Large Acceleration of α-Chymotrypsin-Catalyzed Dipeptide Formation by 18-Crown-6 in Organic Solvents

Dirk-Jan van Unen, Johan F. J. Engbersen, David N. Reinhoudt

Laboratory of Supramolecular Chemistry and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands; telephone: +31 53 4892980; fax: +31 53 4894645; e-mail: SMCT@ct.utwente.nl

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Abstract: The effects of 18-crown-6 on the synthesis of peptides catalyzed by α-chymotrypsin are reported. Lyophilization of the enzyme in the presence of 50 equivalents of 18-crown-6 results in a 425-fold enhanced activity when the reaction between the 2-chloroethylester of *N*-acetyl-L-phenylalanine and L-phenylalaninamide is carried out in acetonitrile. Addition of crown ether renders the dipeptide synthesis in nonaqueous solvents catalyzed by α-chymotrypsin possible on a preparative scale. The acceleration is observed in different solvents and for various peptide precursors. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **59**: 553–556, 1998.
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INTRODUCTION

The formation of peptide bonds by enzymatic methods offers clear advantages over chemical methods, such as mild reaction conditions, no racemization, and the fact that it renders time-consuming protection and deprotection steps superfluous. Several proteases have been applied in the synthesis of peptides (Blair West and Wong, 1986; Kisee et al., 1988; Kuhl et al., 1990; Margolin et al., 1987; Ricca and Crout, 1989), but the reaction cannot be performed in water because of the hydrolysis of the product. The main disadvantage of enzyme catalysis in organic solvents is the *reduction by two to six orders of magnitude in activity* compared with that in water (Dordick, 1989). This is one of the reasons that this method is still not widely applied. There are a few possibilities to enhance the reactivity. Kitaguchi and Klibanov (1989) showed that the activity of thermolysin could be enhanced by the addition of water-mimics, like formamide and ethylene glycol. Paradkar and Dordick (1994) reported the solubilization of α-chymotrypsin in organic solvents by aerosol-OT, resulting in a high enzyme activity in the synthesis of dipeptides.

We have reported that addition of crown ethers enhances the activity of proteases in the transesterification of amino acid esters (Broos et al., 1991, 1995; Engbersen et al., 1996; Reinhoudt et al., 1989) and tyrosinase (Broos et al., 1996) in organic solvents. The highest activating effect was obtained when crown ether was added before lyophilization (Broos et al., 1995). When pretreated with crown ether the activity of α-chymotrypsin is enhanced by a factor of 600 and only 50 times lower than the hydrolytic activity in water. More recently, Itoh et al. (1996) and Takagi et al. (1996) reported that the enantioselectivity and activity of lipases is also affected by crown ethers (Itoh et al., 1996; Takagi et al., 1996).

In this article we describe the large acceleration of the α-chymotrypsin-catalyzed dipeptide formation by 18-crown-6. The effects of various amounts of crown ether, the influence of the solvent, and the type of amino acid substrates will be discussed. Furthermore, the activation of the enzyme on a preparative scale is demonstrated for the synthesis of *N*-Ac-L-phenylalanyl-L-phenylalaninamide.

MATERIALS AND METHODS

α-Chymotrypsin (E.C. 3.4.21.1), type II, from bovine pancreas (54 U/mg protein; hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at pH 7.8), and the peptide precursors *N*-Ac-L-Phe-OH, L-Phe-NH_2, L-Tyr-NH_2 were obtained from Sigma (St. Louis, MO). D-Phe-NH_2, L-Leu-NH_2, and *N*-Ac-L-Phe-OEt were purchased from Bachem (Bubendorf, Switzerland). The 2-chloroethylester of *N*-Ac-L-Phe-OH was synthesized from *N*-Ac-L-Phe and 2-chloroethanol using Amberlite IR-120 as a catalyst (Newman and Chen, 1973). 18-Crown-6 was a gift from Shell Laboratories (Amsterdam). The solvents were of analytical grade or higher and were from Acros (Geel, Belgium).

Pretreatment of α-Chymotrypsin

α-Chymotrypsin (5 mg/mL) was dissolved in 20 m*M* sodium phosphate buffer, pH 7.8, containing the appropriate amount of 18-crown-6. The equivalents of added crown

Correspondence to: D. N. Reinhoudt
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ether are relative to the moles of enzyme. For comparison studies with the crown ether D-sorbitol (5 mg/mL) was used. The samples were lyophilized, after rapid freezing in liquid nitrogen, for 24 h.

