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Tetrahedron 61 (2005) 6824–6828
Tetrahedron

Enhanced activity of α-chymotrypsin in organic media using designed molecular staples

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Received 24 March 2005; revised 28 April 2005; accepted 28 April 2005

Abstract—We report the enhancement of α-chymotrypsin activity in organic solvents using modified peptides bearing two crown ethers. The transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol was used as model reaction. Co-lyophilization of crown ether modified peptides with α-chymotrypsin prior to use resulted in an increase of enzyme catalytic activity in non-aqueous media. The efficiency of enzyme activation is dependent on the amino acid sequence of peptidic additives and on the positions of the amino acids bearing the crown ligand.

1. Introduction

Enzymes are continuously gaining importance as 'green' bio catalysts in organic processes and useful tools in organic synthesis due to their exquisite enantioselectivity and chemoselectivity.[1–4] Furthermore, enzymes often simplify the synthetic protocol by reducing the overall number of steps required. Thus, enzymes are competitive and economic alternatives for small and large scale production of enantiopure pharmaceutical intermediates and other useful chiral synthons.[5]

Unfortunately, activity of enzymes decreases in non-aqueous media, in which starting materials are soluble in. To overcome such problems, considerable developments have been made in the past to understand enzyme behavior and reactivity in non-aqueous media.[6,7] Several procedures involving additives have been devised to improve efficiency of enzymes in organic media.[8–12] Among these procedures, the use of additives such as crown ether,[13] sorbitol,[14] sugars,[14] polyethylene glycol,[15] cyclodextrins,[16] and salts,[17] represents an attractive method as it is both straightforward and economical.

However, all of these additives are non-specific and their efficiency is unpredictable. Tailor-made devices specifically designed to enhanced stability, activity and solubility of many different enzymes in non-aqueous media, are therefore highly desirable. Towards that goal, Reinhoudt et al. have studied the use of crown ethers.[13] They observed an improvement of chymotrypsin activity up to 500 fold in organic media by co-lyophilization of the biocatalyst with 250 equiv of 18-crown-6. Recent studies by this group, on the mechanism of crown-ether-induced activation of enzymes in non-aqueous media revealed the possibility of a conformational stabilization in organic solvent by crown ethers.[18] On the other hand, Griebenow et al.[20,21] have demonstrated by infrared spectroscopy that no relationship could be established between secondary structure and activity in various subtilisin-crown ether preparations. The crown ether enhancing effect has also been associated with a molecular imprinting effect. Indeed, it has been proposed that the crown ether is not able to stabilize the overall active 3D structure of the enzyme, but rather helps preserve the active site structure.[19]

Towards more efficient crown systems, Shinkai et al.[22] have developed multi-crown ether compounds, 'crowned' arborols. They have tested the efficiency of these arborols to solubilize myoglobin. Their results showed that smaller 'crowned' arborols gave better results for dissolution of myoglobin in DMF. The authors proposed that the larger multiple crown systems were less efficient probably due to their lower ability to cover the myoglobin surface accurately.

On the basis of our groundwork on functional peptidic devices,[23] we sought to exploit bis-crown ether modified peptides for the structural stabilization and solubilization of enzymes in organic media.

Keywords: Peptide nanostructure; Enzyme stabilization; Crown ether; Organic solvent.
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doi:10.1016/j.tet.2005.04.063

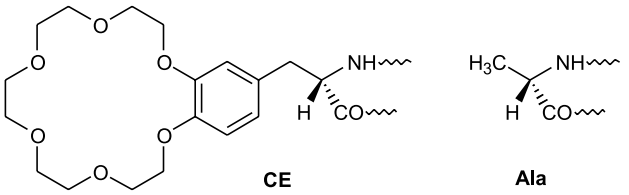
Herein, we report our results on the ability of bis-crown peptides **1**–**3** to act as 'molecular staples' for protein surfaces and their effect on the catalytic activity of α-chymotrypsin in an organic environment.

