Degradation and Aggregation of Human Calcitonin *In Vitro*

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Received October 16, 1998; accepted December 1, 1998

Purpose. To investigate the degradation of human calcitonin (hCT) by enzymes or mucosa from different gastrointestinal (GI) compartments and evaluate the stabilization effect of a synthetic ionizable copolymer on the stability of hCT in an aqueous solution. These data are a prerequisite for the development of a hydrogel based colon-specific hCT delivery system.

Methods. Luminal and brush border membrane (BBM) enzymes from the colon and small intestine (SI) of the rabbit were isolated and their enzymatic activity toward hCT *in vitro* was evaluated. Human fecalase was used to mimic the luminal enzymatic activity in the human colon and its degradation ability was assessed. Excised intact rabbit intestinal tissues from both the colon and the SI were used to study the degradation patterns of hCT by intact mucosa. Detection of intact human calcitonin was performed using gradient elution, reverse phase high-pressure liquid chromatography (RP-HPLC). The structure of the hCT fragments was determined by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis. UV/VIS and fluorescence spectroscopy methods were used to evaluate the influence of a copolymer, possessing the same structure as the primary chains in hydrogels degradable in the colon, on the fibrillation process of hCT.

Results. *In vitro* results showed that isolated luminal enzymes and BBM enzymes from the SI were more potent in degrading intact hCT, as expected. Moreover, BBM enzymes were far more abundant in the SI than in the colon. Compared with rabbit colonic luminal enzymes, the degradation potency of human fecalase was further abated. Intact mucosal studies revealed extensive degradation by the SI mucosa but not by the colonic mucosa. The primary structures of the peptide fragments were identified by MALDI-TOF MS analysis. Fibrillation studies of hCT indicated that acrylic acid-containing polymeric materials were able to decrease the aggregation of hCT in aqueous solutions.

Conclusions. Reduced proteolytic activity suggests that the colon is an advantageous site for peptide delivery. The structures of hCT degradation products were identified and the participation of particular enzymes in the degradation process was suggested. In addition, it was determined that an acrylic acid-containing copolymer improved the physical stability of hCT in aqueous solution.

KEY WORDS: colon specific delivery; human calcitonin; protein degradation; luminal enzymes; brush border membrane enzymes; human fecalase; MALDI-TOF MS.

INTRODUCTION

Human calcitonin is a hypocalcemic hormone secreted by the parafollicular C-cells of the thyroid gland. Though it has been discovered in many species, only human, salmon (sCT), porcine and an eel analogue of calcitonin are in general medical use in several countries. Human CT is an effective therapeutic agent for the treatment of several bone related disorders, such as osteoporosis, Paget's disease, Sudeck's atrophy, and malignant hypercalcemia. Moreover, an analgesic effect has been ascribed to calcitonin, presumably due to a direct hormonal action on calcitonin receptors in the brain (1).

The oral route is the preferred route of administration for most drugs including peptides and proteins. Establishing an oral delivery system for peptide and protein drugs is of great importance, because it is expected, that for treatment of chronic non-life threatening disorders, such as postmenopausal osteoporosis, parenteral administration will result in poor patient compliance and thus restricted utility (2). There are several barriers for the oral delivery of peptide drugs (3–6): chemical and enzymatic degradation during transit, the mucosal barrier, metabolism during transepithelial transport and hepatic elimination. Calcitonin is an excellent candidate for the development of alternate delivery routes due to its size and wide therapeutic index (3).

In the past few years, a new hydrogel system has been developed, which can be used for colon-specific delivery of protein drugs (7–11). These hydrogels are based on biocompatible copolymers of N,N-dimethylacrylamide (DMAA) with *tert.*-butylacrylamide (BuMA; to improve mechanical properties), acrylic acid (AA; to introduce pH-sensitivity), and crosslinking agents containing aromatic azo bonds which are degradable by azoreductase activity in the colon. In the stomach, due to the low pH range, the hydrogels will have a low equilibrium degree of swelling. To avoid the release of hCT in the small intestine, hydrogels with adjustable swelling kinetics were designed and synthesized (11). As the gels pass down the GI tract, swelling will increase due to the ionization of carboxylic acid groups in response to the pH increase. Upon arrival in the colon, a degree of swelling will be reached that will make the crosslinks susceptible to azoreductase activity. The gels will be degraded and the drug released. The hydrogels were designed to protect hCT entrapped inside the capsule, which was coated with the hydrogel, and release the drug specifically in the colon. The rationale for colon-specific delivery of hCT is that the enzymatic activity in the colon is much reduced, thus, the lifetime of the hCT should be prolonged.

A prerequisite for designing an oral delivery system for hCT is the comparison of the proteolytic potentials of different compartments of the GI tract. Numerous enzymes may participate in the degradation of a protein in the GI tract—luminal enzymes and the membrane-associated enzymes found in the brush border membrane (BBM) of mucosal cells lining the GI tract. BBM enzymes are ectoenzymes, which expose their bulk, including the active sites, at the external surface of the GI tract. They are anchored by a short stalk that is inserted into the hydrophobic core of the lipid bilayer. The majority of the BBM enzymes are metalloenzymes though some serine proteases are also present. This is an organized system which efficiently degrades proteins and peptides into amino acids (12). Bacterial activities are predominantly present in the colon. Some of the specificities of bacterial enzymes are unique, nevertheless they also possess proteolytic activity (13). Degradation studies of

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0724-8741/99/0300-0359$16.00/0

hCT by those enzymes would allow us to assess the feasibility of colonic specific delivery of peptide drugs.

