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Effect of Crown Ethers on Structure, Stability, Activity, and Enantioselectivity of Subtilisin Carlsberg in Organic Solvents

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Abstract

Colyophilization or codrying of subtilisin Carlsberg with the crown ethers 18-crown-6, 15-crown-5, and 12-crown-4 substantially improved enzyme activity in THF, acetonitrile, and 1,4-dioxane in the transesterification reactions of *N*-acetyl-L-phenylalanine ethylester and 1-propanol and that of ($\pm$)-1-phenylethanol and vinylbutyrate. The acceleration of the initial rate, V_0 , ranged from less than 10-fold to more than 100-fold. All crown ethers activated subtilisin substantially, which excludes a specific macrocyclic effect from being responsible. The secondary structure of subtilisin was studied by Fourier-transform infrared (FTIR) spectroscopy. 18-Crown-6 and 15-crown-5 led to a more nativelike structure of subtilisin in the organic solvents employed when compared with that of the dehydrated enzyme obtained from buffer alone. However, the high level of activation with 12-crown-4 where this effect was not observed excluded overall structural preservation from being the primary cause of the observed enzyme activation. The conformational mobility of subtilisin was investigated by performing thermal denaturation experiments in 1,4-dioxane. Although only a small effect of temperature on subtilisin structure was observed for the samples prepared with or without 12-crown-4, both 18-crown-6 and 15-crown-5 caused the enzyme to denature at quite low temperatures (38°C and 56°C, respectively). No relationship between this property and V_0 was evident, but increased conformational mobility of the protein decreased its storage stability. The possibility of a “molecular imprinting” effect was also tested by removing 18-crown-6 from the subtilisin-18-crown-6 colyophilizate by washing. V_0 was only halved as a result of this procedure, an effect insignificant compared with the ca. 80-fold rate enhancement observed prior to washing in THF. This suggests that molecular imprinting is likely the primary cause of sub-tilisin activation by crown ethers, as recently suggested.

Keywords

crown ether; enzyme activation; Fourier-transform infrared (FTIR) spectroscopy; nonaqueous enzymology; subtilisin Carlsberg

INTRODUCTION

The application of enzymes under nearly nonaqueous conditions has become common practice in the industrial setting (McCoy, 1999). Many companies now use biocatalysis as a competitive and economic alternative to classical synthetic approaches. For example, BASF Corp. produces optically active amines with a lipase in organic solvents (McCoy, 1999). Many other examples of successful application of enzymes in the synthesis of pharmaceutical intermediates can be found in the literature (see, e.g., Stinson, 1999). This is unsurprising because enzymes offer properties, such as enantio-, prochiral-, and regioselectivity, that are of much value to the synthetic organic chemist (Klibanov, 1990). However, the use of enzymes as a standard catalyst in a chemistry laboratory remains the exception rather than the rule (Dordick, 1992).

One important issue preventing the widespread use of enzymes in organic solvents is the fact that they are usually 10^2 to 10^6 times less active in organic solvents than in water (Klibanov, 1997). To counter this limitation, many methods have been introduced leading to improved enzyme activity in organic solvents. For example, control of the pH value (Yang et al., 1993), colyophilization with lyoprotectants (Dabulis and Klibanov, 1993) and salts (Khmelnitsky et al., 1994; Ru et al., 1999), addition of water-mimicking agents (Almarsson and Klibanov, 1996; Kitaguchi et al., 1990), imprinting with substrates and substrate analogs (Rich and Dordick, 1997; Russell and Klibanov, 1988), immobilization (Orsat et al., 1994; Petro et al., 1996; Ruiz et al., 2000), solubilization (Meyer et al., 1996; Okahata et al., 1995a,b; Paradkar and Dordick, 1994; Wangikar et al., 1997; Xu et al., 1997), mutagenesis (Chen and Arnold, 1993), and solvent precipitation (Dai and Klibanov, 1999) represent methods that have been successful for improving the catalytic activity of enzymes. One of the most successful groups of activating additives identified thus far are macrocyclic compounds, which includes cyclodextrins (Griebenow et al., 1999; Ooe et al., 1999; Santos et al., 1999) and crown ethers (Broos et al., 1995a; Engbersen et al., 1996; Itoh et al., 1996; Reinhoudt et al., 1989; van Unen, 2000; van Unen et al., 1998, 2001). The advantage of their application lies in the simplicity of the preparation of the enzyme by simple colyophilization procedures. However, the mechanism of how the macrocyclic compounds activate enzymes remains incompletely understood. This particularly applies to the activation of enzymes by crown ethers because no enzyme structural data have yet been presented.

Another issue is the enantioselectivity of enzymes under nonaqueous conditions. It has been established that variation of solvent parameters can lead to drastic changes in enzyme selectivity, and many empirical rules have been developed for specific situations (Klibanov, 1990). For example, subtilisin enantioselectivity has been related to the dielectric constant and dipole moment of the solvent (Fitzpatrick and Klibanov, 1991). Other investigations have found relationships between enantioselectivity and hydrophobicity of the solvent (Sakurai et al., 1988; Tawaki and Klibanov, 1991). However, one important issue that has not been studied systematically thus far is the relation of the three-dimensional structure of the enzyme catalysts under nonaqueous conditions to the observed selectivity. For example, the enantioselectivity of subtilisin Carlsberg and a lipase markedly improved in various organic solvents by simple colyophilization of the enzyme with methyl- β -cyclodextrin (Griebenow et al., 1999; Santos et al., 1999). The improved enantioselectivity was shown to

correspond to the degree of nativity of subtilisin secondary structure in the solvents (Griebenow et al., 1999). However, it is also evident that the dynamic properties of the catalyst might influence enantioselectivity. For example, whereas activity was shown to increase upon addition of small amounts of water to subtilisin in THF, enantioselectivity decreased (Santos et al., 1999). Enzyme denaturation is unlikely in this instance because of the first aforementioned fact and thus the changes in dynamic properties of the catalyst might be responsible for the decrease in enantioselectivity. Somewhat differing results have also been presented (Broos et al., 1995b), showing increased enantioselectivity to be associated with increased enzyme flexibility.

Herein, we investigate the activity, enantioselectivity, structure, and stability of subtilisin Carlsberg colyophilized or codried with crown ethers in organic solvents. The strategy pursued was to change the properties of the catalyst by changing the formulation at otherwise constant conditions (e.g., no changes in the solvent parameters) to allow for conclusions on how changes in subtilisin structure and stability are related to catalytic properties.

MATERIALS AND METHODS

Solvents were purchased in their anhydrous form (water content <0.005%) in Sure/Seal bottles (Aldrich). The substrates for the enzymatic assays were from the following sources: ($\pm$)-1-phenylethanol and 1-propanol were from Aldrich; vinylbutyrate was from TCI; and *N*-acetyl-L-phenylalanine ethylester was from Sigma. Lyophilized serine protease subtilisin Carlsberg (EC 3.4.21.62) and the crown ethers (18-crown-6, 15-crown-5, and 12-crown-4) were purchased from Sigma.

