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# Acceleration of enzyme-catalyzed reactions in organic solvents by crown ethers

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**Abstract.** Crown ethers can strongly enhance the transesterification of *N*-acetyl-l-phenylalanine ethyl ester with 1-propanol catalyzed by α-chymotrypsin in various organic solvents. Of the different crown ethers tested, 18-C-6 gave the best results. It was found that a macrocyclic effect is responsible for the observed rate enhancement. It is proposed that the water- and cation-complexing ability of crown ethers plays a key role in the observed rate enhancement.

## Introduction

Recently, the action of enzymes in nearly anhydrous organic media has been gaining increasing interest[1]. The major advantages of these reactions in comparison with reactions in aqueous solution are enhanced (thermal) stability of the enzyme, novel substrate and stereospecificities, and the elimination of side reactions that involve water as the nucleophile[1]. For these enzymatic reactions, it is now known that, for high catalytic activity, the enzyme must be lyophilized from a buffer at optimal pH. Prior addition of high concentrations of ligand (inhibitor) to the buffer solution may result in a further increase of the catalytic activity[2]. It is believed that both the optimal pH and the ligand contribute to the fixation of the enzyme in a catalytically active conformation during lyophilization[2].

Another variable which strongly influences the catalytic activity is the amount of water present during the reaction in an organic solvent. An essential amount of water on the enzyme surface is necessary to retain the catalytically active conformation[2]. This can be achieved by selecting a sufficiently hydrophobic organic solvent (high $\log P$)[3], which does not remove this water mantle. More polar solvents can be used if the enzyme is immobilized on a hydrophilic support, which creates an enriched water concentration in the vicinity of the enzyme[4]. In some cases, water can be replaced by hydrogen-bonding solvents, such as formamide or glycol[5,6]. It has been reported[5] that, under optimized conditions, the catalytic activity of an enzyme suspended in organic solvents is comparable with its activity in aqueous solution. Under these conditions, the suspended enzyme contains up to 30% water. However, it is not clear whether the specific properties of the enzymes in organic solvents are still operative under these conditions.

Enzymatic reactions in organic solvents can also be performed in a "homogeneous" way if the enzyme is (covalently) linked to amphiphiles, such as polyethylene glycol, in order to make the enzyme surface more hydrophobic[7]. These authors reported that the catalytic activities of the modified enzymes are higher in comparison to the native enzyme suspension[8,9].

Some years ago, *Odell* and *Earlam* reported[10] that crown ethers and cryptands cause to dissolve proteins in methanol. It was proposed that these compounds complex with positively charged amino-acid side chains on the enzyme surface, thus making the enzyme more hydrophobic. This is analogous to the ability of crown ethers to dissolve alkylammonium and guanidinium salts in organic solvents[11,12,13]. In addition to cations, crown ethers are also able to complex alkali cations, protons, and water. All these species are present during enzymatic reactions in organic solvents. Therefore, we expected that crown ethers would have an effect on the enzymatic activity in these solvents and we have studied their influence on the transesterification reaction of *N*-acetyl-l-phenylalanine ethyl ester with 1-propanol[2].

## Experimental

### *Instrumental and analytical procedures*

Transesterification reactions were monitored by GLC (Varian 3400), equipped with a fused silica capillary column (J & W, liquid phase: DB-5$^+$, film thickness: 25 μm, 15 m × 0.322 mm).

Conditions: 2 min at 100°C followed by warming up to 190°C at 20°C/min.

Water contents in enzyme preparations and organic solvents were analyzed by a coulometric Karl Fisher method.

Pretreatment of the enzyme was performed by lyophilizing a buffered solution (0.1M phosphate buffer, pH 7.8) containing 0.5% (w/w) enzyme and 0.25% (w/w) *N*-acetyl-l-phenylalanine[2]. The enzyme, pretreated with potassium phosphate buffer, contained 11.8% (w/w) water, with sodium phosphate 6.1%, and with cesium phosphate buffer 4.2%.

