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Inhibition of proteolysis in luminal extracts from the intestine of the brushtail possum

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Abstract

The proteolytic activity of luminal extracts from five regions (duodenum, jejunum, ileum, caecum and colon) of the brushtail possum intestine towards bovine serum albumin (BSA) and human luteinizing hormone releasing hormone (LHRH) was investigated. There were no significant differences in degradation rates between fresh and previously frozen extracts from any region of the possum intestine. The inhibition of degradation of BSA by luminal extracts from two regions (jejunum and ileum) and of LHRH from four regions (jejunum, ileum, caecum and colon) was evaluated. Soybean trypsin–chymotrypsin inhibitor (SBTI), sodium deoxycholate, Carbopol 934P, bacitracin and bestatin significantly inhibited the degradation of both LHRH and BSA ($P < 0.05$). SBTI almost totally inhibited the proteolysis of BSA and the peptidolysis of LHRH in extracts from the small intestine. This finding suggests that serine proteases such as chymotrypsin are responsible for the protein and peptide degradation in luminal extracts. It is concluded that including serine protease inhibitors in a formulation may enhance oral delivery of bioactive peptides and proteins to possums.

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Introduction

Since its introduction in the nineteenth century, the brushtail possum (*Trichosurus vulpecula*) has spread throughout 90% of New Zealand and has become a pest of major economic consequence. The use of immunocontraceptive or chemical sterilant agents is a potential method for controlling the possum (Cowan 1996). Luteinizing hormone releasing hormone (LHRH) is a decapeptide (Figure 1) that regulates the secretion of both luteinizing hormone and follicle-stimulating hormone from the anterior pituitary in a dose-dependent manner (Fink 1988). Its ability to block the release of these gonadotrophins, when administered at high doses by sustained or multiple dosing, is the basis of its use as a female contraceptive and suggests a potential use for pest control (Fink et al 1974). The success of such an approach would rely on the oral delivery of proteins or peptides. The most practical way to deliver the bioactive agents would be via baits for oral delivery. The two major barriers to the successful oral delivery of proteins and peptides are enzymatic degradation within the intestine and blocks to absorption across the gastrointestinal epithelium. The first major enzymatic barrier to absorption is that formed by the proteases secreted into the stomach and intestine, including the pancreatic proteases such as chymotrypsin secreted into the lumen of the intestine. To reduce the metabolic barrier in the gut, various approaches have previously been investigated: (i) the co-administration of proteins and peptides with protease inhibitors (Woodley 1994); (ii) the structural modification of peptides to prevent proteolytic attack (Saffran et al 1986; Haviv et al 1993); (iii) the use of carrier systems that would protect the protein or peptide from luminal digestion and to release the drug at a site in the intestine favourable for absorption (Uchiyama et al 1999); and (iv) the formulation of peptides and proteins in particles to shield them from luminal and mucosal enzymatic attack (Watnasirichaikul et al 2000). Among these, the first approach has been shown both in-vitro and in-vivo to significantly improve the bioavailability of peptides and proteins after oral dosing (Bernkop-Schnürch 1998).

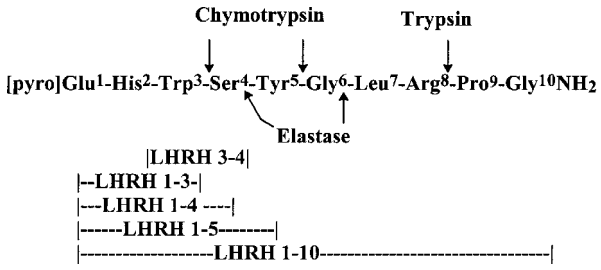

Figure 1 Cleavage sites of luteinizing hormone releasing hormone (LHRH) by serine proteases.