Enzyme Preparation

The enzyme, subtilisin Carlsberg, was prepared by lyophilization as described by Santos et al. (1999). Colyophilization of the enzyme with 18-crown-6, 15-crown-5, and 12-crown-4 was performed in a similar manner, except that the crown ethers (at a 1:4 weight ratio of enzyme:excipient) were added to the buffer solution prior to lyophilization. Preparation of the subtilisin films for FTIR spectroscopy was performed by drying 100 mL of a solution of subtilisin (10 mg/mL) in 10 mM potassium phosphate buffer directly onto CaF₂ windows using dry N₂ gas. The respective crown ethers were added prior to drying at a 1:4 weight ratio of subtilisin to crown ether. Samples for kinetic measurements were prepared similarly, except that they were dried in glass vessels.

Kinetic Measurements

Transesterification of *N*-Acetyl-L-Phenylalanine Ethylester with 1-Propanol—

The anhydrous organic solvents were stored over 3-Å molecular sieves for at least 24 h prior to use. Transesterification was performed, followed by high-performance liquid chromatography (HPLC), and analyzed as described by Griebenow et al. (1999). The reaction was done with 100 mM *N*-acetyl-L-phenylalanine phenylester, 1 M 1-propanol, and 1 mg of subtilisin per milliliter of solvent using an orbital shaker (25°C and 250 rpm).

Transesterification of Vinylbutyrate with ($\pm$)-1-Phenylethanol (Sec-Phenethylalcohol)—After transesterification, gas chromatography was performed and analyzed as described by Griebenow et al. (1999). The reaction was done with 70 mM ($\pm$)-1-phenylethanol, 100 mM vinylbutyrate, and 1 mg of subtilisin per milliliter of solvent on an orbital shaker (45°C and 300 rpm).

FTIR Spectroscopy

FTIR studies were conducted with a Nicolet Magna-IR (System 560) optical bench as described elsewhere (Griebenow and Klivanov, 1995, 1996, 1997; Griebenow et al., 1999). Lyophilized protein powders were measured as KBr pellets (1 mg of protein per 200 mg of KBr). Suspensions of subtilisin powders in organic solvents were prepared by sonication for 2 min in a sonication bath, and measured in an FTIR cell equipped with CaF₂ windows and using 25- or 50- μ m-thick spacers. Enzyme films were prepared, measured, and analyzed as described by Griebenow et al. (1999). Each protein sample was typically measured five times. Spectra were corrected for the solvent background and water vapor contributions in an interactive manner using Nicolet OMNIC 3.1 software to obtain the protein vibrational spectra. All spectra were analyzed by second derivatization in the amide I region for their component composition (Prestrelski et al., 1993). 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 OMNIC 3.1 (Griebenow et al., 1999). We observed no over-deconvolution with the parameters chosen (24 cm⁻¹ for full-width at half-maximum and $k = 2.4$ for the enhancement factor). Gaussian curve-fitting was performed in the amide I region after band-narrowing of the protein vibrational spectra by FSD as described elsewhere (Griebenow and Klivanov, 1997; Griebenow et al., 1999). The band assignment in the amide I region followed those in the literature (Griebenow and Klivanov, 1997; Griebenow et al., 1999), and typical examples are listed in Table I.

RESULTS

It has been established that crown ethers significantly activate various serine proteases (trypsin, α -chymotrypsin, and subtilisin Carlsberg) in both hydrophilic (van Unen et al., 1998, 2000) and hydrophobic (Broos et al., 1995a; Engbersen et al., 1996) organic solvents. Herein we focus on hydrophilic organic solvents because the solubility for many substrates of interest is superior to that of hydrophobic solvents. As the model enzyme we employed the frequently used serine protease subtilisin Carlsberg (see, e.g., Broos et al., 1995a; Griebenow et al., 1999; Santos et al., 1999; Zaks and Klivanov, 1988).

Effect of Crown Ethers on Catalytic Properties of Subtilisin Carlsberg

As model reaction **1** (Scheme 1) we employed the well-established transesterification reaction of *N*-acetyl-L-phenylalanine ethylester with 1-propanol to allow for comparison of our data with literature results (Broos et al., 1995b; Engbersen et al., 1996; Santos et al., 1999; Schmidtke et al., 1996; Zaks and Klivanov, 1988).

When subtilisin Carlsberg was lyophilized from an aqueous phosphate buffer, the suspended enzyme formed the ester product with initial rates (V_0) of 7 and 8 nmol mg⁻¹ subtilisin min⁻¹ in THF and acetonitrile, respectively (Santos et al., 1999). Colyophilization of the enzyme with the crown ethers 18-crown-6, 15-crown-5, and 12-crown-4 at a 1:4 weight ratio (enzyme:additive) in all cases resulted in a substantial increase in V_0 (Table II). The effect was more pronounced in THF, with rate enhancements ranging from 69- to 104-fold, than in acetonitrile (rate enhancement from 5- to 14-fold).

To assess enantioselectivity of the various colyophilizates, we employed the transesterification model reaction **2** (Scheme 2) between ($\pm$)-1-phenylethanol and vinylbutyrate (Fitzpatrick and Klivanov, 1991; Griebenow et al., 1999; Santos et al., 1999).

The initial rate for the formation of the *S*-enantiomer product (V_S) was used to assess the activation of subtilisin by the crown ethers in THF for this reaction. We found that the V_S value increased only fourfold from 14 ± 4 nmol mg⁻¹ min⁻¹ to 56 ± 15 nmol mg⁻¹ min⁻¹ for the additive 18-crown-6. Larger rate enhancements were observed for 15-crown-5 (28-fold) and 12-crown-4 (37-fold). Thus, whereas for reaction **1** the rate enhancements were comparable for the different crown ethers studied, for reaction **2** substantial differences, depending on the crown ether, were observed. We hypothesized that the discrepancy between the activation effect by the crown ethers for the two different reactions studied might have been caused by the different temperatures used in both experiments (25°C for reaction **1** and 45°C for reaction **2**). These temperatures were selected to allow for comparison with the literature (Griebenow et al., 1999; Santos et al., 1999). Thus, reaction **2** was also performed at 5°C and 21°C (Fig. 1A). For all preparations, the increase in the V_S value was less than twofold upon increasing the reaction temperature from 5°C to 21°C. A three- and fourfold increase was then observed for preparations with 15-crown-5 and 12-crown-4, respectively, upon further temperature increase to 45°C. In contrast, the value for the preparation with 18-crown-6 showed an increase by only a factor of 1.5. We conclude that the differences between crown ether activation between reaction **1** and **2** were exaggerated by the differing temperatures in the experiments. However, the remaining differences point to specific effects of crown ether activation in the individual reactions.