Though sCT may have a slightly higher therapeutic potency than hCT, its therapeutic efficacy is hampered by the generation of neutralizing antibodies after prolonged administration (1). On the other hand, the drawback associated with hCT therapy is its physical instability in aqueous solutions, especially near neutral pH. At this experimental condition, hCT has a tendency to self-associate and precipitate in solution. Studies have shown that hCT potency is reduced when fibrillation occurs. Fibrillation can be minimized by avoiding highly concentrated hCT solutions and by using 0.001% acetic acid rather than high salt content buffers (14,15).

Here, the degradation of hCT by luminal, brush border membrane enzymes, and by intact intestinal mucosa from the rabbit SI and colon was studied. The degradation products were analyzed by MALDI-TOF MS and their structures determined. Human fecalase was used to mimic the human colon luminal enzymes. In addition, the stabilizing effect of an acrylic acid-containing copolymer on the fibrillation onset time of hCT solutions was determined.

MATERIALS AND METHODS

Chemicals

Mannitol, phosphate buffered saline tablets, trypsin, α-chymotrypsin, elastase, Ala-Ala-Ala p-nitroanilide (AAA-NAp), succinyl-Ala-Ala-Ala *p*-nitroanilide (Suc-AAA-NAp), N-CBZ-L-Phe chloromethyl ketone (ZPCK), Nα-benzoyl-L-arginine *p*-nitroanilide (Bz-R-NAp), and Trizma® base were purchased from Sigma (St. Louis, MO), α-glucosidase was from Fluka (Ronkonkoma, NY), and succinyl-Phe-Leu-Phe *p*-nitroanilide (Suc-FLF-NAp) was from Bachem (London, UK). Acetonitrile and water (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Human calcitonin (hCT, H-[Cys-Gly-Asn-Leu-Ser-Thr-Cys]-Met-Leu-Gly-Thr-Try-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2) was a generous gift from Suntory, Ltd. (Tokyo, Japan).

Isolation of Rabbit Brush Border Membrane Enzymes

The research using experimental animals adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and was approved by the University of Utah IACUC.

New Zealand white rabbits (male 2 kg) were fasted overnight and sacrificed by intravenous injection of a sodium pentobarbital overdose. The small intestine and colon were surgically removed. Luminal contents were washed out with cold 0.01 M phosphate buffered saline (pH 7.4) and used for luminal enzyme separation. The intestinal segments of both the colon and small intestine were cut open and everted spontaneously; they were further sliced into 3 cm segments and immediately frozen.

Brush border membrane enzymes were isolated from the frozen everted small intestinal and colonic segments of the same length using the divalent cation precipitation technique (16). Mucosal cells were released from the intestinal tissue by thawing and vibrating at maximum speed for 1 min using a vortex. The cells were disrupted by homogenization in a blender. $CaCl_2$ was added to the suspension to make a 10 mM solution in order to precipitate the nuclei, mitochondria, microsomes and other cellular debri. The cellular debri were removed as a pellet following low speed centrifugation at 2,800 × *g* for 15 min at 4°C (Model Biofuge 15R, Heraeus Instruments, Germany). The BBM enzymes were isolated from the supernatant as a pellet by high speed centrifugation at 28,500 *g* for 30 min at 4°C (Model J2 HS, Beckman Instruments, USA). The pellet was then homogenized and suspended in 50 mM mannitol 2 mM Tris buffer and divided into aliquots and frozen for further use.

Isolation of Rabbit Luminal Enzymes

The isolated (washed out) luminal contents of the small intestine and colon were diluted with PBS to obtain a 10% (w/v) slurry for the SI and a 20% (w/v) slurry for the colonic contents followed by purging with nitrogen. The suspension was rapidly homogenized using a blender and centrifuged at 2800 × g (Model Biofuge 15R, Heraeus Instruments, Germany) for 5 min to obtain the supernatant and pellet (particulate material) fractions. The pellet fraction was re-suspended in PBS buffer to give the same final volume as the supernatant fraction. Both the supernatant and pellet fractions were divided into aliquots and stored frozen.

Isolation of Human Fecalase

Human feces were collected from a healthy adult on a normal Western diet. The fresh human feces were suspended in PBS buffer, to obtain a 10% (w/v) suspension. The suspension was rapidly homogenized using a blender and centrifuged at 2800 × *g* (Model Biofuge 15R, Heraeus Instrument, Germany) for 15 min to obtain the supernatant and pellet (particulate material) fractions. The pellet was re-suspended with an equal volume of PBS. The suspensions were divided into aliquots and stored frozen.

Characterization of the Enzymes

The protein content of the isolated luminal enzymes, BBM enzymes and human fecalase were evaluated by a modified Lowry assay. The homogeneity of the suspensions was examined by determination of their dry weights after lyophilization. The activities of the colon and SI BBM enzymes were assessed by a glucosidase assay and the activities of luminal enzymes, human fecalase and pancreatic enzymes were estimated by monitoring the cleavage of enzyme specific substrates including Bz-R-NAp, Suc-AAA-NAp, AAA-NAp, and Suc-FLF-NAp. ZPCK (1 μM) was added to the final trypsin and elastase incubation solutions to eliminate unwanted chymotrypsin activity due to impurities. The cleavage rate was calculated by monitoring the *p*-nitroanalide concentration at 410 nm using a UV/VIS spectrometer (Lambda 6/PECSS, Perkin-Elmer, USA).

