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Influence of cyclodextrins on the stability of the peptide salmon calcitonin in aqueous solution

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Abstract

The influence of β-cyclodextrin (βCD), and various βCD derivatives, on both the chemical and the physical stability of the polypeptide hormone salmon calcitonin (sCT) in aqueous solutions was investigated at elevated temperature (55°C). Also, the influence of various βCD derivatives on the enzymatic degradaton of sCT was evaluated. At pH 6, the effect of CDs on the chemical stability of sCT was negligible at CD concentrations below 5% (w/v). The only exception was the negatively charged carboxymethyl-βCD (CMβCD), which increased sCT stability. The charged CDs, i.e. 2-hydroxytrimethylammonio-propyl-βCD (TMAβCD) and CMβCD, promoted degradation in concentrated solutions of sCT at pH 6. 2-Hydroxypropyl-βCD (HPβCD) and randomly methylated βCD (RMβCD) not only inhibited aggregation, they also solubilised dimers formed in the test solutions, thereby increasing the physical stability. All the CDs tested accelerated the α-chymotryptic degradation of sCT. Maltosyl-βCD (G_2βCD), HPβCD and RMβCD showed an inhibition of leucine aminopeptidic and tryptic degradation.

Keywords: Cyclodextrins; Salmon calcitonin; Stability; Aqueous solution; Enzymatic degradation; Aggregation

1. Introduction

Calcitonin (CT) is a water-soluble polypeptide hormone, discovered by Copp and colleagues in 1961 (Copp, 1992; Hardman et al., 1996). It is produced by the parafollicular cells of the thyroid gland in mammals and the ultimobranchial gland of birds and fish. It decreases blood calcium levels and inhibits bone resorption by directly affecting osteoclast activity, and it is suggested to have an anabolic osteoblastic effect on bone (Hardman et al., 1996). CTs of a different origin are used therapeutically in the treatment of osteoporosis and Paget's disease, and in the management of hypercalcaemia. Salmon CT (sCT) is about 40 times more potent than human CT in lowering blood calcium levels and has a longer duration of action. It is therefore widely used for treatment of bone diseases. The amino acid sequence of CT varies between species, but all CTs consist of 32 amino acids in a single chain, with a disulphide linkage between cysteine residues in positions 1 and 7, forming a loop at the N-terminus, and with a proline amide at the C-terminus.

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Cyclodextrins (CDs) have been extensively studied over the last decade as pharmaceutical excipients. They are mainly used as complexing agents to solubilise lipophilic drugs, thereby increasing their bioavailability, and as stabilising agents (Szejtli, 1988; Frömming and Szejtli, 1994; Loftsson and Brewster, 1996, 1997). Many publications have been devoted to absorption enhancement by CDs of proteins and peptides through mucosal membranes, with special emphasis on the nasal mucosa (Shao et al., 1992; Watanabe et al., 1992b; Schipper et al., 1993; Shao et al., 1994; Matsubara et al., 1995; Marttin et al., 1996a,b). Increased bioavailability of insulin in rats and rabbits after intranasal administration with CDs has been reported (Merkus et al., 1991; Watanabe et al., 1992a). Schipper et al. (1995) and Yetkin et al. (1997) have shown that methylated CDs enhance absorption of sCT from nasal formulations in rats and rabbits. Absorption enhancement may be due either to modification of the mucosal barrier by CDs, or to their stabilising effects on the peptide. It has been shown that various β-cyclodextrin (βCD) derivatives can increase the chemical stability of peptides in aqueous solution, as well as in lyophilised powder (Ressing et al., 1992; Haeberlin et al., 1996). CDs may form inclusion complexes with hydrophobic side chains of polypeptides, especially aromatic residues, or adhere to the polypeptide chain, in a manner similar to polyhydroxylated alcohols. They can thereby protect susceptible groups from chemical or enzymatic modification and minimise interactions between hydrophobic surfaces. Brewster et al. (1991) showed that even at very low concentrations 2-hydroxypropyl-β-cyclodextrin (HPβCD) was able to solubilise and prevent aggregation of lyophilised interleukin-2 upon reconstitution with water, and that CDs can be used to solubilise ovine growth hormone (Brewster et al., 1991; Simpkins, 1991). Investigations by Tokihiro et al. (1996, 1997) demonstrated that while maltosyl-β-cyclodextrin (G_2βCD) has an inhibiting effect on the aggregation of insulin in aqueous solution, sulphated and highly substituted sulphoalkylated βCDs increase the aggregation. On the other hand, it has been shown that sulphated βCDs decrease the rate of urea-induced unfolding of acidic fibroblast growth factor (Burke et al., 1993).

