Crown ether activation of cross-linked subtilisin Carlsberg crystals in organic solvents

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The activity of cross-linked subtilisin Carlsberg crystals in the catalysis of peptide bond formation can be significantly enhanced by pretreatment of the enzyme crystals with crown ethers. Soaking of the enzyme crystals in a solution of crown ether in acetonitrile followed by evaporation of the solvent results in an up to 13 times enhanced enzymatic activity. The effects of crown ether treatment under various conditions gives support for the hypothesis that removal of bound water molecules from the active site during the drying process is the origin of the observed enzyme activation.

Introduction

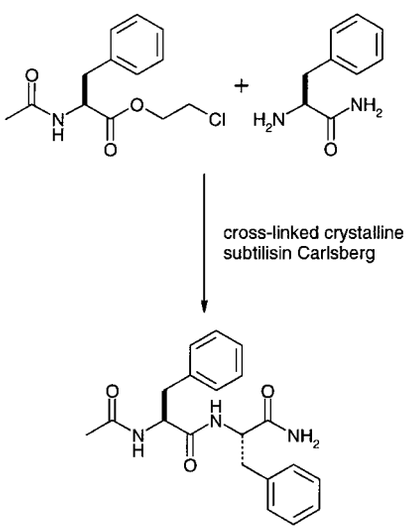
The advantages of using enzymes in non-aqueous organic solvents, like the enhanced thermal stability of the enzyme, a simple recovery of the suspended enzyme by filtration, increased substrate solubility, a shift of the equilibrium in favor of synthesis over hydrolysis, and the altered selectivities of the enzyme, are nowadays well recognized.[1] However, increasing the relatively low activity, and the stability and regeneration of the precious catalysts are still major issues in non-aqueous enzymology.

Enzymes suspended in organic solvents exhibit typically two to six orders of magnitude[2] lower catalytic activity than in aqueous solution. This drawback is one of the major reasons why enzymes in organic media are not yet widely applied. Our earlier studies on enzymes in organic media revealed that lyophilization of the enzymes in the presence of crown ethers gives large relative increases in the enzymatic activity in organic media. With this methodology it is possible to compensate partly for the dramatic reduction of activity commonly observed for enzymes in non-aqueous organic solvents.[3]

A new and promising technology to improve the *operational stability* of enzymes in organic solvents is the use of cross-linked enzyme crystals.[4] Although such crystals usually also have a relatively low catalytic activity in organic media, they are highly resistant against autolysis and thermal inactivation. These cross-linked enzyme crystals can be reused in many reaction cycles without appreciable loss of activity. Recently Halling *et al.*[5] showed that the activity of cross-linked crystals of subtilisin Carlsberg is strongly dependent on the dehydration procedure employed. Klibanov and Xu[6] found that the activity in organic solvents could be tuned by pH control with buffer salts which are soluble in the organic solvent. We have also investigated whether the inherent lower activity of cross-linked enzyme crystals can be enhanced by crown ethers; the activity of enzyme crystals of subtilisin Carlsberg in peptide bond formation in organic media was used as a model reaction. It appeared that simple soaking of the enzyme crystals in a solution of 18-crown-6 in acetonitrile followed by drying, resulted in a more than tenfold increase of the enzymatic activity.

Results and discussion

The effects of crown ethers on cross-linked crystalline enzymes were investigated using the peptide bond formation between the 2-chloroethyl ester of *N*-acetyl-L-phenylalanine and L-phenylalaninamide as a model reaction (Scheme 1). Cross-linked



Scheme 1 Protease-catalyzed peptide bond formation.

crystals of thermolysin and subtilisin Carlsberg catalyze peptide bond formation reactions.[7] Because subtilisin Carlsberg forms crystals[8] relatively easily and because we have previously determined the effects of crown ethers on the activity of an amorphous powder of this same enzyme as suspensions in organic solvents, we have used cross-linked enzyme crystals of this enzyme.[3f]

