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Degradation of proteins by guinea pig intestinal enzymes

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Summary

Insulin, insulin B-chain, albumin and azoalbumin were used as model proteins to evaluate the proteolytic activity of different gastrointestinal tract sections in guinea pigs. Brush border membrane enzymes and luminal enzymes (supernatant and pellet) were isolated from the small intestine and colon of guinea pigs and the cleavage of the above mentioned proteins studied. The results clearly indicate that small intestine brush border and luminal enzymes possess a higher proteolytic activity compared to the colon. The total proteolytic activity of the small intestine toward insulin and insulin B chain was 4.4 and 12 times greater, respectively, than of the colon. The degradation patterns of insulin and insulin B chain cleavage by small intestinal luminal enzymes at about 50% degradation were compared with those of chymotrypsin, trypsin and elastase. While the insulin degradation occurred by all three enzymes, the insulin B chain was predominantly cleaved by trypsin. The degradation rate of unfolded proteins, i.e., insulin B chain and azoalbumin was much faster than the degradation rates of insulin and albumin, both with luminal and brush border membrane enzymes. The results obtained support the hypothesis that the colon demonstrates decreased enzymatic activity (compared to SI) and is a route for possible oral administration of protein drugs.

Introduction

The development of recombinant technologies has produced many of peptides and proteins available for pharmaceutical use. Currently, 132 biotechnologically engineered peptides and proteins await FDA approval (Eddington, 1991). Consequently, many research groups are studying the possibility of delivering peptides and proteins by means of other than parenteral, such as oral, pulmonary, ocular, buccal, vaginal, rectal and transdermal routes (Eppstein and Longenecker, 1988; Wearley, 1991). The oral route is the most attractive due to its convenience and high patient compliance. However, the bioavailability of peptides and proteins following oral administration is normally very low, due to absorption barriers and

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Abbreviations: BBM, brush border membrane; Bz, benzoyl; GI, gastrointestinal; NAp, *p*-nitroanilide; P, *N*-(2-hydroxypropyl)methacrylamide copolymer backbone; SI, small intestine; TFA, trifluoroacetic acid.

enzymatic degradation in the gastrointestinal tract, as well as first pass liver metabolism (Lee and Yamamoto, 1990; Woodley, 1991).

One of the prerequisites for the successful design of oral peptide/protein delivery systems is an understanding of catabolic processes occurring in the gastrointestinal (GI) tract (Ikesue et al., 1991). The enzymatically catalyzed hydrolysis of peptides and proteins in the GI tract occurs at several sites: luminally, at the membrane associated enzymes found on the brush border membrane (BBM) of mucosal cells lining the GI tract, and intracellularly.

We are systematically studying the relationship between the structure of macromolecules and their susceptibility to enzymatically catalyzed hydrolysis (Kopeček and Rejmanová, 1983; Kopeček and Ulbrich, 1983). Recently, we have reported on the relationship between the structure of *N*-(2-hydroxypropyl)methacrylamide copolymers containing oligopeptide side-chains terminated in *p*-nitroaniline and their degradability by BBM enzymes isolated from rat small intestine (Kopečková et al., 1991) and guinea pig luminal and BBM enzymes (Kopečková et al., 1992).

The purpose of this study is to obtain information about the relative potential of proteolytic activity at different regions along the GI tract of one animal species. To this end, brush border membrane (BBM) and luminal (supernatant and pellet) enzymes from the small intestine and colon of guinea pigs were isolated and the degradation of peptides and proteins (insulin B chain, insulin, albumin and azoalbumin) was evaluated in vitro. Insulin degradation by luminal and BBM enzymes was also studied in the presence of inhibitors to estimate the contribution of individual enzymes in the digestive process. The susceptibility of insulin and insulin B chain to degradation by pancreatic proteolytic enzymes (chymotrypsin, trypsin, and elastase) was also studied.