Degradation Study of Human Calcitonin by Isolated Enzymes and Pancreatic Enzymes

Human calcitonin solutions were freshly prepared prior to experiments conducted at a concentration of 1 mg/ml. All luminal enzymes, BBM enzymes, and human fecalase enzyme suspensions were diluted to 0.075 mg protein/ml of the final

incubation solution. The final concentrations of the pancreatic enzymes (trypsin, chymotrypsin, elastase) were 0.1 μM. The degradation mixtures were incubated at 37°C for various time intervals. Trifluoroacetic acid (0.6%) was added at the end of the incubation period to lower the pH to 2 and to quench the enzymatic activity. The solution was filtered through a 0.2 μm filter, frozen immediately in dry ice and stored in refrigerator for chromatography analysis.

Degradation of Human Calcitonin by Excised Intact Intestinal Mucosa

Special reflection cells (modified from ref. 17) were made in-house using Plexiglas. The basic design included a top lid with two holes for gas inlet and outlet, a bottom base with a silicone rubber gasket that can be attached to the middle piece by four screws. The bottom surface facing the buffer solution had an area of 3 cm^2; the volume of the incubation mixture was 3 ml. SI and colon segments were surgically removed from the rabbit immediately after sacrifice and mounted in the reflection cells with the mucosal side facing the solution. The tissues in the reflection cells were equilibrated with a Universal Ringer buffer at 37°C for 3 min, then replaced with a 0.4 mg/ml hCT solution or a substrate solutions in Ringer. Suc-AAA-NAp, AAA-NAp, Bz-R-NAp, and Suc-FLF-NAp solutions (1 mM) were used to evaluate enzymatic activity. A binary 95%O_2/5% CO_2 gas mixture was bubbled into the solution to provide circulation and maintain intestinal tissue viability. The cells were then placed into a shaking water bath (Model YB-521, American Scientific Products, USA) at 37°C. At various time intervals up to 1.5 h, 100 μl of samples were withdrawn from the solution. The sample was mixed with 50 μl of 0.6% TFA and filtered through a 0.2 μm filter, and frozen immediately for further analysis.

Chromatography Analysis

Reverse phase high performance liquid chromatography (RP-HPLC) was used to determine the concentration of hCT solutions. The HPLC apparatus consisted of a Rainin gradient system (HPLC pump model SD-200) with a Rainin UV detector (model UV-D2). The C_{18} column (15 cm, Microsorb-MV, Rainin Instruments, USA) was equilibrated with 90% eluent A (10% acetonitrile, 89.9% water, and 0.1% trifluoroacetic acid) and 10% eluent B (80% acetonitrile, 19.9 % water, and 0.1% trifluoroacetic acid). A gradient method starting at 90% eluent A and 10% eluent B to a mixture of 80% eluent B and 20% eluent A over a 40 min period at a flow rate of 1 ml/min was used. The absorbance of the eluent was monitored at 214 nm. The column was calibrated with hCT solutions of known concentrations ranging from 0.001 to 1 mg/ml.

Mass Spectrometry Analysis

The peptide fragments from the degradation study with intact mucosa in Ringer buffer were subjected to MALDI-TOF MS analysis. All measurements were performed by a MALDI-TOF mass spectrometer (Voyager-DE STR Biospectrometry Workstation, PerSeptive Biosystems, Inc., Farmingham, MA). Sinapinic acid (*trans*-3-(4-hydroxy-3,5-dimethoxyphenyl)acrylic acid) was used as a matrix for the peptide samples. The mass spectrometer was equipped with a pulsed nitrogen laser at wavelength of 337 nm and appropriate UV-optics. The delay time was varied to achieve the highest mass resolution. All spectra were obtained in linear mode with the negative ions off. Calibration was performed by using standard peptides.

Synthesis of Linear Copolymer Used for the Fibrillation Study

The copolymer, poly(DMAA-*co*-BuMA-*co*-AA-*co*-MA-Gly-Gly-aminopropanol) was synthesized as previously described (9). This copolymer has the same structure as the backbone (primary chains) of the hydrogels developed for colon-specific delivery (7–11). The polymer was synthesized by radical precipitation copolymerization of N,N-dimethylacrylamide (DMAA), N-tert-butylacrylamide (BuMA), acrylic acid (AA), and N-methacryloylglycylglycine *p*-nitrophenyl ester (molar ratio of monomers 37:20:40:3) in acetone. The reactive ester groups were inactivated by aminolysis with 1-amino-2-propanol. The M_w of polymer was 65,000, and M_w/M_n was 1.6 (determined by SEC using Superose 6 column calibrated with polyHPMA fractions). The content of carboxyl groups was 3.93 mmol/g polymer as determined by NaOH titration.

UV/VIS Spectroscopic Human Calcitonin Fibrillation Studies

The fibrillation of hCT solutions were examined spectroscopically at 340 nm. The onset time of fibrillation was determined by extrapolation of the absorbance vs. time dependence to the baseline. For long-term fibrillation studies, periods of visual examinations were utilized.

Fluorescence Studies of Human Calcitonin Fibrillation

Fluorescence measurements of anisotropy were performed on a photon-counting spectrofluorometer PC-1 equipped with a Glan-Thompson polarizer (ISS, Inc., Champaign, IL). The fluorescence excitation and emission wavelengths were 278 and 300 nm, respectively. Spectral bands of the excitation and emission monochromator were 16 nm. A freshly prepared 1 mg/ml hCT solution in PBS (pH 7.4) buffer was placed in quartz cuvettes and the anisotropy and intensity of fluorescence were measured immediately. After 10 h incubation at room temperature, the anisotropy value and fluorescence intensity of the fibrillated hCT solution was determined.