In the present study, bovine serum albumin (BSA) and LHRH were used as a model protein and a model peptide, respectively, to investigate the effectiveness of inhibitors in preventing their enzymatic degradation in luminal extracts from the possum intestine. So far, inhibitors that prevent the degradation of LHRH and BSA have been reported in cell culture and in several vertebrate species, including rats, rabbits and salmon (Advis et al 1982; Baricos et al 1987; Han et al 1995; Ambroso et al 1997; Reis et al 1998; Yang et al 1998; Walker et al 1999). However, there are no data available concerning the protection of BSA and LHRH from degradation in the intestine of marsupials. In a previous study, we showed that luminal extracts from the small intestine of the possum are largely responsible for the degradation of proteins and peptides (Wen et al 2002b). We also identified the relative contribution of the pancreatic enzymes and concluded that chymotryptic activity was the most important (Wen et al 2002b). This suggested that the use of inhibitors of chymotrypsin, such as soybean trypsin–chymotrypsin inhibitor (SBTI) or tosyl phenylalanyl chloromethyl ketone (TPCK), would protect peptides and proteins from digestion. Antibiotic peptides such as bacitracin and bestatin and formulation aids such as sodium deoxycholate (SDA) and Carbopol 934P (C934P) inhibit peptidolysis in possum mucosal homogenates (Wen et al 2002c). We proposed to study the effects of these agents on luminal extracts. The present study aimed to determine the rates of enzyme catalysed degradation of LHRH and BSA in intestinal luminal extracts, identify which enzymes play major roles in BSA and LHRH degradation in possum luminal extracts, and determine the extent to which protease and peptidase inhibitors could protect BSA and LHRH from degradation by intestinal luminal extracts.

Materials and Methods

Materials

Human LHRH (purity 99%) was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). BSA fraction V was purchased from SERVA (Feinbiochemica GmbH & Co., Heidelberg, Germany). Bowman-Birk type SBTI from soybean, TPCK, deoxycholic acid sodium salt, trifluoroacetic acid (anhydrous protein sequencing grade), bacitracin, chitosan and bestatin were all obtained from Sigma Chemical Company (St Louis, MO, USA). C934P was a gift from BF Goodrich (Cleveland, OH, USA). All other chemicals were of analytical grade and were purchased from Ajax Chemicals (Auburn, New South Wales, Australia). Sample tubes of 0.5 mL and 1.5 mL were purchased from Eppendorf (Eppendorf Netheler Hinz GmbH, Hamburg, Germany). Tissue extracts were homogenized by a Ystral homogenizer (Ystral GmbH, D-7801, Dottingen, Germany). Deionized water was obtained from a reverse osmosis Milli-Q Reagent Water system and was used for the preparation of all solutions.

Animals

Adult, male brushtail possums that had been live-captured in the Otago (latitude 45° S) region of New Zealand were used in this study. At the time of capture, each animal was screened for health status and only large ($\geqslant$ 2.5 kg live weight), apparently healthy animals were included. Before use, animals were group housed (n = 5−12/group) in environmentally enriched pens under conditions of natural daylight and temperature. A mixed diet of fresh fruit, specially formulated cereal-based pellets and bread was provided daily. Fresh water was available *ad libitum*. Under these housing conditions, possums gain weight from the time of capture and mortality rates are extremely low (McLeod et al 1997). All experimentation had prior approval from the AgResearch Invermay Animal Ethics Committee working under the Animal Protection (Codes of Ethical Conduct) Regulations 1989.

Collection and preparation of intestinal extracts

The possums were killed by intra-cardiac injection of barbiturate (4 mL Euthal; Delta Veterinary Laboratories, Hornsby, New South Wales, Australia), administered while they were under halothane-induced anaesthesia (Fluothane: ICI New Zealand Ltd, Lower Hutt, New Zealand). A midline incision was made, the intestine removed and the duodenum, jejunum, ileum, caecum, proximal colon and distal colon identified and sectioned as described by Lönnberg (1902). The luminal extracts of each region were carefully expelled under gentle pressure, weighed and transferred to labelled centrifuge tubes. All extracts were diluted with a minimal volume of 0.125 M NaCl solution, homogenized on ice and then centrifuged at 3000 *g* for 15 min at 4°C. The supernatants were collected, transferred into a graduated tube and made up to 10 mL with 50 mM phosphate buffer (pH 7). Aliquots (200 μL) were transferred to labelled 0.5-mL tubes and either used immediately or stored at −80°C until required. All equipment and solutions used for the procedure were kept at 4°C, to minimize enzyme degradation.

Analysis of proteins and peptides

The concentrations of soluble protein in the luminal extracts and acid soluble peptides in luminal digests were determined by the modified Lowry assay using BSA as the reference standard (Peterson 1977).