The effect of 18-crown-6 on the activity of cross-linked enzyme crystals was first investigated by two methods that we have previously reported for enzyme activation, *viz.* the direct addition of the crown ether to the enzymatic reaction medium[3b] and the pretreatment by lyophilization of the protein in the presence of the crown ether.[3c,f] Addition of 18-crown-6 to the reaction medium appeared to have no effect on the rate, v_0, of the enzyme-catalyzed peptide bond formation (Table 1). This result is in contrast with our earlier studies on α-chymotrypsin suspensions[3b] where the rate of transesterification could be enhanced by a factor of twenty upon the addition of crown ether to the solvent. Lyophilization of the enzyme crystals in the presence of 18-crown-6, however, showed some interesting effects (Table 1). Whereas lyophilization in the absence of crown ether resulted in a *decrease* to less than 10% of the original enzyme activity, the presence of 50 μmol of 18-crown-6 during the lyophilization process resulted in a sample with an 8.5 times larger activity, values which were both compared to the activity

Table 1 Effects of 18-crown-6 on cross-linked crystalline subtilisin Carlsberg by crown ether addition to the reaction mixture or by pretreatment with lyophilization. Conditions: see Experimental, taking into account the following difference; the reactions were performed with an enzyme crystal concentration of 2.5 mg ml^{-1}

Pretreatment	Amount of 18-crown-6 (μmol)	v_0/nmol min^{-1} mg^{-1}
Addition to the reaction medium	0	0.45
	12.5	0.50
	50.0	0.43
Lyophilization	0	0.03
	12.5	1.07
	50.0	3.80

Table 2 Effects of soaking and drying cross-linked crystalline subtilisin Carlsberg from a solution of crown ether in acetonitrile on the rate of peptide bond formation. Conditions: see Experimental. Enzyme crystals (1 mg ml^{-1}) were dried from acetonitrile which contained 15 mM of crown ether

Crown ether	v_0/nmol min^{-1} mg^{-1}	v_0(crown ether)/v_0
None	0.43	—
18-Crown-6	4.11	9.6
Monoaza-18-crown-6	4.82	11.2
15-Crown-5	1.25	2.9
Decyl-18-crown-6	0.54	1.2
Dicyclohexyl-18-crown-6	1.64	3.8
Dibenzo-18-crown-6	0.81	1.9
Dibenzo-24-crown-8	0.38	0.9
Pentaglyme	1.04	2.4

of the non-pretreated crystals. The reduction of the activity after lyophilization of the enzyme crystals might originate from a distortion of the enzyme conformation by crystallization of the water in the solvent-filled channels of the crystals. The presence of 18-crown-6 might prevent this process and in this respect 18-crown-6 may act as a lyophilization protecting agent. However, the enhancement by a factor of 8.5 indicates that 18-crown-6 must have an additional role as an activator of the enzyme catalysis (*vide infra*).

Another way of pretreating the cross-linked enzyme crystals with crown ethers is by soaking them in a crown ether solution of acetonitrile, followed by gentle evaporation of the solvent. The enzymatic activities given in Table 2 for the rates of peptide bond formation of *N*-Ac-L-Phe-L-Phe-NH_2 show that subtilisin Carlsberg crystals can be significantly activated by this procedure. The most effective crown ethers are 18-crown-6 and monoaza-18-crown-6, with rate enhancements of about ten times, whereas treatment with more hydrophobic and sterically hindered crown ethers, like dibenzo- and decyl-18-crown-6, proved to be less effective. The observed increase in rate of enzyme activity is first of all a *macrocyclic* effect, as pretreatment with pentaglyme, a linear analog of 18-crown-6, gives only a twofold rate enhancement.

Recently, Margolin *et al.*[9] reported that drying of buffer-soaked cross-linked crystals of lipase and alcalase by rinsing with surfactant solutions in organic solvents enhances the enzyme activity. However, after storage of the activated enzymes for two weeks the activation was lost in the case of most surfactants. Our cross-linked crystalline subtilisin Carlsberg activated by 18-crown-6, on the other hand, did not show any loss of activity even after storage for four weeks at 4 °C.

