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Activity of pancreatic endopeptidases towards luteinizing hormone-releasing hormones

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Abstract

LHRH and its analogues have low oral bioavailability; this is in part due to their degradation by peptidases present in the intestinal lumen. To determine the appropriate inhibitors to co-administer with LHRH oral formulations, the peptidases involved in their digestion have to be identified. Human (hLHRH) and salmon (sLHRH) LHRH analogues contain a number of potential cleavage sites for the lumenal pancreatic secreted serine endopeptidases: chymotrypsin, trypsin and elastase. The rate of LHRH degradation by equimolar concentrations of chymotrypsin, trypsin and elastase were examined separately in vitro, at pH 8.0, 15°C. At a molar ratio of 1:1000 (enzyme:LHRH), both LHRH analogues were rapidly hydrolysed by α-chymotrypsin with half-lives of 2.5 ± 0.3 and 2.7 ± 0.4 min (mean ± S.D., $n = 3$), respectively, whereas in the presence of elastase both LHRH analogues were slowly hydrolysed with half-lives of 90 ± 15 and 114 ± 21 min (mean ± S.D., $n = 3$), respectively. Trypsin had no activity towards either LHRH analogues after 2 h incubation. The degradation of the LHRH analogues by elastase is likely to be a property of the chymotrypsin impurity. It is concluded that protection of the LHRH analogues from α-chymotrypsin is a requirement for the development of oral absorbable product.

Keywords: Luteinizing hormone-releasing hormone; Pancreatic endopeptidases degradation; Oral delivery; Subsite specificity

1. Introduction

Recent developments in the biotechnology industry have enabled the large-scale production of peptides and novel peptides drugs (Banga and Chein, 1988). The major challenge to the pharmaceutical scientist is to deliver these drugs orally. Analogues of luteinizing hormone-releasing hormone (LHRH) are currently used in the treatment of several diseases including, prostate and breast cancers, and endometriosis (Filicori and Flamigni, 1988). The pancreatic serine endopeptidases, chymotrypsin, trypsin and elastase present in high concentrations in the lumen of the small intestine have been shown to represent a major metabolic

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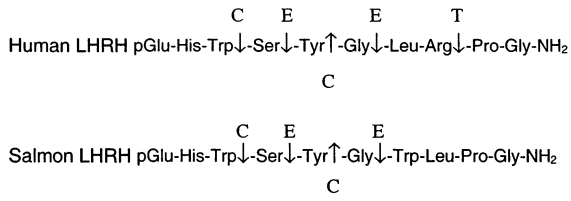

Fig. 1. Potential cleavage sites of human and salmon LHRH by trypsin (T), chymotrypsin (C) and elastase (E).

barrier to the delivery of peptide drugs (Langguth et al., 1997). These peptidases are specific for a range of amino acid bonds; trypsin is specific towards lysine and arginine residues (Keil-Dlouhá et al., 1971), chymotrypsin is specific towards phenylalanine, tryptophan and tyrosine (Baumann et al., 1970) and elastase has a broad specificity, targeting alanine, serine, glycine and valine amino acids (Atlas et al., 1970). Based on their substrate specificity, it was predicted that all three pancreatic enzymes would degrade hLHRH. However, sLHRH should be hydrolysed by chymotrypsin and elastase but stable against trypsin activity because it does not contain lysine or arginine residues (Fig. 1). Human and salmon LHRH are C-terminally and N-terminally blocked and are therefore, not expected to be hydrolysed by the exopeptidases, carboxypeptidases A and B and the aminopeptidases present in the intestinal lumen (MacCann, 1977).

A number of investigators have included serine protease inhibitors in oral formulations to increase the metabolic stability of the peptide drug, thereby improving their opportunity for oral absorption (Kidron et al. 1982; Nishiata et al., 1983). The most commonly used inhibitors are aprotinin (Nishiata et al., 1983; Morishita et al. 1992) and the Bowman–Birk type inhibitor isolated from soybean (Kidron et al., 1982). Aprotinin and the Bowman–Birk type inhibitor are strong inhibitors of both chymotrypsin and trypsin (Kassell et al., 1965; Seidl and Liener, 1972). Interestingly, among the many Bowman–Birk inhibitor-type inhibitors, only Garden Bean inhibitor II (Wilson et al., 1973) and soybean inhibitor C-II (Odani and Ikenaka, 1977) inhibit pancreatic elastase, which is expected to hydrolyse both LHRH analogues (Fig. 1). Further, the broad specificity of elastase makes it potentially more damaging towards peptide drugs than trypsin and chymotrypsin. Previous studies by Langguth et al. (1997) showed that the LHRH analogue buserelin (Pyr-His-Trp-Ser-Tyr-D-Ser(Bu^t)-Leu-Arg-Pro-ethylamid) was degraded by all three endopeptidases. However, each peptidase was assayed at different concentrations and therefore, their hydrolytic activity towards the peptide could not be compared directly.

