate. This indicates that complexes were formed between the enzyme and the polymer and that these complexes were smaller than 5 μm in size. It was also shown that the amount of solubilized albumin increased with increasing concentration of ethyl cellulose (Figure 1).

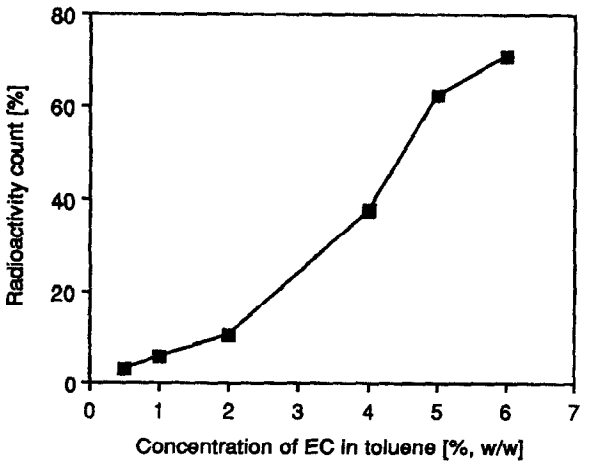

Figure 1. Solubilization of albumin in toluene by ethyl cellulose. Radioactivity count in the filtrate after filtration (5 μm filter) of toluene containing different concentrations of ethyl cellulose and labelled albumin. The total radioactivity in albumin was set at 100 %. (From ref. 8).

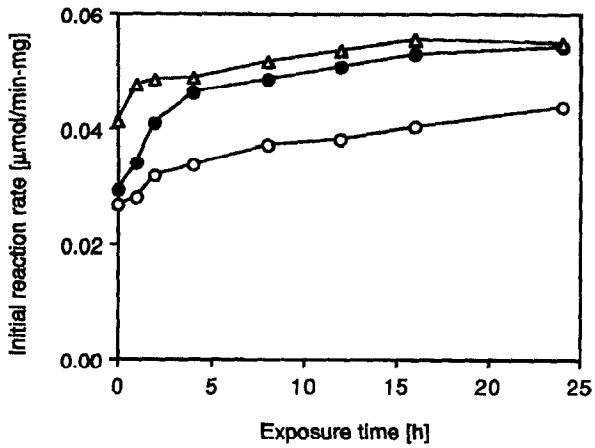

Figure 2. Initial activity of chymotrypsin suspended in toluene containing ethyl cellulose (5 %) with 0.5 %(v/v) water (m) or buffer (l), or freeze dried chymotrypsin–ethyl cellulose suspended in toluene with 0.5 % (v/v) water (Δ). The esterification of N-acetyl-L-phenylalanine (10 mM) with ethanol (1 M) was used as model reaction to measure the activity of samples after different exposure time in the solvent. (Recalculated from ref. 8).

It is a drawback that the formation of the complexes requires a long time. In an effort to promote complex formation, chymotrypsin and ethyl cellulose were freeze-dried together from an aqueous solution. When exposed to toluene the freeze-dried preparation showed higher activity than an enzyme powder exposed to toluene containing ethyl cellulose (Figure 2). However, when the enzyme powder contained a suitable amount of buffer salts the activity gradually increased and after 24 h it was equal to that of the freeze-dried ethyl cellulose–chymotrypsin preparation. This shows that complexes can be formed by just adding an enzyme powder containing suitable salts in a solution of the polymer in the organic solvent. However, the formation of the soluble complexes is speeded up by a prior freeze-drying of the enzyme together with the polymer.

Complexes between enzymes and polymers can be solubilized in many different solvents. In addition to toluene which was initially used, chloroform, ethyl acetate, 2-butanone, ethanol and others have been successfully used for dissolving complexes of chymotrypsin and ethyl cellulose. Furthermore, several polymers have been shown to solubilize chymotrypsin, for example poly vinyl butyral (average molecular weight: 36 kDa), polymethyl methacrylate (BDH, Poole, England; melt flow index: 4.0), polyvinyl methyl ketone (melting temperature: 160 °C) and polyethylene glycol (molecular weight: 40 kDa).[9] This shows that complex formation between proteins and polymers is a rather wide-spread phenomenon that can be utilized to dissolve proteins in organic solvents. A prerequisite probably being that the polymer itself should be soluble in the solvent.