RESULTS

Degradation of hCT by Rabbit Luminal Enzymes

The activities of the luminal enzymes were evaluated using specific substrates including Bz-R-NAp, Suc-AAA-NAp, AAA-NAp, and Suc-FLF-NAp (data not shown). The protein concentrations were determined by Lowry assay and normalized for the incubation study. To test the uniformity of the luminal enzyme suspensions, 3 ml aliquots were lyophilized and the dry masses were weighed. The deviations of the dry masses were less than 10% (data not shown). The rate constants calculated by fitting the data to a pseudo-first order model using a non-linear least square were 3.47×10^{-2}, 1.47×10^{-2}, 6.30×10^{-3}, and 1.12×10^{-2} min^{-1} for the SI luminal supernatant

(SILS), SI luminal pellet (SILP), colon luminal supernatant (CLS) and colon luminal pellet (CLP), respectively (Table 1). The potency of isolated luminal enzymes from the colon was less than that of the SI for both the pellet and the supernatant fractions.

Degradation of hCT by BBM Enzymes

The normalized activities of the BBM enzymes from the glucosidase assay (using *p*-nitrophenyl-α-glucoside as a substrate) were 0.27 and 0.09 IU (1 IU = hydrolysis of 1 mmol substrate/min at 37°C) for the SI and colon, respectively. The total protein content of the isolated BBM enzymes from the intestinal tissues (31 cm) was determined to be 14.6 and 0.4 mg for the SI and colon, respectively. The amount of BBM enzymes isolated from the SI was 36 times greater than that from the colon segment of the same length. The pseudo-first order rate constants of the degradation of hCT by BBM enzymes and hCT half-lives are shown in Table 1.

Degradation of hCT by Human Fecalase

The protein contents of human fecalase were determined by the Lowry protein assay for both the supernatant and pellet fractions. The homogeneity of the suspensions was evaluated using the lyophilization method described above, and no significant deviations were observed. The pseudo-first order rate constants of the degradation of hCT by human fecalase and hCT half-lives are presented in Table 1. The half-lives of hCT in human fecalase were compared with the half-lives in rabbit luminal enzymes (Table 1). Under the experimental conditions used, human fecalase appeared to possess the lowest enzymatic activity.

Degradation of hCT by Pancreatic Enzymes

Trypsin, α-chymotrypsin and elastase are the major digestive enzymes originating from the pancreas and secreted into the GI. The pseudo-first order rate constants of the degradation of hCT by pancreatic enzymes and hCT half-lives are shown in Table 1. The enzymatic activity of stock solution of enzymes was assessed by cleavage studies using *p*-nitroanilide-containing substrates: Bz-R-NAp for trypsin, Suc-FLF-NAp for α-chymotrypsin, Suc-AAA-NAp and AAA-NAp for elastase (data not shown). Due to the presence of α-chymotrypsin in trypsin and elastase, 1 μm ZPCK was added to specifically inhibit the chymotrypsin activities. At this concentration, ZPCK did not influence the activities of trypsin and elastase. The products from the degradation were subjected to MALDI-TOF MS analysis and the hCT cleavage sites for the individual enzymes are summarized in Table 2. As expected, trypsin cleaved only one bond originating in Lys_{18}; the major cleavage sites during incubation with α-chymotrypsin were Tyr_{12} and Phe_{19}, whereas the cleavage points for elastase were bonds originating in Ala_{26}, Ile_{27}, and Gly_{28}.

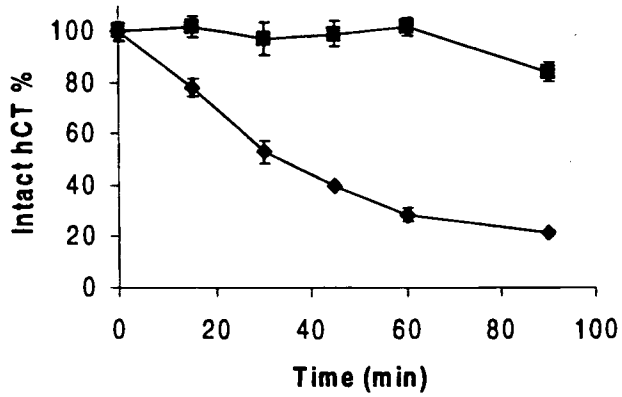


Fig. 1. Degradation of hCT by intact intestinal mucosa using reflection cells. SI (■), and colon (◆). Each cells contained 3 ml of 0.4 mg/ml hCT in universal Ringer buffer (7.4) at the beginning of the experiment. Data shown are averages of duplicates.

Table 1. Degradation of Human Calcitonin

Enzyme	k_{app} (min^{-1})	hCT half-lives (min)
Rabbit SI luminal supernatant	0.0347 ± 0.0001	20 ± 0
Rabbit colon luminal supernatant	0.0063 ± 0.0001	110 ± 3
Human fecalase supernatant	0.0046 ± 0.0001	150 ± 4
Rabbit SI luminal pellet	0.0147 ± 0.0008	47 ± 3
Rabbit colon luminal pellet	0.0112 ± 0.0001	62 ± 0
Human fecalase pellet	0.0053 ± 0	132 ± 0
Rabbit SI BBM enzymes	0.0055 ± 0.0004	239 ± 17
Rabbit colon BBM enzymes	0.0029 ± 0.0003	128 ± 13
Trypsin	0.0845 ± 0.0002	8 ± 0
α-Chymotrypsin	0.0321 ± 0.0001	22 ± 0
Elastase	0.0338 ± 0.0001	21 ± 0

Note: Pseudo-first order degradation rate constants and half-lives of hCT (0.5 mg/ml) were determined for rabbit SI luminal enzymes, colon luminal enzymes, human fecalase (0.075 mg protein/ml), and pancreatic enzyme solutions (0.1 μM). The results are means of duplicates. The rate constants of degradation were estimated by using a non-linear least square fitting method with a pseudo-first order model. $t_{1/2} = \ln 2/k_{app}$.

