Improved Enzyme Activity and Enantioselectivity in Organic Solvents by Methyl-β-cyclodextrin

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Abstract: The use of enzymes in organic solvents to introduce chirality to a number of relevant organic compounds has been well documented. However, there are still major drawbacks in such applications, in particular the frequently much lower enzyme activity under nonaqueous conditions. In addition, the reaction outcome (substrate enantioselectivity and reaction rates) cannot be accurately predicted. To overcome these limitations, herein we introduce methyl-β-cyclodextrin (MβCD) as a new macrocyclic additive to simultaneously enhance the activity and enantioselectivity of dehydrated subtilisin Carlsberg suspended in neat organic solvents. MβCD was efficient in dramatically increasing the activity and significantly improving the enantioselectivity of subtilisin in co-lyophilizates when compared to the powder lyophilized from buffer alone. The initial rate determined for the transesterification between *sec*-phenethyl alcohol and vinyl butyrate increased by up to 164-fold and the enantioselectivity could be doubled. In addition, marked solvent effects were noted. To investigate the possible relationship between enzyme structure and these kinetic data, the secondary structure of subtilisin was investigated by Fourier transform infrared (FTIR) spectroscopy under all relevant conditions. Using the α-helix content determined from the amide I vibrational band as the main quantitative parameter, we found that MβCD is partially efficient in ameliorating dehydration-induced structural perturbations. Suspension of the subtilisin−MβCD co-lyophilizate in the various solvents revealed solvent-induced structural perturbations in some of them (e.g., acetonitrile), while no such changes were observed in others (e.g., THF and 1,4-dioxane). For the first time the results demonstrated that enantioselectivity and structural intactness in the various solvents were clearly related. Increase in the enzyme activity in contrast is mainly caused by increased structural mobility of subtilisin in the solvents by MβCD. We conclude that it is important to carefully select the additive and the solvent system to achieve high enantioselectivity and activity in such applications. Simultaneous improvement of both enzyme properties requires careful optimization of the enzyme formulation and proper selection of a suitable solvent. FTIR spectroscopy has proven to be a very valuable methodology to structurally guide such an optimization procedure.

Introduction

Enantiomeric purity in synthetic organic reactions has become very important, in particular in the synthesis of pharmaceuticals.[1] Enzymes are extremely valuable catalysts in this context because of their properties, which include high specificity and enantio- and prochiral selectivity. These natural catalysts have also been shown to be very useful for the introduction of chirality under nonaqueous conditions.[2] However, enzymes are frequently poorly active in organic media when compared to their respective activities in their natural aqueous environment.[3] It is well documented that lyophilization (the most frequent method of enzyme preparation) causes pronounced structural perturbations for most proteins, including the model enzyme in this study, subtilisin Carlsberg.[4] Such lyophilization-induced structural perturbations could contribute to the observed loss of enzymatic activity and in particular enantioselectivity when employing suspended, dehydrated enzyme powders in such applications. Furthermore, possible solvent-induced denaturation

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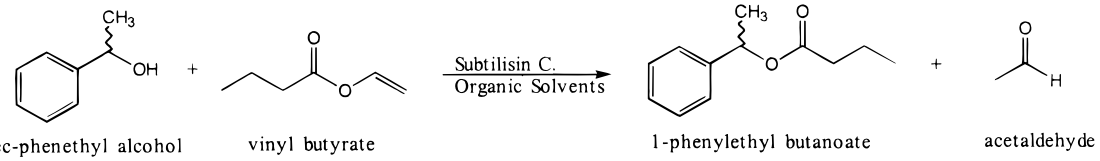
Scheme 1



of the suspended enzyme catalysts might also contribute to the loss in activity and enantioselectivity.

To overcome these problems and make enzymes as asymmetric catalysts more appealing to the organic chemist, many strategies increasing their potency in organic solvents have been explored. These include the mode of enzyme preparation,[5a] the control of the pH value,[5b] co-lyophilization with lyoprotectants[5c] and salts,[5d,e] addition of water-mimicking agents,[5f] imprinting with substrates and substrate analogues,[5g,h] immobilization,[5i,j] solubilization,[5k−p] mutagenesis,[5q] formation of substrate salts,[5r] and cross-link crystallization.[2c] One of the most successful groups of activating additives identified thus far are crown ethers.[6] While the mechanism of activation is still somewhat speculative, co-lyophilization of various enzymes with the crown ether 18-crown-6 in particular resulted in highly active and enantioselective formulations in organic solvents. It is reasonable to assume that such crown ethers bind to surface amino acid residues (in particular the ϵ-amino group of lysine) and that this binding alters the enzyme properties beneficially. For example, due to the amphiphilic character of the crown ethers they could increase the flexibility of the enzymes in the solvents as a result of such binding. Such increased structural mobility has been demonstrated by Broos et al. (1996) as a possible mechanism of how some solvents enhance the enzyme catalytic rate.[7] Alternatively, crown ethers could also activate enzymes by facilitating the removal of water molecules from the active site upon substrate binding.[6b]