Those results indicate varying influences of different CD derivatives on the stability of different peptides and proteins. It is therefore necessary to carefully select a combination of CDs and peptide based on appropriate experimentation. In this study, the influence of the natural β- and γCDs and various βCD derivatives on the chemical and physical stability of sCT in aqueous solution was investigated. Also, the influence of γCD and βCD derivatives on the stability of sCT towards enzymatic degradation was evaluated.

2. Experimental procedures

2.1. Materials

Synthetic salmon calcitonin (sCT) from Mallinkrodt Chemicals (Missouri, USA) was kindly donated by Hexal AG, Holzkirchen, Germany. Cyclodextrins were obtained from various sources: β-cyclodextrin (βCD) from Nihon Shokuhin Kako Co. (Tokyo, Japan); γ-cyclodextrin (γCD), carboxymethyl-β-cyclodextrin (CMβCD, DS 0.5), 2-hydroxypropyl-β-cyclodextrin (HPβCD, DS 0.7), randomly methylated β-cyclodextrin (RMβCD, DS 1.8) and hydroxytrimethylammonio propyl-β-cyclodextrin (TMAβCD, DS 0.5) from Wacker Chemie GmbH (München, Germany); and maltosyl-β-cyclodextrin (G_2βCD, MS 1) from Pharmatec. Inc. (Florida, USA). Purified enzymes were purchased from Sigma Chemical Co. (St. Louis, USA): Leucine aminopeptidase L-5006, EC 3.4.11.2; α-chymotrypsin C-4129, EC 3.4.21.1; trypsin T-4665, EC 3.4.21.4. Solutions and buffers were prepared with distilled water and all other materials were commercial products of analytical grade.

2.2. HPLC method

The HPLC system consisted of an isocratic pump (ConstaMetric 3200, LDC Analytical), a variable wavelength UV detector operated at 210 nm (SpectroMonitor 3200, LDC Analytical), an automated injection system (AS-2000A, Merck-

Hitachi) with a 20 μl sample loop, and an integrator (D-2500, Merck-Hitachi). Reversed-phase chromatography was conducted with a Phenomenex Prodigy C_{18}, 75 × 4.6 mm, 3 μm column, with a Supelguard C_{18}, 20 × 4.6 mm, 5 μm guard column, both maintained at 40°C in an air-bath column thermostat (SpH 99, Spark-Holland). The mobile phase consisted of 28% (v/v) acetonitrile (HPLC-grade, Rathburn) in an aqueous solution of 0.1% (w/v) triethylamine adjusted to pH 2.5 with phosphoric acid. The flow rate was 1.5 ml/min, and the retention time was approximately 11 min.

2.3. Measurement of total protein

The total protein concentration in aggregated samples was determined with UV absorption measurements at 280 nm in aqueous buffer using a Perkin-Elmer Lambda 3A spectrophotometer. Samples were diluted tenfold in 100 mM acetate buffer. Concentration of test solutions was calculated compared to standards of sCT, treated in the same manner.

