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α-Chymotrypsin-catalyzed degradation of desmopressin (dDAVP): influence of pH, concentration and various cyclodextrins

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Abstract

Desmopressin [1-(mercaptopropanoic acid)-8-D-arginine vasopressin; dDAVP] is a vasopressin analogue with a selective antidiuretic effect. The oral bioavailability of desmopressin is limited due both to its high hydrophilicity leading to a low intestinal permeability and to low enzymatic stability. The degradation of desmopressin was investigated in aqueous buffer solutions (pH 6.00–9.00) containing the enzyme α-chymotrypsin at a concentration of 0.50 mg/ml at 37°C. The degradation of desmopressin was also studied in solutions containing α-chymotrypsin in the concentration range 0.10–1.00 mg/ml (pH 7.40 and 37°C). The rate of degradation was shown to be highly dependent on both enzyme concentration and pH. Maximal α-chymotrypsin activity was observed in the pH range 7.40–8.00. It was observed that phenylalanine was formed during the degradation of desmopressin. Phenylalanine was formed in the amount of 20% in 120 min. In the same time period 95% of desmopressin was degraded. The formation of phenylalanine can be explained from the substrate specificity of α-chymotrypsin. Cyclodextrins are known to stabilize drugs including peptides against both chemical and enzymatic degradation. In this study it was shown that hydroxypropyl cyclodextrins (α, β and γ) stabilized desmopressin against α-chymotrypsin-catalyzed degradation. The stabilization was by a factor of 3, 9 and 8 at the concentration 12.5% (w/v) for hydroxypropyl-α-cyclodextrin, hydroxylpropyl-β-cyclodextrin and hydroxypropyl-γ-cyclodextrin.

Keywords: α-Chymotrypsin; Cyclodextrin; Desmopressin (dDAVP); Enzyme kinetics; Hydrolysis; Stability

1. Introduction

Desmopressin [1-(mercaptopropanoic acid)-8-D-arginine vasopressin; dDAVP] (Fig. 1) is a syn-

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thetic analogue of the antidiuretic hormone vasopressin containing a deamidated N-terminal. In addition, L-arginine has been substituted with D-arginine. Desmopressin is active in the treatment of central diabetes insipidus and nocturnal enuresis (Vilhardt, 1990).

Desmopressin is administered perorally and intranasally with bioavailabilities of only 1% and 2–10%, respectively (Harris et al., 1986; Vilhardt and Lundin, 1986; Köhler and Harris, 1988). Low lipophilicity of desmopressin (Lundin and Artursson, 1990; Lundin et al., 1991; Kahns et al., 1993) together with enzymatic degradation at the site of administration (Saffran et al., 1988; Lundin et al., 1989; Morimoto et al., 1991) contribute to the poor bioavailability of the therapeutic agent. When administered perorally desmopressin is degraded by the proteolytic enzyme α-chymotrypsin (Kahns et al., 1993). α-Chymotrypsin catalyzes the cleavage of peptide bonds in which the reactive carbonyl group belongs to the L-amino acids tryptophan, tyrosine, phenylalanine and to a lesser extent leucine and methionine (Hess, 1971). The enzyme also catalyzes the hydrolysis of amides and esters of aromatic amino acids (Hess, 1971). The substrate specificity of α-chymotrypsin therefore suggests cleavage of the Tyr–Phe and/or the Phe–Gln bond in desmopressin (Fig. 1).

Cyclodextrins are known to form inclusion complexes with a variety of chemical substances enabling enhanced solubility, protection against enzymatic degradation and absorption enhancement (Brewster et al., 1991; Haeberlin et al., 1996; Stella and Rajewski, 1997). The extent of protection of cyclodextrin peptide complexes against enzymatic degradation is dependent on the nature of the peptide and the chosen cyclodextrin (Haeberlin et al., 1996). Most peptides are too large to fit into the interior cavity of cyclodextrins. Instead, cyclodextrins form complexes with accessible hydrophobic amino acid residues. They probably interact with unfolded amino acid side-chains (Matsuyama et al., 1987; Szejtli, 1991). It has been reported that the side-chains of the aromatic amino acids Tyr^2 and Phe^3 are directed away from the ring structure of desmopressin (Kihlberg et al., 1995).