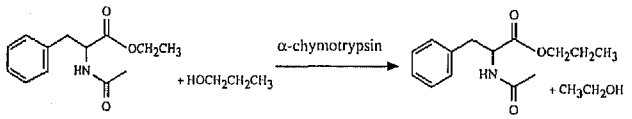
All reactions were performed at least twice on a 5-ml scale in vials with magnetic stirring (350 rev./min). Conditions: 2.5mM *N*-acetyl-l-phenylalanine ethyl ester, 1M 1-propanol, 0.5 mg pretreated enzyme/ml, 25°C. No reaction occurred under these conditions in the absence of enzyme.

*Materials*

The following solvents were used without further purification (% water v/v): toluene p.a. (0.006% $H_2O$), cyclohexane p.a. (0.002% $H_2O$), 2-methyl-2-butanol p.a. (0.02% $H_2O$) and 1-propanol (0.06% $H_2O$), all from Merck; octane c.p. (0.002% $H_2O$) (Janssen) and dibutyl ether (0.4% $H_2O$) (BDH). THF (Janssen) was freshly distilled from sodium benzophenone ketyl, followed by addition of 1% water.
Bovine pancreatic α-chymotrypsin (type II) with specific activity of 46 units/mg of solid was purchased from Sigma.
*N*-Acetyl-1-phenylalanine ethyl ester (Bachem), *N*-acetyl-1-phenylalanine (Sigma), 18-C-6 (Shell), dibenzo-18-C-6 (Aldrich), dicyclohexyl-18-C-6 (Aldrich), dibenzo-24-C-8 (Fluka), 15-C-5 (Merck), decyl-18-C-6 (Merck) and kryptofix 22 didecyl (Merck) were used as such. Monoaza-18-C-6 was synthesized according to the method of *Maeda* et al.[4].
Pentaglyme (hexaoxaoctadecane)[15] was synthesized from pentaethylene glycol (Aldrich) by reaction with sodium hydride and methyl iodide. The crown ethers contained less than 0.3% w/w water so that the ultimate concentration of water in the solution was not seriously affected by addition of crown ethers.

## Results and discussion[16]

The study of the effect of crown ethers on enzyme-catalyzed reactions in organic solvents was initially carried out with 18-crown-6 (18-C-6), because its complexing behaviour towards various charged and neutral compounds has been well documented[17]. The influence of 18-C-6 on the α-chymotrypsin-catalysed transesterification of *N*-acetyl-1-phenylalanine ethyl ester with 1-propanol was tested in various solvents (reaction 1).




Reaction 1

Furthermore, the dependence of 18-C-6 concentration on the kinetics and the effect of variation of the buffer cation on the influence of 18-C-6 were determined. Finally, we studied the influence of several other crown ethers on the reaction as well as that of the linear analog of 18-C-6.
In Table I, the initial velocities ($V_0$) in the absence and presence of 2mM 18-C-6 in different solvents are presented. The solvents are listed with decreasing log$P$ values. As shown for the blank reactions, $V_0$ is very dependent on the solvent used, in accordance with the literature[2]. Of the different solvent parameters indicating hydrophobicity, only the log$P$ value gives a good correlation with enzyme activity[3]. This is rationalized by the ability of the solvent to strip off the essential water layer from the enzyme. However, apart from the amount of water present on the catalyst, other solvent factors also appear to determine the activity, such as direct interactions between solvent and enzyme and/or the changing dielectric constant of the reaction medium[2]. This is demonstrated by the values of $V_0$ determined in dibutyl ether and toluene (Table I, entries 5 an 7).
Addition of 2mM 18-C-6 has a pronounced effect on the $V_0$ in all of the solvents tested, especially in the more hydrophobic ones[18]. The different effect of 18-C-6 in the various solvents can only be explained if more is known about the mechanism responsible for enhancement of the reaction. For this reason, we also studied the influence of several other parameters on the reaction.
The effect of variation of the 18-C-6 concentration on the $V_0$ at 18-C-6 concentrations of 0 to 4mM in toluene was determined. The results are plotted in Figure 1.