Table 2. Degradation of hCT by Pancreatic Enzymes: Trypsin, α-chymotrypsin, and Elastase

Bond cleaved	Fragment detected	Molecular weight	
		MALDI	Calculated
	Trypsin		
Lys_{18}-Phe_{19}	Phe_{19}-Pro_{32}	1442	1442
Lys_{18}-Phe_{19}	Cys_1-Lys_{18}	1995	1995
None	Cys_1-Pro_{32}	3418	3419
	α-Chymotrypsin		
Tyr_{12}-Thr_{13}	Cys_1-Tyr_{12}	1261	1261
Tyr_{12}-Thr_{13}	Thr_{13}-Pro_{32}	2175	2176
Phe_{19}-His_{20}	Cys_1-Phe_{19}	2142	2142
None	Cys_1-Pro_{32}	3418	3419
	Elastase		
Ala_{26}-Ile27	Cys_1-Ala_{26}	2925	2925
Ile_{27}-Gly_{28}	Cys_1-Ile_{27}	3038	3038
Gly_{28}-Val_{29}	Cys_1-Gly_{28}	3194	3194
None	Cys_1-Pro_{32}	3419	3419

Note: All experiments conducted in PBS buffer (pH 7.4) at 37°C, [E] = 0.1 μM, [hCT] = 0.5 mg/ml.

Degradation of hCT by Excised Intact Intestinal Mucosa

To evaluate the enzymatic activity of the excised intact intestinal mucosa, Bz-R-NAp, Suc-AAA-NAp, AAA-NAp, and Suc-FLF-NAp were used. The *p*-nitroaniline release rates by intact rabbit SI mucosa were 3.44, 3.20, 0.54 and 0.24 μM/min, for substrates Bz-R-NAp, Suc-FLF-NAp, AAA-NAp and Suc-AAA-NAp (1 mM), respectively. These rates were considerably higher than those obtained monitoring the *p*-nitroaniline release by intact colonic mucosa. In the latter case, the rates were 0, 0.01, 0.07 and 0.02 μM/min, for substrates Bz-R-NAp, Suc-FLF-NAp, AAA-NAp and Suc-AAA-NAp, respectively. These data are consistent with the degradation of hCT (Fig. 1). HPLC chromatograms of the SI hCT degradation products displayed multiple peaks which corresponded to various peptide fragments, while the intact hCT peak was the only major peak in the colon hCT degradation mixture (data not shown).

MALDI-TOF MS analysis revealed the structures of degradation products with molecular weights greater than 1000 Daltons (Fig. 2, Table 3). The values of the peaks from the MALDI-TOF mass spectra represented the peptide molecular weight divided by the charge plus one H^+. The observed accuracy of the MALDI-TOF MS analysis was greater than 0.1% of the total molecular weights of the analyzed compounds (mol. weight range 1000 to 4000). Often, the two adjacent peaks of a particular peptide fragment were the sodium and potassium salts formed during analysis in PBS. The MALDI-TOF MS peak values were used to match the molecular weight of a potential peptide sequence. The detected fragments from the 30 min hCT degradation by SI mucosa and 60 min degradation by colonic mucosa are shown in Fig. 2. Table 3 lists the detected fragments from the 10, 30, and 60 min degradation by SI mucosa. The cleavage points from the 30 min SI degradation and the 60 min colon degradation of hCT were compared in Fig. 3.

UV/VIS and Fluorescence Spectroscopy Studies of hCT Aggregation

The water soluble copolymer, poly(AA-co-DMAA-co-BuMA-co-MA-Gly-Gly-aminopropanol) which has the structure of the primary chains (backbone) of biodegradable hydrogels for colon-specific peptide delivery (9) was used as a stabilizer. One gram of the copolymer contained 3.93×10^{-3} mol of acrylic acid equivalent, as determined by acid-base titration. Copolymer solutions in PBS were prepared to achieve final acrylic acid equivalent concentrations of 0.01%, 0.1%, and 1%. The final pH values were 7.3, 7.1, and 5.8, respectively. The onset times of hCT (1 mg/ml PBS) fibrillation were determined spectroscopically at 340 nm using both quartz and polystyrene cuvettes. The fibrillation onset times ranged from 5 h (polystyrene cuvettes, 0% copolymer) to 30 days (quartz cuvettes, 1%) (Fig. 4).