The present work was undertaken to study in more detail the influence of pH and enzyme concentration on the α-chymotrypsin-catalyzed degradation of desmopressin. In addition, the potential stabilizing effect of different cyclodextrins

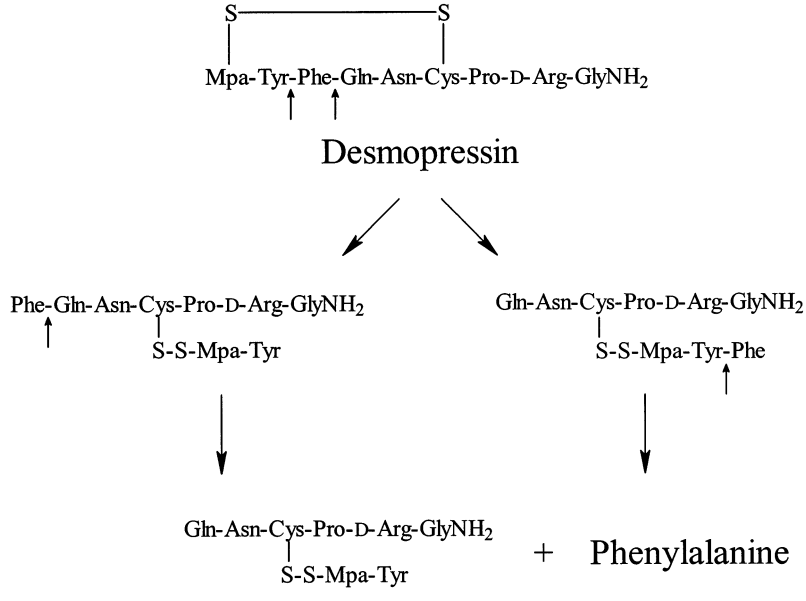

Fig. 1. Structure of desmopressin and the possible sites of cleavage by α-chymotrypsin. In addition, the proposed degradation of desmopressin into two intermediates and the further degradation of these leading to the formation of phenylalanine are shown.

on the α-chymotrypsin-catalyzed degradation of desmopressin was investigated.

2. Materials and methods

2.1. Chemicals

Desmopressin was kindly provided by Ferring A/S (Malmö, Sweden). α-Chymotrypsin (type II, from bovine pancreas; 50 U/mg) was obtained from Sigma Chemical Co. (St. Louis, USA). Phenylalanine was purchased from Calbiochem-Novabiochem, Switzerland. Hydroxypropyl-α-cyclodextrin (HP-α-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Buffer substances and solvents were of reagent grade.

2.2. Apparatus

High-performance liquid chromatography (HPLC) was carried out using a Shimadzu LC-6A pump and a Shimadzu SPD-6A UV detector equipped with a Rheodyne 7125 injection valve with a 20-μl loop. A Inertsil ODS-2 column (250 × 4.6 mm; 5-μm particles) from Chrompack (Middelburg, The Netherlands) was used for desmopressin. Phenylalanine analysis was performed using a column (120 × 4.6 mm) packed with Spherisorb ODS-2 (5-μm particles). Readings of pH were done on a Radiometer PHM 83 Autocal instrument.

2.3. Chromatographic conditions

In case of desmopressin a mobile phase consisting of 20% (v/v) acetonitrile in 0.1% (v/v) H_3PO_4 was used. Triethylamine was added in the concentration 10^{-3} M to prevent tailing. The flow rate was 1.0 ml/min and the column effluent was monitored at 220 nm. The described HPLC procedure was capable of separating desmopressin from its degradations products. For analysis of phenylalanine the mobile phase consisted of 0.1% (v/v) H_3PO_4 with 10^{-3} M triethylamine added to improve the peak shape. The pH of the mobile phase was adjusted to 2.5 with 2 M NaOH. The flow rate was 1.2 ml/min and the column effluent was monitored at 220 nm. Quantitation of desmopressin and phenylalanine was done by measuring the peak heights in relation to standards analyzed under the same conditions.

2.4. Kinetic measurements

The experiments were carried out in triplicate. Degradation studies of desmopressin (10^{-4} M) were performed at 37°C in 0.1 M phosphate buffer with pH adjusted to 6.00, 6.50, 7.00, 7.40, 8.00, 8.50 and 9.00. The α-chymotrypsin concentration was 0.50 mg/ml. For the study of the influence of enzyme concentration on the rate of degradation of desmopressin at pH 7.40, additional α-chymotrypsin concentrations of 0.10, 0.25 and 1.00 mg/ml were employed. At appropriate intervals, samples of 250 μl of reaction solution were withdrawn and added to Eppendorf tubes containing 250 μl of 5% (v/v) perchloric acid. The acidity of the mixture afforded total inactivation of the enzyme. After mixing and centrifugation at 13 000 rpm for 3 min, the supernatant was analyzed by HPLC using the conditions described above. The stability of desmopressin in 0.1 M phosphate buffer was also investigated.

The influence of cyclodextrins on the α-chymotrypsin-mediated degradation of desmopressin was studied at pH 7.40 using an α-chymotrypsin concentration of 0.50 mg/ml (37°C). The cyclodextrin (HP-α-CD, HP-β-CD and HP-γ-CD) concentrations were 2.5, 5.0, 7.5, 10.0 and 12.5% (w/v).