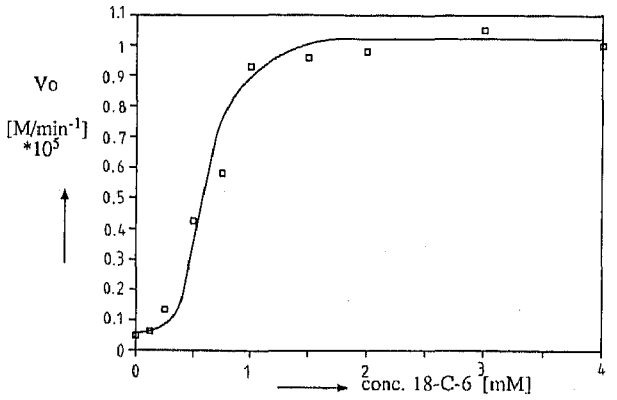



*Fig. 1. Influence of 18-C-6 concentration on initial velocity of reaction 1*[a].

[a] Conditions: 2.5mM ester, 1M PrOH in toluene, 0.5 mg enzyme/ml (pretreated with potassium phosphate buffer), 25°C.

Figure 1 shows that strong rate acceleration occurs in the range 0–1mM 18-C-6 and that a plateau is reached at 18-C-6 concentrations above approx. 2mM.
In Table II, the effects of various other crown ethers on the $V_0$ of the transesterifcation reaction in toluene are presented.

*Table I Influence of 18-C-6 on reaction 1 in various solvents*[a].

| Entry | Solvent | 18C6 (mM) | $V_0$ (M/min$^{-1}$) * $10^7$ | $V_0$(18C6)/$V_0$(blank) |
|---|---|---|---|---|
| 1 | octane | 0 | 54 ± 6 | 29 |
| 2 | | 2 | 1545 ± 80 | |
| 3 | cyclohexane | 0 | 42 ± 8 | 19 |
| 4 | | 2 | 805 ± 95 | |
| 5 | dibutyl ether | 0 | 1.7 ± 0.1 | 31 |
| 6 | | 2 | 53 ± 4 | |
| 7 | toluene | 0 | 5.0 ± 0.2 | 20 |
| 8 | | 2 | 98 ± 13 | |
| 9 | 2-methyl-2-pentanol | 0 | 0.20 ± 0.01 | 2 |
| 10 | | 2 | 0.40 ± 0.02 | |
| 11 | THF + 1% $H_2O$ | 0 | 1.8 ± 0.0 | 8 |
| 12 | | 2 | 14.5 ± 0.5 | |

[a] Conditions: 2.5mM ester, 1M PrOH, 0.5 mg enzyme/ml (pretreated with potassium phosphate buffer), 25°C.

*Table II Influence of various crown ethers on reaction 1 in toluene*[a].

| Entry | Crown ether | $V_0$(crown ether)/$V_0$(blank) |
|---|---|---|
| 1 | 18-C-6 | 19.6 |
| 2 | pentaglyme | 1.4 |
| 3 | monoaza-18-C-6 | 11.3 |
| 4 | decyl-18-C-6 | 1.9 |
| 5 | dicyclohexyl-18-C-6 | 4.0 |
| 6 | dibenzo-18-C-6 | 6.0 |
| 7 | dibenzo-24-C-8 | 1.8 |
| 8 | 15-C-5 | 1.8 |
| 9 | kryptofix 22 didecyl | 2.6 |

[a] Conditions: 2.5mM ester, 1M PrOH, 2mM crown ether, 0.5 mg enzyme/ml (pretreated with potassium phosphate buffer), 25°C.

Very strikingly, the linear analog of 18-C-6, pentaglyme $[CH_3-O-(-CH_2-CH_2-O-)_5-CH_3]$ applied under the same conditions as 18-C-6, has very little effect on the kinetics of the reaction. This is a strong indication that a macrocyclic effect is responsible for acceleration of the reaction. Furthermore, this experiment demonstrates that it is not solely the hydrogen-bond-accepting properties of 18-C-6 which cause the rate-enhancing effect. This effect has been proposed to explain the rate enhancement which results from addition of water analogs, such as glycol or formamide[5].

The most effective catalysis effect was obtained for the 18-membered crown ethers, especially for 18-C-6. This, together with the observation that a macrocyclic effect is operative, indicates that species which preferably form complexes with 18-C-6 must play a role in acceleration of the reaction. Species that meet this condition are alkali metal ions, especially potassium, water, and alkylammonium ions. Potassium ions were present in our reaction mixtures as a result of pretreatment of the enzyme with potassium phosphate buffer. In order to study the influence of this cation, we have also pretreated the enzyme with sodium and cesium phosphate buffers[19].