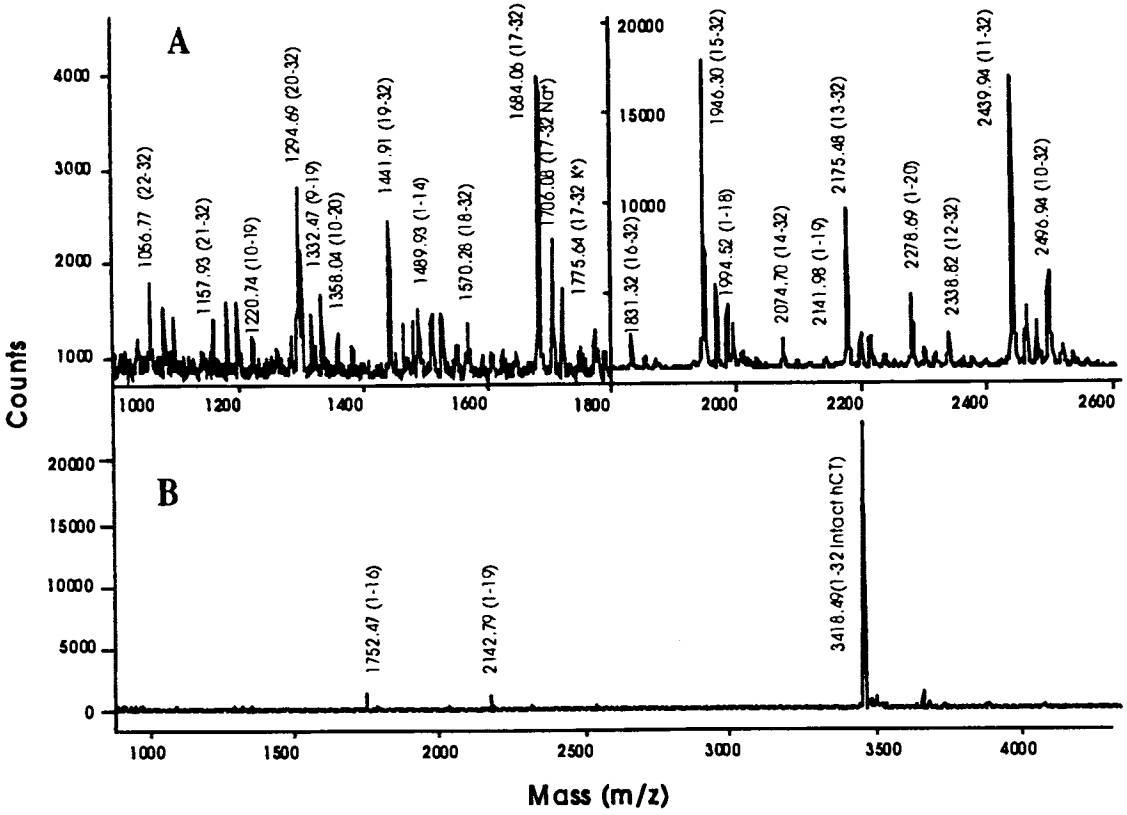


Fig. 2. Degradation of hCT by rabbit intestinal mucosa. The supernatant was analyzed by MALDI-TOF MS. The numbers in brackets indicate the amino acid sequence of the fragments. Na^+ and K^+ salts are marked only for fragment 17–32. For comparison of molecular weights of the fragments obtained from MALDI-TOF MS and the theoretical values. A) Rabbit SI mucosa, incubation time 30 min at 37°C. B) Rabbit colonic mucosa, incubation time 60 min at 37°C.

Table 3. Degradation of hCT by Isolated Rabbit SI Mucosa

Fragment	10 min	30 min	60 min	1st cleavage	2nd cleavage	MALDI value	Calculated value
Cys_1-Met_8			Yes	None	Met_8	827	827
Phe_{22}-Pro_{32}		Yes	Yes	Thr_{21}	None	1057	1057
Thr_{21}-Pro_{32}	Yes	Yes	Yes	His_{20}	None	1158	1158
Gly_{10}-Phe_{19}		Yes		Leu_9	Phe_{19}	1221	1220
His_{20}-Pro_{32}	Yes	Yes	Yes	Phe_{19}	None	1295	1295
Leu_9-Phe_{19}	Yes	Yes		Met_8	Phe_{19}	1332	1333
Gly_{10}-His_{20}		Yes		Leu_9	His_{20}	1358	1357
Phe_{19}-Pro_{32}	Yes	Yes		Lys_{18}	None	1442	1442
Cys_1-Gln_{14}		Yes		None	Gln_{14}	1490	1491
Lys_{18}-Pro_{32}		Yes		Asn_{17}	None	1570	1571
Asn_{17}-Pro_{32}	Yes	Yes		Phe_{16}	None	1684	1685
Cys_1-Phe_{16}		Yes		None	Phe_{16}	1753	1753
Phe_{16}-Pro_{32}	Yes	Yes		Asp_{15}	None	1831	1832
Asp_{15}-Pro_{32}	Yes	Yes	Yes	Gln_{14}	None	1946	1946
Cys_1-Lys_{18}		Yes		None	Lys_{18}	1995	1995
Gln_{14}-Pro_{32}		Yes		Thr_{13}	None	2075	2074
Cys_1-Phe_{19}		Yes		None	Phe_{19}	2142	2142
Thr_{13}-Pro_{32}	Yes	Yes	Yes	Tyr_{12}	None	2175	2176
Cys_1-His_{20}	Yes	Yes		None	His_{20}	2279	2280
Tyr_{12}-Pro_{32}	Yes	Yes		Thr_{11}	None	2339	2339
Thr_{11}-Pro_{32}	Yes	Yes	Yes	Gly_{10}	None	2440	2440
Gly_{10}-Pro_{32}	Yes	Yes	Yes	Leu_9	None	2497	2497
Cys_1-Ile_{27}			Yes	None	Ile_{27}	3039	3038
Cys_1-Pro_{32}[a]	Yes	Yes	Yes	None	None	3419	3419

[a] Not shown in Fig. 2.

The fluorescence anisotropy values determined by fluorescence spectroscopy studies showed the anisotropy average of free hCT as 0.08, while the fully fibrillated hCT possessed a value of 0.50. These data were consistent with the reported literature values (14).