2.4. Measurement of free amino groups in degraded samples

The ninhydrin reaction was used to measure free amino groups in degraded samples. Samples were diluted tenfold in 1 M acetate buffer at pH 5.2. Test solutions were prepared by shaking 0.5 ml of diluted sample with 1 ml of ninhydrin reagent solution (Sigma Chemical Co., N-1632) in a test tube. The mixture was heated at 100°C for 15 min. After cooling, the test solutions were diluted tenfold in acetate buffer, and absorbance of the solutions was measured at 570 nm using a Perkin-Elmer Lambda 3A spectrophotometer. Concentration of test solutions was calculated compared to standards of sCT, treated in the same manner.

2.5. Gel electrophoresis

A PhastSystem gel electrophoresis system from Pharmacia (Uppsala, Sweden) was used. Native PAGE was performed with reversed polarity in an acidic buffer system, using high density homogenous gels from Pharmacia (separation range 1–100 kDa). The bands were visualised by staining with Coomassie Brilliant Blue G-250.

2.6. Kinetic studies

Stock solutions of the CDs were prepared at the desired pH values in a Teorell-Stenhagen universal buffer. The buffers were prepared by mixing 20 ml of a solution containing 3.54 g boric acid, 3.27 g phosphoric acid, 7.00 g citric acid and 13.72 g sodium hydroxide, with the appropriate volume of 0.1 M HCl, and filling up to 100 ml with water. The pH of the CD-buffer solutions was adjusted to the desired value at 55°C before sterilisation in an autoclave. The exact pH was determined after autoclaving. Stock solutions of sCT were 1 mg/ml in 10 mM phosphate buffer at pH 3. Test solutions were prepared by diluting the sCT stock solution to a final concentration of 0.05 mg/ml sCT in CD-buffer solution. Equal aliquots were transferred to five glass vials, which were then sealed tightly and put in an incubator at 55.0 ± 0.5°C. At predetermined time intervals, one vial was removed from the incubator and frozen. All vials (for each CD derivative at each pH value) were defrosted at the same time, and their sCT contents determined by HPLC. sCT concentrations were expressed as percentage of the initial concentration. The observed first-order rate constants (k_{obs}) were obtained by linear regression of the natural logarithm of percentage sCT remaining versus time plots.

2.7. Dimerisation studies

CD-buffer solutions were prepared as described above. Test solutions of sCT were made to a concentration of 10 mg/ml sCT in CD-buffer solution. Test solutions were filtered through a sterile 0.20 μm cellulose acetate membrane to ensure clarity of solutions before incubation. Each test solution was divided into three vials, which were then incubated at 55.0 ± 0.5°C. At a 5 day interval, one vial was removed from the incubator and frozen. All vials were defrosted at the same time and filtered through a 0.20 μm cellulose

acetate membrane. The protein concentration in the filtrate was assessed by absorption at 280 nm and absorption at 570 nm after reaction with ninhydrin. The amount of soluble dimers was assessed by native gel electrophoresis. The remaining sCT was determined by HPLC.

2.8. Enzymatic studies

Stock solutions of CDs were prepared in 50 mM Tris buffer, with 10 mM $MgCl_2$ and adjusted to pH 7.4 at 37.0 ± 0.2°C before sterilisation in an autoclave. The exact pH of the buffer solutions was determined after autoclaving. The enzymes were diluted in 50 mM Tris buffer, 1.5 U/ml for LAP and trypsin, and 0.5 U/ml for α-chymotrypsin. 800 μl CD stock solution and 100 μl enzyme solution were mixed and allowed to equilibrate at 37.0 ± 0.2°C for 15 min. The reaction was initiated by adding 100 μl of preheated sCT stock solution to the reaction mixture. Initial concentration of sCT was 0.5 mg/ml. A 100 μl sample was drawn and diluted in 400 μl of quenching solution (0.2% (v/v) TFA in water) and frozen. At predetermined intervals a sample was drawn from the reaction mixture and treated in the same manner. All samples were defrosted at the same time and their remaining sCT contents measured by HPLC. The pseudo first-order rate constants were determined as before (Section 2.6).