It should be noted that also low molecular weight substances can be useful in solubilizing enzymes in organic media. The surfactant didocecyl glucosylglutamate was used to solubilize a *Pseudomonas* lipase.[10] The complexes had a molecular weight of about 130 kD. In later studies, several surfactants, e.g. dialkyl amphiphiles and several lipases have been used.[11] The complexes formed spontaneously when aqueous solutions of enzyme and surfactant were mixed. The complexes were not soluble in polar solvents like ethanol or water. Good solubility and enzymatic activity was observed in diisopropyl ether, benzene, toluene and hexane. In chlorinated hydrocarbons no activity was detected although the complexes were soluble.

Physical Characterisation of Enzyme–Polymer Complexes

Ethanol was used as solvent for spectrophotometric studies of the complexes. It was shown that dissolution of chymotrypsin in a solution of ethyl cellulose in ethanol resulted in an increase in absorbance similar to that caused by chymotrypsin dissolved in water (wavelength: 200–300 nm).[9] When the chymotrypsin powder was suspended in pure ethanol, no absorbance due to the enzyme was detected. This shows that ethyl cellulose was needed to dissolve chymotrypsin in the solvent.

The chymotrypsin–ethyl cellulose complexes were further characterized by light scattering measurements. The range of apparent molecular weights determined was 65–149 kD indicating that the complexes were small, containing just a few enzyme and polymer molecules.[8] An increase in the total concentration of ethyl cellulose–chymotrypsin

preparation resulted in an increase in apparent molecular weight of the complexes.

Influence of the water content

As with all other types of enzyme preparations used in organic media, the amount of water present in the reaction mixture is of great importance for the enzymatic activity. The best measure of the water content of the reaction mixture is the thermodynamic water activity. This parameter is most conveniently measured in the gas phase but at equilibrium it is equal in all phases. If pure water is chosen as the standard state the water activity in the gas phase is equal to the ratio of the actual partial pressure of water and the partial pressure of water above pure water. A certain water activity implies a certain hydration of the enzyme, irrespective of the presence of water absorbing substances. Basically, it is the hydration of the enzyme which governs its catalytic activity. In the reaction mixture there will be a distribution of water between the different phases present. The enzyme hydration depends not only on the total amount of water present, but also on the solubility of water in the reaction medium and the amount of water adsorbed by other components of the reaction mixture. When controlling the enzymatic activity in organic media, the water activity is thus a much more useful parameter than the water concentration.

However, the convenient vapour phase equilibration method used to achieve fixed water activity is not applicable when volatile, water soluble substrates like ethanol are used. Instead, experiments were carried out with fixed total amounts of water. The different components of the reaction mixture were dried (freeze-drying of enzyme preparations and drying with 4 Å molecular sieves for the solvent) so that their water content could be neglected in comparison with the amount of water added to the reaction mixture (normally 0.2–1.0 %, by vol.).[8] For chymotrypsin in toluene, the activity decreased with increasing amount of water in the range studied (Figure 3). When chymotrypsin was used in a solution of ethyl cellulose in toluene, an activity maximum was observed at 0.5 % water addition. Apparently, the presence of ethyl cellulose increased the amount of water required for optimal enzymatic activity. Probably water was bound directly to the polymer, thus reducing the amount of water available for the enzyme. When an enzyme–polymer preparation formed by freeze-drying was used in toluene, the catalytic activity increased with increasing amount of water in the range studied. It is interesting that the preformed complexes require even more water for optimal activity than complexes formed by dissolving the enzyme in polymer containing solvent.

Enzyme Stability in Enzyme–Polymer Complexes

The influence of the complex formation on enzyme stability was investigated. The residual activity of chymotrypsin after incubation for 1 h at 50 °C under different conditions was measured.[8] The presence of ethyl cellulose caused an increase in residual activity (Figure 4).

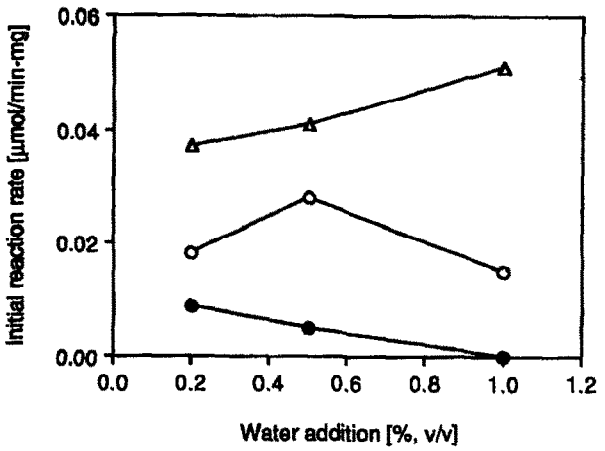

Figure 3. Initial reaction rate obtained with chymotrypsin preparations in toluene with different amounts of water added: enzyme powder in pure toluene (l), enzyme powder in toluene containing ethyl cellulose (m) and freeze dried chymotrypsin–ethyl cellulose preparation in toluene (Δ). The model reaction was the same as in Figure 2. (Recalculated from ref. 8).

