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Facile α-chymotrypsin-catalyzed degradation of the HIV inhibitor [D-Ala^1]-Peptide T amide

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Summary

[D-Ala^1]-Peptide T amide is an octapeptide presently undergoing clinical evaluation as a potential agent for the treatment of AIDS. As a part of studies aiming at obtaining an oral delivery form of the peptide, its stability towards α-chymotrypsin was examined. The peptide was found to be rapidly degraded by this pancreatic enzyme, the degradation being due to cleavage of the Tyr–ThrNH$_2$ bond. At pH 7.4 and 37°C the peptide showed a K_m value of 1.3×10^{-4} M, a k_{cat} value of 560 min^{-1} and a specificity constant (k_{cat}/K_m) of 4.3×10^6 M^{-1} min^{-1}. At physiological concentrations of α-chymotrypsin and at pH 6.0–7.4 the half-life of degradation of the peptide was calculated to be less than 5 s. It is thus concluded that a means to protect [D-Ala^1]-Peptide T amide against cleavage by α-chymotrypsin is an absolute requirement for the development of an orally absorbable product. The peptide proved highly stable toward aminopeptidase as well as in human plasma.

Introduction

[D-Ala^1]-Peptide T amide (PT) (Fig. 1) is an octapeptide which prevents binding of the human immunodeficiency virus (HIV) to its CD4 receptor, a surface protein present on T4 lymphocytes and other cells (Pert et al., 1986, 1988; Ruff et al., 1987). The peptide is undergoing evaluation as a potential therapeutic agent for the neuropsychiatric and neurological sequelae of acquired immune deficiency syndrome (AIDS) (Wetterberg et al., 1987; Brenneman et al., 1988). The compound is given by intravenous infusion in all clinical trials.

As a part of studies currently being performed in this laboratory on designing prodrug derivatives of bioactive peptides to improve their delivery (Bundgaard, 1991), we are interested in the feasibility of developing an orally absorbable preparation of PT. Su et al. (1990) recently predicted from an intestinal wall permeability study that the peptide could have good oral bioavailability in human. However, these authors also noted that PT is subject to degradation by intestinal enzymes. We herein report that PT is extremely rapidly degraded by the pancreas enzyme α-chymotrypsin and that a means to protect the peptide against α-chymotrypsin is mandatory for the development of an orally absorbable prepara-

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tion. Data on the stability of PT in human plasma and in the presence of aminopeptidase are also given.

Materials and Methods

Apparatus

High-performance liquid chromatography (HPLC) was carried out with a Shimadzu system consisting of an LC-6A pump, an SPD-6A variable-wavelength UV detector and a Rheodyne 7125 injection valve with a 20 μl loop. Separations were performed with a deactivated Supelcosil LC-8-DB reversed-phase column (33 × 4.6 mm, 3-μm particles) in conjunction with a Supelguard precolumn (both from Supelco Inc., U.S.A.). Readings of pH were carried out on a Radiometer Type PHM 83 Autocal instrument.

Chemicals

[D-Ala^1]-Peptide T amide, *N*-benzyloxycarbonyl-[D-Ala^1]-Peptide T amide (Z-PT) and [D-Ala^1]-Ser-Thr-Thr-Thr-Asn-Tyr were kindly provided by Carlbiotech A/S, Copenhagen. α-Chymotrypsin (type II; from bovine pancreas) (56 U/mg) and leucine aminopeptidase (cytosol, type III-CP from porcine kidney) were obtained from Sigma Chemical Co. (MO, U.S.A.). Chemicals and solvents used in the kinetic studies were of reagent grade.