Materials and Methods

Chemicals

The chemicals used were as follows. Proteins: insulin (from bovine pancreas; 0.5% zinc content), oxidized insulin B chain, albumin (bovine) and azoalbumin. Enzyme inhibitors: phosphoramidon, EDTA, amastatin and soybean trypsin inhibitor. Enzymes: chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), and elastase (EC 3.4.21.36) were from Sigma. *p*-Nitroanilide substrates: Gly-Leu-Phe-NAp, P-Gly-Gly-Leu-Phe-NAp, P-Gly-Leu-Ala-Ala-Ala-NAp, and P-Gly-Leu-Ala-Ala-Leu-NAp were prepared as previously described (Kopečková et al., 1992); other oligopeptide *p*-nitroanilides were from Sigma. All other chemicals were of analytical or reagent grade.

Isolation of guinea pig intestinal enzymes

Isolation of brush border membrane (BBM) enzymes. Brush border membrane enzymes were isolated from frozen everted small intestinal and colonic segments of guinea pigs using the divalent cation precipitation technique (Kessler et al., 1978). Mucosal cells were released from the tissue after thawing by brief vibration. The cells were disrupted by homogenization in a blender. After addition of $CaCl_2$, the nuclei, mitochondria, microsomes, and other cell debri precipitated and were removed as a pellet at low speed centrifugation. The BBM enzymes (vesicles) were isolated as a pellet by high speed centrifugation. Proteins were assayed using the Lowry method, as modified by Peterson (1983), and Zn^{2+} resistant α-glucosidase was used as a marker for BBM enzymes (using *p*-nitrophenyl-α-glucoside as a substrate) (Peter et al., 1972). The content of protein in the final BBM preparation isolated from the small intestine was 1.1 mg/ml. The content of protein in BBM preparation isolated from the same amount of colonic material was 0.25 mg/ml. The specific activity of α-glucosidase was 131 mU/mg protein in SI preparation (enrichment factor 9.5) and less than 5 mU/mg in colonic preparation. The BBM enzymes were stored at -20°C in 1 ml portions.

Isolation of small intestinal and colonic luminal contents. Guinea pigs, Hartley strain, 320–350 g body weight, were fasted overnight before the experiment (Kopečková et al., 1992). The animals were killed by an overdose of diethyl ether vapours and the intestine removed. The contents of the small intestine and colon were washed out

with cold PBS buffer pH 7.4. The suspension was sparged with nitrogen and diluted with PBS to obtain a 10% (w/v) slurry for SI and 20% for colon contents. The suspension was rapidly homogenized using a blender and centrifuged at $2800 \times g$ for 5 min to yield supernatant and pellet (particulate material) fractions. The pellet fraction was resuspended in PBS buffer to give the same final volume as the supernatant fraction. The fractions were stored frozen in 2 ml portions. The protein contents determined by the Lowry method were: 0.15, 0.10, 0.13 and 0.43 mg/ml for SI/supernatant, SI/pellet, colon/supernatant, and colon/pellet, respectively.

Degradation of proteins

Protein degradation by BBM enzymes. Protein substrates were incubated with BBM enzymes in 0.1 M Tris buffer pH 7.4 with 0.2% Triton X-100 at 37°C. Stock solutions of protein substrates (20 mg/ml) were prepared in Tris buffer; insulin and azoalbumin were first dissolved in 0.2 ml of 0.01 M NaOH followed by immediate dilution with 0.8 ml of Tris buffer. The incubation mixture contained 0.25 ml of protein substrate stock solution, 0.65 ml buffer and 0.1 ml BBM enzyme preparation. The final concentrations were: 5 mg/ml protein substrate (if not otherwise stated) and 0.11 (SI) or 0.03 (colon) mg/ml of BBM protein content.

Protein degradation by luminal enzymes. Protein substrates were incubated with luminal contents (supernatant and pellet fractions) in 0.1 M phosphate buffer pH 7.4 under conditions similar to those described for BBM enzymes. The final concentration of enzymes, expressed as mg/ml of protein content of enzyme preparation, was 0.015 for small intestinal supernatant and pellet and 0.013 for colonic supernatant and pellet.

Degradation of insulin in the presence of inhibitors. The enzyme preparations were incubated with inhibitors for 15 min prior to the addition of insulin. The incubation mixture contained 0.17 mM of insulin in Tris buffer (BBM enzymes) or in phosphate buffer (luminal enzymes). The concentrations of inhibitors are denoted in Table 5.