In the current study we present data and mechanistic insights on enzyme activation by another class of macrocyclic excipients, cyclodextrins. They are versatile cyclic compounds that can form inclusion complexes with a number of guest molecules. As a result, they are useful in a number of applications ranging from chiral separations to drug delivery and as enzyme mimics.[8] These diverse applications of cyclodextrins motivated us to study their effect on the activity of subtilisin in organic solvents. It has been shown recently that cyclodextrins when added to a buffer solution containing a protein reduce its mean unfolding temperature.[9] The author concluded that cyclodextrins form inclusion complexes with the side chains of buried hydrophobic residues of the protein, shifting the equilibrium in favor of the unfolded polypeptide. The potential binding of cyclodextrins to amino acid residues is very intriguing in light of the enzyme activation by crown ethers in organic solvents.[6] Therefore, we decided to investigate their effect on the serine protease subtilisin suspended in organic solvents, and in particular, on the reaction rates and the enzyme enantioselectivity. After finding the additive methyl-β-cyclodextrin (MβCD) very efficient in enhancing the enzyme performance, we also addressed simultaneously the influence of this additive on enzyme structure and rigidity under various conditions leading to new mechanistic insights.

Results and Discussion

Subtilisin, our model enzyme, was lyophilized from an aqueous buffer solution (pH 7.8) with or without various cyclodextrins. The preparations were suspended in various typical organic solvents (THF, acetonitrile, 1,4-dioxane, toluene, dichloromethane, and octane). The kinetics of the product formation in the well-characterized and well-understood transesterification between *sec*-phenethyl alcohol and vinyl butyrate was followed by gas chromatography (Scheme 1).[2g,10]

No enhancement effect on the enzyme enantioselectivity or the reaction rate was observed when subtilisin was prepared in the presence of α- and γ-cyclodextrin. However, a marginal increase in the enzyme enantioselectivity and catalytic rate was observed when the enzyme was prepared in the presence of β-cyclodextrin (βCD), at a 1:1 weight ratio (data not shown). The effect resembles those found with crown ethers where the size of the cavity determines the efficiency of the additive in enzyme activation.[6] For example, α-chymotrypsin activity is enhanced more by 18-crown-6 than by 15-crown-5 or 12-crown-4.[6] Regarding the chemical similarity of α-, β-, and γ-CD, the difference in activation suggests specific macrocycle−enzyme interactions. The cavity sizes of α-, β-, and γ-CD are ca. 0.47−0.53, 0.6−0.65, and 0.75−0.83 nm, respectively.[8g]

Since it has been reported in the literature that the magnitude of the enzyme enhancement in organic solvents by macrocyclic

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Table 1. Enantioselectivity, Initial Rate, and α-Helix Content of Subtilisin Carlsberg in Different Organic Solvents

solvent		lyophilized[a,b]	MβCD co-lyophilizate[a,c]	enhancement	α-helix (%)[d]
THF	V_S[e]	0.014 ± 0.004	2.3 ± 0.6	164	32 ± 1
	V_R	0.00043 ± 0.00015	0.039 ± 0.009	91	
	E[f]	32 ± 2	59 ± 4	1.8	
1,4-dioxane	V_S	0.034 ± 0.021	3.8 ± 1.5	112	30 ± 1
	V_R	0.0011 ± 0.0005	0.085 ± 0.03	77	
	E	30.7 ± 8.9	45 ± 1.5	1.5	
CH_2Cl_2	V_S	0.0014	0.027 ± 0.002	19	26 ± 1
	V_R	0.00015	0.0026 ± 0.0002	17	
	E	9.5	10.2 ± 0.03	1.1	
acetonitrile	V_S	0.0050 ± 0.0026	0.085 ± 0.006	17	22 ± 1
	V_R	0.0012 ± 0.0007	0.016 ± 0.003	13	
	E	4.6 ± 0.6	5.4 ± 0.8	1.2	
toluene	V_S	0.0035 ± 0.0005	0.04 ± 0.027	11	26 ± 1
	V_R	0.00035 ± 0.00005	0.0062 ± 0.0023	18	
	E	11.8 ± 0.6	7.4 ± 1.4	0.63	
octane	V_S	0.015 ± 0.005	0.058 ± 0.033	4	29 ± 0
	V_R	0.0015 ± 0.0005	0.010 ± 0.003	6	