N-BOC-Ala-Ala-Ala-CE-Ala-CE-Ala-NH-CH_2-CH_2-CH_3 **1**

N-BOC-Ala-Ala-CE-Ala-Ala-CE-Ala-NH-CH_2-CH_2-CH_3 **2**

N-BOC-Ala-CE-Ala-Ala-Ala-CE-Ala-NH-CH_2-CH_2-CH_3 **3**

N-BOC-Ala-Phe-Ala-Ala-Ala-Phe-Ala-NH-CH_2-CH_2-CH_3 **4**



Macrocyclic ligands of the 18-crown-6 family are known to complex Na^+, K^+, and R-NH_3^+ ions. Hence, we envisioned that bis-crown peptides like **1**–**3** could potentially bind tightly at the surface of globular proteins through cooperative complexation of two adjacent charged groups from exposed Lys, Arg, Asp, and Glu residues (Fig. 1).

The hydrophobic nature of the peptidic framework and the neutralization of charged groups at the surface should enhanced solubility in low polarity solvents. Likewise, the 'linkage' of distant functional groups by bis-crown ether devices should also stabilize the active 3D structures of the enzyme. In other words, peptidic devices **1**–**3** could possibly acts as flexible but rigidifying 'staples' that allow stabilization of enzyme active conformation without significant lost of mobility. To demonstrate the proof of concept, we investigated the model reaction consisting of transesterification of *N*-Ac-L-Phe ethyl ester with *n*-propanol catalyzed by α-chymotrypsin in cyclohexane (Scheme 1). This transformation was used previously by several groups for enzyme activity studies.[13]

We have already described the synthesis, conformational

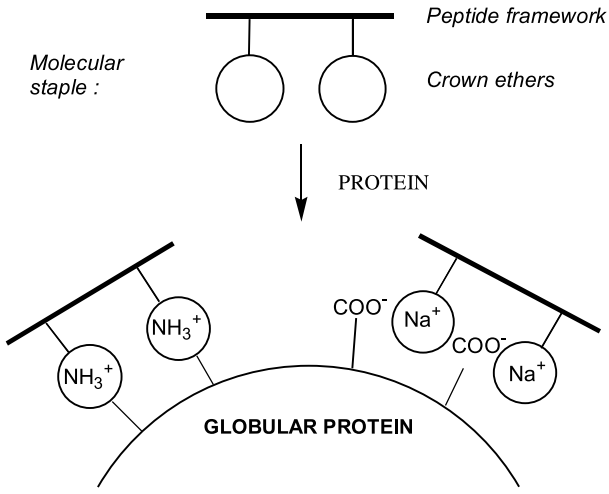
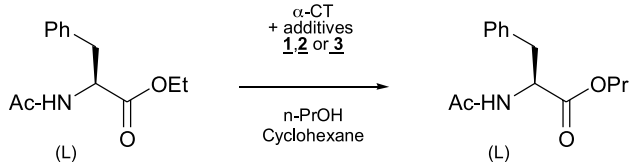


Figure 1. Proposed complexation mode of molecular staples on the surface of a globular protein.



Scheme 1. Transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester catalyzed by α-chymotrypsin used as model system in the present studies.

behavior, and ion binding ability of peptides **1**–**3**. These compounds were shown to complex Cs^+ ions selectively by forming 'sandwich' type complexes with one Cs^+ by the cooperative action of the two 18-crown-6 rings.[24] Furthermore, they were also shown to bind selectively and efficiently diammonium compounds.[25] The alanine based heptapeptide frameworks were chosen for their ability to adopt several different conformations, hence to facilitate the cooperative complexation of two charged groups at variable distances on the surface of α-chymotrypsin. The crown ether residue was synthesized from L-DOPA and the peptides were prepared by solid phase peptide synthesis using the oxime resin.[24]

2. Results and discussion

α-Chymotrypsin was coated with variable amount of **1**, **2** or **3** by adding the bis-crown additive to an aqueous solution (pH 7.8) of the biocatalyst, and then lyophilizing to dryness. As control, we also prepared the enzyme coated with twice as much of 18-crown-6 by the same procedure.