Kinetic measurements

Degradation studies of PT and Z-PT were performed at 37°C in a 0.1 M phosphate buffer solution of pH 6.0–7.4 containing α-chymotrypsin at concentrations of 0.0025–0.01 mg ml^{-1}. The initial concentration of the peptides was 5×10^{-5} or 4×10^{-4} M. At appropriate intervals, samples of 250 μl were withdrawn and added to 250 μl of a 5% (v/v) aqueous solution of perchloric acid to stop the reaction. After mixing and centrifugation for 3 min at 13000 rpm, 20 μl of the supernatant was analyzed by HPLC. A reversed-phase Supelcosil LC-8-DB column was eluted at ambient temperature with a mobile phase consisting of acetonitrile-0.1% phosphoric acid (3 : 97 v/v) with triethylamine added at a concentration of 10^{-3} M to improve peak shape. For the analysis of Z-PT the concentration of acetonitrile in the mobile phase was 15% v/v. The column effluent was monitored at 215 nm, the flow rate being 1.5 ml min^{-1}. Quantitation of the compounds was performed by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

Degradation studies of PT in 80% human plasma as well as in 0.1 M phosphate buffer

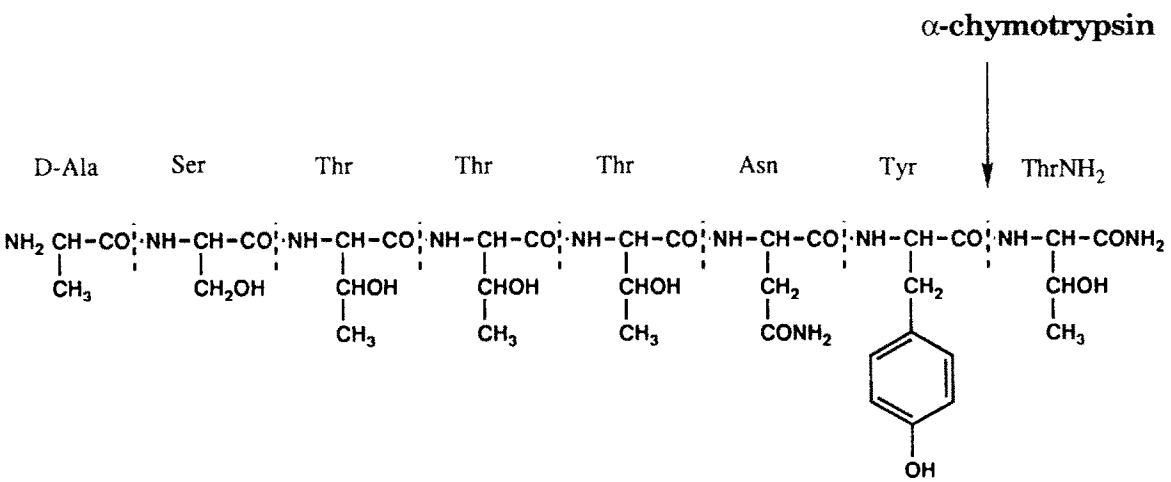

Fig. 1. Structure of [D-Ala^1]-Peptide T amide and site of cleavage by α-chymotrypsin.

solution (pH 7.4) containing leucine aminopeptidase were carried out in the same manner as for the α-chymotrypsin reactions. The initial concentration of PT was 5×10^{-5} M.

Results and Discussion

α-Chymotrypsin-catalyzed hydrolysis

α-Chymotrypsin is an endopeptidase (serine protease) which catalyzes the hydrolysis 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). As expected from this substrate specificity, [D-Ala^1]-Peptide T amide (PT) was found to be readily cleaved by α-chymotrypsin at the bond between Tyr and $ThrNH_2$ (Fig. 1). When PT (5×10^{-5} M) was incubated at 37°C in a 0.1 M phosphate buffer solution of pH 7.4 containing 0.005 mg ml^{-1} of α-chymotrypsin, the disappearance of PT was found to be accompanied by the stoichiometric formation of [D-Ala^1]-Ser-Thr-Thr-Thr-Asn-Tyr as revealed by HPLC analysis of the reaction solution. Under the HPLC conditions described above, PT showed a retention time of 3.2 min whereas [D-Ala^1]-Ser-Thr-Thr-Thr-Asn-Tyr had a retention time of 4.0 min. No peaks other than those due to these compounds were seen in the chromatograms (Fig. 2). As seen from Fig. 3, PT was quantitatively hydrolyzed at its Tyr–$ThrNH_2$ bond by α-chymotrypsin.