The enantioselectivity of lyophilized subtilisin Carlsberg for reaction **2** in THF at 45°C has been determined by our group to be 32 ± 2 (Griebenow et al., 1999), in agreement with the literature data of Noritomi et al. (1996) and Fitzpatrick et al. (1991). Only a modest increase in enantioselectivity was observed for subtilisin colyophilized with 15-crown-5 ($E = 42 \pm 1$) and 12-crown-4 ($E = 39 \pm 1$). Enantioselectivity was even slightly lower in the presence of 18-crown-6 ($E = 28 \pm 3$). In the presence of crown ethers, enantioselectivity showed some temperature dependence (Fig. 1B). Enantioselectivity was higher at 21°C for all crown ethers, particularly for 18-crown-6, than at 5°C or 45°C. However, crown ethers did not improve enantioselectivity substantially in contrast to the macrocyclic compound M β CD (Griebenow et al., 1999; Santos et al., 1999), a fact that has also been reported for α -chymotrypsin colyophilized with 18-crown-6 (Broos et al., 1995b).

Intuitively, one would expect enantioselectivity to decrease with increasing temperature, as often observed, but in many instances the temperature behavior of enantioselectivity is

complex (Noritomi et al., 1996; Philipps, 1992). For example, Noritomi et al. (1996) reported a strong dependence of temperature behavior on the method of enzyme preparation. Enantioselectivity could increase or decrease with temperature in the same solvent for different preparations. This might indicate that rather subtle differences in the enzyme's active site conformation influence enantioselectivity.

Stability of Subtilisin Carlsberg Colyophilized with Crown Ethers

The comparably low values for V_S and the low enantioselectivity obtained for reaction **2** at 45°C in the presence of 18-crown-6 could in general be due to enzyme deactivation at elevated temperature; for example, due to temperature-induced structural alterations in subtilisin (Griebenow et al., 1999). Therefore, stability studies were carried out. Herein, subtilisin Carlsberg colyophilized with crown ethers was stored at 5°C and 25°C for various periods of time prior to use in reaction **1** (Fig. 2). The most sensitive sample proved to be the subtilisin colyophilizate with 18-crown-6. When stored at 5°C, activity decreased in a linear fashion over time. Storage at 25°C resulted in a complete loss of activity within the detection limits of the method (ca. 1 nmol mg⁻¹ min⁻¹) in 2 days. The colyophilizate with 15-crown-5 proved more stable; however, activity decreased linearly upon storage at 5°C and exponentially upon storage at 25°C. The most stable preparation was the colyophilizate with 12-crown-4. The data for the latter sample in particular, but also for the others, scattered significantly. Because the data were obtained with various batches of the enzyme the subtle sample differences may have caused significant differences in the catalytic activity of subtilisin colyophilized with crown ethers.

Secondary Structure of Lyophilized Subtilisin Suspended in THF and Acetonitrile

It has been suggested that crown ethers activate enzymes by keeping the structure more nativelike (van Unen, 2000). In principle, this could occur at the global level (preservation of the three-dimensional structure) or at the local level (preservation of active site conformation). Unlike in the aqueous environment, enzymes are restricted in their conformational mobility in organic solvents kinetically prohibiting large structural alterations (Griebenow and Klibanov, 1996; Partridge et al., 1999). Thus, under nonaqueous conditions, both scenarios could apply. Preservation of overall enzyme structure was probed by FTIR spectroscopy to investigate the first possibility. Note that significant changes in protein tertiary structure are usually accompanied by changes in the secondary structure. For example, in thermal denaturation experiments, both processes occur simultaneously (see, e.g., Carrasquillo et al., 2000). Thus, an unperturbed secondary structure composition usually indicates a largely unperturbed tertiary structure. However, under certain circumstances, highly ordered molten globule states with a nativelike secondary structure and a changed tertiary structure are possible.

It has been established that suspension of amorphous subtilisin powder obtained by lyophilization from an aqueous phosphate buffer in many organic solvents caused only minor structural changes in addition to those caused by the dehydration procedure itself (Griebenow and Klibanov, 1997; Griebenow et al., 1999). This concept has held true for a variety of other proteins upon suspension in methylene chloride (Carrasquillo et al., 1998, 1999, 2000). In this work, for purposes of comparison, we performed experiments with

lyophilized subtilisin prior to and after suspension in THF (Fig. 3B). The main spectral difference was that the bands at ca. 1680 and 1670 cm^{-1} were not resolved in the spectrum in THF. Otherwise, little change was observed in the spectrum, in agreement with Griebenow and Klibanov (1997).

The secondary structure of subtilisin colyophilized with crown ethers could not be tested for the lyophilized solids. As a control, we dried subtilisin with 18-crown-6 on a KBr window as film and obtained an amide I infrared (IR) spectrum comparable to that shown in Figure 5C. When this sample was used to produce a KBr pellet, significant denaturation occurred and the spectrum appeared similar to that of denatured subtilisin (Fig. 5D). Thus, due to the reduction in subtilisin's conformational stability by the additives, the method itself caused protein denaturation and aggregation.

The spectra of the colyophilizates of subtilisin with the three crown ethers employed in this work are shown in Figure 3C–E. The spectra of subtilisin colyophilized with the crown ethers were different from those of subtilisin lyophilized in phosphate buffer and suspended in THF (Fig. 3B). When we quantified overall structural changes by calculating the spectral correlation coefficient for the amide I second derivative spectra (Prestrelski et al., 1993), the lyophilized powder in THF had a similarity of 0.59 ± 0.04 (mean $\pm$ SD) to that of subtilisin in aqueous buffer. Correlation coefficients obtained for the crown ether-containing samples were 0.72 ± 0.01 for 18-crown-6, 0.69 ± 0.01 for 15-crown-5, and 0.57 ± 0.03 for 12-crown-4. Thus, the samples containing 18-crown-6 and 15-crown-5 had a secondary structure more similar to that of native subtilisin, whereas the sample with 12-crown-4 had a structure as unlike that of native subtilisin as the lyophilized powder suspended in THF. Quantitative data obtained by Gaussian curve-fitting led to the same conclusion. The α -helix content was the same for subtilisin colyophilized with 18-crown-6 and 15-crown-5 in THF as for subtilisin in aqueous solution, whereas that for subtilisin colyophilized with 12-crown-4 was the same as for the lyophilized powder in THF. It is of interest to note that the spectral features of the samples obtained with 18-crown-6 and 15-crown-5 were very similar to the spectrum of subtilisin dissolved in glycerol (Xu et al., 1997). For all samples, the β -sheet component at ca. 1630 cm^{-1} was shifted to ca. 1637 cm^{-1} and the spectrum was simplified to three main peaks. Such spectral changes are reminiscent of the changes observed for lyophilized subtilisin upon dissolving it in glycerol (Xu et al., 1997).

Next, we investigated the effect of the solvent on the structure of subtilisin colyophilized with 18-crown-6 and 15-crown-5. It was found that the structures of both enzyme preparations were less native in acetonitrile than in THF (Table III). Thus, the nature of the solvent does affect the structure of subtilisin in crown ether colyophilizates.