[a] Enzyme concentration 1 mg/mL in all experiments. [b] Lyophilized from aqueous phosphate buffer, pH 7.8. [c] Lyophilized from aqueous phosphate buffer containing MβCD at a 1:6 weight ratio of subtilisin to excipient. [d] α-helix content determined for the suspended subtilisin−MβCD co-lyophilized by Gaussian curve fitting of the amide I IR spectra after resolution enhancement by Fourier-self-deconvolution (FWHM 24 cm^{-1}, k = 2.4). [e] Initial rates in μmol mg^{-1} min^{-1}. [f] $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S$ (enzyme enantioselectivity).

compounds depends on the ratio of additive to enzyme,[6b] we increased the concentration of the additive. It was necessary to employ methyl-β-cyclodextrin (MβCD) in this instance, due to the limited solubility of βCD in water. MβCD is highly soluble in water, and yet it shares similar chemical properties with its counterpart βCD. When MβCD was employed at a 1:6 weight ratio of enzyme to excipient, a significant activation of subtilisin occurred when compared with the data for the enzyme lyophilized from buffer alone (Table 1). The initial rates were larger for the MβCD formulation under all solvent conditions tested. The activation of the enzyme was in particular pronounced in the solvents THF and 1,4-dioxane. The initial rates obtained for the "*S*" enantiomer using this new enzyme preparation were 164- and 112-fold larger in THF and 1,4-dioxane, respectively, than for the enzyme lyophilized without the additive. In addition, enzyme enantioselectivity of this new preparation was also significantly higher in THF (2 times) and in 1,4-dioxane (1.5 times) than for the enzyme lyophilized without MβCD. In other solvents enantioselectivity was not significantly influenced by the additive (in CH_2Cl_2 and acetonitrile) or even dropped (in toluene and octane). We excluded nonenzymatic cyclodextrin mediated transesterification in control experiments. No reaction was observed in the absence of the enzyme. In an additional control experiment, subtilisin was lyophilized from buffer and suspended in THF, and MβCD was added next to the reaction mixture at the 1:6 weight ratio as before. No effect of the additive on the initial rates and enantioselectivity was noted in these experiments. Two conclusions can be derived from this. First, MβCD must indeed interact in a specific way with the enzyme, probably by binding to surface residues. This behavior must be the result of the pretreatment of the enzyme (lyophilization in the presence of the additive). Therefore, the situation resembles those found with crown ethers where highly active preparations can also only be obtained when the enzymes are co-lyophilized with the additive.[6b] Second, substrate−MβCD inclusion complexes formed in the solvent can be excluded to contribute to the improvement in enantioselectivity. In principle, preferential binding of one enantiomer over the other by MβCD in the solvent could also have caused changes in the enantioselectivity.

Next, we tried to relate the determined enantioselectivity values with various solvent parameters. In some previous works, enantioselectivity has been shown to be related to solvent hydrophobicity,[11] dipole moment,[2g] and dielectric constant.[2g] However, in each of these examples specific reasons were hypothesized to account for the observed effects, in particular differences in water replacement from the active site by the different enantiomers.[12] In the case of the substrate chosen in this work, no correlation was found with any of the preparations (powder lyophilized from buffer alone or MβCD−enzyme formulation), between the enzyme enantioselectivity and any of the solvents physicochemical properties mentioned above.[2g]

To further investigate the somewhat puzzling observation that reaction rates improved under all conditions while enantioselectivity increased or decreased depending on the solvent, FTIR studies were conducted to investigate possible structural contributions. We determined the effect of the additive MβCD on the secondary structure of subtilisin in the lyophilized state and suspended in organic solvents. It is well established that lyophilization causes significant structural changes in subtilisin.[4] Significant spectral changes occurred in subtilisin upon lyophilization from aqueous buffer solution (Figure 1). The amplitude of all spectral components increased relative to the α-helix band at ca. 1658 cm^{-1}. Quantitative data revealed a significant decrease in the α-helix content from 34% to 25% and an increase in the β-sheet content from 15% to 32% in agreement with previous data.[4b] Co-lyophilization of subtilisin with MβCD prevented these structural changes to some extent. In particular, the bands at ca. 1646 and 1631 cm^{-1} did not show a significant intensity increase when compared to the aqueous spectrum (Figure 1). However, significant amide I spectral changes still occurred at wavenumbers above 1658 cm^{-1}. Quantitative data agree with the reduced extent of lyophilization-induced structural perturbations in the MβCD co-lyophilizate. The decrease in the α-helix content (30 ± 1%) was approximately halved compared to that in the case of no additive.