Degradation by chymotrypsin, trypsin and elastase. Insulin (0.83 mM) was incubated in phosphate buffer (pH 7.4; 37°C) with 2 μM chymotrypsin, 1.9 μM trypsin or 19 μM elastase. Insulin B chain (1.7 mM) was incubated with 0.4 μM chymotrypsin, 0.7 μM trypsin or 0.4 μM elastase.

FPLC chromatography of degradation mixtures

Incubation mixtures were analyzed on a Fast Protein Liquid Chromatography system (Pharmacia). The degradation of albumin and azoalbu-

TABLE 1

Isolation characteristics of BBM and luminal enzymes [a]

Enzyme source:	One guinea pig (340 ± 25 g)					
Compartment:	Small intestine			Colon		
Weight of compartment:	8 ± 1.6 g tissue	4 ± 0.8 g content		4 ± 0.5 g tissue	5 ± 1.8 g content	
	BBM	Supernatant	Pellet	BBM	Supernatant	Pellet
Final volume of enzyme preparation (ml)	4	40	40	2	25	25
Protein content (mg/ml)	1.1 ± 0.2	0.15 ± 0.03	0.10 ± 0.02	0.25 ± 0.02	0.13 ± 0.03	0.43 ± 0.15
Specific peptidase activity (nmol/min per mg) [b] using substrates						
Ac-Ala-Ala-Ala-NAp	660	n.d.	n.d.	29	n.d.	n.d.
Bz-Arg-NAp	< 2	407	n.d.	n.d.	10	n.d.
Gly-Leu-Phe-NAp	360	116	123	4	15	12

[a] Values are means of three isolation procedures (four animals per isolation) ± standard deviation.
[b] nmol *p*-nitroaniline released/min per mg of protein content in enzyme preparation.

min was evaluated by size exclusion chromatography on a Superose 12 column (HR 10/30; Pharmacia) in Tris buffer (0.05 M Tris + 0.5 M NaCl; pH 8.0) with spectroscopic detection (280 or 400 nm). The degree of degradation was calculated from the ratio of protein and degradation fragment peak areas. The degradation of insulin and insulin B chain was followed by gradient chromatography on a PepRPC (C_2/C_{18}) Pharmacia reverse phase column with buffer A: 0.3% TFA/H_2O and buffer B: 0.3% TFA/CH_3OH. The samples withdrawn from the incubation mixture were diluted with 0.3% TFA/H_2O and a diluted sample (50 μg) applied onto the column. The separation was performed under a linear gradient (0–75%) B over 50 min at a flow rate of 0.5 ml/min with UV detection at 225 nm (detector UVIS 204 Linear).

Results and Discussion

Numerous studies have been performed to describe proteolytic degradation processes occurring in the GI tract (Shilling and Mitra, 1990, 1991; Lee and Yamamoto, 1991; Lee et al., 1991; Bai and Amidon, 1992; Mrsny, 1992), but only a few have been devoted to the comparison of mutual proteolytic potentials in different compartments of the GI of one animal species (Gibson et al., 1989; Longer et al., 1989; Kopeček et al., 1991; Kopečková et al., 1991; Woodley 1991). Results of such studies are a prerequisite for designing oral delivery system for insulin and other protein drugs. It is difficult to design in vitro assays that reflect actual enzyme concentrations along the GI tract in vivo. Table 1 describes typical results of the isolation protocol, showing the amount of enzymes (mg protein/ml preparation) isolated from one guinea pig. Low molecular weight oligopeptide *p*-nitroanilides were used to evaluate the specific peptidase activity (Table 1) of different preparations, expressed in *p*-nitroaniline release per min and per mg of protein content in the enzyme preparation. The substrate Gly-Leu-Phe-NAp which has an unprotected α-amino group was rapidly cleaved by SI BBM enzymes, indicating a high activity of aminopeptidases in this location. Besides aminopeptidase activity, there is significant endopeptidase activity (elastase-like) as demonstrated in the fast cleavage of protected trialanine *p*-nitroanilide.

