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# Stability of several LHRH antagonists against proteolytic enzymes and identification of degradation products by mass spectrometry

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In this study stabilities of several LHRH antagonists against proteolytic enzymes are compared. For the enzymatic tests 15 proteases which differ in both substrate specificity and pH optimum were selected. The cyclic and two linear antagonists proved to be extraordinarily stable against the enzymes used over an incubation time of 50 h. Some degradation products were identified by high-performance liquid chromatography combined with mass spectrometry.

## 1. Introduction

The decapeptide LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$) is a primary regulator of the reproductive cycle. LHRH analogues are used in medicine for the treatment of infertility, hormone sensitive prostate and premenopausal breast cancer and for a variety of other hormone-dependent diseases [1–3]. To increase the stability of LHRH against proteolytic degradation and to enhance its biological activity, LHRH analogues were modified in positions 1, 2, 3, 6 and 10. LHRH antagonists were synthesized with bulky hydrophobic D-amino acids in position 1, 2 and 3 such as D-Nal(2)[1], D-Phe(4Cl)[2] and D-Pal(3)[3] [4]. A substitution with D-Ala-$NH_2$ in position 10 of LHRH causes an improvement of the antagonistic potency compared with Gly-$NH_2$ in this position. Replacements of $Gly^6$ in LHRH antagonists with hydrophilic non-basic D-amino acids such as D-Cit and D-Hci produce potent antagonists with low risk of inflammation at the injection site, which is characterized as an allergic response associated with histamine release [5]. Equally potent antagonistic compounds with additionally improved water solubility are obtained by substitution in this position with strongly polar hydrophilic modified D-amino acids as D-Glu(Tris) or D-Ser(Rha) [6, 7]. Replacement with substituted basic D-amino acids, e.g. D-Lys(Nic) in position 6, is another possibility for the synthesis of antagonistically active LHRH compounds, because only the free base causes histamine release [8]. Arg or Lys(iProp) in position 8 are used for antagonists which successfully suppress an allergic response [9]. One might consider synthesizing proteolytically stable antagonists by cyclization, but both the C- and N-termini of the peptide chain should be free for good receptor binding affinity [10].

The involvement of the $Tyr^5$-$Gly^6$ bond in the initial degradation of LHRH has been proven by a number of studies on the products of LHRH hydrolysis by whole homogenates of brain, pituitary, liver, lung, kidney, ovaries and testes [11–16]. The degradation in prostatic cells does not seem to be similar to that by various peptidases which were suggested to be involved in the degradation of LHRH in different tissues. These peptidases are endopeptidase 24.15 (EC 3.4.24.15; splitting $Tyr^5$-$Gly^6$), post-proline cleaving endopeptidase or prolyl endopeptidase (EC 3.4.21.26; splitting $Pro^9$-$Gly^{10}$-$NH_2$), pyroglutamic-aminopeptidase (EC 3.4.11.8; splitting $pGlu^1$-$His^2$), angiotensin converting enzyme (ACE 3.4.15.1; splitting $Trp^3$-$Ser^4$) and the endopeptidase 24.11 (EC 3.4.24.11; splitting $Gly^6$-$Leu^7$), which is present in brain homogenates and in peripheral tissues [17–21]. Recently, a neutral metallo-endopeptidase was also found in the prostate [15]. Metabolism might occur within the lumen of the rectal, nasal, buccal or vaginal cavities, at the surfaces of these membranes or within the cells [22].

In addition to these investigations, the action of other enzymes like chymotrypsin, thermolysin, nagarse, pronase E and subtilisin on LHRH and its analogues has also been studied [7, 23–26].

In the present paper we compare the stability of several LHRH antagonists against a series of proteolytic enzymes. Peptide structures are given in chapter 3.1. Some of the compounds studied proved to be potent antagonists and will be clinically tested. For the enzymatic tests we selected 15 proteases, which differ in both substrate specificity and pH optimum. The extent of degradation products resulting from the incubation of LHRH antagonists with proteolytic enzymes was determined by HPLC. Some of them were identified by MS.