We also tested whether colyophilization with a crown ether has any effect on the structure of subtilisin Carlsberg upon storage for several days. For this experiment, we used the sample showing the most significant storage instability and employed the subtilisin-18-crown-6 colyophilizate. It was difficult to employ the powder incubated at 25°C due to aggregation, and thus there were difficulties in achieving the fine suspensions necessary to allow for filling of the IR cell. However, when the colyophilizate was stored at 5°C for 7 days, the α -helix content remained unchanged ($35 \pm 1\%$) and the β -sheet content increased only slightly

from $14 \pm 3\%$ to $18 \pm 4\%$. Thus, no significant structural changes accompanied the approximately threefold activity decrease for this preparation upon storage (see Fig. 2).

Molecular Imprinting Effect

We also tested whether a “molecular imprinting effect” (Klibanov, 1997) could be responsible for subtilisin activation by crown ethers. Herein, we first colyophilized subtilisin with 18-crown-6 and then removed the additive by extensive washing with the solvent THF. We determined the crown ether remaining after the washing procedure by FTIR spectroscopy. The fact that the amide I protein IR band does not contain crown ether contributions, and that the crown ether band at ca. 838 cm^{-1} contains only a negligible protein contribution, can be utilized in this instance. Herein, the ratio obtained by dividing the area of the protein amide I IR band (1720 to 1600 cm^{-1}) by the area of the crown ether band at ca. 838 cm^{-1} (820 to 850 cm^{-1}) is proportional to the protein:crown ether ratio in the sample. Using the known molar ratio of 344 mol crown ether per mole of subtilisin (corresponding to the 4:1 weight ratio) for the sample prior to washing, we found that a maximum of 11 mol of crown ether per mole of subtilisin was still present in the sample after the washing procedure. From titration experiments, it has been well established that, at such low molar ratios, 18-crown-6 does not activate enzymes in organic solvents to a significant extent (van Unen, 2000). As a result of the washing procedure, subtilisin activity was approximately halved compared with that of colyophilize prior to washing (Table II), but remained substantially higher than for the powder lyophilized without the crown ether. These results coincide with those of molecular imprinting data (Klibanov, 1995). Thus, crown ethers likely preserve the active center structure without necessarily preventing overall protein structural perturbations. A similar conclusion has recently been reported by van Unen et al. (2000), who found that the activity of α -chymotrypsin was not changed significantly after removing the crown ether.

A molecular imprinting effect was also indicated for subtilisin dried as film (see later) in the presence of 18-crown-6 at a 1:0.7 weight ratio of subtilisin to crown ether. Depending on the buffer salt (Na-phosphate or K-phosphate buffer) the initial rate, V_S , for the formation of the product ester decreased by two- to fourfold after removal of the crown ether. However, the enzyme was still approximately four- to eightfold more active than the films obtained without the crown ether. Details on these results will be published elsewhere.

Crown Ether–Induced Changes in Conformational Mobility of Subtilisin

The denaturation of subtilisin in the presence of 18-crown-6 upon production of KBr pellets indicated that restrictions in the conformational mobility of lyophilized protein powders (Carrasquillo et al., 1998, 1999, 2000; Griebenow and Klibanov, 1996, 1997) are reduced as a result of the presence of the crown ethers. Similarly, the storage stability data obtained support a reduction in conformational stability for subtilisin colyophilized with 15-crown-5 and, in particular, 18-crown-6. Such variations in conformational stability should lead to significant changes in the thermal stability of subtilisin, as reported for subtilisin–cyclodextrin in thermal denaturation experiments followed by FTIR spectroscopy (Griebenow et al., 1999). To measure thermal denaturation curves and to follow them in organic solvents, those with quite high boiling points are needed and we have previously

identified 1,4-dioxane as a suitable polar solvent system (Griebenow et al., 1999). Furthermore, we found it difficult to perform these measurements with suspended subtilisin powders and thus subtilisin films were obtained by simple drying procedures and employed instead (Griebenow et al., 1999).

First, we determined the kinetics for reaction **1** for subtilisin films in dioxane. Subtilisin dried from buffer alone had a very low activity (below the detection limit of our method within 48 h of measurement) and thus the kinetic rate was $<1 \text{ nmol mg}^{-1} \text{ min}^{-1}$. For films codried with crown ethers, the following initial velocities were obtained: 18-crown-6, $8 \text{ nmol mg}^{-1} \text{ min}^{-1}$; 15-crown-5, $35 \text{ nmol mg}^{-1} \text{ min}^{-1}$; and 12-crown-4, $34 \text{ nmol mg}^{-1} \text{ min}^{-1}$. When we studied reaction **2** with dry subtilisin films, V_S was determined to be $1.4 \pm 0.1 \text{ nmol mg}^{-1} \text{ min}^{-1}$ at 45°C . Codrying of the enzyme with 18-crown-6 at a 1:4 weight ratio resulted in a 3.6-fold rate enhancement. More efficient rate enhancements were obtained with 15-crown-5 (13-fold), and in particular with 12-crown-4 (121-fold).

Enantioselectivity for subtilisin dried as film without crown ether was 17 ± 2 and similar values were found for films with 18-crown-6 ($E = 16 \pm 1$) and 12-crown-4 ($E = 20 \pm 2$). For the sample dried with 15-crown-5, enantioselectivity was increased slightly to 32 ± 5 . Thus, subtilisin activity in 1,4-dioxane was markedly influenced by crown ethers in the dry films, whereas enantioselectivity was not, as was the case for lyophilized powders in THF. From these results, we can conclude that subtilisin films in 1,4-dioxane constitute acceptable models for activation of lyophilized subtilisin, because results obtained were qualitatively comparable.

The films proved useful in assessing whether the more natively like structure of subtilisin colyophilized with crown ethers and subsequently suspended in THF and acetonitrile (Table III) was likely due to a reduction of dehydration-induced structural alterations prior to suspension in the solvents or due to crown ether-induced refolding upon suspension in the solvents. Thus, we assessed the secondary structure of subtilisin after drying as films and after subsequent exposure to 1,4-dioxane. When subtilisin dried from buffer was exposed to dioxane, very little spectral change occurred (Table IV). The α -helix content determined for the dried subtilisin (28%) was similar to that found after solvent exposure (27%). Drying of subtilisin in the presence of the three crown ethers as film led to a reduction in dehydration-induced structural perturbation (Table IV). Only slight spectral changes were observed when exposing subtilisin co-dried with 18-crown-6 and 15-crown-5 to dioxane, and the α -helix and β -sheet content remained unchanged and similar to that of subtilisin in aqueous buffer (Table IV). Thus, for these two crown ethers, the more natively like secondary structure was caused by reduction of dehydration-induced structural alterations in subtilisin. It is likely that the more natively like structure for the subtilisin colyophilizes with 15-crown-5 and 18-crown-6 in THF and acetonitrile are due to minimization of lyophilization-induced structural perturbations. Somewhat dissimilar to this, subtilisin codried with 12-crown-4 underwent some solvent-induced denaturation as indicated by the decrease in the α -helix content (Table IV). This finding again might explain the secondary structure of the 12-crown-4 colyophilize suspended in THF, which was the least natively like of the crown ether colyophilizes.