Table 2 compares the total peptidase acivities of BBM enzymes and the supernatant and pellet of luminal contents, as determined by low and high molecular weight synthetic substrates. Macromolecular substrates based on *N*-(2-hydroxypropyl)methacrylamide copolymers back-

TABLE 2

Total peptidase activity of BBM and luminal enzymes characterized by p-nitroanilide substrates [a]

Substrate	*p*-Nitroaniline released (nmol/min)					
	Small intestine			Colon		
	BBM	Supernatant	Pellet	BBM	Supernatant	Pellet
Low-molecular weight *p*-nitroanilides						
Ac-Ala-Ala-Ala-NAp	2900	n.d.	n.d.	15	n.d.	n.d.
Bz-Arg-NAp	8	2440	n.d.	0	33	n.d.
Gly-Leu-Phe-NAp	1580	696	492	2	49	129
Polymeric *p*-nitroanilides						
P [b]-Gly-Gly-Leu-Phe-NAp	2	227	160	0.6	11	30
P-Gly-Leu-Ala-Ala-Ala-NAp	19	84	44	0.1	17	38
P-Gly-Leu-Ala-Ala-Leu-NAp	4	138	107	0.1	22	51

[a] Calculated from specific activity (nmol *p*-nitroaniline released/min per mg of protein content in enzyme preparation) multiplied by total enzyme content in given compartment.

[b] P denotes *N*-(2-hydroxypropyl)methacrylamide polymeric backbone carrying oligopeptide side chains of denoted structure, terminating in *p*-nitroanilide.

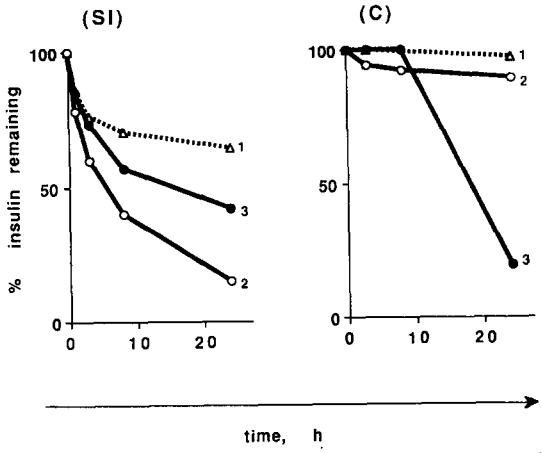

Fig. 1. Degradation of insulin by intestinal enzymes. (SI) Small intestine; (C) colon. (1) BBM enzymes; (2) supernatant of luminal contents; (3) pellet of luminal contents. Insulin (0.83 mM) was incubated at pH 7.4 with BBM enzymes in Tris buffer or luminal enzymes in phosphate buffer under the conditions described in Materials and Methods. The results are an average from two independent experiments.

bone containing oligopeptide side chains terminating in *p*-nitroaniline (Table 2) have to be cleaved first with an endopeptidase since the oligopeptides are attached to the polymeric backbone via their α-amino groups (Kopečková et al., 1992). The total peptidase activity of SI was greater than colonic activity for both low and high molecular weight synthetic substrates.

The time dependence of insulin degradation (as determined by the disappearance of the intact protein peak) by BBM enzymes, supernatant and pellet of luminal contents both from SI and colon is shown in Fig. 1. The degradation of insulin by enzymes isolated from the SI is relatively fast, whereas, of the three enzymatic preparations isolated from the colon, only the luminal pellet showed substantial activity. It is not clear to what extent this relatively high activity is due to true extracellular proteolytic activity or to cell lysis (Gibson et al., 1989), since there is evidence that large amounts of proteases are released by aging bacterial colonic cultures (Gibson and Macfarlane, 1988). It is important to note that the enzymatic activity in the colonic preparations may be due mostly to peptidases of bacterial origin and, to a lesser extent, residual pancreatic enzyme activity reaching the colon (Macfarlane et al., 1986).