Next, we suspended the MβCD−subtilisin co-lyophilizate in the organic solvents used in the kinetic experiments. It has been established previously that suspension of subtilisin lyophilized from a phosphate buffer in nonprotein dissolving organic solvents caused very small spectral changes.[4b] In the aforemen-

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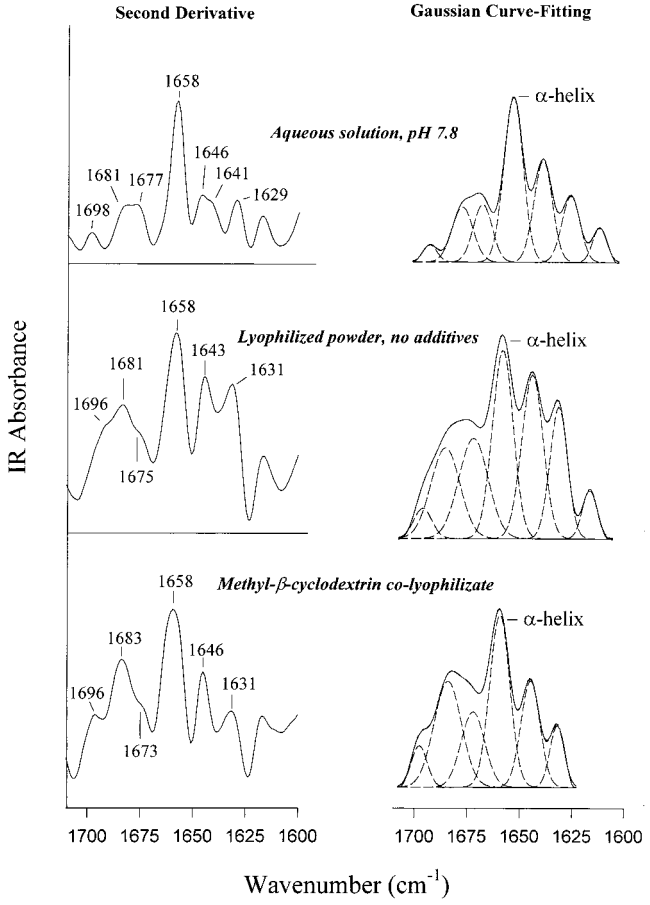

Figure 1. Amide I FTIR spectra of subtilisin under various conditions: (left) after second derivatization and (right) Fourier self-devonvoluted FTIR spectra and Gaussian curve-fitting. The solid lines represent the superimposed FSD spectra and the results of the curve fitting; individual Gaussian bands are shown with a dashed line.

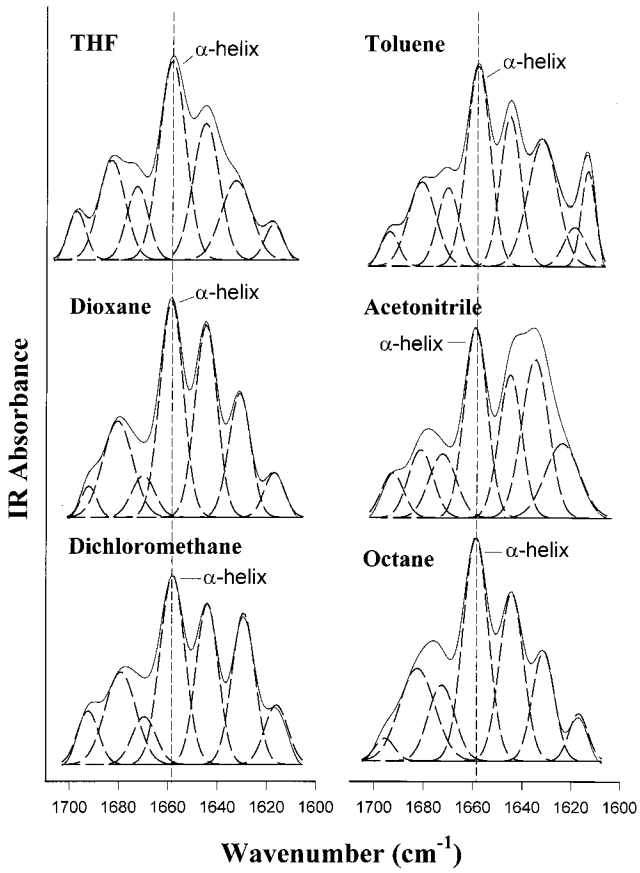

Figure 2. Fourier self-devonvoluted FTIR spectra and Gaussian curve-fitting of subtilisin Carlsberg co-lyophilized with MβCD and suspended in various solvents in the amide I spectral region. The solid lines represent superimposed FSD spectra and the results of the curve-fitting; individual Gaussian bands are shown with a dashed line.