Studies on Effects of 18-crown-6 on Rate of Dipeptide Formation

The enzyme preparation, peptide precursors, and solvents were equilibrated at a thermodynamic water activity of 0.113 above a saturated LiCl solution for 24 h. Reactions were performed in duplicate on a 1-mL scale with magnetic stirring at 500 rpm. Typical conditions were: 2.5 mg/mL α-chymotrypsin, 50 m*M* *N*-Ac-L-Phe-OEtCl, and 50 m*M* L-Phe-NH_2 in acetonitrile at 30°C. The reactions were terminated by the addition of 4 volumes of dimethylsulfoxide. The reaction mixture was analyzed by HPLC. Initial rates were calculated from conversions of <5%.

Enzymatic Synthesis of *N*-Ac-L-Phe-L-Phe-NH_2

α-Chymotrypsin (100 mg) was lyophilized from a 20 m*M* sodium phosphate buffer, pH 7.8, with 52.8 mg of 18-crown-6 overnight. The enzyme preparation was incubated with 2.5 mmol of *N*-Ac-L-Phe-OEtCl and 5.0 mmol L-Phe-NH_2 in 50 mL of acetonitrile (stored over molecular sieves of 0.3 nm). After 24 h of incubation the solvent was evaporated under reduced pressure. The residue was washed with 1 *M* HCl, 0.5 *M* $NaHCO_3$, and water, respectively, and was twice dried azeotropically with methanol, yielding 87% of the dipeptide *N*-Ac-L-Phe-L-Phe-NH_2. The product was pure according to ^{1}H-NMR spectroscopy, FAB mass spectroscopy, and HPLC. No traces of 18-crown-6 were detected in the isolated product.

HPLC Analysis

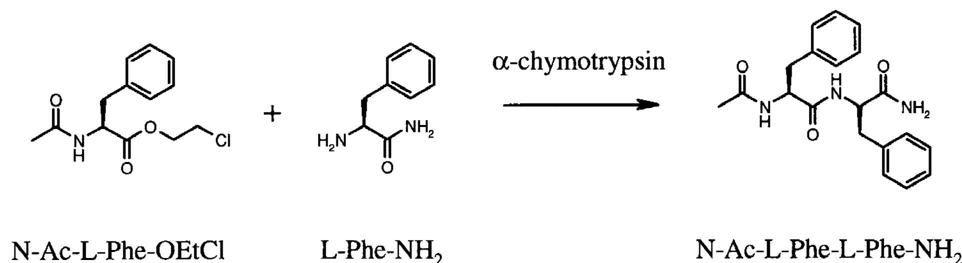
HPLC analysis was performed with Waters HPLC equipment consisting of a 600 gradient pump, a 717 autosampler, and 996 photodiode array detector, with Millenium software. A Waters μBondapak C_{18} 12.5-nm, 10-μm (300*3.9 mm) reversed-phase column was eluted at a flow rate of 1 mL/min with 30% acetonitrile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

RESULTS AND DISCUSSION

Our initial studies revealed that trypsin, subtilisin Carlsberg, and α-chymotrypsin are suitable catalysts for the peptide bond formation between the 2-chloroethylester of *N*-acetyl-L-phenylalanine and L-phenylalaninamide (Scheme 1). Freeze drying of the proteases with 500 equivalents of 18-crown-6 is very effective in the acceleration of the enzymatic peptide bond formation. After pretreatment with 18-crown-6 the initial enzyme activities of trypsin, α-chymotrypsin, and subtilisin Carlsberg were 110, 125, and 17 times higher, respectively. Although the activity of pretreated subtilisin Carlsberg was the highest, a serious drawback of this enzyme was that, under the conditions used, 20 mol% of the hydrolysis product, *N*-acetyl-L-phenylalanine, was also formed. With α-chymotrypsin and trypsin no trace of hydrolysis was detected. Because α-chymotrypsin gave the highest activity and acceleration this protease was used for our further investigations.

For the optimal amount of 18-crown-6 in the pretreatment the rate of dipeptide formation was determined with enzyme preparations lyophilized with different amounts of crown ether (Fig. 1). The largest acceleration was observed with 50 to 100 equivalents of 18-crown-6. Under these conditions, the enzyme activity is 450 times larger than that of the untreated enzyme. This corresponds to a specific activity in acetonitrile of 0.7 U/mg of protein.