To affect the transesterification, coated enzymes were suspended at room temperature in cyclohexane in the presence of *N*-acetyl-L-phenylalanine ethyl ester and *n*-propanol. The rate of transesterification was followed by high performance liquid chromatography (HPLC) measuring the appearance of *N*-Ac-L-Phe-O-*n*-Pr. Initial rates (V_o) were calculated from conversion $<10\%$. In order to define the most efficient conditions, experiments with α-chymotrypsin coated with 5, 10, 25, and 50 equiv of bis-crown peptides **1**–**3**, as compared to enzyme, were performed. Results are shown in Table 1.

It has to be noted that almost no activity was observed with α-chymotrypsin alone under the reaction conditions used. For α-chymotrypsin coated with 5 equiv of each additive, an increase of enzymatic activity was observed. However, no differences could be noted between biocatalyst stabilized with bis-crown ether additives **1**, **2**, **3**, and 18-Crown-6.

When α-chymotrypsin coated with 10 equiv of peptide additives were used, the initial rate increased by 40 fold compared to α-chymotrypsin without additives, but only two-fold compared to 18-Crown-6. The conversion of the substrate after 30 min is more than 30% for the systems with peptide **1** and with 18-Crown-6, and 15% for both **2** and **3** (Table 1). Interestingly, in this case α-chymotrypsin coated with bis-crown peptide **1** is a little more active then α-chymotrypsin coated with peptide **2** and **3** and 18-Crown-6. This difference could be due to the better enzyme surface

Table 1. Effect of peptides **1–3** on the activity of α-chymotrypsin in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester in cyclohexane/1 M 1-PrOH[a]

Additive	Mole additive (mole α-chymotrypsin)	V_o (+additive) (10^{-5} M min^{-1})	V_o (+additive) / V_o	Conversion (%) 30 min	Conversion (%) 2 h
None	0	12	1	<5	<10
Peptide **1**	5	125	10	<10	20–25
Peptide **2**	5	95	7	<10	15–20
Peptide **3**	5	90	7	<10	15–20
18-Crown-6	10	65	5	<10	15–20
Peptide **1**	10	515	41	30–35	60–70
Peptide **2**	10	420	33	15–20	35–40
Peptide **3**	10	200	16	15–20	20–25
18-Crown-6	20	285	23	25–30	55–60
Peptide **1**	25	2600	208	80	>95
Peptide **2**	25	755	60	30–35	60
Peptide **3**	25	450	36	20–25	40–50
18-Crown-6	50	420	34	40	60–70
18-Crown-6	50	3.3[b]	41[b]		
Peptide **1**	50	3175	254	70–80	> 95
Peptide **2**	50	3695	295	70–80	>95
Peptide **3**	50	1255	100	60–70	70–80
18-Crown-6	100	10	<1	<5	<5

[a] Conditions: 2.5 mM substrate, 1 mg mL^{-1} enzyme powder (not corrected for buffer salts weight), 22 °C.
[b] Results obtained by Reinhoudt and co-workers under the same conditions.[13]

complexation of bis-crown peptide **1** that has the two crown ether residues separated by only one alanine.

The most pronounced effect was observed with 25 equiv of **1** (Table 1). In only 30 min, the conversion of the substrate reached 80% and completion after 1 h with α-chymotrypsin coated with this additive compared to 40% and even less when using 18-Crown-6, peptides **2** or **3**. An improvement of chymotrypsin activity up to 200 fold is observed with peptide **1**, compared to 30 fold for 18-Crown-6. So, the peptide chain allows a six-fold increase of enzyme activity.

Although, not optimized crown peptide additives compared favorably then with commonly used additives (Table 2). These new additives could eventually be used with other enzyme or mixed with other additives like KCl to increase enzyme activation.

Using α-chymotrypsin coated with 50 equiv of peptide **1** lead to a modest improvement of rate compared to reaction with 25 equiv of **1**. Improvement was more important with 50 equiv of peptide **2** and **3** but without a good reproducibility. This is probably due to difficulties of additive dissolution in phosphate buffer with a larger amount of peptide **2** or **3**. Indeed in all other cases, experiments were highly reproducible in duplicate runs. When 100 equiv of 18-Crown-6 were colyophilized with α-chymotrypsin, the rate enhancement of the biocatalyst thus obtained, decreased dramatically. This behavior has also been reported when using more then 250 equiv of 18-Crown-6 with α-chymotrypsin.[18] The important lost of activity is probably due to enzyme denaturation during co-lyophilization.