## 2. Investigations, results and discussion

The results of our studies on the stability of several LHRH antagonists against proteolytic enzymes are summarized in Figs. 1 and 2. Only proteases which split more than 5% of the corresponding antagonist are taken into consideration. Fig. 1 shows the extent of proteolysis of 6 antagonists, including two cyclic compounds and differing in position 5, 6, 7 or 8. Three of them, [$Nle^7$]-SB-75, Cyclorelix and Cyclo-SB-88, proved to be rather stable against proteases. In contrast, the analogues D-22620, A-75998 and Cetrorelix$^{INN}$ were unstable against some of the endopeptidases used. In a previous work it was shown that 0.8%–1.5% of Cetrorelix$^{INN}$ was degraded by chymotrypsin, pronase and nagarse after an incubation time of 50 h [24].

In the search for other antagonistically acting analogues of Cetrorelix$^{INN}$, position 6 has been substituted with varying D-Lys derivatives, resulting for instance in compounds D-22620 and A-75998. Modification of a basic amino acid in position 6 should in principle suppress inflammation at the injection site, characterized as an allergic response associated with histamine release. However, the antagonist D-22620, which contains an extensively modified D-Lys residue, was substantially split by chymotrypsin (45%) and clostripain (41%) and additionally by proteinase K and pronase E. The antagonist A-75998 contains $Me\text{-}Tyr^5$, D-$Lys(Nic)^6$ and $Lys(iProp)^8$. As a result of $Me\text{-}Tyr^5$, water solubility was decisively improved, and the allergic response was repressed by $Lys(iProp)^8$ [10]. A-75998 was very unstable against the non-specifically cleaving pronase E and degraded up to 53%. To a minor extent it was also

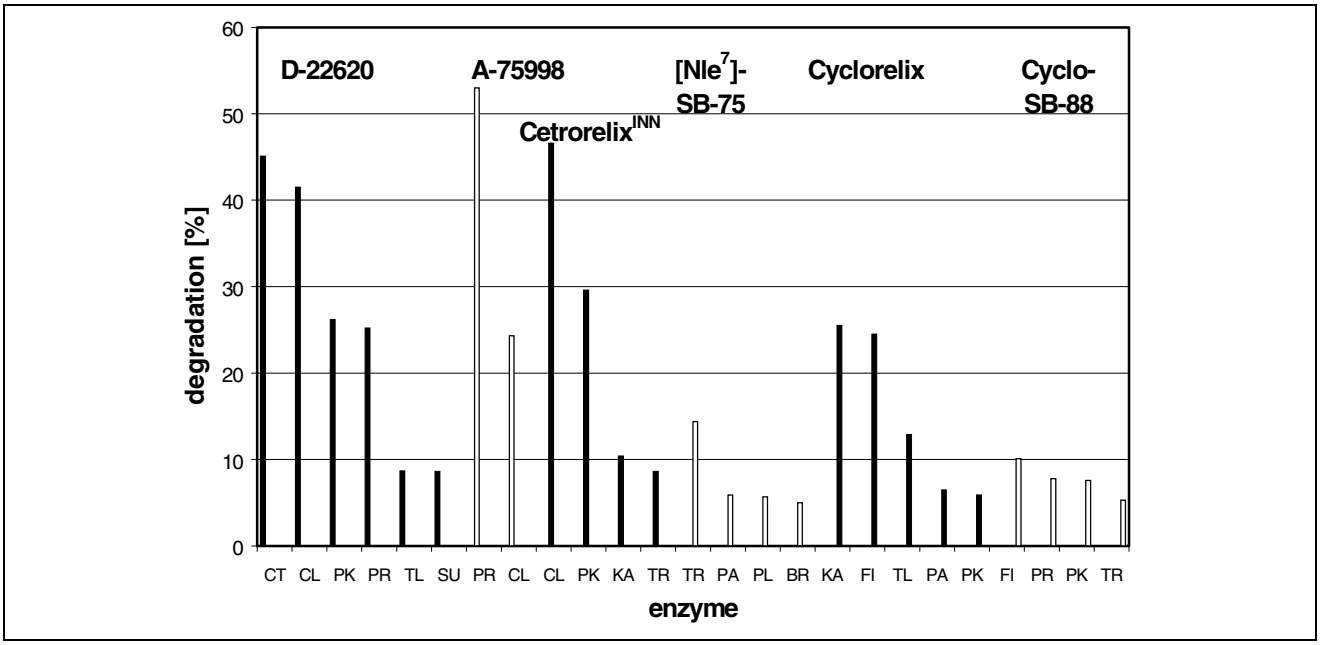


Fig. 1: Enzymatic degradation of D-22620, A-75998, Cetrorelix$^{INN}$, [Nle$^7$]-SB-75, Cyclorelix and Cyclo-SB-88 with different proteases

split by clostripain. Cetrorelix$^{INN}$, however, was intensively degraded only by clostripain (47%) and proteinase K (30%).