3. Results and discussion

3.1. pH-rate profile and influence of CDs

The degradation rate profile for sCT in aqueous solution has previously been determined, and it was shown that sCT exhibits a first-order overall degradation in the pH range from 2 to 8 (Lee and Chien, 1991; Lee et al., 1992; Windisch et al., 1997). The influence of three different βCD derivatives, i.e. CMβCD, RMβCD and TMAβCD, at 5% (w/v) concentration on the pH-rate profile of sCT was evaluated. Fig. 1 is a representative figure of sCT degradation in aqueous CD solutions. The degradation of sCT in the aqueous CD solutions was in all cases first-order. The influence of 0.5% (w/v) βCD and 5% (w/v) γCD, G_2βCD and HPβCD at pH 3 (pH of maximum stability) and pH 6 (approximate pH of nasal mucus) at 55°C was also investigated (Table 1).

sCT shows greatest stability between pH 3 and 3.5. As shown in Fig. 2, the CDs alter the profile, generally increasing the degradation rate. The effect is greatest between pH 2 and 4, especially for CMβCD. Around neutral pH the influence is smaller, and CMβCD and RMβCD slightly stabilise the peptide at pH 6 and 7, respectively. The main degradation products of sCT in the pH range from 2–6 have been identified (Lee et al., 1992; Windisch et al., 1997). Reduced sCT has been detected as the only degradation product in aqueous buffer at pH 2.2, but at pH 3 deamidation products are dominant. At pH 4, hydrolysis of the peptide bond between cys[1] and ser[2] is the most prominent. The negative charges on CMβCD appear to promote the degradation, especially in the case of deamidation products. At

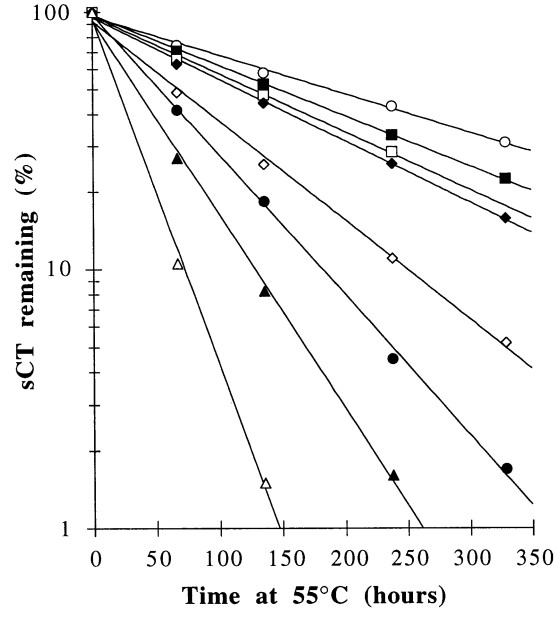

Fig. 1. Determination of degradation rate constants for sCT. The semi-logarithmic plot of remaining sCT versus time in samples containing 5% (w/v) CMβCD at various pH after incubation at 55°C for 0 and up to 329 h. pH 2.0 (●); pH 3.0 (○); pH 3.5 (■); pH 4.0 (□); pH 5.0 (◆); pH 6.0 (◇); pH 7.0 (▲); pH 8.0 (△).

Table 1
Degradation rate constants (k_{obs}) of sCT at two different pH values and 55 ± 0.5°C, in solutions containing different CDs (5% w/v)

Cyclodextrin	pH 3			pH 6		
	k_{obs} (h^{-1})	R^2	Inhibition (%)	k_{obs} (h^{-1})	R^2	Inhibition (%)
None	0.00042	0.9514	0	0.00476	0.9971	0
0.5% βCD[a]	0.00044	0.9644	−7	0.00554	0.9991	−16
5% γCD	0.00043	0.9654	−4	0.00596	0.9967	−25
5% CMβCD	0.00150	0.9950	−261	0.00386	0.9970	19
5% G_2βCD	0.00041	0.9914	0	0.00546	0.9988	−15
5% HPβCD	0.00038	0.9770	7	0.00440	0.9992	8
5% RMβCD	0.00080	0.9647	−92	0.00496	0.9979	−4
5% TMAβCD	0.00052	0.9984	−26	0.00499	0.9996	−5

[a] The βCD concentration was 0.5% (w/v), because of its limited water solubility.

higher pH, degradation pathways mainly involve dimers and polymerisation (Cholewinski et al., 1996; Windisch et al., 1997).