DISCUSSION

To achieve an effective oral administration of a protein drug, such as hCT, its extensive degradation in the GI tract must be significantly suppressed or circumvented. Common attempts to reduce the degradation and transport barriers include analogues, conjugates, formulations such as microparticles and emulsions, stimuli sensitive polymers, co-administration of enzyme inhibitors (4,18), and reversible lipidization (19). The rationale for the design of colon-specific protein delivery systems is based on the decreased proteolytic activity in this compartment and on the presence of bacterial activities with unique specificities (8,20,21). Several approaches using polymeric delivery systems have been proposed for colonic delivery of peptides/proteins (7–11,21–23). Their optimization, however, should be based in part on an exact knowledge of the degradation processes occurring in the GI tract. To this end, we have studied in detail the degradation of hCT in conditions modeling the GI tract.

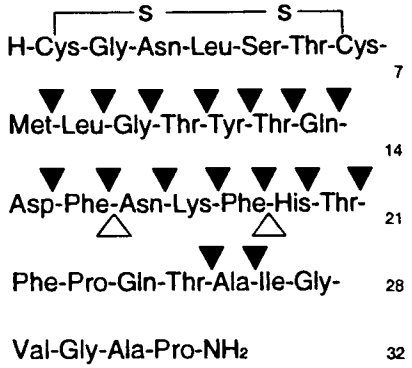


Fig. 3. Human CT cleavage sites during 30 min incubation with intact rabbit SI mucosa (▼) and 60 min incubation with intact rabbit colonic mucosa (Δ) as determined from MALDI-TOF mass spectra.

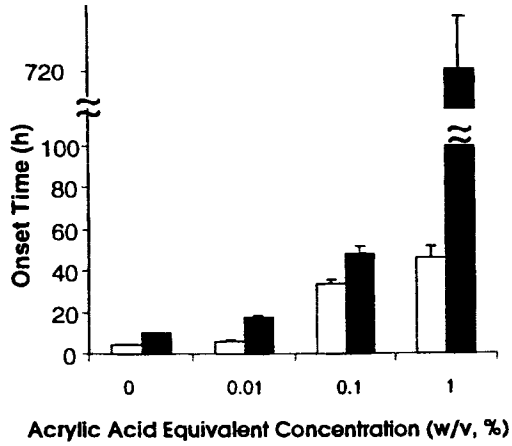


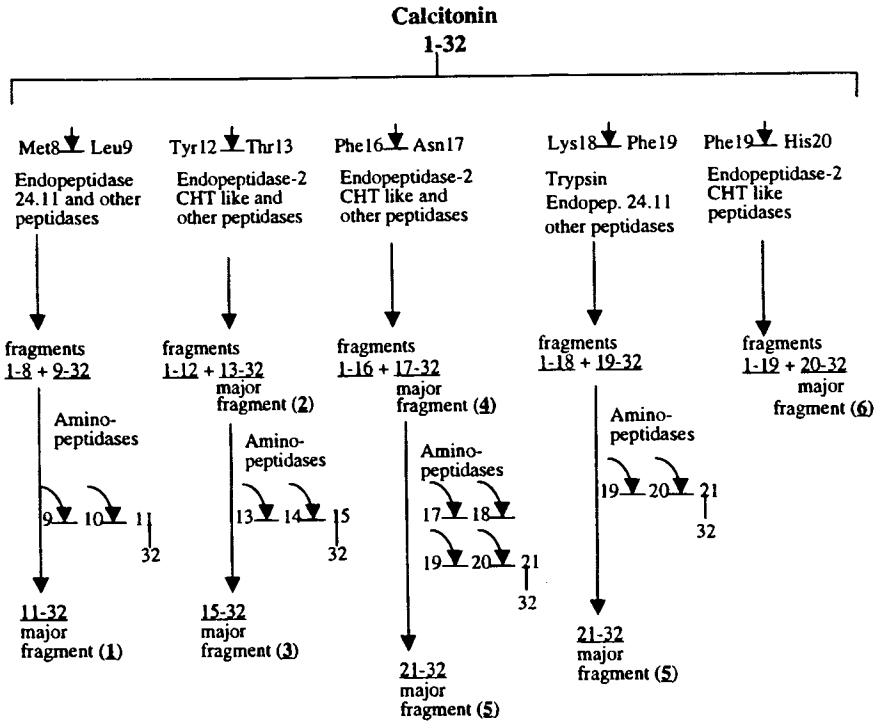
Fig. 4. Estimation of hCT fibrillation onset time in the presence of different concentrations of acrylic acid containing copolymer. The amounts of polymer stabilizer added were expressed as acrylic acid equivalent concentration. Polystyrene cuvettes (□) and quartz cuvettes (■). Data shown are averages of duplicates.

The small intestinal lumen is the most hostile environment for peptides and proteins. The quantities of chymotrypsin, trypsin, elastase, and carboxypeptidases A and B that are daily secreted by the human pancreas are in the gram range (12). There are individual variations in enzyme output. Contrasting data have been published on the ratio of the individual enzymes secreted (12). Part of the pancreatic enzymes may survive and be transported in the colon. Consequently, we have studied hCT degradation by pure pancreatic enzymes, by isolated luminal contents from SI and colon, and by BBM enzymes. The pancreatic enzymes cleaved hCT very efficiently, as expected (Table 1). The half-lives of hCT degradation by SI luminal enzymes were shorter than that of colon luminal enzymes (Table 1). The colonic pellet possessed about two times higher proteolytic activity than the colonic supernatant. Similar results were obtained when insulin B-chain was incubated with colonic enzymes from guinea pigs (24). Other authors also observed an increased proteolytic activity associated with colonic particulate material (25). On the other hand, our data with human fecalase showed approximately the same proteolytic activity associated with soluble material and pellet (Fig. 3). One possibility may be that the residual pancreatic activity is minimal after colon transit when fecalase was isolated. The reduction of the residual pancreatic activity is probably due to the digestion of these enzymes by bacterial proteases.