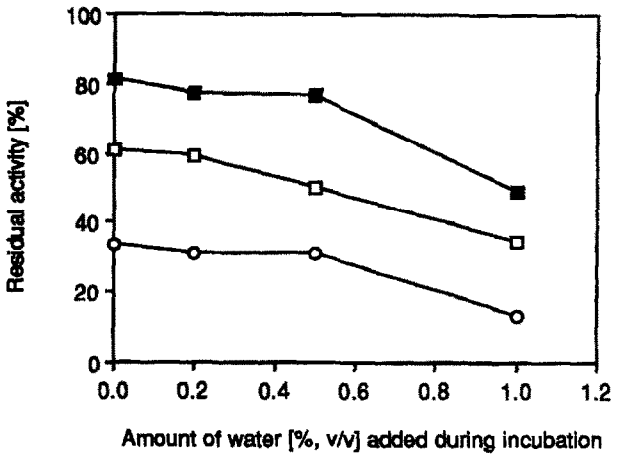

Figure 4. Residual activity of chymotrypsin preparations after incubation in toluene at 50 °C for 1 h: chymotrypsin powder in pure toluene (m), freeze dried chymotrypsin–ethyl cellulose preparation made with 10 mM sodium phosphate buffer, pH 7.8 (o) and freeze dried chymotrypsin–ethyl cellulose preparation made with 100 mM buffer (n). The activity was measured at 25 °C using the model reaction in Figure 2. (Recalculated from ref. 8)

Furthermore, the stability increased with increasing amounts of buffer present in the preparation. It is clear that buffer salts play an important role in the formation of active and stable complexes. The residual activity decreased with increasing amounts of water present during incubations. This agrees with previous studies on enzyme stability in organic media with different water contents. Water increases the internal flexibility of the enzyme and participates in several mechanisms of irreversible enzyme inactivation.[12]

The enzyme stability was further studied by differential scanning calorimetry. The denaturation of chymotrypsin in toluene was investigated.[13] Dry enzyme powder in toluene did not show any denaturation in the temperature range used up to 100 °C (Figure 5). However, after addition of water denaturation was observed. With 2 % water present, the denaturation temperature was 60 °C. When 10 % ethyl

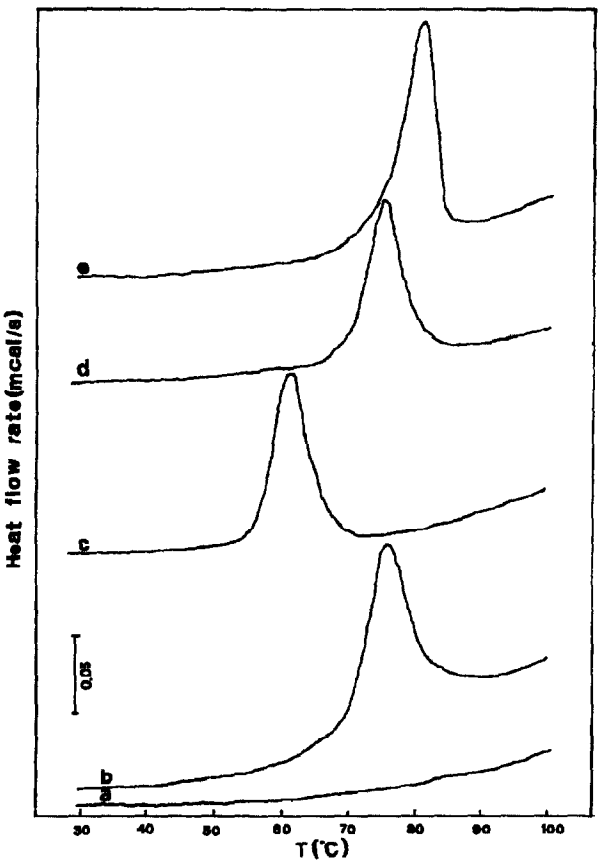

Figure 5. Thermograms of chymotrypsin in toluene: (a) without water addition, (b) with 1 % (v/v) water, (c) with 2 % (v/v) water, (d) with 10 % ethyl cellulose and 2 % (v/v) water, (e) freeze dried chymotrypsin–ethyl cellulose preparation and 2 % (v/v) water. (From ref. 13).

cellulose was present in the mixture with the same water content, a considerable stabilisation was obtained and denaturation temperature was increased from 60 to 75 °C. A thorough study showed that the denaturation temperature increased with increasing ethyl cellulose concentration up to about 15 % polymer (Figure 6). When a freeze-dried complex was studied an even higher denaturation temperature of 82 °C was observed. These data again show that water has a destabilising effect and that ethyl cellulose stabilises the enzyme.