tioned work, the α-helix content for the lyophilized protein was determined at 26%. For suspensions of this powder in the following solvents the α-helix contents were determined as follows in octane (28%), toluene (26%), THF (27%), dioxane (26%), and acetonitrile (27%). In contrast, we found significant solvent-dependent structural changes in the MβCD co-lyophilizate. While suspension of the co-lyophilizate in THF, dioxane, and octane (Figure 2) caused very small spectral changes when compared to the spectrum of the co-lyophilizate in the dry state (Figure 1), suspension of it in toluene, dichloromethane, and in particular acetonitrile caused significant solvent-induced spectral and thus structural alterations. Quantitative analysis of the FTIR spectra obtained supports this observation (Table 1). The α-helix content determined for the subtilisin−MβCD co-lyophilizate was the same in suspensions in THF, dioxane, and octane as prior to suspension. A minor increase in the β-sheet content was noted (data not shown). In contrast, significant decreases were diagnosed for the α-helix content when the co-lyophilizate was suspended in toluene, dichloromethane, and acetonitrile. Next, we investigated whether these solvent-induced protein structural perturbations and activity could be related. No correlation is evident when plotting the initial rate for the "*S*" enantiomer (V_S) versus the α-helix content as indicator for the overall structure of the enzyme (Table 1). However, in the two cases where significant enzyme activation occurred (in THF and dioxane), no solvent-induced denaturation occurred and enzyme structure was the same as for the co-lyophilizate with MβCD. This might be an indication that preservation of enzyme structure is essential in obtaining highly active enzyme preparations in organic solvents. However, the lack of correlation between the enzyme structure and initial rates indicates that there must be other factors which also contribute significantly to the reaction rates.[3] Such factors include solvent-dependent variations in enzyme flexibility and substrate desolvation.[3] The latter observation is particularly evident when analyzing the situation in acetonitrile. Even though the FTIR spectra (Figures 1 and 2) and α-helix content (Table 1) indicate significant solvent-induced structural perturbations in the MβCD co-lyophilizate, activity is still higher than for the powder lyophilized from phosphate buffer. These results are in agreement with the data reported by Griebenow and Klibanov[4b] and Dong et al.[4d] where no correlation between enzyme structure and initial rates was found.

The larger susceptibility of the subtilisin−MβCD co-lyophilizate to solvent-induced structural alterations, when compared to the enzyme without the additive, indicates a reduction in the conformational stability ("rigidity") of subtilisin caused by the additive. Similar conclusions have been derived when employing crown ethers as additives[6] and also when the molecular lubricant water was added to the organic solvent.[13] To further investigate differences in the stability of subtilisin in the presence and absence of MβCD we conducted thermal inactivation experiments. In the first set of experiments the lyophilized subtilisin samples were incubated at 45 °C in the organic solvent THF (which was chosen because the subtilisin

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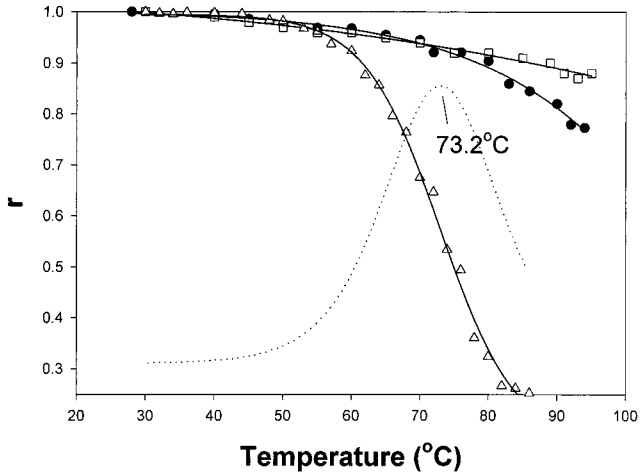

Figure 3. Thermal denaturation curves for subtilisin: (△) in D_2O, pD 7.8; (●) dried from aqueous buffer solution, pH 7.8, and exposed to 1,4-dioxane; and (□) co-dried from aqueous buffer with MβCD at a 1:6 weight ratio of protein−additive in 1,4-dioxane. The spectral correlation coefficient (r) was calculated using the second derivative amide I FTIR spectra of the samples at 30 °C and at elevated temperatures. A decrease in the correlation coefficient to values <1 indicates temperature-induced protein structural perturbations. The dotted curve is the inverted first derivative of the thermal denaturation of subtilisin in D_2O (△), and the T_m can be obtained from its maximum,[25] as indicated.