The effect of CD concentration on the stability of sCT at 55°C and pH 6 was determined by incubating sCT in buffered solutions with CD concentrations of 0.5 to 10.0% (w/v). Fig. 3 shows the of inhibition of sCT degradation by various CDs at different concentrations. Generally, the influence of CD concentration is negligible at pH 6, with the exception of CMβCD.

3.2. Dimerisation of sCT

A concentrated solution of sCT, 10 mg/ml, in 5% (w/v) CD solution, buffered to pH 6, was incubated at 55°C. The CDs tested were CMβCD, HPβCD, RMβCD and TMAβCD. After only 1 day in the incubator, fibrillation and deposits could be observed in some of the sample vials. This was most prominent for solutions containing CMβCD, but could also be seen in solutions containing TMAβCD. Solutions containing RMβCD remained clear throughout the 10 day period. The appearance of sample solutions when removed from the incubator is described in Table 2. The samples were filtered and native-PAGE was performed to visualise the aggregates. Lanes from electrophoretic gels are shown in Fig. 4. In lanes containing samples after 5–10 days of incubation, three to five different species of aggregates are visible, the band with R_f 0,55 most pronounced. Those are most likely dimers of different charges. sCT is a basic peptide (it has an isoelectric point above pH 9, as determined by isoelectric focusing on PhastSystem), and has an abnormal electrophoretic mobility in a native system. Molecular weight determinations compared to the

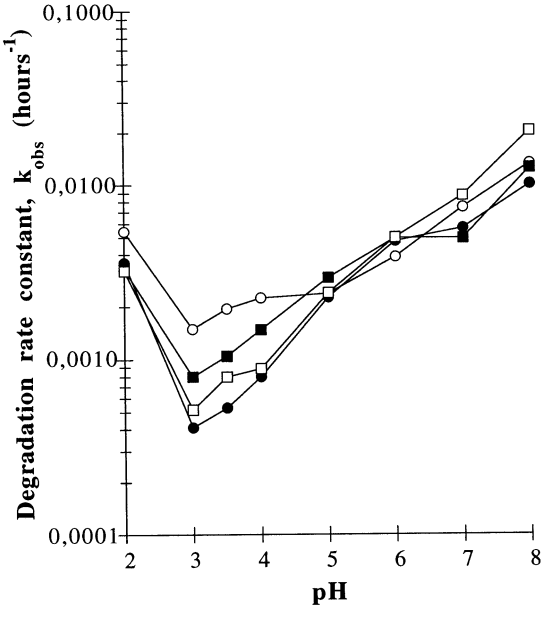

Fig. 2. The pH-rate profile of sCT, determined in citrate-phosphate-borate buffer with and without 5% (w/v) CDs, at 55°C in the pH range from 2 to 8. Initial sCT concentration was only 0.05 mg/ml, and therefore dimerisation is negligible. Without CD (●); CMβCD (○); RMβCD (■); TMAβCD (□).