We then assessed the thermal stability of the various subtilisin films by performing FTIR experiments. The background-corrected amide I protein vibrational spectra obtained at increasing temperatures were used to calculate second derivative spectra. The spectral correlation coefficients (Prestrelski et al., 1993) were calculated using those second derivative spectra at elevated temperatures and that at 28°C. A correlation coefficient of 1 shows spectral and thus structural identity at various temperatures. A decrease in the value of the correlation coefficient is a measure of temperature-induced protein structural perturbations. The results of these measurements are shown in Figure 4.

For subtilisin in D₂O at pD 7.8 we determined the denaturation temperature, T_d , from the minimum of the first derivative spectra at 73°C (Griebenow et al., 1999). The temperature-induced structural transition was characterized by a decrease in the α -helix band at ca. 1658 cm⁻¹ and the appearance of a strong band at ca. 1621 cm⁻¹ (Fig. 5A,B). Such a band is frequently found in the thermal denaturation of proteins and has been associated with the formation of intermolecular “aggregation” β -sheets (Dong et al., 1996). Thus, subtilisin unfolds upon heating in D₂O and the unfolded molecules form aggregates.

We next studied the susceptibility of subtilisin films to thermal denaturation as obtained from buffer alone or in the presence of crown ethers in 1,4-dioxane. A slight decrease in spectral correlation coefficient was observed for subtilisin dried from aqueous buffer solution, but no typical sigmoidal denaturation curve was observed up to 95°C (Griebenow et al., 1999). Only slight changes in the α -helix and β -sheet content were observed (Table IV). Thus, conformational mobility of subtilisin in dried films was severely restricted, which leads to high thermostability (Griebenow et al., 1999). The same was found for the sample obtained with 12-crown-4, and conformational mobility of subtilisin codried with 12-crown-4 changed little. The spectra shown in Figure 5G,H at 29°C and 93°C indicate some temperature-induced structural changes (leading to some reduction in the correlation coefficient), but they were much less pronounced than for subtilisin in aqueous solution. Some changes in the secondary structure composition can be seen (Table IV), comparable to those for the subtilisin film obtained from buffer.

Significant effects of the crown ether on thermostability were noted for subtilisin codried with 18-crown-6 and 15-crown-5. The sample with the most significant thermal transition was that of subtilisin codried with 18-crown-6 (T_d ca. 38°C). Although the sample appeared to have a spectrum with a dominant peak due to α -helices at 28°C (Fig. 5C), at 42°C strong bands at ca. 1695 cm⁻¹ and 1625 cm⁻¹ indicated intermolecular β -sheet formation and thus denaturation of subtilisin. A marked decrease in the α -helix content and increase in the β -sheet content was evident from Gaussian curve-fitting analysis (Table IV). The sample codried with 15-crown-5 denatured at a higher temperature of ca. 56°C. The structural changes (Fig. 5E,F and Table IV) were comparable to those observed for the sample codried with 18-crown-6. Thus, subtilisin codried with 18-crown-6 has a less rigid structure than subtilisin codried with 15-crown-5.

The results from the thermal denaturation experiments relate to the stability data obtained with lyophilized subtilisin samples. Stability was lowest for the sample with the lowest T_d (i.e., subtilisin colyophilized with 18-crown-6). Subtilisin colyophilized with 15-crown-5

with a higher T_d was more stable, and the sample colyophilized with 12-crown-4 the most stable. Furthermore, subtilisin colyophilized with 18-crown-6 and 15-crown-5 was susceptible to the KBr-pellet method in FTIR experiments and samples showed pronounced signs of intermolecular β -sheet formation (data not shown), similar to those found in thermal denaturation experiments.

There was also a rough correlation between the thermal stability of subtilisin codried with crown ethers and the catalytic activity in reaction **2** at 45°C. The film codried with 12-crown-4 proved to be the sample with the highest activity and thermostability. The sample codried with 15-crown-5 had medium thermostability and activity, and the sample codried with 18-crown-6 was the least stable and active sample. It is likely that these differences were caused by temperature-induced denaturation of the enzyme, which highlights the need to conduct stability studies when employing new enzyme formulations.

Effect of Crown Ether on Salt Matrix

It is common knowledge that crown ethers bind specific cations depending on the size of their cavity; specifically, 12-crown-4 forms inclusion complexes with Li^+ , 15-crown-5 with Na^+ , and 18-crown-6 with K^+ . It is clear that the activation results (see, e.g., Table II) cannot be explained by specific binding of salt ions by the crown ethers, because all three crown ethers employed did activate subtilisin. However, because subtilisin was colyophilized from potassium phosphate buffer, an effect of 18-crown-6 on the salt matrix may have occurred, leading to dissolving of the salt into the organic solvent. Thus, we also performed experiments with subtilisin codried with 18-crown-6 from sodium phosphate buffer. We found that the initial rate, V_S , for subtilisin codried with the crown ether approximately doubled when using Na^+ phosphate buffer, but the same effect was observed for the film dried from buffer alone. Thus, the effect of 18-crown-6 on the buffer salt matrix was negligible.

DISCUSSION

It is first important to note that it is well established that the amounts of 15-crown-5, but particularly 12-crown-4, are reduced during the dehydration process (Broos et al., 1995a). Thus, the variations in subtilisin properties described in this work might partially be caused by variations in the effective crown ether concentration. However, the goal of the present work was to influence subtilisin properties by macrocyclic compounds in one solvent system in order to be able to investigate the effect of the macrocycle (and not the solvent) on the protein properties. This approach enables comparison of structural and dynamic properties of the enzyme catalyst with kinetic parameters. Also, in practical terms, it would be difficult to control the final concentrations of 15-crown-5 and 12-crown-4 in any preparation. From the point of view of storage stability and enzyme activation, in this work we found 12-crown-4 to be the most efficient excipient to activate serine protease subtilisin, regardless of the fact that it is efficiently removed from the preparation during the lyophilization step (Broos et al., 1995a). We examine this matter in what follows (see “Molecular Imprinting” subsection).

Enantioselectivity

We were surprised to find that enantioselectivity data did not correlate with structural or conformational stability data. Regardless of how native subtilisin structure or how affected the conformational mobility was as a result of the additive, enantioselectivity was comparable to that of the untreated enzyme dehydrated from buffer without crown ethers. Thus, rate enhancements were comparable for the *R* and *S*-enantiomers in agreement with data presented for α -chymotrypsin colyophilized with 18-crown-6 (Broos et al., 1995b).

Subtilisin Structure and Dynamics in Organic Solvents

Understanding enzyme catalysis under nonaqueous conditions and the improvement of catalyst performance by additives is a notoriously difficult task because of the enormous complexity of the system. For example, differences in catalytic performance may be related to changes in: (a) structural and dynamic properties of the protein; (b) substrate–enzyme interactions due to changes in solvent properties; (c) solvation of the transition state of the reaction; (d) and diffusional limitations, among others (Klibanov, 1997). Many experiments have been performed to understand why crown ethers improve the enzyme performance of proteases and lipases under nonaqueous conditions (see Introduction). However, data regarding the effect of crown ethers on protein structure and stability have not yet been presented. In what follows, we present a model based on all findings made thus far in combination with results obtained in this work. We have excluded any analysis of studies describing increased enzyme activity by crown ethers in aqueous solutions (see, e.g., Itoh et al., 1996).