Enzyme recovery

From a practical point of view it is often important that the enzyme and the product can be separated, so that a pure product can be obtained and the enzyme can be re-used. When using the enzyme–polymer complexes, this can easily be achieved by just adding water to the system. Water addition causes phase separation, and the enzyme can be recovered from the aqueous phase while hydrophobic products partition to the organic phase.

Kinetic Evaluation of Enzyme–Polymer Complexes

It is difficult to compare directly the results obtained with enzyme preparations of different types. Each type of preparation can be optimized separately with respect to water content, substrate concentration, amount of enzyme

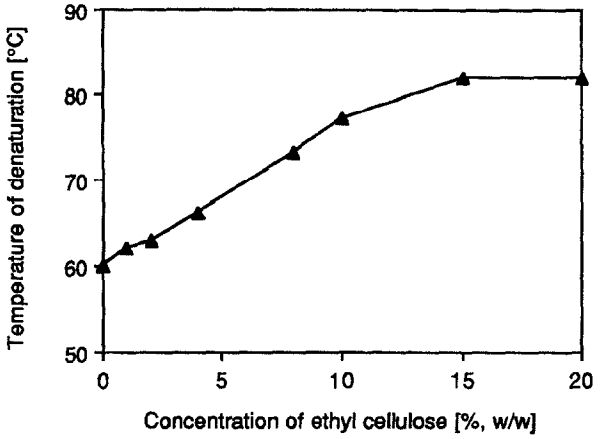

Figure 6. Denaturation temperature of chymotrypsin in toluene containing 2 % (v/v) water as a function of the concentration of ethyl cellulose. (Data from ref. 13).

relative to amount of support or complex former, etc. In Table 1 a few values obtained with the same reaction, in the same solvent and with the same substrate concentration are listed. All the specific activities are in the same range but the polymer complexes seem to give preparations with somewhat higher activity. A thorough investigation of kinetic constants (K_m and k_{cat} values) for the different preparations with controlled water activity is needed before definite conclusions can be made. An investigation of this kind has recently been published concerning lipase-catalyzed transesterification.[14] In this study the highest V_{max} value was obtained with a PEG-modified preparation and the Celite immobilized preparation yielded considerably higher activity than enzyme powder. Enzyme–polymer complex was not included in the investigation. However, this indicates that the highest reaction rates can be obtained with highly dispersed enzyme preparations like covalently modified enzymes or enzyme–polymer complexes. These two types of enzyme preparations seem to have quite similar properties. It was recently shown that subtilisin covalently modified with polyethylene glycol formed aggregates in solvents like toluene.[15] The hydrodynamic diameters of these aggregates were around 300 nm. Compared to covalent modification, noncovalent complex formation has the important advantage of being a considerably simpler technique. Furthermore, the risk of enzyme inactivation during the biocatalyst preparation is much smaller.

Table 1. Specific activity obtained with different chymotrypsin preparations in the esterification of N-acetyl-L-phenylalanine (10 mM) with ethanol (1 M) in 2 mL toluene. The amount of enzyme in each experiment was 1.6 mg and the total water content was 1.0 % (v/v).

Enzyme preparation	specific activity μmol/min mg	references
support: celite	0.016	Ref. 16
support: celite + EC [1]	0.026	Ref. 16
EC complex [1]	0.051	Ref. 8
PVB complex [2]	0.063	Ref. 9

[1] EC = ethyl cellulose
[2] PVB = polyvinyl butyral

Conclusions

Noncovalent complex formation between enzymes and polymers is a promising technique for the preparation of highly active biocatalysts for use in organic media. Mass transfer limitations can be assumed to be very low which is of special importance when fast reactions are carried out. By chosing a proper polymer the microenvironment around the enzyme can be tailored to suit the particular application. The complexes form transparent solutions in many organic solvents. This can be utilized for spectroscopic studies which can increase our knowledge about the properties of enzymes in organic media.

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