The stabilities of the LHRH antagonists [Cit$^6$]-SB-75, Antarelix$^{TM}$, D-21973 and D-21744 are illustrated in Fig. 2. The splitting products of these 4 antagonists were identified by LC-MS. Introduction of a D-amino acid in position 6 obviously enhances the stability against proteolytic action. Whereas [Cit$^6$]-SB-75 was degradated by endoproteinase Arg-C and pepsin to 35% and 29%, respectively, Cetrorelix$^{INN}$ containing D-Cit$^6$ (Fig. 1) was not cleaved by these enzymes. [Cit$^6$]-SB-75 was hydrolysed by thermolysin and subtilisin to an even greater extent.

Antarelix$^{TM}$ represents an antagonist which contains N$^6$-isopropyllysine in position 8. It turned out to be unstable against chymotrypsin and endoproteinase Arg-C, the latter specifically cleaving the peptide bonds at the carboxylic site of Arg. In this case the Lys(iProp)$^8$-Pro$^9$ bond may be involved.

D-21973, an antagonist with D-Lys(kic) in position 6, was considerably split by chymotrypsin (37%) and to a lower extent by pronase E (16%).

The Me-Ser$^4$ containing antagonist D-21744 proved to be very stable against enzymatic degradation.

Some proteolysis products of the LHRH antagonists shown in Fig. 2 were identified by LC-MS. The results are summarized in Tables 1 and 2.

[Cit$^6$]-SB-75 containing L-Cit in position 6 was hydrolysed by various enzymes (Fig. 2). Pepsin prefers aromatic amino acids such as Tyr and Phe in the $P_1$-position. [Cit$^6$]-SB-75 was split by this protease, in accordance with the substrate specificity, after the aromatic amino acids Tyr and D-Pal(3). We identified two hydrolysis products (**1** and **2**, Table 1). Clearly, D-Pal(3) as an unnatural and modified