*Table III Effect of buffer cations on acceleration of 18-C-6 on reaction 1*[a].

| Cation | 18C6 (mM) | $V_0$(M/min) * $10^7$ | $V_0$(18C6)/$V_0$(blank) |
|---|---|---|---|
| $K^+$ | 0 | 54 ± 6 | 29 |
| | 2 | 1545 ± 80 | |
| $Cs^+$ | 0 | 28.5 ± 0.4 | 13 |
| | 2 | 368 ± 42 | |
| $Na^+$ | 0 | 19.2 ± 0.7 | 6 |
| | 2 | 108 ± 10 | |

[a] Conditions: 2.5mM ester, 1M PrOH in octane, 0.5 mg enzyme/ml, 25°C.

In Table III, the reactivities of these enzyme preparations in the transesterification reaction are shown. We must be cautious in comparing the absolute velocities of the different enzyme preparations with each other, because they contain different quantities of water. It is clear, however, that, also in these pretreated enzymes, addition of 18-C-6 had a pronounced effect on the $V_0$ of the transesterification. When the buffer cation is taken into account, the effect decreases in the order $K^+ > Cs^+ > Na^+$. The same sequence was found for the decrease in complexation ability of these ions with 18-C-6 in various solvents. At the molecular level, it is probable that, during lyophilization of the enzyme from a buffered solution, part of the active sites are blocked with buffer cations. Removal of these cations by complexation with crown ethers will result in more catalytically active enzymes. The different complexation ability of 18-C-6 towards the different cations, and the results presented in Table III, are in agreement with this assumption. However, an acceleration of 28 times in the presence of 18-C-6, as determined for $K^+$, means that, in the absence of 18-C-6, not more than 3–4% of the enzyme would be active. This is very low compared with the reported value of 60–70% catalytically active sites in a similarly prepared enzyme sample[2,20]. Thus, it seems very likely that crown ethers (also) have an effect on the rate-determining step of the transesterification reaction.

Complexation of water by crown ethers in organic solvents has been known for a long time[21]. In a systematic study, *Golovkova* et al.[22] determined the complexation ability of several crown ethers with water in $CDCl_3$. Complexation decreased in the order dicyclohexyl-18-C-6 > 18-C-6 > dibenzo-18-C-6 > dibenzo-24-C-8 > 15-C-5. Apart for dicyclohexyl-18-C-6, the same sequence was observed for the effect of the crown ethers on the enzymatic activity. The active site of α-chymotrypsin can contain up to 16 water molecules[23]. These water molecules must be displaced by the substrate before reaction can occur. Part of the water present in the bulk can form complexes with crown ethers[24]. This can result in partial dehydration of substrate and/or active site by a shift in the solvation equilibrium. Assuming that this process is (part of) a rate-determining step, a shift of equilibrium will lead to enhanced catalysis. Complexation of water in the organic solvent will not automatically mean that the protein becomes dehydrated, resulting in inactivation. Since crown ethers have comparable affinity for polar and apolar media (log*P* of simple crown ethers like 18-C-6 is about zero)[25], the complexation of charged groups on the enzyme surface by hydrated crown ethers seems to be very likely[10]. α-Chymotrypsin has 14 lysine side chains at the enzyme surface[26] which can be complexed. Of all the crown ethers tested, 18-C-6 has the highest affinity for these alkylammonium cations[27]. Complexation of buffer cations by crown ethers may also result in better accessibility of the substrate to the active site, as does the removal of water, which formerly complexed with these cations.

In conclusion, we present here a simple and inexpensive method for strongly enhancing enzymatic reactions in dry organic solvents. This was especially observed for several 18-membered crown ethers. It has been shown that a macrocyclic effect is responsible for the observed enhancement. Furthermore, the known behaviour of crown ethers towards alkylammonium cations, water, and alkali metals agrees with the proposed activation mechanism and the presented data. Work is in progress to further elucidate the mechanism of the observed rate enhancement by crown ethers.

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[19] The enzyme was also pretreated with lithium phosphate, *N*-ethylmorpholine · HBr and tris(hydroxymethyl)aminomethane buffers (0.1M, pH 7.8). However, the lyophilized samples were too hygroscopic and/or sticky to perform accurate measurements.
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