activation was most pronounced in it, Table 1). After various incubation times the activity and enantioselectivity were determined. We found that the initial rates for the lyophilized powder decreased exponentially by factors of 6 (*S* enantiomer) and 5 (*R* enantiomer) within the time period of 100 h. For the subtilisin−MβCD co-lyophilizate initial rates decreased by a factor of more than 20 for both enantiomers within less than 50 h. This highlights the increased susceptibility of subtilisin in THF toward thermal inactivation in the presence of MβCD. Similarily, we observed a drop in activity for lyophilized subtilisin in 1,4-dioxane by factors of 4 (*S* enantiomer) and 1.6 (*R* enantiomer) when increasing the temperature from 45 to 85 °C.[14] Under the same experimental conditions, activity of the subtilisin−MβCD co-lyophilizate decreased by a factor of ca. 450 and 131, respectively. Again, these experiments suggest that the additive MβCD caused a significant decrease in the conformational stability of the enzyme. Finally, thermal denaturation experiments were conducted in conjunction with FTIR spectroscopy. For these experiments subtilisin was prepared as a thin dry film on a CaF_2 window in the absence and presence of MβCD. Measurements were performed exposing these films to 1,4-dioxane.[14] From plots of the spectral correlation coefficient r[4e,f] as an indicator for overall structural changes versus the temperature, it is evident that the thermal stability of subtilisin is significantly decreased by MβCD (Figure 3). This indicates that the conformational flexibility of dehydrated subtilisin is enhanced by MβCD in 1,4-dioxane. However, thermal stability still was much higher than for subtilisin in aqueous solution where the T_m determined was 73.2 °C at pD 7.8.

It is intriguing that the only relationship between structural and dynamic properties of enzymes in organic solvents and their activity identified thus far is that between the structural mobility

(14) 1,4-Dioxane was chosen as the solvent in the thermal denaturation experiments because of its relatively high boiling point (101.3 °C). The enhancements in catalytic rate and enantioselectivity in this solvent when employing the MβCD−subtilisin co-lyophilizate were not as excellent as in THF, but still significant.

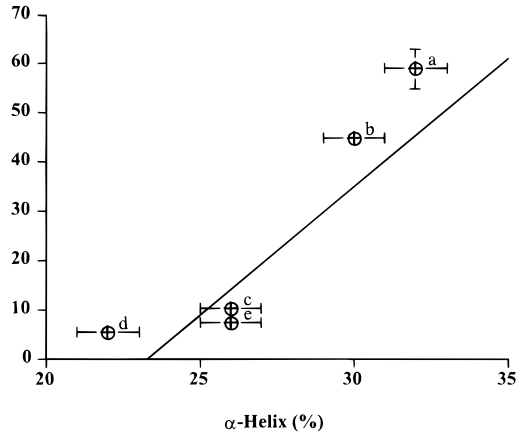

Figure 4. Dependence of the enzyme enantioselectivity on the α-helix content for the subtilisin−MβCD formulation. Solvents: (a) THF, (b) 1,4-dioxane, (c) CH_2Cl_2, (d) acetonitrile, and (e) toluene.

("flexibility") and the reaction rates.[7] Therein, it has been reported that for lyophilized subtilisin suspended in different organic solvents, kinetic data and fluorescence anisotropy data could be related.[7] High reaction rates and high enzyme enantioselectivity were extrapolated to higher flexibility of the enzyme catalyst. According to Bross et al., the more flexible the enzyme is, the greater the probability that the enzyme achieves a more active and enantioselective conformation. This concept is very attractive, in particular, in light of recent investigations involving thermostable enzymes.[16] It has been shown that at optimum activity level, the mesophilic and thermophilic isoenzymes 3-isopropylmalate dehydrogenase adopt a very similar conformational flexibility. At room temperature the thermophilic form is nearly inactive and conformational mobility largely restricted. All results on the conformational stability of subtilisin reported in this work indicate decreased rigidity of subtilisin in the organic solvents in the presence of MβCD. Therefore, we conclude that activation of subtilisin by MβCD is likely primarily due to increased flexibility of the enzyme in the solvents. It can be excluded that enzyme structural alterations in typical applications largely contribute to differences in enzyme activity. It is exciting to note that the same delicate balance between stability and flexibility seems to play an important role for enzyme function in organic solvents as well. To achieve high reaction rates at moderate temperatures it seems to be necessary to adjust the structural mobility. However, further investigations similar to those conducted in aqueous solution[16] measuring the molecular mobility and activity at various temperatures are certainly necessary to validate this point of view.