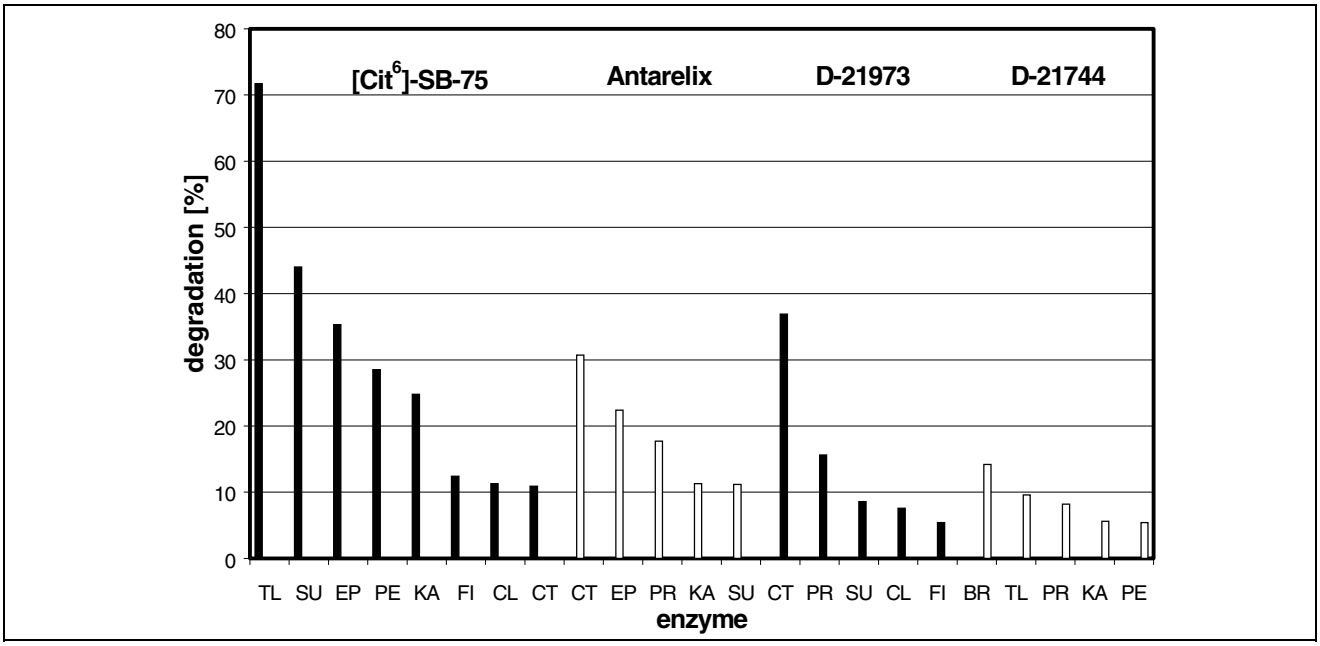


Fig. 2: Enzymatic degradation of [Cit$^6$]-SB-75, Antarelix$^{TM}$, D-21973 and D-21744 with different proteases

**Table 1: Structural assignement by LC-MS of proteolysis products of [Cit$^6$]-SB-75**

| Enzyme | LHRH derivative and its proteolysis products | | Sequence No. | $M_r$ (calc.)[a] | m/z[b] $MH^+$ | m/z[b] $MH_2^{2+}$ |
|---|---|---|---|---|---|---|
| | **[Cit$^6$]-SB-75** Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | | 1–10 | 1429.7 | 1430.6 | 715.9 |
| Pepsin | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | Ser-Tyr-Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | (**2**) | 4–10 | 861.5 | 862.5 | |
| Ficin | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.3 | |
| | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit | (**3**) | 1–6 | 993.4 | 994.5 | 497.7 |
| | Leu-Arg-Pro-D-Ala-$NH_2$ | (**4**) | 7–10 | 454.3 | 455.5 | |
| Clostripain | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit | (**3**) | 1–6 | 993.4 | 994.5 | 497.8 |
| | Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | (**5**) | 6–10 | 611.4 | 612.3 | |
| Endoproteinase Arg-C | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit | (**3**) | 1–6 | 993.4 | 994.4 | 497.6 |
| | Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | (**5**) | 6–10 | 611.4 | 612.4 | |
| | Leu-Arg-Pro-D-Ala-$NH_2$ | (**4**) | 7–10 | 454.3 | 455.4 | |
| Kallikrein | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser | (**6**) | 1–4 | 673.2 | 674.4 | |
| | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit | (**3**) | 1–6 | 993.4 | 994.4 | 497.7 |
| | D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**7**) | 2–5 | 597.2 | 598.3 | |
| | D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit | (**8**) | 2–6 | 754.3 | 755.2 | |
| Chymotrypsin | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | (**5**) | 6–10 | 611.4 | 612.5 | |

[a] Monoisotopic masses; [b] m/z values for the $[M+H]^+$ or $[M+2H]^{2+}$ ions in the ESI mass spectra

D-amino acid was accepted by pepsin, but we did not find hydrolysis of the bond between D-Phe(4Cl) and D-Pal(3).
The protease ficin accepts in the $P_1$-position both small amino acids such as Gly or Ala and aromatic amino acids such as Phe and Tyr [28]. The degradation products found are the result of splitting after Tyr (**1**) and also after Cit (**3** and **4**). A ficin cleavage product containing Cit in $P_1$ was not observed until now, but it is known from the literature that ficin hydrolyses the peptide bond after Arg in insulin [29]. Thus, structural similarity between Arg and Cit residues may account for this hydrolysis result.

Using clostripain we unexpectedly obtained the hydrolysis products **1** and **5**. Normally, this thiolprotease acts with a restricted substrate specificity for Arg-Xaa bonds. This had been demonstrated early with glucagon and the insulin B chain as substrates [30]. Product **3** could again be explained by Arg and Cit similarities, but hydrolysis between Tyr$^5$ and Cit$^6$ is clearly different from the known substrate specificity of this protease.
Endoproteinase Arg-C similarly gave the peptides **1**, **3** and **5** and additionally the hydrolysis product **4** resulting from cleavage after Cit$^6$.