It has been speculated that enzyme activation by colyophilization with certain additives, such as polyols, is caused by them working as a lyoprotectant (Dabulis and Klibanov, 1993). A larger fraction of the enzyme would be locked in a conformational state more suitable for catalysis. However, van Unen et al. (1996) argued that crown ether activation cannot be explained simply by such effects, because colyophilization with the established lyoprotectant D-sorbitol resulted in a rate enhancement by only a factor of 8. In this work, we provide spectroscopic evidence that some crown ethers are efficient in reducing dehydration-induced structural perturbations in subtilisin. This leads to a more nativelike structure in the organic solvents employed herein for subtilisin in the presence of 18-crown-6 and 15-crown-5. However, our data show that all crown ethers employed substantially accelerated the initial rates in both reactions **1** and **2**. If subtilisin secondary structure were related exclusively to the observed catalytic rates, under the conditions employed in this work one would expect that 18-crown-6 and 15-crown-5 in general should have the largest rate enhancement effect, because the structure was most nativelike in THF and 1,4-dioxane. For subtilisin codried with 12-crown-4, kinetic data should be close to those obtained with subtilisin lyophilized from buffer due to the similar structure. This was certainly not the case; subtilisin codried with 12-crown-4 accelerated reactions at least as well as preparations obtained with the other two crown ethers (Table II), even though the structure of subtilisin was similar to that without the additive in the solvents studied. Thus, there was no relationship between preservation of an overall more nativelike subtilisin structure and its catalytic activity in the organic solvents.

The reason for this somewhat astonishing finding may be quite simple. Like nearly any spectroscopic method, FTIR spectroscopy is not able to report the fate of individual molecules, but rather provides an average picture. Thus, observed structural perturbations may indicate that all molecules were structurally perturbed to some extent or that two populations exist consisting of natively and denatured molecules (or a combination of both). Presently, it seems that the second interpretation is correct. In particular, when performing active site titrations, it has been shown that dehydration followed by solvent exposure disrupts about less than half of the active centers and thus three-dimensional-structure α -chymotrypsin and subtilisin (Burke et al., 1992; Schmitke et al., 1996). If this disruption could cause complete unfolding of the molecule, then the subtilisin the α -helix content should accordingly decrease to ca. 18%. However, because even thermally denatured subtilisin still has some α -helix content (Fig. 5B), the expected value should be higher. Thus, the values for α -helix content, determined by FTIR spectroscopy for lyophilized subtilisin to be around 25% herein and in other works, coincide with this possibility. If this model holds true, structural preservation would produce rate enhancements by only a factor of ca. 2. Because rate enhancements caused by crown ethers (as reported here and elsewhere) are often much larger than this, one cannot find any relation between the overall structure and catalytic rates. Indeed, this is what has been observed in this work as well as in other studies (Dong et al., 1997; Griebenow and Klivanov, 1997; Griebenow et al., 1999). Thus, we conclude that global structural preservation can account only for minor variations in the catalytic rates observed for suspended enzyme powders.

Another factor that may contribute to the low enzyme activity of subtilisin Carlsberg suspended in polar solvents is the reduced conformational mobility (Klivanov, 1997; Schmitke et al., 1996). Indeed, addition of small amounts of water as molecular lubricant (Barron et al., 1997) activates lyophilized subtilisin in such solvents (Santos et al., 1999; Zaks and Klivanov, 1988), but at about one order of magnitude the effect still is rather small. Furthermore, addition of denaturing cosolvents such as dimethylsulfoxide (DMSO) has been shown to improve catalytic performance of subtilisin in organic solvents (Almarsson et al., 1996). Again, the effect was substantial, but the rate enhancements were within one order of magnitude. In this work we did find a relationship between “macroscopic” dynamic properties of subtilisin films in 1,4-dioxane as quantified by measuring thermal denaturation curves and kinetic data. The low activity for subtilisin codried with 18-crown-6 at 45°C was likely caused by increased thermoinstability. Increased enzyme “flexibility” or molecular mobility by the crown ether was also related to lower storage stability. However, no relationship was found between catalytic activity at room temperature and dynamic properties of subtilisin. All crown ethers activated the enzyme to a substantial extent, regardless of the macroscopic structural mobility. Thus, again this effect cannot be responsible for the crown ether activation of subtilisin. It is of interest to note that 18-crown-6 does not influence the microscopic protein structural mobility as measured by time-resolved fluorescence anisotropy (Broos et al., 1995b), further indicating that dynamic changes are not responsible for the crown ether activation.

Binding to Lysine or Other Surface-Exposed Amino Acid Residues

An alternative explanation of the activation of enzymes by crown ethers (Engbersen et al., 1996; van Unen et al., 1998) and methyl- β -cyclodextrin (Griebenow et al., 1999; Santos et al., 1999) involves binding of the macrocycle to amino acid residues. According to this theory, crown ethers might form complexes with the quaternary ammonium group and therefore prevent inter- and intramolecular salt bridges from being formed. This would prevent the enzyme from being locked in a catalytically less active conformation (van Unen et al., 1998, 2000). However, this activation only seems relevant for the effect of 18-crown-6 on enzymes when the crown ether is added *after* the enzyme has been lyophilized (Engbersen et al., 1996; Reinhoudt et al., 1989; van Unen, 2000). The activation achieved in this manner is up to approximately tenfold smaller than the activation achieved in colyophilization experiments. Both 12-crown-4 and 15-crown-5 do not activate enzymes when added to the reaction medium after the lyophilization process (van Unen, 2000), but we found significant enzyme activation by both additives when used in colyophilization experiments. In addition, van Unen (2000) was able to achieve significant rate enhancements employing trypsin and acetylated trypsin colyophilized with 18-crown-6 at a 1:50 crown ether:enzyme molecular ratio for reaction **1** in cyclohexane. Furthermore, activation was the same within the error range and approximately 50-fold in both cases. In agreement with these data we found that crown ether activation is efficient for all three crown ethers employed, independent of the macrocyclic cavity. This excludes binding to specific amino acid residues from being responsible for the observed activation effect.

Similarly, the results obtained by us in employing the three different crown ethers also exclude the possibility of a significant contribution to subtilisin activation by influencing the buffer salt matrix due to metal ion complexation. This was highlighted by the insignificant change in catalytic activity for subtilisin-18-crown-6 when replacing K^+ phosphate buffer with Na^+ phosphate buffer.