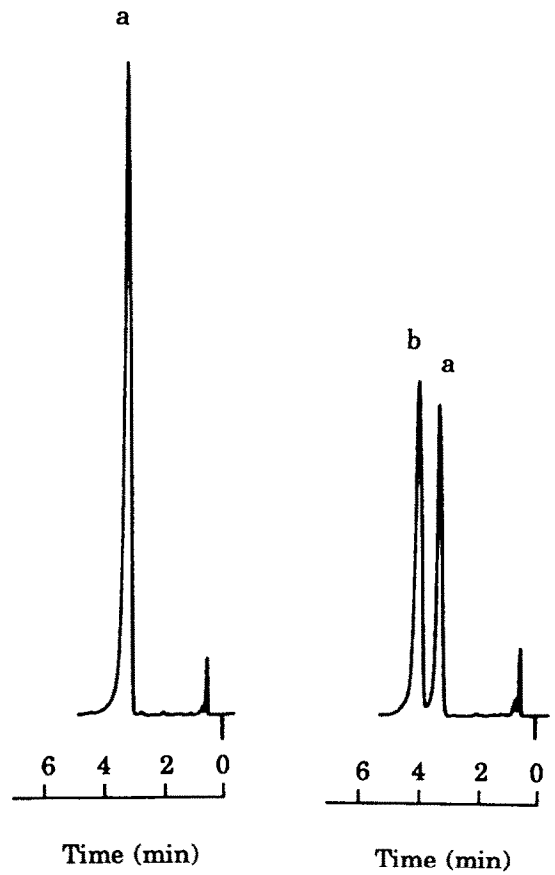

Fig. 2. Chromatograms showing the separation of [D-Ala^1]-Peptide T amide (a) and [D-Ala^1]-Ser-Thr-Thr-Thr-Asn-Tyr (b) in 0.1 M phosphate buffer solution of pH 7.4 containing 0.005 mg ml^{-1} of α-chymotrypsin (at 37°C).

At an initial substrate concentration of 5×10^{-5} M, the α-chymotrypsin-catalyzed hydrolysis of PT proceeded according to strict first-order kinetics. First-order plots obtained at various pH values are shown in Fig. 4. The half-lives of hydrolysis calculated from the slopes of these plots are listed in Table 1 along with the half-lives observed at pH 7.4 at various concentrations of α-chymotrypsin. The data show that the rate of the α-chymotrypsin-catalyzed hydrolysis of PT increases with pH in the pH range 6.0–7.4 which is in accordance with the normal maximum activity of the enzyme occurring near pH 8 (Hess, 1971). It is further apparent that the rate of reaction is directly proportional to the enzyme concentration in the range investigated. In the absence of α-chymotrypsin, PT proved highly stable. Thus, less than 5% degradation occurred upon incubation of PT in 0.1 M phosphate buffer solution of pH 7.4 at 37°C for 50 h.

In order to obtain data for the specific enzymatic rate parameters, the degradation of PT was followed at a relatively high initial concentration (4×10^{-4} M). Under these conditions and with an α-chymotrypsin concentration of 0.005 mg ml^{-1}, corresponding to 2×10^{-7} M (the molecular weight of the enzyme being 25000 (Hess, 1971)), the progress of degradation of PT followed mixed zero- and first-order kinetics. As seen from Fig. 5, the rate of degradation initially

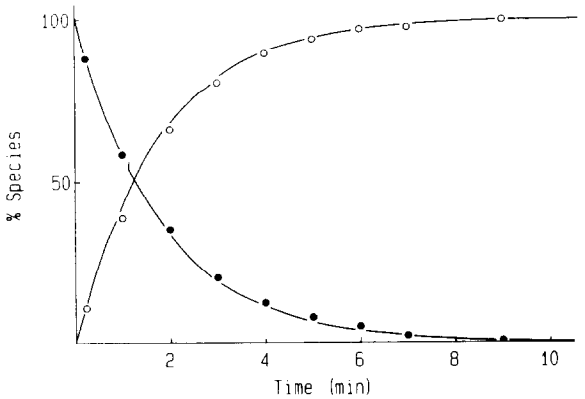

Fig. 3. Plots showing the time courses of degradation of [D-Ala^1]-Peptide T amide (●) and formation of [D-Ala^1]-Ser-Thr-Thr-Thr-Asn-Tyr (○) in 0.1 M phosphate buffer solution containing 0.005 mg ml^{-1} of α-chymotrypsin (at 37°C).