3. Results and discussion

3.1. Hydrolysis in buffer solutions containing α-chymotrypsin

The stability of desmopressin was studied in aqueous buffer solutions with different pH values containing various concentrations of α-chymotrypsin. All stability studies were performed at 37°C and were done in triplicate. The results are

Table 1
Half-lives ($t_{1/2}$, min) for the α-chymotrypsin-catalyzed hydrolysis of desmopressin in 0.1 M phosphate buffer at 37°C: variation of pH[a]

α-Chymotrypsin (mg/ml)	pH						
	6.00	6.50	7.00	7.40	8.00	8.50	9.00
0.50	105.6 ± 1.8	46.7 ± 2.0	24.5 ± 0.2	20.2 ± 0.3	20.5 ± 0.1	29.3 ± 1.6	35.5 ± 3.8

[a] Experiments were done in triplicate and results are given as mean ± S.D.

stated as the mean ± S.D. In most cases, standard deviations below 5% were observed. The rate of degradation was derived by following the disappearance of desmopressin. At an initial desmopressin concentration of 10^{-4} M and a constant concentration of α-chymotrypsin the hydrolysis of desmopressin followed strict pseudo first-order kinetics for several half-lives. Pseudo first-order rate constants for the hydrolysis were determined from the slopes of linear plots of the logarithm of residual desmopressin against time.

As can be seen from Tables 1 and 2, the degradation of desmopressin is highly dependent on pH and the concentration of α-chymotrypsin. The data show that the rate of the α-chymotrypsin-catalyzed hydrolysis increases with pH in the pH range 6.00–7.40 (Table 1). As shown in Fig. 2, maximal reactivity is seen between pH 7.40 and 8.00. This is in accordance with previous studies, where the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan showed the same pH optimum (Hess, 1971). Pseudo first-order degradation is observed in the enzyme concentration range studied and the rate constant is proportional to the enzyme concentration ($r = 0.998$). In the absence of α-chymotrypsin, no degradation was found after incubation of desmopressin in 0.1 M phosphate buffer pH 7.40 for 2 days (Table 2).

The normal concentration of the pancreatic enzyme α-chymotrypsin in the gut lumen, including the stool, is about 0.5 mg/ml or 2×10^{-5} M (Kahns et al., 1993). With that enzyme concentration, a pH in the duodenum of 7 and a transit time of 1 h, 80% of a peroral dose of desmopressin will be degraded during transit through the duodenum. So the α-chymotrypsin concentration and the pH in the gut lumen are important factors determining the amount of desmopressin available for absorption.

3.2. Formation of phenylalanine

The substrate specificity of α-chymotrypsin suggests cleavage of the Tyr–Phe and/or the Phe–Gln bond in desmopressin (Fig. 1), resulting in two intermediates with a supposed affinity to α-chymotrypsin (Phe-Gln-Asn-Cys(S-S-Mpa-Tyr)-Pro-D-Arg-GlyNH_2 and Gln-Asn-Cys(S-S-Mpa-Tyr-Phe)-Pro-D-Arg-GlyNH_2). If these two intermediates are further degraded by α-chymotrypsin this would lead to formation of phenylalanine (Fig. 1). This means that desmopressin might be degraded by a parallel reaction to two intermediates and these intermediates might degrade further by a consecutive reaction to phenylalanine. Desmopressin (10^{-4} M) was incubated in 0.1 M phosphate buffer pH 7.40 containing 0.5 mg/ml α-chymotrypsin. As seen from Fig. 3, phenylalanine is produced and its concentration increases gradually with time. Phenylalanine is not formed simultaneously during the degradation of desmopressin. After 120 min, 95% of desmopressin was degraded whereas only 20% of phenylalanine was formed. This must mean that the further degradation of the intermediates to phenylalanine is slower than the initial degradation of desmopressin. Another explanation for the low formation (non-equimolar) of phenylalanine might be that one of the two initially formed intermediates do not have affinity to α-chymotrypsin and therefore are not degraded further.

Table 2
Half-lives ($t_{1/2}$, min) for the α-chymotrypsin-catalyzed hydrolysis of desmopressin in 0.1 M phosphate buffer at 37°C: variation of α-chymotrypsin concentration[a]

pH	α-Chymotrypsin concentration (mg/ml)				
	0.00	0.10	0.25	0.50	1.00
7.40	Stable[b]	115.6 ± 5.2	53.2 ± 2.0	20.2 ± 0.3	10.5 ± 0.3

[a] Experiments were done in triplicate and results are given as mean ± S.D.
[b] No degradation was seen for 2 days.