Table 1 Specific proteolytic activity of fresh and frozen luminal extracts from five regions of the possum intestine determined towards bovine serum albumin (BSA) and luteinizing hormone releasing hormone (LHRH) in phosphate buffer, pH 7.0, at 37°C.

Luminal extract	Specific activity towards LHRH (μg h^{-1} (mg protein)$^{-1}$)		Specific activity towards BSA (μg h^{-1} (mg protein)$^{-1}$)	
	Fresh	Frozen	Fresh	Frozen
Duodenum	148.80±7.00	156.90±5.00	–	–
Jejunum	666.00±42.65	643.5±11.65	4968±219	5467±1041
Ileum	263.90±14.50	308.00±10.25	5124±549	4973±561
Caecum	33.01±4.25	37.95±3.00	–	–
Proximal colon	22.7±7.01	36.40±5.05	–	–

Specific activities represent the mean±s.e.m. of three experiments. –, no significant digestion.

Analyses of acid soluble peptides from the degradation of BSA were performed in microtitre plate wells in a total volume of 250 μL and absorbance (750 nm) was read using a Microplate Spectrophotometer (Spectra MAX 340, CA, USA) controlled by an external computer with SOFT-max PRO software (Molecular Devices Corporation, CA, USA). The amount of peptide in each sample was determined by comparison with the BSA standard curve.

LHRH was assayed by reversed phase high-performance liquid chromatography (HPLC) using a Phenomenex Luna C_{18} column (250×4.6 mm, 5 μm particle size) fitted with a C_{18} guard column (10×3 mm). A mobile phase of 0.1% trifluoroacetic acid in 21% v/v acetonitrile in water (adjusted to pH 2.5 with sodium hydroxide) was used at flow rate of 1.0 mL min^{-1}, and eluates were detected at 215 nm (Wen et al 2002a).

Determination of the proteolytic activity in luminal extracts

BSA (900 μL, 1.0 mg mL^{-1} in phosphate buffer, pH 7.0) was warmed to 37°C over 15 min and then added to fresh or frozen luminal extracts (100 μL, 200 μg mL^{-1} of protein in phosphate buffer, pH 7.0) that had been pre-warmed to 37°C. The mixture was incubated at 37°C for 180 min. Similarly, pre-warmed LHRH (40 μL, 1.5 mg mL^{-1} in phosphate buffer) was incubated with luminal extracts (210 μL, 24 μg mL^{-1}) for 240 min. Aliquots of the BSA incubates (100 μL) or LHRH incubates (25 μL) were withdrawn at pre-determined times and transferred into 0.5-mL Eppendorf tubes containing either 5 μL trichloroacetic acid (50%) to precipitate tissue protein and terminate proteolysis of BSA, or 25 μL HCl (0.2 M) to stop hydrolysis of LHRH. The final mixture was allowed to stand for 15 min at room temperature and then centrifuged at 20 000 *g* for 10 min. Proteolytic activity of BSA digesta (Table 1) was determined by measuring the rate of formation of acid soluble peptides. LHRH peptidolytic activity (Table 1) was evaluated by measuring the concentration of intact LHRH using HPLC.

Inhibition of luminal degradation of BSA and LHRH

Inhibition of proteolytic digestion of BSA and LHRH was evaluated in-vitro by incubating the protein or peptide in luminal extracts in either the presence or absence of selected inhibitors. Four inhibitors (SBTI, SDA, bestatin and bacitracin) were selected for use with BSA (Table 2), and six inhibitors (SBTI, TPCK, SDA, bestatin, bacitracin and C934P) were selected for use with LHRH (Table 3). In addition, the stability of LHRH in the presence of mixtures of SBTI with SDA, bestatin or C934P was evaluated (Table 3).

Intestinal luminal extracts (20 μL, 1000 μg mL^{-1}) were incubated at 37°C for 30 min in either 80 μL of 50 mM phosphate buffer, pH 7.0 (control), or in 80 μL of a solution of inhibitor dissolved in phosphate buffer. After addition of pre-warmed BSA (900 μL, 1.0 mg mL^{-1}), mixtures were incubated for 180 min at 37°C. The final concentration of

Table 2 Percentage inhibition of bovine serum albumin (BSA) proteolysis by soybean trypsin–chymotrypsin inhibitor (SBTI), sodium deoxycholate (SDA), bestatin and bacitracin when BSA (0.9 mg mL^{-1}) was incubated with luminal extracts (20 μg mL^{-1}) in phosphate buffer, pH 7.0, at 37°C.