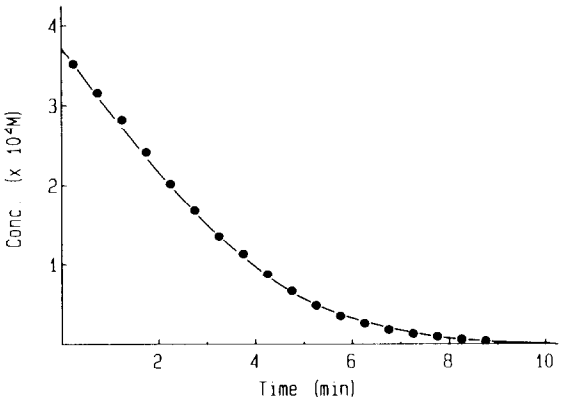

Fig. 5. Plot showing the rate of degradation of [D-Ala^1]-Peptide T amide in 0.1 M phosphate buffer solution of pH 7.4 (●) containing 0.005 mg ml^{-1} of α-chymotrypsin (at 37°C). The curve is calculated from Eqn 2 and the rate parameters given in the text.

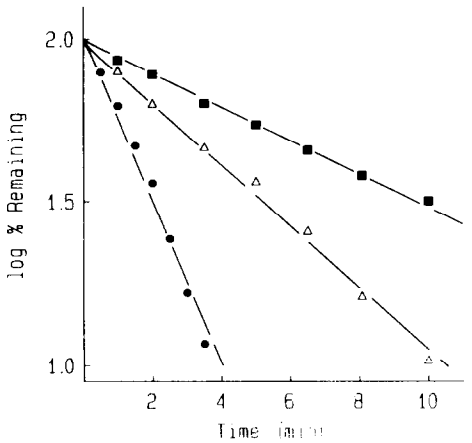

Fig. 4. Plots showing first-order kinetics of degradation of [D-Ala^1]-Peptide T amide in 0.1 M phosphate buffer solution of pH 6.0 (■), pH 6.5 (△) and pH 7.4 (●), respectively, containing 0.005 mg ml^{-1} of α-chymotrypsin (at 37°C).

TABLE 1

Half-lives for the α-chymotrypsin-catalyzed hydrolysis of [D-Ala^1]-Peptide T amide in 0.1 M phosphate buffer solutions at 37°C

[α-Chymotrypsin] (mg ml^{-1})	$t_{1/2}$ (min)		
	pH 6.0	pH 6.5	pH 7.4
0.0025			2.3
0.005	6.0	2.8	1.1
0.01			0.6
0.5			< 0.1

followed zero-order kinetics, and as the substrate depleted, it changed to follow first-order kinetics. This behaviour is typical for enzyme-catalyzed reactions following Michaelis-Menten kinetics in which the initial substrate concentration is greater than the Michaelis constant K_m (Segel, 1975).

The differential form of the Michaelis-Menten equation is

$$-\frac{dS}{dt} = \frac{k_{cat} E_0 S}{K_m + S} \quad (1)$$

where k_{cat} is the turnover constant, K_m represents the apparent binding constant, E_0 is the enzyme concentration and S denotes the substrate concentration at time t. Integration of Eqn 1 gives (Thomas et al., 1987):

$$k_{cat} E_0 t = S_0 - S + K_m \ln(S_0/S) \quad (2)$$

where S_0 is the initial substrate concentration. By analyzing the curve in Fig. 5 according to Eqn 2, using iterative nonlinear regression analysis (Thomas et al., 1987), a good fit of this equation to the curve was obtained. The following values of the rate parameters K_m and k_{cat} were obtained: $K_m = 1.3 \times 10^{-4}$ M; $k_{cat} = 560$ min^{-1}; $k_{cat}/K_m = 4.3 \times 10^6$ M^{-1} min^{-1}.