Molecular Imprinting

Having excluded global protein structural and conformational mobility changes as being responsible for crown ether activation of subtilisin, together with effects on the salt matrix, it became apparent that the most likely explanation involves a very local effect that influences the active site structure. Van Unen (2000) performed studies in which α -chymotrypsin was colyophilized with 18-crown-6 followed by crown ether removal by washing with organic solvent. The activity of the enzyme was comparable prior to and after washing, an effect that closely resembles the molecular imprinting phenomenon (Braco et al., 1990; Klibanov, 1995). From these experiments, van Unen concluded that the enzyme structure must be more nativelike in the organic solvents. However, van Unen (2000) was not able to distinguish between local and global structural effects because no enzyme structural data were collected. Although we confirmed that subtilisin colyophilized with 18-crown-6 and 15-crown-5 is indeed more native in the organic solvents than lyophilized powder, the results with 12-crown-4 do not support that a nativelike structure is needed to achieve enzyme activation. Thus, it is highly likely that our interpretation has to be modified to suggest a more nativelike active site structure in the presence of the crown ethers. Because enzymes are more rigid in organic solvents, this interpretation (unlike in aqueous medium) cannot be

dismissed as a valid possibility. The crown ethers might bind to the active site during the dehydration step, thus keeping it nativelike, but not necessarily the remaining part of the molecule. That such local structural effects can occur in the presence of imprinting agents has been proven by the de novo introduction of catalytic activity to the protein bovine serum albumin (BSA) by colyophilization with a transition state analog (Slade and Vulfson, 1998). Similarly, imprinting with various agents has been reported to induce even larger structural alterations in various proteins upon lyophilization (Mishra et al., 1996). Thus, whereas local structure permitted the respective function after imprinting (e.g., binding of certain substances), global structure was largely perturbed. In agreement with this, Griebenow and Klibanov (1997) found no correlation between overall structure of subtilisin imprinted with various ligands and rate enhancements due to this imprinting. The investigators concluded from this that effects other than global structural preservation must cause the activation; for instance, structural conservation only in the active site region. The imprinting hypothesis also might provide an explanation for the similar activation of subtilisin by the various crown ether colyophilizates in THF and acetonitrile. It has been reported that 15-crown-5 and 12-crown-4 (particularly the latter) are decreased substantially in concentration during the lyophilization process (Broos et al., 1995a). However, because this is likely to be happening at stages in the secondary drying step, when the bulk of the water is already sublimated, it might not affect the active site structure in a significant manner because the protein has already been rigidified (Carrasquillo et al., 2000).

CONCLUSIONS

We have shown that the main factor leading to the activation of subtilisin by crown ethers in organic solvents is likely to be a “molecular imprinting effect” (Klibanov, 1995). No relationship could be established between secondary structure and activity in the various subtilisin–crown ether preparations. Independent of overall structural preservation, activity was the best for the colyophilizate obtained with 12-crown-4, the sample with the least native structure. Also, crown ether–dependent changes in conformational mobility caused some changes in subtilisin stability, but again no relationship with the activation was observed. Also excluded from contributing largely to the enzyme activation were: (a) crown ether effects on the buffer salt matrix; and (b) crown ether binding to lysin (or other) amino acid residues.

In summary, a molecular imprinting effect locally preserving the active site structure under conditions of possible enzyme structure impairment seems the most likely explanation for the observed enzyme activation. This can be visualized as a process in which active site structure is locally preserved during the dehydration process by the presence of the crown ethers. Exposure to the organic solvents leads to release of the crown ethers, but the active site structure remains intact. A likely explanation for this phenomenon is that the enzyme structure is kinetically but not thermodynamically stable in organic solvents (Griebenow and Klibanov, 1996; Partridge et al., 1999). Results obtained in this work for subtilisin Carlsberg largely support and coincide with the findings obtained with α -chymotrypsin (van Unen, 2000).

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Figure 1.

Initial rates for the formation of *S*-enantiomer (A) and enantioselectivity $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S$ (B) for the transesterification of (±)-1-phenylethanol with vinylbutyrate in THF at various temperatures catalyzed by subtilisin Carlsberg lyophilized with crown ethers. Reaction conditions: 70 mM (±)-1-phenylethanol; 100 mM vinylbutyrate; enzyme concentration 1 mg/mL; 45°C, 300 rpm on an orbital shaker.

Figure 2. Initial rates for the transesterification of *N*-acetyl-L-phenylalanine ethylester with 1-propanol after storage of the subtilisin-crown ether colyophilizates for various times at 5°C or 25°C.

Figure 3.

Fourier self-deconvoluted amide I spectra and Gaussian curve fitting of subtilisin Carlsberg in: (A) aqueous solution at pH 7.8; (B) lyophilized powder suspended in THF; (C) 12-crown-4 colyophilized powder suspended in THF; (D) 15-crown-5 colyophilized powder suspended in THF; and (E) 18-crown-6 colyophilized powder suspended in THF. The solid lines represent the resolution-enhanced spectra superimposed with the result of the Gaussian curve-fitting. The individual Gaussian bands are shown as broken lines. Spectrum (A) has already been presented by our group (Griebenow et al., 1999), but was included to allow for comparison.

Figure 4.

Thermal denaturation of various subtilisin Carlsberg samples followed by FTIR spectroscopy: in D_2O at pD 7.8 (■); dried as film from buffer and exposed to 1,4-dioxane (○); and codried with 18-crown-6 (●), 15-crown-5 (△), and 12-crown-4 (◆), and exposed to 1,4-dioxane. The correlation coefficient is a measure for overall structural perturbations and was determined from the second derivative amide I spectra at 28°C and at elevated temperature. A correlation coefficient of 1 demonstrates spectral and thus structural identity; lower correlation coefficients indicate spectral and thus structural differences. Data for subtilisin using full symbols have already been published by our group (Griebenow et al., 1999) but were included to allow for comparison. Approximate midpoints of the thermal denaturation (T_d values) were calculated from the minimum of the first derivative spectra calculated for the fitted curved shown.

Figure 5.

Inverted second derivative spectra of subtilisin Carlsberg dissolved in D₂O at pD 7.8 (A,B) and dried in the presence of various crown ethers (18-crown-6, 15-crown-5, and 12-crown-4) as thin film followed by subsequent exposure to 1,4-dioxane (C–H). Spectra shown on the left are for subtilisin prior to thermal denaturation. Spectra on the right show the results of the thermal denaturation experiments after the structural transition was complete (see Fig. 4) or the highest temperature possible in 1,4-dioxane was reached (ca. 93°C).

Scheme 1.

Scheme 2.

Table I

Band positions, areas, and assignments of the component bands resolved by Gaussian curve-fitting in the amide I region for representative subtilisin Carlsberg samples.