Degradation of insulin B-chain (Fig. 2) followed the same pattern. The rate of degradation, however, was faster in all cases due to the less organized structure of insulin B-chain in solution when compared to insulin. However, these conclusions are only semiquantitative, since in vivo, the interaction of proteins with the BBM enzymes will be hindered by the diffusion barrier of the mucus and the unstirred water layer (Thompson and Dietschy, 1984). Nevertheless, these values clearly indicate the potential of the respective compartments to degrade proteins.

The degradation potential of the small intestine and colon for insulin and insulin B chain. is shown in Table 3. First, the specific proteolytic activity was calculated (nmol of insulin degraded/min per unit of enzyme concentration, expressed in mg of protein content in enzyme preparation). This specific activity was calculated

TABLE 3

Total peptidase capacity of BBM and luminal enzymes for the degradation of insulin and insulin B chain [a]

Substrate	Degradation capacity (nmol degraded/min)							
	Small intestine				Colon			
	BBM	Sup [b]	Pel [c]	Total	BBM	Sup	Pel	Total
Insulin	55	1200	550	1800	no degr.	69	336	405
Insulin B chain	1300	37800	16200	55400	2	510	3900	4400

[a] Calculated as specific activity multiplied by total enzyme content in given compartment. Specific activity (nmol protein degraded/min per mg of protein content in enzyme preparation) was determined from the intial time interval when a linear dependence of % degradation vs time was observed.

[b] Supernatant of luminal content.

[c] Pellet of luminal content.

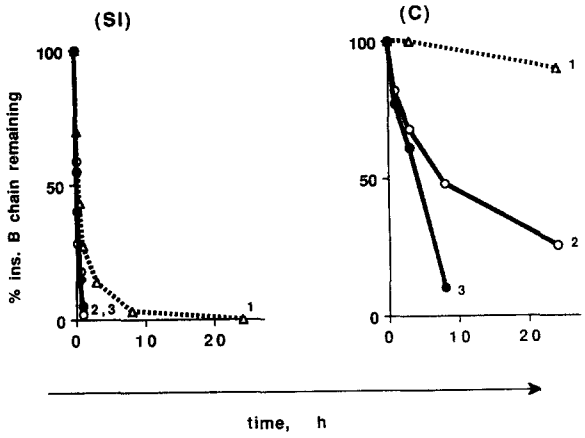

Fig. 2. Degradation of insulin B chain by intestinal enzymes. (SI) Small intestine; (C) colon. (1) BBM enzymes; (2) supernatant of luminal contents; (3) pellet of luminal contents. Insulin B chain (1.7 mM) was incubated at pH 7.4 with BBM enzymes in Tris buffer and luminal enzymes in phosphate buffer. The results are an average from two independent experiments.

for the time interval when almost linear dependence of % degradation vs time was obtained (0–30%). By multiplying the former with the total amount of enzyme expressed in mg of protein, the values of total peptidase activity were obtained. It appears that the difference in the rate of degradation between SI and colon is much higher for the unfolded insulin B-chain when compared with the folded insulin molecule.

Half-life determination of proteins incubated with BBM and luminal enzymes isolated from the SI and colon (Table 4) also correlated with the lower proteolytic activity in the colonic region of the gastrointestinal tract. Chemical modification of albumin to azoalbumin rendered the latter more susceptible to degradation. Apparently, the changes in the conformation of the protein molecule induced by chemical modification facilitated enzymatic cleavage by making the peptide bonds more accessible to proteases (Creighton, 1984). The results of decreased enzymatic activity in the colon are in the agreement with studies performed by Gibson et al. (1989) on the cleavage of azoalbumin with human feces and by Kopečková et al. (1992) using synthetic macromolecular substrates.

To estimate the participation of individual enzymes in the cleavage of insulin, degradation experiments in the presence of inhibitors were performed (Table 5). Phosphoramidon (an inhibitor of BBM endopeptidase-24.11 (Danielson et al., 1980)), amastatin (an inhibitor of aminopeptidases (Rich et al., 1984)), EDTA (an inhibitor of metalloproteinases) and soybean trypsin inhibitor

TABLE 5

Degradation of insulin by small intestinal BBM and luminal enzymes in the presence of inhibitors

Inhibitor	% inhibition after 8 h incubation	
	BBM	Luminal [a]
Phosphoramidon (10 μM)	22	2
EDTA (1 mM)	83	0
Amastatin (10 μM)	92	38
Soybean trypsin inhibitor (1 μM)	0	71

[a] Supernatant of luminal content.