Having identified possible rationales to explain the subtilisin activation by MβCD, we then focused on the possible relationship between enantioselectivity and protein structural integrity. When plotting the enantioselectivity E versus the α-helix content, a clear correlation between enzyme structure and function emerged (Figure 4). The higher the α-helix content (and thus, the less perturbed the enzyme structure), the higher the enzyme enantioselectivity. The only exception is for the data point obtained in octane. However, in this solvent the limited

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solubility of MβCD is a likely explanation. Due to insignificant solubility the additive might partially block the active site of the enzyme preventing the substrate from "correct" binding. Consequently, substrate binding would be less specific and therefore enantioselectivity lower than expected from the structural analysis. The sluggish increase in activity in octane also indicates the partial blockage of the active site by MβCD in this solvent (Table 1). The MβCD additive is soluble in the other solvents studied.[17]

That enantioselectivity and structure are related is not unexpected because of the need to preserve the three-dimensional structure of the enzymes' active site to achieve high enantioselectivity. Differences in enantioselectivity arise from differences in the binding of the *S* and *R* enantiomer.[12] Similar conclusions were derived recently when comparing lyophilized subtilisin with subtilisin cross-linked enzyme crystals (CLECs).[18]

As mentioned earlier, several factors that contribute to the solvent dependence of enzyme enantioselectivity have been identified. These factors include the desolvation of the substrate tetrahedral intermediate, exclusion of water from the active site, the flexibility of the catalyst, and the structural integrity of the suspended catalyst. Our data provide evidence that structural preservation is a criterion of utmost importance when trying to improve enantioselectivity by solvent engineering. If the catalyst undergoes solvent-induced structural changes, then the latter influence the enantioselectivity strongly. Consequently, solvent effects on enantioselectivity cannot be studied under such conditions. In this light, it is expected that in this work we would not find a correlation between any of the solvent parameters and the enantioselectivity. This is indeed the case: enantioselectivity does not correlate with any of the solvent parameters tested (hydrophobicity, dielectric constant, dipole moment, and polarity). Our results and those reported by others imply that CLECs should be primarily employed in mechanistic investigations of the effect of the solvent on enantioselectivity and other selectivity because solvent effects on enzyme structure are prohibited, or at least minimized, due to structural constraints.[18−20] However, while there may be advantages in such mechanistic investigations to employ CLECs, there are also significant drawbacks. For example, subtilisin CLECs do not have a much higher activity in organic solvents than the lyophilized powder.[3] Currently no efficient strategies for the activation of such crystals are available.[21]

Last, we addressed the question whether the improvements in enzyme activity and enantioselectivity afforded by MβCD are also found with a different enzyme. We studied the kinetics of the model transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate catalyzed by *Candida rugosa* lipase in THF. Our results show that the effect of MβCD on the lipase is similar to that observed with subtilisin. When the lipase was co-lyophilized with MβCD, a 16.8-fold increase in the initial rate of the "*R*" enantiomer and a 2.7-fold increase in the enzyme enantioselectivity was observed **(Table 2**). Therefore, enzyme enhancement by MβCD seems to be of a general nature.

Table 2. Enantioselectivity and Initial Rates of *Candida rugosa* Lipase Suspended in THF

	lyophilized (buffer only)[a]	lyophilized with MβCD[a]
V_S[b]	0.00082	0.0051
V_R	0.0019	0.032
E[c]	2.3	6.3

[a] Enzyme concentration 1 mg/mL in all experiments. [b] Initial rates in μmol mg^{-1} min^{-1} [c] $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S$ (enzyme enantioselectivity)

Experimental Section

Materials. The solvents were purchased in the anhydrous form (in Aldrich Sure/Seal bottles, water content below 0.005%). All solvents used for this study were predried prior to their use as recommended (THF and dioxane were dried by distilling them from Na, and CH_3CN from CaH), and they were transferred to the reaction vial under N_2.

βCD and MβCD were purchased from Aldrich. The enzymes, subtilisin Carlsberg (EC 3.4.21.62) and the lipase from *Candida rugosa* (EC 3.1.1.3), were purchased as lyophilized powders from Sigma Chemicals, Inc.

Methods. (a) Enzyme Preparation. The enzyme, subtilisin Carlsberg, was prepared as follows: The lyophilized enzyme powder was dissolved (5 mg/mL) in a 20 mM phosphate buffer (at pH 7.8, the enzyme's optimum pH). This solution was then rapidly frozen (in liquid N_2, to avoid destruction of the enzyme due to self-hydrolysis) and lyophilized for 48 h. The lipase was prepared as described for subtilisin, but it was lyophilized from a buffer at pH 7.5. Co-lyophilization of the enzymes with MβCD was performed in the same manner, except that MβCD (at a 1:6 weight ratio of enzyme to MβCD) was added to the buffer solution prior to lyophilization.

(b) Cyclodextrin Solubility Experiments. The cyclodextrin (MbCD or βCD), 50 mg, was placed in a 1.0 mL vial; then the solvent (THF, 1,4-dioxane, acetonitrile, toluene, CH_2Cl_2, or octane) was added (5 mL portions) until the cyclodextrin was dissolved.