Remarkably, the effect of 18-crown-6 decreases sharply when larger amounts are added. As the amount of crown ether present in the enzyme powder can readily dissolve in acetonitrile it is not likely that the lower enzyme activation is caused by an increase of the physical barrier for the substrate to enter the active site of the enzyme. Khemelnitsky et al. (1995) reported that the salt matrix of the enzyme powder is an important factor for the enzymatic activity in organic solvents. Therefore, we have investigated whether the decrease in enzyme activity at high crown ether concentrations is due to crown ether promoted dissolution of the buffer salt (sodium phosphate) from the enzyme preparation into the organic solvent. Analysis of the sodium concentration in the organic solvent showed that, in the presence of 50 equivalents of 18-crown-6, 2.2% of the



Scheme 1.

buffer salt is dissolved in the organic medium and, in the presence of 1000 equivalents of 18-crown-6, this is 8.7%. Such low percentages of dissolved buffer salt rule out the possibility that the observed decline in enzyme activation is caused by a substantial stripping of the buffer salt from the enzyme preparation. An alternative explanation for the lower enzyme activation at >100 equivalents of 18-crown-6 could be that the nucleophilic amine function of the substrate is complexed by 18-crown-6, reducing the rate of deacylation of the enzyme. However, no significant decrease in activity was observed when an activated enzyme preparation (lyophilized in the presence of 50 equivalents of 18-crown-6) was incubated with a 50 m*M* *N*-Ac-L-Phe-OEtCl and L-Phe-NH_2 solution, containing as many as 1000 equivalents of 18-crown-6 with respect to the enzyme. This excludes the possibility that the decreasing activity is caused by complexation of the nucleophile by 18-crown-6 as well as the possibility of reversible inhibition of the enzyme by the excess of crown ether. Because the decrease in activity upon using larger amounts of 18-crown-6 is only observed while present during lyophilization this indicates that this effect is caused by a denaturing process during the lyophilization process.

The activating effect of crown ethers on enzyme activity can be attributed to several factors. The effect of lyoprotection of α-chymotrypsin under these conditions was investigated with the well-known lyoprotectant, D-sorbitol (Dabulis and Klibanov, 1995). Lyophilization with this compound resulted in only eightfold higher initial activity of the enzyme. However, when 50 equivalents of 18-crown-6 were also present during lyophilization a 450-fold increase in enzyme activity was observed. Together with the observation that 18-crown-6 is also capable of activating α-chymotrypsin when added to the enzyme suspension in organic media (Broos et al., 1991) this clearly points out that the lyoprotecting role of 18-crown-6 provides only a minor contribution to the activation process. As acetylated trypsin is not activated by lyophilization with crown ether, in contrast to normal trypsin (Broos et al., 1995), an important effect of the crown ethers might be that they form complexes with the quarternary ammonium groups (Gokel, 1991) of lysine residues of the enzyme, thereby preventing salt-bridge formation in the enzyme powder. In organic solvents, the formation of inter- or intramolecular salt bridges between ammonium groups and anionic sites of the protein may destabilize the enzyme (Zheng and Ornstein, 1996), and lock it into a less catalytically active conformation. Prevention of this salt-bridge formation by complexation with crown ether molecules may result in a more active enzyme. In addition, facilitated transport of water molecules (Gokel, 1991) from the active site by complexation with 18-crown-6 may contribute to enhanced substrate binding and, consequently, to higher enzyme activity (Engbersen et al., 1996).

Because enzymes have different activity levels in various organic solvents (Zaks and Klibanov, 1988) this crown ether effect was investigated in a number of solvents with different polarities. Table I shows that enzyme activity is en-

Table I. Initial rate of peptide bond formation, V_o (nanomoles per minute per milligram of protein), between *N*-Ac-L-Phe-OEtCl with L-Phe-NH_2 catalyzed by α-chymotrypsin, pretreated with or without 18-crown-6, in different solvents.

Solvent	V_o (no 18-crown-6)	V_o (50 equivalents of 18-crown-6)	Acceleration
Dioxane	0.19	64.1	337
Acetonitrile	1.70	678.5	400
2-Butanone	4.29	501.1	117
3-Pentanone	4.85	277.5	57
tert-Amylalcohol	1.27	58.2	46
Toluene	5.33	70.8	13

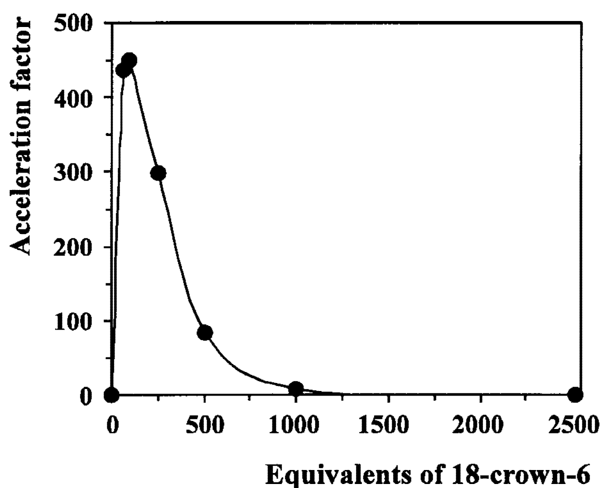


Figure 1. Crown ether acceleration [V_o(18-crown-6)/V_o] for the peptide bond formation catalyzed by α-chymotrypsin between *N*-Ac-L-Phe-OEtCl and L-Phe-NH_2 in acetonitrile as a function of the amount of 18-crown-6 added before lyophilization.