In order to make this procedure more convenient for synthetic chemists, reactions were performed using conventional glassware and magnetic stirring comparatively to mechanical stirring frequently used with biocatalytic processes. Interestingly, no significant changes were observed in the results between reactions carried out with magnetic or mechanical stirring for reaction times of 2 h or less. However, for reactions requiring longer period of time (>2 h) to attain a reasonable conversion level, it is preferable to use mechanical stirring. Along the same lines, it is important to note that very high conversion of the substrate can be achieved with only 10 equiv of peptidic additive **1** after 24 h (>95% with magnetic stirring and >99% with mechanical stirring).

These results show the efficiency of crown ether peptide to enhance the α-chymotrypsin activity in cyclohexane. However, more work is required to understand the mechanism of enzyme activation by peptide **1–3**. CD and IR studies could not be investigated due to the chiral nature of additives **1–3**. Hence, it is not possible to study their effect on structural stability of chymotrypsin as their own

Table 2. Rate enhancement α-chymotrypsin colyophilized with different additives

Entry	Additives	Enhancement	Reference
1	Crown peptide **1** (25 equiv)	208	
2	18-C-6 (50 equiv)	41	13
3	KCl 94% w/w[a]	51	17
4	Various cyclodextrins[b]	2–40	16
5	Substrate analog[c]	264	14
6	Sorbitol[c]	19	14

[a] For transesterification reaction of *N*-Ac-L-Phe-OEt in anhydrous hexane.
[b] For transesterification reaction of *N*-Ac-L-Tyr-OMe in various organic solvent/water (97:3).
[c] For transesterification reaction of *N*-Ac-L-Phe-OEtCl in anhydrous carbon tetrachloride.

spectrum interferes with the one of the enzyme. The different enhancing ability between **1**–**3** demonstrates that the peptidic framework plays a functional role in the stabilization of the enzyme structure. Therefore, it is fair to say that bis-crown peptides with other amino acid sequences could be engineered to stabilize numerous enzymes of industrial and synthetic interest.

To evaluate the influence of bis-crown peptide **1** on the enantioselectivity of α-chymotrypsin, the transesterification reaction was studied with *N*-Ac-D-Phe-OEt and *N*-Ac-D,L-Phe-OEt as substrates using α-chymotrypsin coated with 10 equiv of **1** as catalyst. After 2 h, almost no conversion was observed for the experiment with the D substrate, whereas around 40% conversion was measured when using the racemic substrate. With the latter reaction, we have determined the enantiomeric excess of product and remaining substrate (ee_p and ee_s) to calculate the enantiomeric ratio (*E*) of the bis-crown peptide enzyme system.[26] An *E* value >200 was obtained; no D-enantiomer could be detected by chiral HPLC even after four days. These results clearly demonstrate that the enantioselectivity of the enzyme is not altered significantly by the presence of crown peptide.

The activity of α-chymotrypsin stabilized with 25 equiv of bis-crown peptide **1** was also studied at different temperature in cyclohexane (Table 3). In comparison with room temperature (22 °C), the activity decreased slightly at 30 °C and significantly at 40 and 50 °C. Therefore, for practical purpose the actual synthetic procedure should be carried out at room temperature.

Table 3. Effect of increasing temperature on catalytic activity of α-chymotrypsin coated with 25 equiv of bis-crown device **1** in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester in cyclohexane/1 M 1-PrOH[a]

T (°C)	V_o (+additive) (10^{-5}M min^{-1})	Conversion (%) 30 min	Conversion (%) 2 h
22	2600	80	>95
30	2210	50	65
40	770	25	30–35
50	45	<10	10–15

[a] Conditions: 2.5 mM substrate, 1 mg mL^{-1} enzyme powder.