In this study, we determine the relative contribution of each pancreatic endopeptidase to the lumenal degradation of the LHRH analogues by incubating the LHRH analogues with equal amounts of the individual pancreatic serine endopeptidases. We show that the human and salmon LHRH analogues are highly susceptible to the activity of α-chymotrypsin, while in the presence of elastase and trypsin they are relatively stable. This suggests that to improve oral absorption of LHRH analogues they have to be protected from the activity of chymotrypsin. We discuss factors that may influence the stability of LHRH analogues to the hydrolytic activity of elastase and trypsin.

2. Materials and methods

2.1. Materials

Human luteinizing hormone-releasing hormone (hLHRH), salmon luteinizing hormone-releasing hormone (sLHRH), α-chymotrypsin (EC 3.4.21.1, TLCK-treated, Type VIII from bovine pancreas), trypsin (EC 3.4.21.4, TPCK-treated type XIII from bovine pancreas), elastase (EC 3.4.21.36, Type IV from porcine pancreas), *N*-α-benzoyl-DL-Arg *p*-nitroanilide, *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide, L-leucine *p*-nitroanilide, *N*-benzoyl-L-Tyr ethyl ester, *N*-α-benzoyl-L-Arg ethyl ester, *N*-α-benzoyl-L-Arg, hippuryl-Arg, hippuryl-Phe, hippuric acid, light mineral oil with a specific activity of 0.875–0.885 at 25°C were all obtained from Sigma Chemical Company (St Louis, MO, USA). All other chemicals were of analytical grade and were purchased from Ajax chemicals (Auburn, NSW, Australia).

2.2. Enzyme assays

The activity of trypsin, chymotrypsin and elastase towards their respective synthetic substrates, *N*-benzoyl-L-arginine ethyl ester, *N*-benzoyl-L-tyrosine ethyl ester and *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide were assayed using the methods recommended by the Sigma Chemical Company.

Degradation of the LHRH analogues by equal amounts of pancreatic enzymes was determined as follows. Each pancreatic enzyme (1 mg) was prepared in 50 mM sodium phosphate buffer pH 8.0 (1 ml total volume). The enzyme solution (2.5 μl) was transferred to a 0.5 ml sample tube and 50 mM sodium phosphate buffer pH 8.0 was added to give a total volume of 0.2 ml. The enzyme solution and the LHRH (0.5 mM) stock prepared in 50 mM sodium phosphate buffer pH 8.0 were equilibrated at 15°C for 10 min. To initiate the reaction, 50 μl of the LHRH stock was added to the enzyme solution and mixed. Samples of 40 μl were taken from the chymotrypsin incubate at 0.5, 1.0 and 2.0 min and from the trypsin and elastase incubates at 0.5, 15, 30 and 120 min. Samples were mixed with an equal volume of 0.2 M HCl to stop the reaction and stored at −80°C. Thawed samples were centrifuged at 20 000 × *g* for 5 min and 20 μl of the supernatant was diluted with 60 μl deionised water and analysed by capillary electrophoresis.

2.3. Capillary electrophoresis

The CE system was a Dionex CES I (Dionex Corporation, Sunnyvale, CA, USA) interfaced to a computerised data handling system (Dionex AI-450 Chromatography Automated Software v 3.3.2). Data were collected at the rate of 5 Hz and electrophoresis was carried out towards the cathode. A fused silica capillary column of 50 μm i.d. and 60 cm total length was prepared and installed as described in the Dionex (1989)User's manual. A cooling jacket covered 33 cm of the capillary. A Neslab RTE-111 (NESLAB Instruments, Inc. Portsmouth, USA) recirculating bath maintained the temperature of the water in the capillary cooling jacket at 20 ± 0.1°C. CE was carried out in 10 mM sodium acetate at pH 4.0 containing 75 mM NaCl. NaCl was added to the buffer stocks prior to pH adjustment with glacial acetic acid. All buffers were passed through a 0.45 μm filter before use. Samples were transferred to silanised Dionex micro-vials (0.5 ml) and covered with approximately 1 mm layer of light mineral oil to prevent evaporation. Sample vials containing the capillary inlet were elevated to 150 mm higher than the destination capillary outlet then lowered to the height of the destination outlet; the loading process took 60 s to complete. Electrophoresis was carried out at constant current by ramping the current from 0 to 60 μA over 1 min and holding the current at 60 μA for the remainder of the run. The peptides were detected by a UV detector set at 215 nm. Prior to each run the capillary, source and destination reservoirs were rinsed with the run buffer as described above. The rinse time at 6 psi for the capillary was 120 s, the rinse and refill times for the source and destination vial was 6 s (Walker et al., 2000).