At a low substrate concentration, i.e. $S \ll K_m$, the enzymatic degradation of PT is first order with the rate equal to

$$-\frac{dS}{dt} = \frac{k_{cat} E_0}{K_m} S \quad (3)$$

Under these conditions, the half-life of degradation is:

$$t_{1/2} = 0.693/(k_{cat} E_0/K_m) \quad (4)$$

Using the values of k_{cat} and K_m listed above, the half-life of degradation of PT at an α-chymotrypsin concentration of 0.005 mg ml^{-1} corresponding to 2×10^{-7} M can be calculated to be 0.8 min. This corresponds well with the value (1.1 min) determined in a run with an initial PT concentration of 5×10^{-5} M (Table 1).

The normal concentration of α-chymotrypsin in the gut including the stool is about 0.5 mg ml^{-1} or 2×10^{-5} M (Goldberg et al., 1968; Dockter et al., 1986). At this enzyme concentration PT is degraded extremely rapidly. At a PT concentration less than 10^{-4} M, where first-order kinetics prevail, the half-life of degradation can be calculated from Eqn 4 to be only 0.5 s at pH 7.4 and 37°C. If pH is 6.0 the calculated half-life (cf. Table 1) is 0.06 min or 4 s. Under saturation conditions, i.e. when $[PT] \gg K_m$, the zero-order rate constant for the degradation equals $k_{cat} E_0$ or 0.011 M min^{-1} at pH 7.4 and 37°C. Thus, at an initial concentration of PT of 10^{-3} M, the half-life of its degradation is approx. 3 s.

The high value (4.3×10^6 M^{-1} min^{-1}) of the specificity constant (k_{cat}/K_m) for the α-chymotrypsin-catalyzed hydrolysis of PT reflects the remarkably high sensitivity of the peptide to the enzyme. A comparison of this value with that for other peptides hydrolyzed by α-chymotrypsin (Bauer, 1976; Bauer et al., 1976; Morihara and Oka, 1977; Bizzozero et al., 1982) indicates that PT is probably the most sensitive peptide substrate described to date.

Blocking of the free N-terminal amino group in PT by *N*-benzyloxycarbonylation to give Z-PT does not reduce the susceptibility of the Tyr–$ThrNH_2$ bond towards α-chymotrypsin. Thus, at an enzyme concentration of 0.005 mg ml^{-1} and at pH 7.4 and 37°C, the first-order half-life of degradation of Z-PT was found to be 0.8 min which is similar to the value (1.1 min) observed for PT under similar conditions. Bioreversible derivatization of PT at its free amino group is therefore not a suitable means to protect the peptide against α-chymotrypsin.

Inclusion complexation with a cyclodextrin appears also to be of no value as a means to afford stabilization of PT against degradation by α-chymotrypsin. Thus, when the reaction solution (pH 7.4, 37°C) of α-chymotrypsin (0.005 mg ml^{-1}) and PT (5×10^{-5} M) contained 2% 2-hydroxypropyl-β-cyclodextrin, a half-life of PT degradation of 1.2 min was observed which is quite similar to the half-life (1.1 min) in solutions without the cyclodextrin.

Stability of PT in human plasma and in the presence of aminopeptidase

When PT (5×10^{-5} M) was incubated at 37°C in a 0.1 M phosphate buffer solution of pH 7.4 containing leucine aminopeptidase at a concentration of 200 U ml^{-1}, no degradation was observed after 24 h. This is also to be expected since the N-terminal alanine residue in the peptide is of D-configuration. Similarly, PT proved to be quite stable in solutions containing 80% human plasma. No degradation was observed following incubation for 5 h at 37°C.

Conclusions

The results obtained show that the HIV-inhibiting octapeptide [D-Ala^1]-Peptide T amide is very rapidly cleaved at its Tyr–$ThrNH_2$ bond by α-chymotrypsin. Under conditions similar to those prevailing in vivo, the half-life of degradation of peptide can be estimated to be less than 5 s. It can therefore be concluded that the development of a means to protect the peptide against α-chymotrypsin is an essential step in any attempts to develop an orally absorbable formulation of the compound. Various means of bioreversible derivation of the peptide at its tyrosine phenol

group in an effort to protect the Tyr–ThrNH$_2$ bond against α-chymotrypsin (Kahns and Bundgaard, 1991) are presently being pursued.

Acknowledgements

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