**Table 2: Structural assignement by LC-MS of proteolysis products of D-21973, D-21744 and Antarelix™**

| Enzyme | LHRH derivatives and their proteolysis products | | Sequence No. | $M_r$ (calc.)[a] | m/z[b] $MH^+$ | m/z[b] $MH_2^{2+}$ |
|---|---|---|---|---|---|---|
| | **D-21973** Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys(kic)-Leu-Arg-Pro-D-Ala-$NH_2$ | | 1–10 | 1512.7 | 1513.8 | 757.4 |
| Pronase E | Arg-Pro-D-Ala-$NH_2$ | (**9**) | 8–10 | 341.2 | 342.2 | |
| Chymotrypsin | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr | (**1**) | 1–5 | 836.3 | 837.4 | |
| | D-Lys(kic)-Leu-Arg-Pro-D-Ala-$NH_2$ | (**10**) | 6–10 | 694.4 | 695.5 | |
| | **D-21744** Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Me-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | | 1–10 | 1443.7 | 1444.8 | 722.9 |
| Bromelain | Me-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala-$NH_2$ | (**11**) | 4–10 | 875.5 | 876.4 | 438.8 |
| | **Antarelix** Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Hci-Leu-Lys(iProp)-Pro-D-Ala-$NH_2$ | | 1–10 | 1457.7 | 1458.7 | 729.9 |
| Chymotrypsin | Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3) | (**12**) | 1–3 | 586.2 | 587.2 | |

[a] Monoisotopic masses; [b] m/z values for the $[M+H]^+$ or $[M+2H]^{2+}$ ions in the ESI mass spectra

A differing substrate specificity was also found for the protease kallikrein which usually accepts Arg in the $P_1$ position and Phe, Leu and other hydrophobic amino acids in $P_2$ position. We identified three fragments with Ser, Tyr and Cit in $P_1$, and two fragments with D-Phe(4Cl) in the $P'_1$ position. The latter may be formed by hydrolysis of **1** and **3** after the Ac-D-Nal(2) residue. In view of the known substrate specificity, formation of **1** is rather unexpected, whereas the occurrence of **3** might be quite reasonable by analogy between Arg and Cit.

Chymotrypsin reacted in a foreseeable manner giving only compounds **1** and **5**.

The LHRH antagonist D-21973 was substantially hydrolysed by chymotrypsin and pronase E (Fig. 2). Whereas after action of pronase E only **9** could be identified (Table 2), chymotrypsin cleaved according to its substrate specificity at the C-site of Tyr giving peptides **1** and **10**.

The antagonist D-21744, although relatively stable against proteolytic degradation, was hydrolysed by bromelain at the D-Pal(3)-Me-Ser bond giving **11**. Apart from the fact that bromelain accepted a D-amino acid in this case, its specificity for an aromatic side chain in $P_1$ has also been proven in the examples with bradykinin and angiotensin [28].

Antarelix™ was mainly susceptible to the action of chymotrypsin, and in agreement with its substrate specificity product **12** was identified.

In conclusion, the examined LHRH antagonists show a broad spectrum in their stabilities against the action of various proteases. The LHRH analogue [Cit$^6$]-SB-75 with an L-amino acid in position 6 is very unstable against the endoproteases used and was hydrolysed substantially. On the other hand, both cyclic LHRH derivatives as well as D-21744 and [Nle$^7$]-SB-75 mainly resisted hydrolytic attack.

The degradation products correspond in some cases to the known substrate specificities of the proteases used. However, the individual structures of the LHRH antagonists containing several D- and modified amino acids also give rise to some unexpected proteolysis fragments.