(c) Kinetic Measurements. Product formation was followed by gas chromatography (GC). The GC instruments (a Varian 3350 and a HP 6890, with Chirasil CB columns, FID detectors, He as carrier gas) were calibrated with the chiral esters, synthesized as reported.[2g] The enzyme powder (about 5 mg) was placed in a 2-mL screw-cap scintillation vial fitted with a mininert cap. The organic solvent (1.0 mL) and the substrates (alcohol and vinyl butyrate substrates) were then added to initiate the reaction. The vial was sealed and subjected to careful sonication using a sonication bath to homogenize the suspension. The mixture was then placed in a controlled-temperature shaker and agitated vigorously (at 45 °C, at 300 rpm). Periodically 0.5 μL of the reacting solution was withdrawn and analyzed by chiral-GC. Under all conditions kinetic experiments were terminated before 10% of the product had been formed. The enzyme enantioselectivity was determined by measuring the initial rates of enzymatic reactions (from plots of product formation vs time) of both enantiomers. The enzyme enantioselectivity for either substrate is equal to the ratio: $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S = V_R[S]/V_S[R]$.[5r] Note that this relationship is valid only when a racemic mixture of the substrates is being studied (as in our experiments), so that both chiral substrates are competing for the binding site simultaneously.

(d) FTIR Spectroscopy. FTIR studies were conducted with a Nicolet Magna-IR System 560 optical bench as described.[4b,c,13,15] A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. Lyophilized protein powders were measured as KBr pellets (1 mg of protein per 200 mg of KBr).[4b,c] Enzymes were suspended in organic solvents, sonicated for 2 min, and measured in a FTIR cell equipped with CaF_2 windows and 50-μm thick spacer. Each protein sample was measured at least five times. When necessary, spectra were corrected for the background and water vapor contributions in an interactive manner using the Nicolet OMNIC 3.1 software to obtain the protein's vibration spectra.[4b,c,13]

(17) Solubility of MβCD in 1,4-dioxane, THF, CH_2Cl_2, and acetonitrile: more than 1 g of MβCD/mL of solvent; in toluene: 0.07 g of MβCD/mL of toluene; in octane: not soluble within detection limits. Note that the additive βCD is not soluble in 1,4-dioxane, THF, and acetonitrile and that only a marginal increase in the enzyme enantioselectivity and in the reaction rates was observed with this additive.

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(21) Thus far, other than adjusting the water activity to its optimum[22a] and controlling the pH[22b] no method has been established to achieve activation of CLECs in organic solvents.

(e) FTIR Data Aanalysis. All spectra were analyzed by second derivatization in the amide I region for their component composition.[4b,c,15,23,24] Second derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}). Fourier self-deconvolution (FSD) was applied to the background- and water vapor-corrected spectra to enable quantification of the secondary structure in the amide I region by Gaussian curve-fitting using the program OMNIC 3.1.[4b,c,15,23,24] We did not observe any over-deconvolution with the parameters chosen (24 cm^{-1} for the full-width-at-half-maximum and $k = 2.4$ for the enhancement factor). Note that FSD alters the band shapes, but preserves the integrated band intensities when over-deconvolution is avoided.[23b,24a] Gaussian curve fitting was performed in the amide I region after band narrowing of the protein vibrational spectra by FSD as described.[13,15,24] The band assignment in the amide I region followed those in the literature.[13,15,24] Data obtained in this work for the aqueous solution and subtilisin lyophilized from buffer are within experimental error of those reported previously.[4b] However, since in this work only the α-helix content is used as the structural parameter describing the enzyme integrity, only the area contribution of the band at ca. 1657 cm^{-1} assigned to α-helices is given.

(f) Thermal Denaturation Experiments. Thin films were produced by drying 100 μL of a solution of 10 mg/mL of subtilisin in 10 mM phosphate buffer (pH 7.8) under a stream of dry nitrogen gas for at least 30 min on a CaF_2 window. Films were produced with or without MβCD at a 1:6 weight ratio of enzyme to additive. The CaF_2 window was then mounted in a model HT-32 liquid cell using a 50 μm thick spacer (Spectra Tech) and the cell filled with 1,4-dioxane. Spectra of subtilisin in D_2O were measured at 50 mg/mL of protein concentration using 50 μm thick spacers and the above liquid cell. The temperature was controlled with a microprocessor programmable controller (Spectra Tech). Overall structural changes were quantified by calculating the correlation coefficients from the amide I (1700−1600 cm^{-1}) second derivative spectrum of the sample at 30 °C and those at elevated temperatures.[4e,f] Melting temperatures could not be determined from the thermal denaturation curves in 1,4-dioxane because the experiment could only be performed up to 95 °C and the protein structural transition was not finished.

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