TABLE 4

Half-life of protein degradation

Protein	$t_{0.5}$					
	Small intestine			Colon		
	BBM	Sup [a]	Pel [b]	BBM	Sup	Pel
Insulin	> 50 h	7 h	12 h	n.c.	> 100 h	17 h
Insulin B chain	25 min	9 min	14 min	> 100 h	7 h	3 h
Albumin	> 80 h	24 h	75 h	n.d.	n.c.	n.c.
Azoalbumin	5 h	20 min	29 min	n.d.	> 70 h	13 h

[a] Supernatant of luminal content.
[b] Pellet of luminal content. n.d., not determined; n.c., not cleaved.

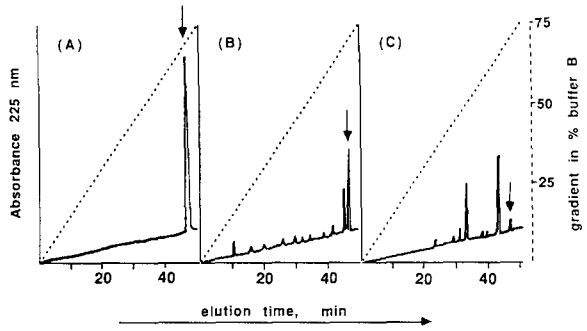

Fig. 3. Reverse phase chromatography of degradation mixtures of insulin B chain (1.7 mM) after 30 min incubation with SI luminal and BBM enzymes. (A) Original insulin B chain; (B) degradation by BBM enzymes; (C) degradation by supernatant of luminal enzymes. A 50 μg aliquot of sample was applied on the Pep RPC (C_2/C_{18}) column, and separation was performed using a linear gradient 0–75% B over 50 min with buffer A: 0.3% TFA/H_2O and buffer B: 0.3% TFA/CH_3OH; Arrow denotes insulin B chain peak position.

(an inhibitor of serine proteinases) were used. The results of the inhibition of the activity of BBM enzymes indicated the participation of metalloproteinases and aminopeptidases in the cleavage of insulin. Only a partial inhibition of BBM activity by phoshoramidon indicates the participation of peptidases other than endopeptidase-24.11. The inactivity of soybean trypsin inhibitor was expected; this indicated the absence of luminal enzymes in the BBM enzymes preparation used. The results on the effect of inhibitors on insulin degradation by SI luminal supernatant (Table 5) indicated the importance of chymotrypsin and trypsin, as well as the minor participation of aminopeptidases in the cleavage process.

A typical chromatogram of the separation of degradation mixture of insulin B-chain (Fig. 3) with SI enzymes demonstrates the sensitivity of the method. The activity and specificity of luminal content enzymes for insulin and insulin B chain cleavage were compared to degradation patterns obtained for chymotrypsin, trypsin and elastase. It is known that the composition and concentration of individual fragments during degradation of insulin with chymotrypsin and trypsin is continuously changing as degradation proceeds (Shilling and Mitra, 1991). We have analyzed the degradation mixtures when approximately half of the original protein was cleaved. Concentration of enzymes were chosen to achieve mutually comparable incubation times. In the digestion of insulin with SI luminal contents (Fig. 4), fragments corresponding to the activity of chymotrypsin (peaks eluted at 28, 34, and 50% of buffer B), elastase (peaks at 16, 28, 46, and 50% of buffer B), and trypsin (20, 42% B) were detected. All these fragment appeared in the digest in comparable amounts, in spite of the fact that a 10-fold concentration of elastase (when compared to chymotrypsin and trypsin) had to be used to achieve comparable rates of degradation with individual enzymes. On the other hand, degrada-