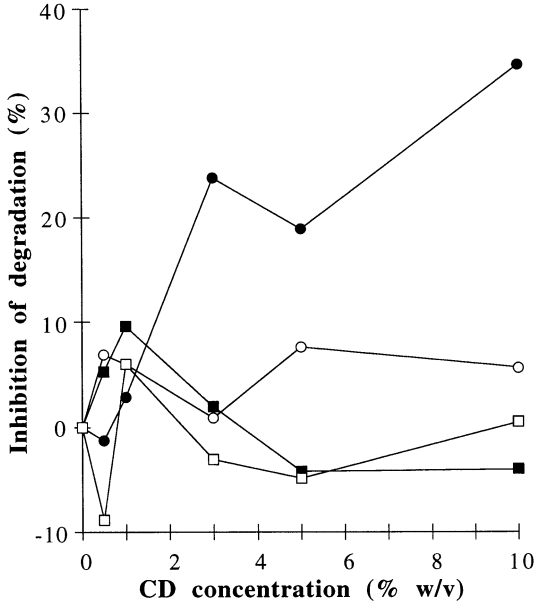

Fig. 3. The inhibition effect of various CD derivatives on the degradation of sCT in pH 6 citrate-phosphate-borate buffer. Initial sCT concentration was only 0.05 mg/ml, and therefore dimerisation is negligible. CMβCD (●); HPβCD (○); RMβCD (■); and TMAβCD (□).

standards available are therefore inaccurate. Table 3 shows the sCT concentration compared with the measured total protein concentration in filtered samples.

Table 2
The appearance of solutions with initial sCT concentration of 10 mg/ml and 5% (w/v) CD at pH 6, incubated for up to 10 days at 55 ± 0.5°C[a]

CD derivative	5 days	10 days
None	+	++
CMβCD[b]	++	+++
HPβCD	−	+
RMβCD	−	−
TMAβCD	−	+

[a] Appearance: clear solution (−); clouded solution, little punctuated aggregates visible (+); punctuated aggregates clearly visible (++); and aggregates and large deposits (+++).

[b] Visible fibrillation occurred instantly. The test solution was filtered before incubation to ensure clarity.

This data indicates the capacity of RMβCD and HPβCD to retard aggregation of sCT, and to solubilise dimers and aggregates of sCT. On the other hand, the charged CDs, i.e. CMβCD and TMAβCD, seem to promote the aggregation and precipitation of sCT.

3.3. Enzymatic degradation of sCT

Lang et al. (1996) studied the degradation of sCT in excised bovine nasal mucosa, and suggested that enzymatic degradation of sCT was primarily due to α-chymotryptic and tryptic activity. CDs have been shown to inhibit the enzymatic degradation of a modified sCT (Haeberlin et al., 1996), and inhibition of enzymatic degradation may be one of the factors contributing to enhanced absorption by CDs. The influence of γCD, CMβCD, G_2βCD, HPβCD, RMβCD and TMAβCD on the enzymatic degradation of sCT in vitro by trypsin, α-chymotrypsin and leucine aminopeptidase was investigated (Table 4). None of the CDs tested showed an inhibiting effect on the α-chymotryptic degradation. In fact, all the CDs accelerated the degradation. G_2βCD, HPβCD and RMβCD showed an inhibition of LAP and tryptic degradation of sCT, 20–30% inhibition for LAP and 54–75% for trypsin.

4. Conclusion

CDs do not greatly affect the chemical stability of sCT in aqueous solution, when the CD concentration is 5% (w/v) or less. On the other hand, HPβCD and RMβCD, significantly increased the physical stability of sCT. In vitro tests with purified enzymes showed an inhibition of tryptic degradation with RMβCD and HPβCD, while α-chymotryptic degradation was enhanced. It may be concluded, that the absorption enhancing effect of RMβCD on sCT, as reported by Schipper et al. (1995) and Yetkin et al. (1997), is probably not due to stabilising of the peptide. Most likely, absorption enhancement may be contributed to the interactions of CDs with components of biological membranes