Sample/state	Gaussian curve-fitting ^a		Assignment
	Position (cm ⁻¹)	Area (%)	
Aqueous solution (pH 7.8)	1694 ± 3	4 ± 1	β-sheet
	1680 ± 2	14 ± 1	other ^b
	1672 ± 1	10 ± 2	other ^b
	1658 ± 1	35 ± 2	α-helix
	1644 ± 1	23 ± 1	random coil ^c
	1631 ± 1	15 ± 1	β-sheet
Colyophilized with 18-c-6 and suspended in THF	1693 ± 1	9 ± 2	β-sheet
	1681 ± 0	15 ± 2	other ^b
	1673 ± 1	15 ± 2	other ^b
	1659 ± 0	35 ± 0	α-helix
	1644 ± 0	20 ± 1	random coil ^c
	1637 ± 0	5 ± 0	β-sheet
Colyophilized with 18-c-6 and suspended in acetonitrile	1623 ± 0	2 ± 0	side chain
	1692 ± 0	9 ± 1	β-sheet
	1677 ± 0	29 ± 2	other ^b
	1659 ± 0	32 ± 1	α-helix
	1645 ± 0	14 ± 1	random coil ^c
	1637 ± 0	13 ± 1	β-sheet
Colyophilized with 15-c-5 and suspended in THF	1622 ± 0	4 ± 0	side chain
	1694 ± 0	8 ± 1	β-sheet
	1679 ± 0	26 ± 2	other ^b
	1660 ± 0	34 ± 1	α-helix
	1646 ± 0	16 ± 1	random coil ^c
	1637 ± 0	12 ± 1	β-sheet
Colyophilized with 12-c-4 and suspended in THF	1622 ± 0	4 ± 0	side chain
	1695 ± 1	12 ± 1	β-sheet
	1681 ± 0	18 ± 3	other ^b
	1672 ± 2	7 ± 2	other ^b
	1660 ± 0	29 ± 2	α-helix
	1648 ± 0	20 ± 2	random coil ^c
	1639 ± 1	10 ± 1	β-sheet
	1628 ± 2	4 ± 1	β-sheet

^aThe amide I region was resolution-enhanced by Fourier-self deconvolution prior to Gaussian curve-fitting analysis; see Materials and Methods for details.

^bOther secondary structures include contributions from various turns; for example, β -turns (Dong et al., 1995).

^cThe expression “random coil” is traditionally used to describe elements of a protein secondary structure not having a regular hydrogen-bonding network involving amide backbone groups. However, for any given protein, the random coil portion is actually highly ordered but the order cannot be described accurately.

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Table II

Initial rates for transesterification of *N*-acetyl-L-phenylalanine ethylester with 1-propanol catalyzed by subtilisin Carlsberg lyophilized with and without different crown ethers in THF and acetonitrile.^a

Additive	Solvent	V_0^b	Rate enhancement ^c
None ^d	THF	7 ± 1	0
	Acetonitrile	8 ± 1	0
18-c-6 ^e	THF	538 ± 96	77
	THF ^f	264 ± 38	38
	Acetonitrile	60 ± 15	8
15-c-5 ^e	THF	482 ± 80	69
	Acetonitrile	36 ± 6	5
12-c-4 ^e	THF	729 ± 49	104
	Acetonitrile	113 ± 11	14

^a Reaction conditions: 100 mM *N*-acetyl-L-phenylalanine ethylester, 1 M 1-propanol, and enzyme concentration 1 mg/mL (25°C, 250 rpm on an orbital shaker).

^b Initial rates are in nmol of product formed per milligram of enzyme and minute.

^c Rate enhancement defined as: $[V_0 \text{ with crown ether}/V_0 \text{ without crown ether}]$.

^d Powder obtained by lyophilization from aqueous potassium phosphate buffer (10 mM, pH 7.8); data from Santos et al. (1999).

^e Powder obtained by lyophilization from aqueous potassium phosphate buffer (10 mM, pH 7.8) containing the corresponding crown ether at a 1:4 weight ratio of subtilisin to crown ether.

^f 18-Crown-6 was removed from this sample by washing it thrice with 2 mL of THF. The sample was suspended in THF by sonication for 20 sec, shaken for 10 min at 250 rpm, and pelleted by centrifugation (20 min, 8000 rpm). A very similar procedure has been reported to efficiently remove 18-crown-6 from chymotrypsin (van Unen, 2000a).

Table III

Secondary structure of subtilisin Carlsberg lyophilized with or without different crown ethers and subsequently suspended in THF and acetonitrile.

Sample	Solvent	α -helix (%) ^a	β -sheet (%) ^a
Subtilisin	Buffer (pH 7.8)	35 $\pm$ 2	19 $\pm$ 2
None ^b	THF	27 $\pm$ 1	22 $\pm$ 1
	Acetonitrile	23 $\pm$ 1	31 $\pm$ 2
18-c-6 ^c	THF	35 $\pm$ 0	14 $\pm$ 3
	Acetonitrile	32 $\pm$ 1	22 $\pm$ 2
15-c-5 ^c	THF	34 $\pm$ 1	20 $\pm$ 1
	Acetonitrile	30 $\pm$ 2	26 $\pm$ 5
12-c-4 ^c	THF	29 $\pm$ 2	26 $\pm$ 2

^a α -Helix and β -sheet content determined by Gaussian curve-fitting of the amide I IR spectra after resolution enhancement by Fourier self-deconvolution (FWHM 24 cm⁻¹, $k = 2.4$) (Griebenow et al., 1999).

^b Powder obtained by lyophilization from 10 mM aqueous potassium phosphate buffer (pH 7.8), and subsequently suspended in the solvents THF or acetonitrile.

^c Powder obtained by lyophilization from aqueous potassium phosphate buffer (10 mM, pH 7.8) containing the correspondent crown ether at a 1:4 weight ratio of subtilisin:crown ether and suspended in the solvents THF or acetonitrile.

Table IV

Secondary structure of subtilisin Carlsberg dried with and without crown ethers in the dehydrated state and after subsequent exposure to 1,4-dioxane at various temperatures.

Sample	Solvent	Temperature (°C)	α -helix (%) ^a	β -sheet (%) ^a
Subtilisin	Buffer (pH 7.8)	28	35 ± 2	19 ± 2
Film ^b	None	30	28	23
	1,4-Dioxane	30	28	23
	1,4-Dioxane	95	24	27
	1,4-Dioxane	95	24	27
18-c-6 ^c	None	28	34	14
	1,4-Dioxane	28	32	15
	1,4-Dioxane	44	25	39
	1,4-Dioxane	54	22	41
15-c-5 ^c	None	27	33	18
	1,4-Dioxane	27	33	21
	1,4-Dioxane	71	20	44
12-c-4 ^c	None	29	34	19
	1,4-Dioxane	29	28	20
	1,4-Dioxane	95	22	26

^a α -Helix and β -sheet content determined by Gaussian curve-fitting of the amide I IR spectra after resolution enhancement by Fourier self-deconvolution (FWHM 24 cm⁻¹, $k = 2.4$) (Griebenow et al., 1999). The data do not distinguish between intra- and intermolecular β -sheets.

^b Subtilisin dried as film from 10 mM potassium phosphate buffer (pH 7.8).

^c Subtilisin dried as film from 10 mM potassium phosphate buffer (pH 7.8) containing the corresponding crown ether at a 1:4 weight ratio of subtilisin to crown ether.