3. Results

Before measuring the enzymatic degradation of the LHRH analogues the activity of each pancreatic peptidase was measured and compared to the activity stated by the supplier. The specific activities obtained for each peptidase were not significantly different ($P < 0.05$) to that stated by the Sigma Chemical Company.

Under the in vitro conditions described, chymotrypsin (TLCK-treated) rapidly degraded both LHRH analogues; the rate of decrease in peak height of the human and salmon LHRH in the electrophoretograms (Fig. 2A Fig. 3A) followed pseudo-zero kinetics, with half-lives of 2.5 ± 0.3 and 2.7 ± 0.4 min (mean ± S.D., $n = 3$), respectively. Chymotryptic digestion of hLHRH resulted in the formation of two primary fragments, which migrated at 8.2 and 9.4 min, with the native hLHRH eluting after 10.7 min (Fig. 2A). Salmon LHRH eluted after 18.6 min with a primary chymotryptic metabolite eluting after 14.5 min (Fig. 3A).

In contrast, both LHRH analogues were stable in the presence of trypsin (TPCK-treated) after 2

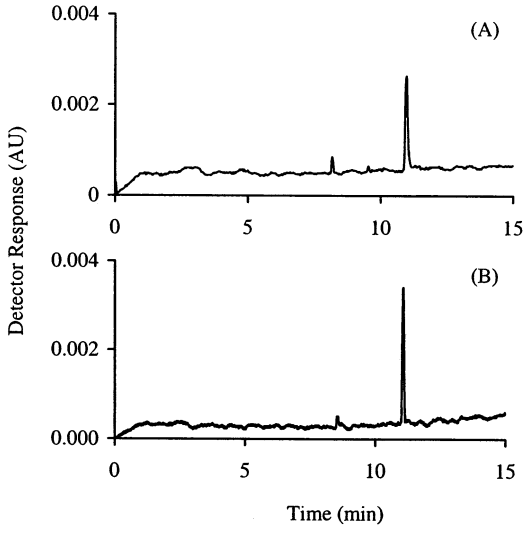

Fig. 2. Capillary electrophoretograms of hLHRH chymotrypsin (A) and elastase (B) digests. hLHRH (0.1 mM) was incubated with the endopeptidase (0.1 μM) in 50 mM sodium phosphate buffer pH 8.0 at 15°C.

h (data not shown) while in the presence of elastase from porcine pancreas both LHRH analogues were slowly hydrolysed, with half-lives of 90 ± 15 and 114 ± 21 min (mean $\pm$ S.D., $n = 3$), respectively. Elastase digestion of hLHRH resulted in the formation of metabolite migrating after 8.18 min, with the native eluting after 10.7 min (Fig. 2B). Elastase digestion of sLHRH resulted in the formation of metabolite migrating after 14.3 min, with the native sLHRH eluting after 18.4 min. The principal metabolites of elastase generated from the respective LHRH analogues had similar electrophoretic mobilities to those metabolites generated by chymotrypsin (Fig. 2 Fig. 3). This suggests that the elastase metabolites may not be due to the activity of elastase but to the activity of contaminant chymotrypsin. To identify the presence of contaminant chymotrypsin activity the commercial elastase preparation was assayed for activity towards the chymotryptic synthetic substrate *N*-benzoyl-L-tyrosine ethyl ester (BTEE). The porcine elastase preparation had a specific activity of 2.6 U per mg protein, which equates to approximately 6% of the α-chymotrypsin (TLCK-treated) preparation.

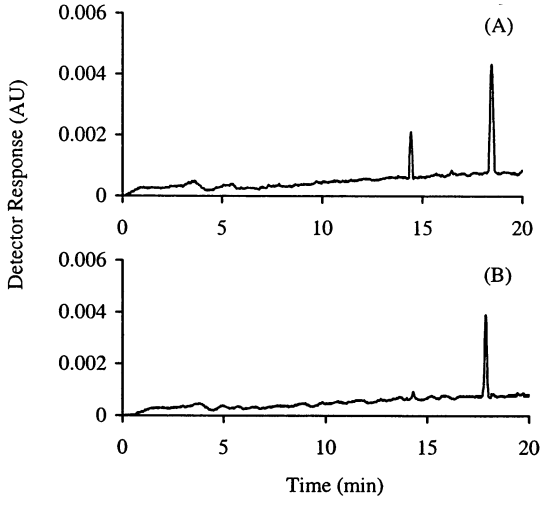

Fig. 3. Capillary electrophoretograms of sLHRH chymotrypsin (A) and elastase (B) digests. sLHRH (0.1 mM) was incubated with the endopeptidase (0.1 μM) in 50 mM sodium phosphate buffer pH 8.0 at 15°C.