Inhibitor	Concentration	Jejunum	Ileum
SBTI	12.5 μg mL^{-1}	95.46±1.16	96.53±0.65
	25 μg mL^{-1}	95.59±1.99	97.04±1.07
	50 μg mL^{-1}	96.44±1.06	98.00±0.55
SDA	0.2 mM	41.92±6.17	32.07±1.18
	0.5 mM	52.86±4.19	48.8±2.69
	1.0 mM	54.02±6.59	50.16±5.17
Bestatin	0.35 mM	25.28±3.99	18.95±5.84
Bacitracin	0.35 mM	–	–
	0.90 mM	28.50±4.12	31.70±18.16

Inhibition was expressed as (1−specific activity of experimental group/specific activity of control group)×100 and represents the mean±s.e.m. of replicates from three possums. –, no inhibition.

Table 3 Percentage inhibition of luteinizing hormone releasing hormone (LHRH) peptidolysis by soybean trypsin–chymotrypsin inhibitor (SBTI), tosyl phenylalanyl chloromethyl ketone (TPCK), sodium deoxycholate (SDA), bestatin, bacitracin or Carbopol 934P (C934P), or mixtures of SBTI with SDA, bestatin or C934P, when LHRH (0.24 mg mL^{-1}) was incubated with luminal extracts (20 μg mL^{-1}) in phosphate buffer, pH 7.0, at 37°C.

Inhibitor	Concentration	Jejunum	Ileum	Caecum	Colon
SBTI	0.13 mg mL^{-1}	88.81±1.19	61.68±4.69	41.34±7.81	41.09±8.03
	0.26 mg mL^{-1}	89.65±1.24	74.82±2.12	48.80±6.50	42.04±3.48
	1 mg mL^{-1}	91.83±1.29	81.34±2.37	51.38±5.17	46.78±4.44
TPCK	0.1 mM	70.85±1.37	40.82±6.02	–	–
	0.5 mM	42.74±4.89	60.48±1.64	–	–
SDA	2 mM	33.58±2.95	51.25±4.36	23.40±11.13	17.17±7.50
	5 mM	37.66±3.36	56.84±5.43	45.76±13.64	43.02±18.09
	10 mM	50.21±3.01	69.37±4.84	50.83±10.50	46.56±9.99
Bestatin	3.5 mM	37.81±6.02	48.52±6.77	26.67±6.58	6.74±6.3
Bacitracin	3.5 mM	11.94±3.51	22.61±0.82	–	–
	9 mM	50.80±5.67	33.96±2.4	–	–
C934P	3.5 mg mL^{-1}	43.08±4.36	59.45±6.18	64.80±11.13	61.25±9.99
SBTI+SDA	1 mg mL^{-1}+10 mM	95.30±0.79	90.40±1.12	–	–
SBTI+bestatin	1 mg mL^{-1}+3.5 mM	93.50±1.01	80.74±1.51	–	–
SBTI+C934P	1 mg mL^{-1}+3.5 mg mL^{-1}	93.76±0.44	86.43±1.22	–	–

Inhibition was expressed as (1 − specific activity of experimental group/specific activity of control group)×100 and represents the mean±s.e.m. of replicates from three possums. –, not determined.

BSA was 0.9 mg mL^{-1}, of luminal extracts was 20 μg mL^{-1}, and of inhibitor in the incubates was as follows: SBTI, 12.5, 25 or 50 μg mL^{-1}; SDA, 0.2, 0.5 or 1.0 mM; bestatin, 0.35 mM; or bacitracin, 0.35 or 0.90 mM. The sampling protocol and analytical procedure described above was used for the estimation of acid soluble peptides. Activity of the luminal contents was calculated from the zero order rates of formation of soluble peptides.