The cleavage of hCT by SI BBM enzymes was about two times faster than the cleavage by colonic BBM enzymes when identical amounts of BBM protein in the incubation mixtures were used (Table 1). In addition, 36 times more protein was isolated from the same length of SI than from colon. Data from hCT cleavage in reflection cells (Fig. 1) seem to be consistent with the above observation, i.e. the ratio of rates of hCT degradation by the SI and colonic tissues was similar as with isolated BBM enzymes.

An *in vitro* model was developed using intact nasal mucosa to evaluate the degradation processes occurring during the nasal delivery of hCT (17). We adapted the method for the study of hCT degradation by intact rabbit intestinal mucosa. There were six major fragments detected (their counts from MALDI-TOF mass spectrum were several times greater than of other fragments) after 10 min of hCT cleavage by intact SI tissue: 11–32, 13–32, 15–32, 17–32, 20–32 and 21–32. These fragments resulted from the combined action of endopeptidases and exopeptidases from BBM enzymes and probably residual pancreatic enzymes. The specificities of some of the BBM endopeptidases are well known (reviewed in ref. 12). For example, endopeptidase 24.11 has a specificity for hydrophobic amino acid residues preferentially at the P_1' position (nomenclature of ref. 26), endopeptidase 24.18 prefers an aromatic residue either in P_1 or in P_1' positions, and endopeptidase-2 has a "chymotrypsin-like" specificity.

A number of hypothetical degradation pathways could be proposed to explain the formation of the fragments detected by MALDI-TOF MS after incubation of hCT solutions with intact SI mucosa. One of them is presented in Scheme 1. The



Schematic 1. Hypothetical human calcitonin degradation patterns in excised intact rabbit SI mucosa. Fragments (1), (2), (3), (4), (5) and (6), were major fragments detected after 10 min., fragments (1), (2) and (3) after 60 min incubation. The smaller fragments (mol. weight < 1000) which resulted from the cleavage by amino- and carboxypeptidases are not shown in this scheme.

first attack by endopeptidases is followed by consecutive cleavage of fragments by aminopeptidases or dipeptidyl peptidases until a relatively stable fragment is formed. The composition and amount of fragments are changing with the time of contact (Table 3). The analysis of the structure of fragments indicated a higher activity of aminopeptidases when compared to carboxypeptidases in the cleavage of fragments formed during initial cleavage of hCT by endopeptidases.

We are aware that the intensity of peaks detected by MALDI-TOF MS may be molecular weight and composition dependent (27). However, an estimation of the relative amounts of fragments at different degradation times can be made. After 10 min of hCT degradation by excised rabbit SI mucosa, the value of counts for six major fragments (*1* to *6*, Scheme 1) was between 14,000 to 35,000; after 30 min the count numbers dropped to 5,000–25,000, and after 60 min the intensity was between 1500 to 2500 counts. On the contrary, incubation of hCT solution with excised rabbit colonic mucosa produced only very few fragments with low intensity (Fig. 2). These results seem to indicate that hCT degradation in the colon will proceed slowly.

The use of acrylic acid-containing copolymers in the design of colon-specific delivery systems appears to have several advantages: stimuli (pH) sensitivity (7–11), inhibitory activity (28), and stabilization effect. The latter effect was a hypothesis based on the observations of Arvinte et al. that minute amounts of acidic low molecular weight compounds prevent the aggregation of hCT (14,15).

To verify the influence of acrylic acid-containing copolymers on the aggregation of hCT, a modified polymer precursor, namely poly(DMAA-*co*-BuMA-*co*-AA-*co*-MA-Gly-Gly-aminopropanol) which is used in the synthesis of biodegradable hydrogels (9), was used as a stabilizer. The data obtained (Fig. 4) seem to indicate that the acidic microenvironment inside hydrogels of similar structure may protect hCT from aggregation.

It is interesting to note that hCT fibrillation onset times in quartz containers were substantially longer when compared to polystyrene containers under identical experimental conditions. Apparently, hCT adsorbs to the hydrophobic surface of polystyrene cuvettes increasing its local concentration. Since fibrillation is a concentration dependent process, the initiation of fibrillation at the solid-liquid interface may be enhanced. In addition, the solid-liquid interface may serve as a nucleation center. The changes of pH of the incubation solutions indicate that at least part of the effects resulted from changes in the ionization state of amino acid residues in the hCT molecules.

In summary, hCT is metabolized by various enzymes including pancreatic enzymes, and BBM enzymes. However, the data clearly indicate a higher stability of hCT in conditions mimicking the colon compartment than in the SI (Figs. 1 and 3). The increased stability of hCT in the acidic microenvironment bodes well for the development of a colon-specific hCT delivery system. One of the major applications of oral hCT would be the treatment of postmenopausal osteoporosis. Human CT is a strong candidate: it does not generate neutralizing antibodies (29) and there are indications that receptors on bones in mammals selectively recognize calcitonin molecules that do not easily adopt an α-helical structure, i.e., hCT, whereas helical calcitonins, like sCT, seem to bind specifically to the kidney (30).

ACKNOWLEDGMENTS

This work was supported in part by NIH grant GM 50839. We are grateful to Dr. Z. Wu of the University of Utah Mass Spectroscopy Service Facility for performing the MALDI-TOF MS analysis. This facility is supported in part by NIH Grant CA 42014 to the Huntsman Cancer Institute.

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