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Pancreatic secretory trypsin inhibitor in gastrointestinal mucosa and gastric juice

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Abstract

We studied the distribution of pancreatic secretory trypsin inhibitor (PSTI) in the epithelia of the gastrointestinal tract and determined whether PSTI is secreted into gastric juice. PSTI was measured by a specific radioimmunoassay in biopsy specimens taken from the upper (n=8) and lower (n=7) gastrointestinal tract of patients with normal endoscopies. PSTI was present in the stomach, small intestine, and colon. Concentrations (μg/g protein) were highest in the stomach, and significantly higher in the antrum (1240, 670–1700, median and range) than in the gastric body (370, 350–570) ($p<0.01$). Concentrations were similar in the duodenum (180, 80–210) and colon (160, 130–360). PSTI determined by immunohistochemistry was present in mucus secreting gastric foveolar cells, duodenal Paneth cells, and colonic non mucus cells. PSTI was present in gastric juice. The median (range) concentration of PSTI in basal gastric juice from 13 patients with duodenal ulcers was 9 (3–21) μg/l and did not change during stimulation with pentagastrin. The rate of secretion, however, did increase significantly ($p<0.05$) from 1430 (180–2810) ng/h to 4500 (1250–12 770) ng/h during pentagastrin stimulation. PSTI was labile in acid pepsin but stable in the neutral conditions present in the mucus layer. The presence of pancreatic secretory trypsin inhibitor throughout the gut and its secretion into the lumen suggests a hitherto unrecognised mechanism protecting gastrointestinal epithelia against luminal proteases.

Pancreatic secretory trypsin inhibitor (PSTI) is a small protein containing 56 amino acid residues which was originally isolated from bovine pancreas,[1] and human PSTI has now been purified[2-4] and cloned.[5] PSTI is thought to protect the pancreas from prematurely activated proteases but the recent demonstration of PSTI-like immunoreactivity (PSTI-LI) in other regions of the gut[6-8] and its isolation from the human stomach,[9] suggests that PSTI may protect the whole gastrointestinal tract.

The present study was undertaken to determine epithelial concentrations of PSTI using biopsy specimens obtained from the stomach, small intestine, and colon and to study the cellular distribution of PSTI-LI. We also analysed gastric juice to determine whether PSTI is secreted into the lumen.

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Methods

The local ethics committee approved the protocol and all patients gave informed consent.

All chemicals were purchased from BDH (Poole, Dorset) unless otherwise stated.

PURIFICATION AND RADIOIMMUNOASSAY OF PSTI

Pancreatic juice from postoperative pancreatic drains was stored at −20°C until extraction. Purification of PSTI was based on the method of Iwai *et al.*[10] Briefly, pooled juice was mixed with an equal volume of 0·1 M sodium citrate, and the pH adjusted to 2·5. Sodium chloride was then added to a final concentration of 1 M and the mixture maintained at 80°C for 40 minutes, centrifuged at 3500 *g* for 45 minutes at 4°C, and the supernatant concentrated on a C-18 Sep-Pak cartridge (Waters Associates, Milford MA) equilibrated with 0·05% v/v trifluoroacetic acid in water. The cartridge was then eluted with 80% acetonitrile in 0·05% trifluoroacetic acid, and the eluent lyophilised. The eluent was reconstituted in 0·05 M sodium bicarbonate and applied to a 1·5×100 cm column packed with Sephadex G-50 superfine (Pharmacia, Uppsala, Sweden) and eluted with the same buffer. Fractions containing trypsin inhibitor activity were pooled, lyophilised, and further purified by reverse phase high pressure liquid chromatography on a 10×100 mm Dynamax C-8 column (12 μm, 300 Å, Rainin, Woburn MA), eluted with a gradient of 16–30% acetonitrile in 0·1% trifluoroacetic acid. PSTI eluted in a number of fractions as several poorly resolved peaks and a mixture of these fractions was used for immunisation of rabbits. A sample was also applied to a Mono S column (Pharmacia) equilibrated with ammonium acetate 0·1 M, pH 3·5 and eluted with a gradient of ammonium acetate 0·1 M, pH 3·5–4·5 (Fig 1). When the three peaks of trypsin inhibitor that eluted from the Mono S column were rerun on a 4·6×250 mm Dynamax C-8 reverse phase high pressure liquid chromatography column (12 μm, 150 Å) eluted with a gradient from 22–30% acetonitrile in 0·1% trifluoroacetic acid, peaks I and III emerged as single peaks whereas peak II separated into 2 peaks – II_1 and II_2. The molecular masses of the four peaks were determined by a ZAB-SE mass spectrometer (VG Instruments, Altrincham, Cheshire) and the amino acid sequence of peak 1 was analysed by a protein sequencer (Model 470, Applied Biosystems, Foster City, California).