## 3. Experimental

### *3.1. Materials*

The following LHRH antagonists were used:
Cetrorelix$^{INN}$ (SB-75): Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala-$NH_2$; Cyclorelix: c[Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala]; Cyclo-SB-88: c[Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Hci-Leu-Arg-Pro-D-Ala]; Antarelix™/Teverelix$^{INN}$: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Hci-Leu-Lys(iProp)-Pro-D-Ala-$NH_2$; A-75998: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Me-Tyr-D-Lys(Nic)-Leu-Lys(iProp)-Pro-D-Ala-$NH_2$; [Nle$^7$]-SB-75: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Cit-Nle-Arg-Pro-D-Ala-$NH_2$; D-22620: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys(Zu(Z))-Leu-Arg-Pro-D-Ala-$NH_2$; D-21973: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys(kic)-Leu-Arg-Pro-D-Ala-$NH_2$; [Cit$^6$]-SB-75: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-Cit-Leu-Arg-Pro-D-Ala-$NH_2$; D-21744: Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Me-Ser-Tyr-D-Cit-Leu-Arg-Pro-D-Ala-$NH_2$.
All LHRH antagonists were from ASTA Medica AG and synthesized by standard solid-phase methods using MBHA resin and the Boc-strategy [27].
Pepsin (3100 U/mg), bromelain (1600 U/mg), plasmin, trypsin (12700 U/mg), clostripain (50 U/mg), α-chymotrypsin (50 U/mg) and kallikrein (40 U/mg) were purchased from Sigma (Germany), ficin (0.8 DMC-U/mg), proteinase K (8 DMC-U/mg) and elastase (porcine pancreas, 276 U/mg) from Serva (Germany). Pronase E (6.9 U/mg) and subtilisin (49.9 U/mg) were obtained from Fluka (Germany), thermolysin (25 U/mg) and endoproteinase Arg-C (20 U/mg) from Boehringer (Germany) and papain (30000 USP-U/mg) from Merck (Germany). All enzymes were used directly for enzymatic reactions without any modifications.

### *3.2. Proteolysis experiments*

To a Tris-solution (1 μM) of LHRH antagonist in a 0.7-ml vial, a Tris-solution (1 mM) of the particular protease was added at 37 °C. The pH of each solution was adjusted with acetic acid to the pH optimum of the enzyme used, i.e., pepsin (2.0), bromelain (4.6), papain (6.2), ficin (7.0), plasmin, pronase E, proteinase K, subtilisin, thermolysin (7.5), trypsin, clostripain (7.6), α-chymotrypsin (7.8), endoproteinase Arg-C (8.0), kallikrein (8.7) and elastase (8.8). The final concentration of each LHRH antagonist was 0.1 mg/ml and for each enzyme 0.15 mg/ml. The samples were agitated on a thermomixer at 10 Hz for 50 h, then heated to 80 °C for 10 min, cooled to room temperature and centrifuged at 10 000 rpm. Subsequently, pure acetic acid was added by an autosampler to adjust the final pH to 1.5–2. After mixing the components, the samples were centrifuged at 10 000 rpm for 2 min and the clear solution analysed by HPLC. The HPLC analyses were performed using a GAT-system (LC 110 pump, GAT-LCD 503 UVIS detector, LC 1610 autosampler), which was fitted with a Nucleosil 100 C18 precolumn (5 μm, 40 × 4 mm) and a Nucleosil 100 C18 column (5 μm, 250 × 4 mm). Samples were eluted isocratically at a flow of 0.8 ml/min using acetonitrile/water (100/160) containing 0.2% trifluoracetic acid. Compounds were detected at a wavelength of 254 nm and room temperature. The recovery of unreacted LHRH analogues was determined by repeating the procedure with each LHRH antagonist without enzyme. The recovery was 100 ± 1%. The results are the means of two degradations. The reproducibility of results was determined with a variance of 2%.

### *3.3. Identification of degradation products by LC-MS*

The mass spectrometric equipment consisted of a LC-Q-spectrometer from ThermoQuest (USA), with an electrospray ion source operating in the positive-ion mode and a heated transfer capillary at 210 °C. LC separations were performed with a gradient pump system 8890 from Spectra Physics, with a UV-detector LCD 500 from GAT (FRG), and a 125 × 2 mm column packed with Nucleosil 300 C18 (5 μm). Gradient elution in the on-line mode was carried out with premixed mobile phases of acetonitrile/water (A) (5/95) and (B) (95/5), both containing 0.1% trifluoroacetic acid. A linear gradient from 20% B to 90% B over 25 min was applied at a flow rate of 0.3 ml/min. Before entering the electrospray ionization unit, the effluent was split down to a final flow of 30 μl/min.

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