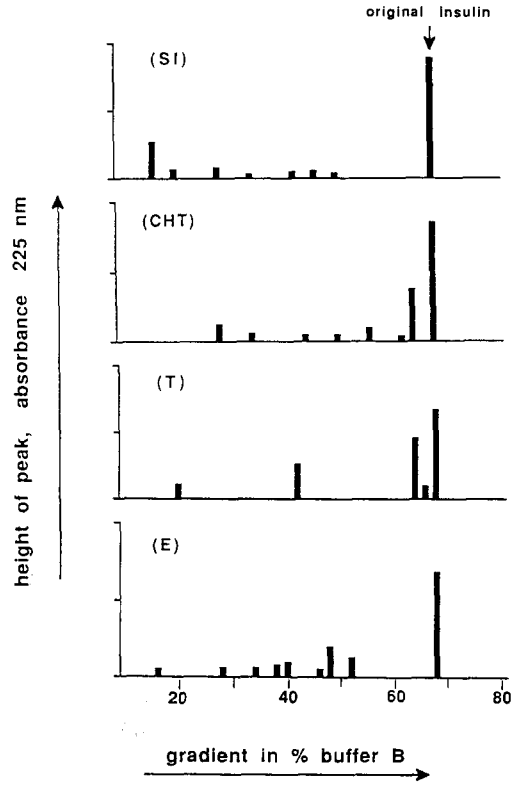

Fig. 4. Degradation patterns of insulin (0.83 mM) cleaved by supernatant of small intestinal content (SI), chymotrypsin (CHT), trypsin (T) and elastase (E), in phosphate buffer pH 7.4. Separation of degradation products was performed as described in the legend to Fig. 3. Incubation times and percentages of insulin degraded: (SI) 10 h and 46%; (CHT) 2 h and 49%; (T) 3.5 h and 60%; (E) 3 h and 59%.

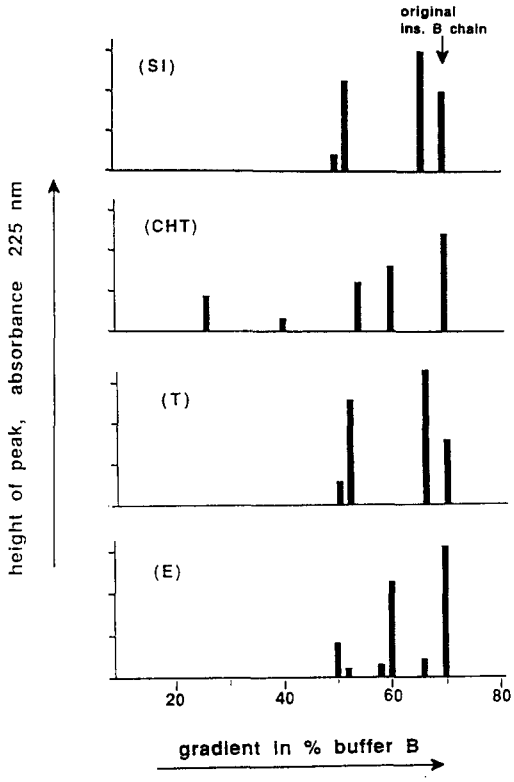

Fig. 5. Degradation patterns of insulin B chain (1.7 mM) cleaved by supernatant of small intestinal (SI) contents, chymotrypsin (CHT), trypsin (T) and elastase (E). For separation conditions see Fig. 3. Incubation times and percentages of insulin B chain degraded: (SI) 15 min and 71%; (CHT) 10 min and 61%; (T) 10 min and 83%; (E) 10 min and 56%.

tion products of insulin B chain cleavage with SI luminal enzymes only contained fragments which were also detected during trypsin cleavage of insulin B chain (Fig. 5). This correlates with the high susceptibility of insulin B chain to trypsin degradation.

The results presented here indicate that the cleavage of a particular peptide/protein in the gastrointestinal tract of guinea pigs is highly dependent on protein structure and location along the GI tract. It appears that the proteolytic activity in the colon is lower when compared to SI. The results obtained support the hypothesis that colon specific protein delivery systems may overcome the enzymatic barrier of the GI tract.

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