LHRH (40 μL, 1.5 mg mL^{-1}) was warmed to 37°C and added to a pre-warmed mixture of luminal extracts (20 μL, 250 μg mL^{-1}) and either 190 μL of 50 mM phosphate buffer, pH 7.0 (control), or 190 μL of inhibitor solution in phosphate buffer. Mixtures were incubated for 240 min at 37°C in a water bath. The final concentration of LHRH was 0.24 mg mL^{-1}, of luminal extracts was 20 μg mL^{-1}, and of inhibitor in the incubates was as follows: SBTI, 0.13, 0.26 or 1.0 mg mL^{-1}; TPCK, 0.1 or 0.5 mM; SDA, 2, 5 or 10 mM; bestatin, 3.5 mM; bacitracin, 3.5 or 9 mM; C934P, 3.5 mg mL^{-1}; or mixtures of SBTI (1.0 mg mL^{-1}) with SDA (10 mM), bestatin (3.5 mM) or C934P (3.5 mg mL^{-1}). The same sampling protocol and analytical procedure as described above was used. Activity was calculated from the zero order rates of hydrolysis of LHRH.

Data analyses

The degradation of BSA (as measured by the formation of acid soluble peptides) and of LHRH (as measured by the decrease in peak area) was plotted against time for a luminal extract from each section of intestine. The specific proteolytic activity was expressed either as μg h^{-1} (mg protein)$^{-1}$ of acid soluble peptide formed from BSA, or as μg h^{-1} (mg protein)$^{-1}$ of LHRH hydrolysed. Activity in the presence of the inhibitor was calculated as: (specific activity of the experimental group/specific activity of the control group)×100, and the percentage inhibition was calculated by subtraction from 100.

Significant differences between fresh and frozen extracts were evaluated using Student's *t*-test and between inhibitors using two-way analysis of variance. A value of $P < 0.05$ was considered significant. All analyses were carried out using MINITAB (release 12; Minitab, Inc., PA, USA).

Results

Proteolytic activity in luminal extracts

The proteolytic activity of luminal extracts from the small intestine was proportional to their protein concentration up to a concentration of 40 μg mL^{-1} (r^2 = 0.999), for both BSA and LHRH. The formation of acid soluble peptides from BSA degradation by luminal extracts from the small intestine (20 μg mL^{-1}) followed pseudo-zero order kinetics for at least 60 min. Degradation of LHRH by luminal extracts followed pseudo-zero order kinetics for at least 30 min in jejunal and ileal preparations, and for at least 90 min in duodenal and hindgut preparations.

There was no significant difference in activity between fresh and frozen luminal extracts from any of the five regions of possum intestine (Table 1). Significant digestion of BSA occurred only in luminal extracts from the small intestine, with no significant difference between the jejunum and ileum. The rank order of peptidolytic activity of the luminal extracts towards LHRH was jejunum ≫ ileum >

duodenum ⩾ caecum ⩾ colon, with an almost 30-fold difference between the least and the most active segment from either fresh or frozen extracts.

The products of LHRH degradation (Figure 1) after incubation with jejunal, ileal, caecal or colonic luminal extracts were determined by measuring the appearance of metabolites using HPLC (Figure 2). The main degradation products of LHRH in jejunal and ileal luminal extracts were LHRH (1–5), LHRH (1–3), LHRH (1–4) and LHRH (3–4) as well as a small amount of an unknown fragment (Figure 2A). In contrast, the degradation rates of LHRH in caecal and colonic luminal extracts were much slower ($P < 0.05$) and LHRH (1–5) was the major metabolite from hindgut incubations (Figure 2B). A small amount of a second unknown fragment was also found after incubation with hindgut luminal extracts (Figure 2B).