Previously, we have proposed two possible contributions to the crown ether activation on amorphous enzyme suspensions in organic solvents.[3f] Either crown ethers might prevent the formation of deactivating inter- and intramolecular salt bridges in organic solvents by complexation of the ammonium functions of lysine residues, or crown ethers may contribute to an enhanced substrate binding and consequently to a higher enzymatic activity by facilitating the transport of water molecules from the active site into the bulk organic solvent. The capability of crown ethers to form complexes with ammonium groups and water molecules by hydrogen bonding is well documented.[10] The effects of both types of complexation are most likely additive in the case of enzyme suspensions in organic solvents and crown ether induced activations up to 500 times have been achieved. In the case of cross-linked crystals, however, most of the accessible lysine amine functions have reacted with the cross-linking agent and are consequently converted into (non-complexing) imine functions. Moreover, unreacted free lysine residues, if present,[11] are most likely shielded and therefore complexation of lysine residues seems to be of minor importance for the crown ether effect in this case.

The most important contribution to the crown ether activation in the case of cross-linked enzyme crystals is most probably the facilitated transport of water molecules from the active site to the bulk organic solvent. Binding of the substrate to the active site requires (partial) dehydration.[12] It is known for α-chymotrypsin that the active site can contain up to sixteen water molecules.[13] In aqueous solutions the transfer of water molecules from the active site to the bulk solvent is entropically favorable due to the increase in translational and rotational freedom.[13] However, in organic solvents, especially when these have a low polarity, dehydration of the active site is an overall unfavorable process. Accompanied by an altered partitioning of the substrate and product molecules between the bulk organic phase and enzyme active site,[14] this will result in a much weaker binding of the substrate by the enzyme. Increased K_m values and thus a lowered enzymatic activity are found upon transfer of enzymes from an aqueous solution to an organic solvent.[15] Even in the polar acetonitrile (log $P = -0.33$) it is likely that water molecules are associated with enzymes suspended in an anhydrous solvent. The published X-ray structure of subtilisin Carlsberg in acetonitrile shows ninety-nine associated water molecules even after extensive washing with the solvent.[16] Crown ethers, that are able to complex water molecules in organic solvents,[17] will facilitate the transport of water from the active site to acetonitrile during the drying process of the enzyme crystals. Complexation constants of crown ether water complexes decrease from dicyclohexyl-18-crown-6 > 18-crown-6 > dibenzo-18-crown-6 > dibenzo-24-crown-8 to 15-crown-5.[18] This is in line with the effects of the crown ethers on the enzyme activity (Table 2), taking into account the differences in partitioning which are related to their hydrophobicity and steric demands.[19]

It is important to note that direct addition of 18-crown-6 to the reaction medium does not result in an increase in rate of the peptide bond formation reaction (Table 1). This shows that enzyme activation must occur during evaporation of the solvent. Most probably, in the direct addition method the crown ether concentration in the vicinity of the enzyme is not high enough to remove the water molecules from the active site. During the evaporation process, on the other hand, the crown ether concentration around the enzyme reaches very high concentrations. While almost equal rates in the case of drying in the presence of 18-crown-6 by solvent evaporation (Table 2, entry 2) or lyophilization (Table 1, entry 6) are obtained this suggests that the same mechanism, *i.e.* dehydration of the active site, is responsible for the observed effects.

Fig. 1 shows that the activity of cross-linked subtiline Carlsberg crystals is 13 times higher after drying the enzyme crystals in the presence of 50 mM 18-crown-6. This concentration corresponds to about 1350 mol equiv. of 18-crown-6 with respect to the enzyme. At this concentration a saturation curve is observed which suggests that 18-crown-6 has removed all water molecules necessary to enable substrate binding.