3.3. Degradation in the presence of cyclodextrins

The ability of different cyclodextrins (HP-α-CD, HP-β-CD and HP-γ-CD) to stabilize desmopressin against cleavage by α-chymotrypsin (0.5 mg/ml) at pH 7.40 and 37°C has been investigated. In all cases pseudo first-order degradation kinetics was found. In Table 3 the half-lives of desmopressin degradation are given. The most effective protection against degradation was achieved by HP-β-CD and HP-γ-CD. At 12.5% (w/v) HP-γ-CD and HP-β-CD provided similar protection resulting in an almost nine-fold decrease in degradation rate compared to that observed in buffer without cyclodextrins added. For the HP-α-CD the maximal protection factor was 3, which was also obtained at 12.5% (w/v). The stabilization effect seen might be due to complex formation between the cyclodextrins and desmopressin. The size of desmopressin (MW 1069) makes formation of inclusion complexes impossible. More likely, complexation may involve the hydrophobic side-chains of the amino acids tyrosine and phenylalanine. The peptide is actually degraded by α-chymotrypsin at these two amino acids, so it is reasonable to assume that the stabilization mediated by the cyclodextrins is due to complexation of these amino acids. Another explanation for the stabilization seen could be inhibition of α-chymotrypsin activity by the cyclodextrins. It has previously been shown that cyclodextrins can inhibit leucine aminopeptidase activity in nasal homogenates and thereby suppress the degradation of insulin (Irie et al., 1992). A recent publication also describes inhibition of the α-chymotrypsin-catalyzed degradation of the

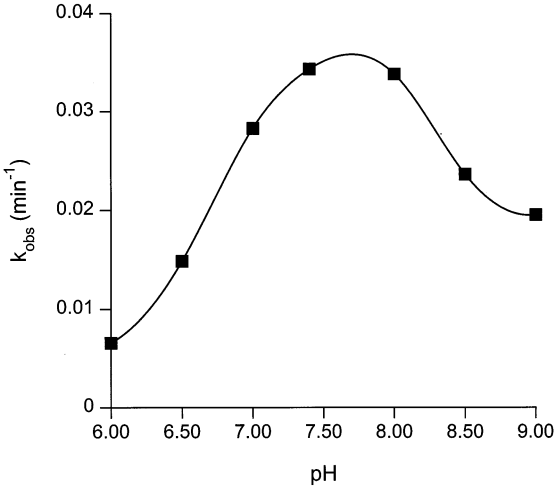

Fig. 2. Plot showing the pH optimum for the degradation of desmopressin in buffer solution containing α-chymotrypsin (0.50 mg/ml) at 37°C.

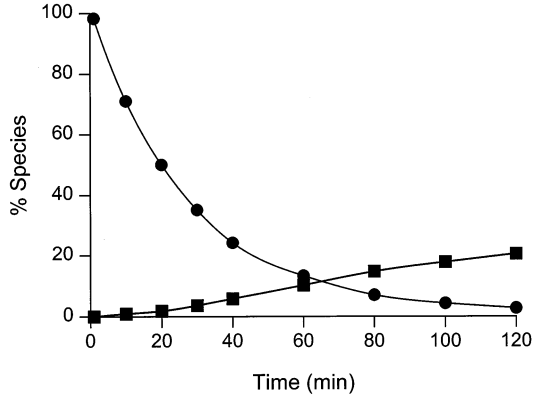

Fig. 3. Formation of phenylalanine during degradation of desmopressin in 0.1 M phosphate buffer pH 7.40 containing α-chymotrypsin (0.50 mg/ml) at 37°C. ●, desmopressin; ■, phenylalanine.

Table 3
Half-lives for the degradation of desmopressin in 0.1 M phosphate buffer pH 7.40 and 0.50 mg/ml α-chymotrypsin solution containing various concentrations of cyclodextrins at 37°C[a]

Concentration of cyclodextrins (%, w/v)	$t_{1/2}$ (min)		
	HP-α-CD	HP-β-CD	HP-γ-CD
0.0	20.2 ± 0.3	20.2 ± 0.3	20.2 ± 0.3
2.5	26.4 ± 0.5	39.1 ± 0.9	41.4 ± 1.6
5.0	35.3 ± 1.5	55.4 ± 0.2	72.0 ± 4.3
7.5	41.8 ± 1.0	66.4 ± 2.2	77.0 ± 2.7
10.0	56.5 ± 1.0	96.9 ± 3.7	128.0 ± 8.7
12.5	65.8 ± 1.1	173.2 ± 1.9	154.6 ± 9.0

[a] Experiments were done in triplicate and results are given as mean ± S.D.

peptide buserelin acetate by maltosyl-β-cyclodextrin (Matsubara et al., 1997). The inhibition was due to both a direct interaction between the enzyme and cyclodextrin and through the formation of a complex between the substrate and cyclodextrin (Matsubara et al., 1997). More studies are needed to determine whether it is complexation and/or inhibition of α-chymotrypsin that is the course of the stabilization seen for desmopressin.

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