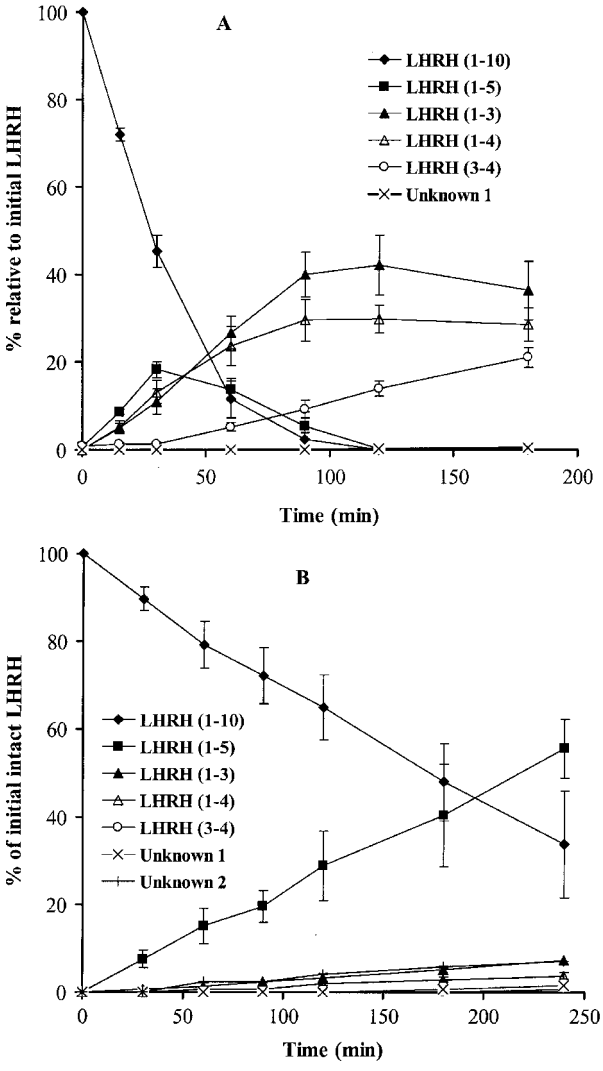

Figure 2 Peptidolysis of luteinizing hormone releasing hormone (LHRH) and the formation of its metabolites by luminal extracts from jejunum (A) and colon (B) in phosphate buffer, pH 7.0, at 37°C. Each point is expressed as % peak area relative to the initial peak area of LHRH and represents the mean±s.e.m. of three experiments.

Effects of inhibitors on proteolysis of BSA and LHRH in luminal extracts

Table 2 summarizes the effects of protease inhibitors on the hydrolysis of BSA in jejunal and ileal luminal extracts. Degradation of BSA was significantly inhibited by SBTI, SDA, bestatin and bacitracin ($P < 0.05$). The rank order of the effectiveness for preventing BSA hydrolysis in luminal extracts of small intestine was SBTI > SDA > bacitracin ⩾ bestatin, with SBTI almost completely inhibiting hydrolysis.

Degradation of LHRH was significantly inhibited by SBTI, TPCK, SDA, bacitracin and C934P in all luminal extracts (Table 3). The rank order of the effectiveness in luminal extracts from the small intestine was SBTI > TPCK > SDA ⩾ bacitracin ⩾ bestatin ⩾ C934P, but in the hindgut the rank order was C934P > SBTI > SDA. There was no significant inhibition by bestatin in colonic luminal extracts ($P > 0.05$).

The formation of metabolic products from LHRH after incubation in jejunal (60 min) or caecal (240 min) luminal extracts, either in the presence or absence of inhibitors, is summarized in Figure 3. In jejunal extracts, SBTI alone or

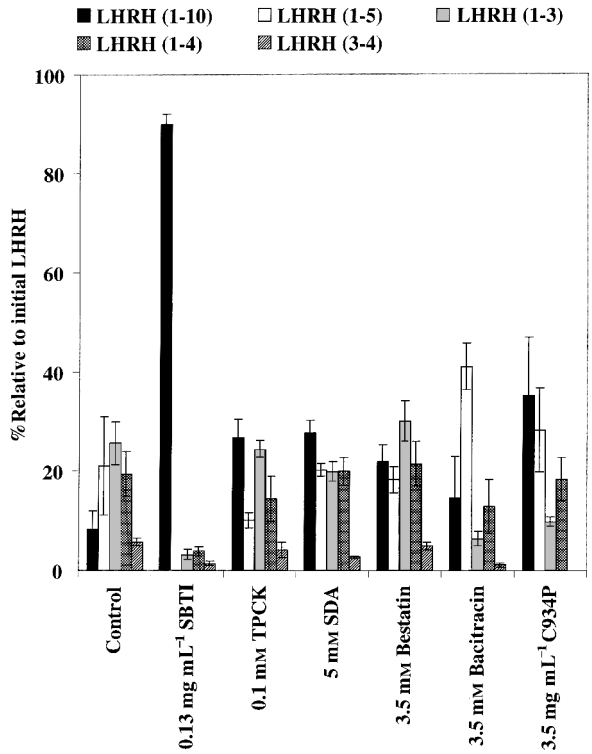

Figure 3 Effects of inhibitors on luteinizing hormone releasing hormone (LHRH) peptidolysis and metabolite formation in luminal extracts from the jejunum of possum in phosphate buffer, pH 7.0, when incubated at 37°C for 60 and 240 min, respectively. Each point is expressed as % peak area relative to the initial peak area of LHRH and represents the mean±s.e.m. of three experiments. C934P, Carbopol 934P; SBTI, soybean trypsin–chymotrypsin inhibitor; SDA, sodium deoxycholate; TPCK, tosyl phenylalanyl chloromethyl ketone.