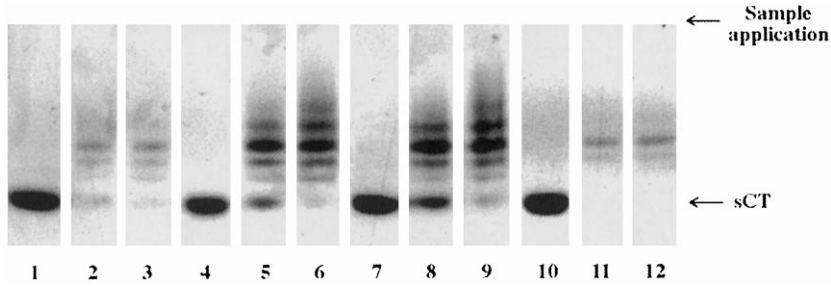

Fig. 4. Native-PAGE of concentrated samples after incubation at 55°C and pH 6. The samples were filtered to remove deposits before electrophoresis. Lanes 1–3 without CDs after 0, 5 and 10 days, respectively; lanes 4–6 with 5% (w/v) HPβCD; lanes 7–9 with 5% (w/v) RMβCD; lanes 10–12 with 5% (w/v) TMAβCD, all after 0, 5 and 10 days, respectively. sCT in samples containing CMβCD could not be detected by this method.

to increase their permeability (Marttin et al., 1995; Irie and Uekama, 1997).

CDs are non-toxic and well tolerated on skin and mucosae, as well as on the cornea of the eye (Irie and Uekama, 1997), and they have been shown to cause reversible and less severe tissue damage than various classical absorption enhancers (Marttin et al., 1996b; Romeijn et al., 1996; Irie and Uekama, 1997). While stabilising effects can not always be obtained by the use of CDs, destabilising effects are frequently small. The presented results, in conjunction with previously published data, indicate that the correct use of CDs as excipients to increase stability and absorption of peptides or peptidomimic drugs can possess an advantage over the classical absorption enhancers.

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Table 3
Total protein concentration and sCT concentration in filtered samples after incubation at 55 ± 0.5°C for up to 10 days[a]

Cyclodextrin	Time at 55°C (days)								
	sCT concentration (mg/ml)			Total protein concentration (mg/ml)					
				Absorbance at 280 nm			Ninhydrin reaction		
	0	5	10	0	5	10	0	5	10
No CD	10.0	0.7	0.5	10.0	4.0	4.3	10.0	3.9	4.6
CMβCD	10.0	0.1	0.1	10.0	7.0	9.0	10.0	0.6	1.0
HPβCD	10.0	3.1	1.2	10.0	9.0	12.0	10.0	6.8	13.8
RMβCD	10.0	3.0	1.1	10.0	12.9	13.9	10.0	12.7	14.0
TMAβCD	10.0	0.4	0.4	10.0	2.6	3.1	10.0	2.5	3.3

[a] sCT was measured by HPLC. The total protein concentration was measured by absorbance at 280 nm, and also by absorbance at 575 nm after reaction with ninhydrin.

Table 4
Degradation rate constants of sCT in reaction mixtures containing various CDs and 0.15 U/ml trypsin, 0.05 U/ml α-chymotrypsin and 0.15 U/ml leucine aminopeptidase, respectively, at pH 7.4 and 37 ± 0.2°C[a]

Cyclodextrin	Trypsin		α-Chymotrypsin		Leucine aminopeptidase	
	k_{obs} (h^{-1})	Inhibition (%)	k_{obs} (h^{-1})	Inhibition (%)	k_{obs} (h^{-1})	Inhibition (%)
None	0.00084		0.00145		0.00061	
γCD	0.00120	−43	0.00281	−94	0.00064	−5
CMβCD	0.00072	14	0.00395	−172	0.00058	5
G_2βCD	0.00039	54	0.00234	−61	0.00045	26
HPβCD	0.00021	75	0.00231	−59	0.00043	30
RMβCD	0.00023	73	0.00186	−28	0.00049	20
TMAβCD	0.00174	−107	0.00240	−66	0.00061	0

[a] sCT was measured by HPLC and values of the degradation rate constants were obtained by linear regression.

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