4. Discussion

This work shows that human and salmon LHRH analogues are highly susceptible to the activity of chymotrypsin, but are stable in the presence of trypsin. The porcine elastase preparation showed very low hydrolytic activity towards both LHRH analogues. We believe that the slow hydrolytic activity of the elastase preparation may not be the property of elastase but due to the activity of contaminant chymotrypsin detected in the porcine elastase preparation. This is further supported by the elastase LHRH metabolites having similar electrophoretic mobilities to those generated by the respective LHRH chymotrypsin digests (Fig. 2 Fig. 3). This work suggests that chymotrypsin is the only pancreatic endopeptidase involved in the digestion of the LHRH analogues. This observation is in contrast to that predicted by the substrate specificity of the endopeptidases (Fig. 1) and work by Langguth et al. (1997), which showed that all three endopeptidases hydrolysed the LHRH analogue buserelin.

We suggest that the elastase and trypsin hydrolytic activity towards the LHRH analogue

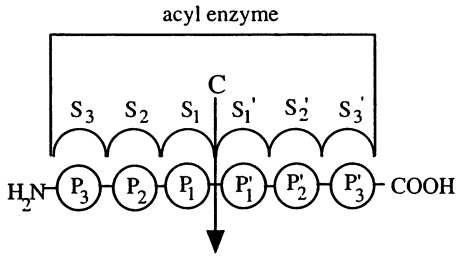

Fig. 4. Schematic representation of enzyme–substrate complex. The active subsites (S) of the acyl enzyme are located on both sides of the catalytic site (C) of the substrate (P).

buserelin observed by Langguth et al. (1997) may be due to contaminant chymotrypsin activity. The commercial preparations of trypsin used by Langguth et al. (1997) contain varying levels of activity towards the chymotrypsin substrate BTEE, as specified by the supplier, the Sigma Chemical Company. In this study, to eliminate the contaminant chymotrypsin activity, a commercial preparation of trypsin that had been pre-treated with the specific chymotrypsin inhibitor TPCK was used. Further, in this work we showed that the commercial porcine elastase used in this study and the work by Langguth et al. (1997) contained contaminant chymotrypsin activity towards BTEE.

Although the substrate specificity of the endopeptidases is commonly assumed to be primarily dependent on a single amino acyl residue, the enzyme activity towards the target bond has also been shown to be influenced by other factors. These factors include the presence of different amino acids in several positions away from the target bond, the length of the peptide chain and its flexibility (Perona and Craik, 1995). The metabolic stability of sLHRH to trypsin was expected, as there are no potential cleavage sites for trypsin. In contrast the Arg^8 residue of hLHRH is a potential cleavage site for trypsin (Fig. 1) yet the hLHRH was stable to trypsin. The active sites of endopeptidases can be divided into subsites that bind residues of the peptide substrate on either side of the scissile bond. Schechter and Berger (1967) introduced a binding site notation to indicate the position of the amino acid residues in relation to the enzyme cleavage site shown by Fig. 4. Using a series of peptides Schellenberger et al. (1993) showed that the presence of a Pro residue in the P′1 site of a substrate is not tolerated in the S′1 subsite of trypsin and thereby prevents the catalytic acyl transfer reaction. This supports the stability of hLHRH (Fig. 1) to trypsin.

The stability of LHRH analogues towards elastase activity maybe attributed to the chain length of the peptide and/or the amino acid residues in the P′1 position of the target site. Kinetic studies on porcine elastase using synthetic peptide substrates showed that at least four residues preceding the cleavage site (P residues) and two residues after it (P′ residues) are necessary for proteolysis to occur optimally (Atlas et al., 1970). However, according to Fig. 1 these substrate-binding requirements of porcine elastase are fulfilled by both LHRH analogues. More recently, Schellenberger et al. (1989) showed that porcine elastase has preference for small amino acids in the P′1 position. From Fig. 1 we see that the P′1 side chain of the predicted elastase cleavage sites of hLHRH include tyrosine and leucine while the corresponding P′1 side chain of sLHRH include tyrosine and tryptophan. Both tyrosine, tryptophan and leucine amino acids have large side chains and are therefore expected to reduce the subsite specificity of the porcine elastase for those sites.

The results obtained from this work show that both hLHRH and sLHRH are rapidly degraded by chymotrypsin. It can therefore be concluded that the development of a means to protect a LHRH analogue from chymotrypsin is an essential step in any attempt to develop a formulation for oral delivery of the compound. Further, this work highlights the importance of considering factors beyond the target site of the peptidase for predicting the metabolic stability of peptide drug in the presence of the peptidase.

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