as a mixture with SDA (10 mM), bestatin (3.5 mM) or C934P (3.5 mg mL^{-1}) abolished the formation of LHRH (1–5) and strongly inhibited the formation of LHRH (1–3) and LHRH (1–4). TPCK (0.1 or 0.5 mM) appeared to selectively inhibit the formation of unknown fragment 1 and of LHRH (1–5). SDA (10 mM) stopped the formation of unknown 1 and selectively inhibited the formation of LHRH (1–5) relative to that of LHRH (1–4), LHRH (1–3) and LHRH (3–4). C934P appeared to stop formation of LHRH (3–4) and inhibit the formation of LHRH (1–3) and LHRH (1–4). However, no inhibition of LHRH (1–5) formation was observed. Bacitracin appeared to inhibit the formation of LHRH (1–3) and LHRH (1–4) rather than LHRH (1–5). Bestatin stopped the formation of unknown 1, but showed only weak inhibition compared with the other inhibitors. Inhibition increased with increasing concentrations of TPCK or SDA in luminal extracts from the small intestine. In caecal extracts, SBTI (1 mg mL^{-1}) inhibited the formation of LHRH (1–5) and LHRH (3–4). SDA (10 mM) stopped the formation of LHRH (3–4), of unknown fragments 1 and 2, and also markedly inhibited the formation of LHRH (1–5), LHRH (1–3) and LHRH (1–4). C934P (3.5 mg mL^{-1}) selectively inhibited the formation of LHRH (3–4) and of unknown fragment 1, and markedly inhibited the formation of LHRH (1–5) and LHRH (1–3). Although bestatin (3.5 mM) stopped formation of LHRH (3–4) and unknown fragment 2, when compared with the control group, the overall inhibition in colonic extracts was not significant ($P > 0.05$).

Discussion

The intestinal lumen contains proteases secreted from the pancreas and peptidases sloughed off the mucosal membrane. The enzymes secreted into the lumen of the intestine are essential to the digestion of dietary proteins and play an important role in the inactivation of proteins and peptides before they reach the systemic circulation. The pancreatic proteases include serine endopeptidases such as trypsin, chymotrypsin and elastase, and exopeptidases such as carboxypeptidases A and B (Woodley 1994). In the present study in which BSA and LHRH were used as a model protein and a model peptide, respectively, there was no difference between either the specific proteolytic activity or metabolic profile of fresh as compared with frozen extracts of luminal extracts from any of the five regions of possum intestine.

Since LHRH does not possess a free C- or N-terminus (Figure 1), we would expect it to be stable in the presence of the exopeptidases, but hydrolysed by the serine proteases such as chymotrypsin or elastase (MacCann 1977; Walker et al 2001). In the present study, we observed that SBTI protected BSA and LHRH from metabolism by serine proteinases in all regions of possum intestine. This agrees with our previous report that chymotryptic activity was responsible for most of the degradation of BSA and LHRH in luminal extracts from possum intestine (Wen et al 2002b).

In all luminal extracts, the initial metabolite was LHRH (1–5), rapidly followed by LHRH (1–3) and LHRH (1–4) in extracts from the small intestine. This suggests that the initial cleavage of the molecule occurs at the Tyr^5-Gly^6, followed by cleavage at the Trp^3-Ser^4 and Ser^4-Tyr^5 peptide bonds. A lag was observed in the formation of LHRH (3–4) in jejunal and ileal extracts. This might result from sequential cleavage of the LHRH (1–4) at His^2-Trp^3 by an unknown peptidase. LHRH was much more stable in hindgut extracts. The major metabolite in hindgut extracts was LHRH (1–5) with some LHRH (1–3), very little LHRH (1–4) and almost no LHRH (3–4) formed. We would expect elastase to cleave LHRH at both Ser^4-Tyr^5 and Gly^6-Leu^7 (Woodley 1994). However, we only obtained LHRH (1–4) and this was present in only small amounts. Two unidentified metabolites (unknown fragments 1 and 2) were observed, one of which could conceivably be LHRH (1–6). These findings are consistent with those reported by Walker et al (2001) on digestion in the salmon intestine.