Because peptides **1**–**3** incorporate derivatives of phenylalanine, their stability to degradation in presence of α-chymotrypsin was verified. Studies were done in phosphate buffer with bis-crown peptide **3** and compared to its phenylalanine analog **4** that gives no enhancement of catalytic activity in cyclohexane. HPLC analysis demonstrated a rapid degradation of peptide **4**, but complete stability of peptide **3** towards hydrolytic degradation by α-chymotrypsin. The crown ether ring attached to the phenyl group therefore prohibits the accessibility to the active site of α-chymotrypsin, leading to enzymatic degradation stability.

3. Conclusions

We have reported the use of peptides bearing two crown ethers as tailor-made additives for the stabilization of the structure and for increasing activity of α-chymotrypsin in organic solvents. Co-lyophilization of α-chymotrypsin with different amount of **1**–**3** lead to coated biocatalysts with enhanced activity that catalyzes efficiently a model transesterification reaction. The best results were obtained with α-chymotrypsin coated with 25 equiv of bis-crown peptide **1** in cyclohexane with complete conversion of the substrate after less than an hour, a tremendous improvement over the control reaction using uncoated α-chymotrypsin. Important differences of efficiency between additives **1**–**3** point out the functional role of the peptidic framework to allow the two binding groups (crown ether) to cooperatively complex and bridge charged groups at the biocatalyst surface. Therefore, it is possible to envision that this stabilization strategy could be applied to numerous enzymes of interest and that efficiency of peptidic devices could be improved rapidly by parallel solid phase synthesis. Although, 'tailor-made' nanoscale additives are presently more expensives then polyethylene glycol, their potential applicability to a wide variety of enzymes not responding to actual additives make them attractive and a valuable alternative. Improvement of **1**–**3** through parallel synthesis and their use with other biocatalysts are currently underway in our laboratories.

4. Experimental

Synthesis, purification, and characterization of bis-crown ether peptides **1**–**3** was done according to the reported procedures.[23] α-Chymotrypsin (E.C. 3.4.21.1), type II, from bovine pancreas and 18-Crown-6 were obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. Distilled cyclohexane over molecular sieves was used. *n*-Propanol was purified by a benzene azeotropic distillation.

4.1. Coating of α-chymotrypsin

α-Chymotrypsin (10 mg, 4E-4 mmol) and the appropriate amount of bis-crown ether peptides or 18-Crown-6 were dissolved in 50 mM sodium phosphate buffer, pH 7.8 (2 mL). The solution was shaken manually (for the solution with 50 equiv of bis-crown ether peptide, a short sonication gave a better dissolution but it was still incomplete). The samples were lyophilized to a white powder after freezing in liquid nitrogen.

4.2. Catalytic activity

Reactions were performed on a 1.5 mL scale with magnetic stirring. In every reaction, 1.5 mg of α-chymotrypsin, free or coated, was used (quantities were adjusted to always have 1.5 mg of α-chymotrypsin content. For example, 3.5 mg of the solid obtained from co-lyophilization of 25 equiv of peptide **1** and α-chymotrypsin were used). The biocatalyst was added to a cyclohexane solution containing *N*-Ac-L-Phe-OEt (2.5 mM) and 1-propanol (1 M) at 22 °C. The transesterification reaction was immediately followed by high-performance liquid chromatography (HPLC) monitoring the appearance of the reaction product ($t_{ret}=5.2$ min). HPLC analyses were performed on an Agilent 1050 HPLC system using an analytical C_{18} reverse phase column (Vydac, Hesperia, CA, USA). Column was eluted isocratic at a flow rate of 1 mL/min with 45% acetonitrile in water

(with 0.1% TFA). 10 μL of reaction mixtures were injected at regular intervals.

Chiral HPLC was performed with a Hypersil Phenylglycine column (250×4.6 mm, 5 μm) using hexane with 1% ethanol as eluent (1.5 mL min^{-1}).

Acknowledgements

The financial support of this work by NSERC of Canada, FQRNT of Quebec, and Université Laval is gratefully acknowledged.

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