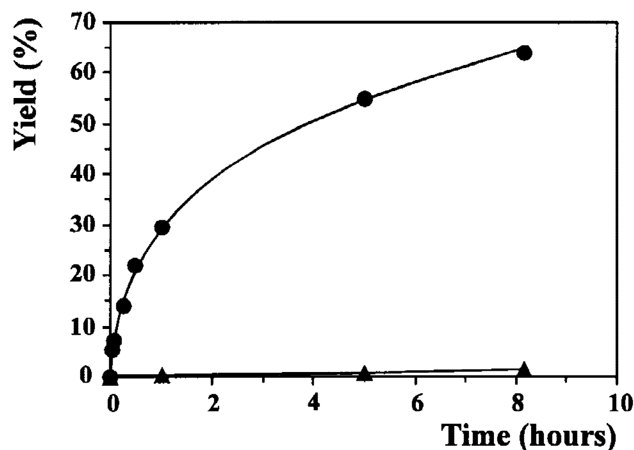


Figure 2. Enzymatic synthesis of *N*-Ac-L-Phe-L-Phe-NH_2 catalyzed by α-chymotrypsin in acetonitrile. Circles: enzyme lyophilized in the presence of 50 equivalents of 18-crown-6; triangles: enzyme lyophilized without 18-crown-6.

Table II. Initial rate of peptide formation by α-chymotrypsin, pretreated with or without 18-crown-16.[a]

Dipeptide	V_o (no 18-crown-6)	V_o (50 equivalents of 18-crown-6)	Acceleration
N-Ac-L-Phe-L-Phe-NH_2	0.22	39.6	181
N-Ac-L-Phe-D-Phe-NH_2	0.29	58.6	204
N-Ac-L-Phe-L-Leu-NH_2	0.54	72.5	135
N-Ac-L-Phe-L-Tyr-NH_2	0.62	65.7	106

[a]Rates are in nanomoles per minute per milligram of protein. Conditions: 5 m*M* of both amino acid precursors, 1 mg/mL α-chymotrypsin, acetonitrile; $a_w = 0.113$, 30°C. The reaction was stopped by the addition of 4 mL of the HPLC eluent.

hanced by 18-crown-6 in all solvents used, with the highest activities in the relatively hydrophilic solvents acetonitrile, 2-butanone, and 3-pentanone. It is noteworthy that crown ether activation was most pronounced in the hydrophilic solvents and less in the more hydrophobic solvents. In our earlier studies on the transesterification of amino acid esters we observed that the optimal crown ether-induced acceleration was found with α-chymotrypsin preparations pretreated with 500 equivalents of 18-crown-6 in hydrophobic solvents, like cyclohexane (Broos et al., 1995). This indicates that the amount of crown ether necessary for optimal enzyme activity enhancement might be dependent on the hydrophobicity of the bulk organic medium.

The importance of the crown ether-induced acceleration on the synthetic aspects of dipeptide formation is shown in Figure 2, which gives the dipeptide yield as a function of time. With crown ether-pretreated α-chymotrypsin almost 70% of the *N*-Ac-L-Phe-L-Phe-NH_2 was formed within 8 h, whereas only 1.5% was formed in the case of a non-pretreated enzyme. On a preparative scale, *N*-acetyl-L-phenylalanyl-L-phenylalaninamide was isolated in 85% yield, starting from *N*-Ac-L-Phe-OEtCl and L-Phe-NH_2, using α-chymotrypsin lyophilized in the presence of 50 equivalents of 18-crown-6 as catalyst.

Table II shows that the crown ether enhancement of the α-chymotrypsin activity in the peptide bond formation between different amino acids is general. Obviously, all the rates of peptide formation were enhanced with factors ranging from approximately 200 in the case of 2-chloroethylester of *N*-acetyl-L-phenylalanine with L- and D-phenylalaninamide to 100 with L-tyrosinamide.

These results demonstrate that the readily available 18-crown-6 partly compensates for the large reduction in activity commonly observed for enzymes in nonaqueous media.

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