To our knowledge, this is the first investigation that has studied the effects of protease inhibitors on the degradation of a model protein or peptide in luminal extracts from any marsupial species. The presence of SBTI alone or as a mixture with SDA, bestatin or C934P in luminal extracts from the small intestine, almost completely inhibited the degradation of BSA and LHRH. Tozaki et al (1998) reported that SBTI significantly inhibited the degradation of a model peptide in luminal extracts from rats. In a previous study, we reported the hydrolysis of the chymotrypsin specific substrate *N*-α-benzoyl-L-tyrosine ethyl ester by luminal extracts (Wen et al 2002b). Accordingly, we concluded that chymotrypsin was the major enzyme responsible for digestion in the lumen of possum intestine. Moreover, TPCK (a chymotrypsin inhibitor) selectively inhibited the formation of LHRH (1–5) in a dose-dependent manner and this is consistent with the work reported by Advis et al (1988) on rats. This is also supported by Walker et al (2001) who demonstrated that LHRH is most susceptible to chymotrypsin. SDA had no significant effect on the formation of LHRH (1–5) or LHRH (1–3) in extracts from the small intestine and, by extrapolation, little effect on chymotrypsin. However, SDA did inhibit the formation of LHRH (1–5) in extracts from the hindgut in a dose-dependent manner. This could be the result of inhibition of mucosal endopeptidase 24.15 sloughed from the mucosal membrane into the lumen, since the major metabolite produced by incubation in mucosal homogenates from the hindgut was LHRH (1–5), with little or no LHRH (1–3) (Wen et al 2002c).

In-vitro experiments have shown that bile salts inhibit proteolytic enzymes of the intestinal lumen in rat (Bai et al 1995). The mechanism of enzyme inhibition by SDA at concentrations above the critical micelle concentration may be due to the entrapment of the peptide within the micelle, thus protecting it from the proteolytic enzymes (Lee et al 1991). However, the mechanism of enzyme inhibition by SDA at concentrations below the critical micelle concentration is not well understood.

Bacitracin weakly inhibited proteolysis of BSA and non-specifically inhibited peptidolysis of LHRH in luminal extracts from small intestine. McKelvy et al (1976) reported that bacitracin inhibited non-specific peptidase activity in

brain extracts from guinea-pigs and rats in-vitro. Bacitracin, a cyclic polypeptide antibiotic, has a broad spectrum of biological activity, including inhibition of endopeptidases, such as subtilisin and papain (Roth 1981), and has been used to protect peptides, such as insulin, buserelin and luteinizing hormone, from degradation (Raehs et al 1988; Yamamoto et al 1994). Although bestatin does not seem as potent an inhibitor as the other inhibitors used in the present study (Tables 2 and 3), it does appear to selectively inhibit the formation of unknown fragment 1. We also found that C934P significantly inhibited the degradation of LHRH in luminal extracts from all regions of possum intestine, but was more effective in preventing the degradation of LHRH in the hindgut than in the small intestine. The inhibition of luminal proteases by C934P has been ascribed either to chelation of calcium or zinc ions from the enzymes (Lueßen et al 1996a, b) or to adsorption and denaturation of the enzymes (Walker et al 1999).

In conclusion, this in-vitro study has demonstrated that serine proteases such as chymotrypsin are involved in the degradation of BSA and LHRH in luminal extracts from possum intestine. SBTI alone or in combination with SDA or C934P almost totally inhibited hydrolysis of BSA and LHRH in luminal extracts from the small intestine of the possum. Moreover, since SDA and C934P are also good permeation enhancers (Lueßen et al 1994; Uchiyama et al 1999), we suggest co-formulation of these with SBTI for the protection of peptide or protein drugs to protect against luminal digestion during transit of the small intestine. We believe such formulations should be targeted to the hindgut of the possum where the observed proteolytic activity of the luminal extracts towards the model protein and peptide was lowest.

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