In conclusion, it is shown that the activity of dry cross-linked subtilisin Carlsberg crystals can be significantly enhanced by pretreatment with crown ethers *via* a soaking and drying pro-

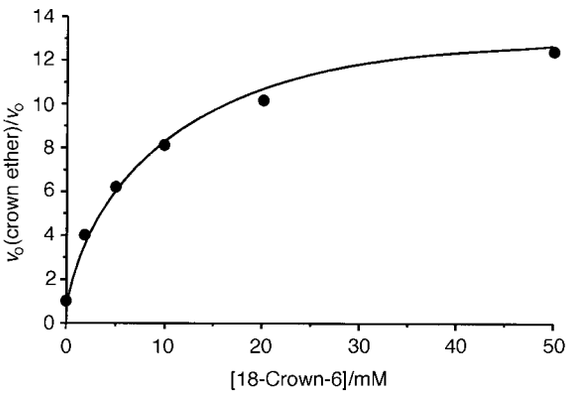


Fig. 1 Crown ether acceleration (v_0(18-crown-6)/v_0) for the peptide bond formation catalyzed by cross-linked subtilisin Carlsberg crystals between *N*-acetyl-L-Phe-OC_2H_4Cl and L-Phe-NH_2 in acetonitrile as a function of the amount of 18-crown-6 added during soaking and drying of the enzyme crystals from acetonitrile.

cedure. Drying of the enzyme crystals from an acetonitrile solution with 18-crown-6 results in an approximate tenfold increase in rate of the dipeptide formation. This method combines a major advantage of the use of cross-linked crystalline enzymes, *i.e.* a high stability, with an easy method of enhancing the enzyme activity. Therefore this method has good prospects for practical applications, as major problems of enzymatic catalysis in non-aqueous media, *i.e.* too low stability and too low enzymatic activity can be overcome by this approach.

Experimental

Cross-linked crystals of subtilisin Carlsberg were prepared according to the method of Schmitke *et al.*[8] The peptide precursors *N*-Ac-L-Phe and L-Phe-NH_2 were obtained from Sigma (St. Louis, MO). The 2-chloroethyl ester of *N*-Ac-L-Phe was synthesized from *N*-Ac-L-Phe and 2-chloroethanol using Amberlite IR-120 as a catalyst. 18-Crown-6 was a gift from Shell Laboratories (Amsterdam, The Netherlands). Other crown ethers were from Aldrich Chemical Co. The solvents were of analytical grade or higher and were from Acros (Geel, Belgium).

Pretreatment of subtilisin Carlsberg cross-linked enzyme crystals

Three different ways of pretreatment were investigated. In the experiments describing the effects of crown ethers on enzyme crystals during lyophilization, 2.5 mg of enzyme crystals were lyophilized for 24 hours in 500 μl 20 mM sodium phosphate buffer, pH 7.8, containing the indicated amount of crown ether, after rapid freezing of the samples in liquid nitrogen. In a second approach, crown ether was added directly to the reaction mixture without any additional pretreatment of the enzyme crystals. As the third approach, a soaking and drying procedure was applied. In this method 0.5 mg of cross-linked crystalline subtilisin Carlsberg was soaked in 500 μl acetonitrile containing the indicated concentration of crown ether. Subsequently the solvent was left to evaporate at room temperature overnight.

Studies on the effect of 18-crown-6 on the rate of dipeptide formation

Enzyme preparations, peptide precursors and solvent were equilibrated at a thermodynamic water activity of 0.113 above a saturated LiCl solution for 24 hours. Reactions were performed in duplicate on a 1 ml scale with magnetic stirring at 500 rev min^{-1}. Typical conditions: 0.5 mg ml^{-1} subtilisin Carlsberg crystals, 50 mM *N*-Ac-L-Phe-OC_2H_4Cl and 50 mM L-Phe-NH_2 in acetonitrile at 30 °C. The reactions were terminated by the addition of 4 volumes of dimethyl sulfoxide. The reaction mixture was analyzed by HPLC. Initial rates were calculated from conversions of <5%.

HPLC analysis

HPLC analysis was performed with a Waters HPLC equipment: 600 gradient pump, 717 autosampler and 996 photodiode array detector with Millenium software. A Waters μBondapak C_{18} 12.5 nm, 10 μm (300 × 3.9 mm) reversed-phase column was eluted at a flow rate of 1 ml min^{-1} with 30% acetonitrile in 10 mM phosphate–triethylamine buffer of pH 2.6. *N*-Ac-L-Phe-OEt was used as internal standard and calibration curves were made with enzymatically prepared *N*-Ac-L-Phe-L-Phe-NH_2.

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