Four New Zealand white rabbits were immunised initially with 60 μg PSTI in 0·5 ml Freund's complete adjuvant (Sigma, Gillingham, Dorset) and subsequently boosted with 30 μg PSTI in 0·5 ml Freund's incomplete adjuvant at four weekly intervals. One produced antiserum T4.

Human PSTI (peak III) was radioiodinated

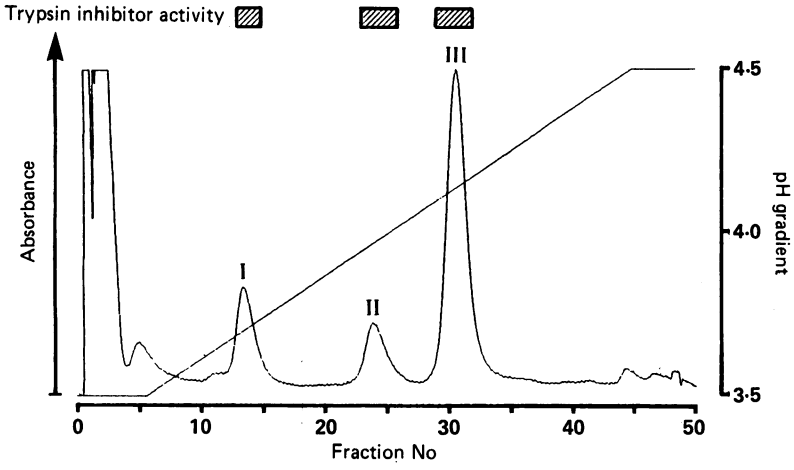

Figure 1: Elution profile of pancreatic secretory trypsin inhibitor (PSTI) extracted from human pancreatic juice from a Pharmacia Mono S column, equilibrated with ammonium acetate (0·1 M, pH 3·5) and eluted with a gradient run from pH 3·5 to 4·5. Elution of the PSTI from the column was observed as optical density at 280 nm and trypsin inhibitor activity.

with ^{125}I by the chloramine T method[11] and tracer, 1500 cpm/tube, was incubated with antiserum T4 (final dilution 1:500 000) together with PSTI standards (0·01–50 ng/ml) or samples in 1 ml of sodium phosphate buffer (0·5 M, pH 7·3) containing 0·15% bovine serum albumin (Sigma) and 0·02% sodium azide. Incubation was at 4°C for 3 days and separation was achieved by adding to each tube at 4°C, 100 µl of ethylenediamine tetra-acetate (EDTA) (0·1 M, pH 7·3), 100 µl of 2% rabbit serum in assay buffer, 100 µl of second antibody (goat antirabbit antiserum, type R 0881, Sigma) diluted 1:5 in assay buffer, and 700 µl of 6% polyethylene glycol 6000 in albumin free assay buffer. The tubes were mixed and incubated at 4°C for 40 minutes before being centrifuged at 3500 *g* and 4°C for 15 minutes. The supernatant was aspirated into separate tubes and both tubes counted.

COLLECTION AND EXTRACTION OF ENDOSCOPIC BIOPSY SPECIMENS

Upper gastrointestinal biopsy specimens were collected during routine endoscopy, using FB25K forceps (Keymed, Southend-on-Sea, Essex), from eight patients, three men and five women, mean age 46 years (range 32–61 years). These patients were under investigation for dyspepsia, but endoscopy and other investigations proved normal and the final diagnosis was non-ulcer dyspepsia. Paired endoscopic biopsy specimens were normal on histological examination. No patients took any drugs within two days of the examination. The mean (SD) weight of the biopsy specimens was 6·8 (2·0) mg.

Lower gastrointestinal biopsy specimens were collected during routine endoscopy, using FG15L forceps (Keymed), from seven patients, four men and three women, mean age 47 years (range 26–65 years). These patients were under investigation for abdominal pain or disturbance of bowel habit, but endoscopy and other investigations showed no abnormality and the final diagnosis was the irritable bowel syndrome. Paired endoscopic biopsy specimens were normal on histological examination. None of the patients took any drugs during the two days before examination except for a bowel preparation, which comprised a low residue diet, two sachets of Picolax (Ferring, Feltham, Middx), and plentiful fluids. The mean (SD) weight of biopsy specimens was 10·6 (3·0) mg.

Biopsy specimens were immediately frozen in liquid nitrogen where they remained until extraction. They were extracted on ice by homogenisation in 200 µl of Tris buffer (10 mM, pH 7·3) for 1 minute. Extracts were centrifuged at 15 850 *g* for 1 minute and supernatants frozen on solid CO_2 and stored at −20°C before radioimmunoassay for PSTI and measurement of protein concentration by a modification of Lowry's method.[12]

GASTRIC JUICE

Gastric juice was collected during routine pentagastrin tests on 18 patients, 13 men and five women, in whom duodenal ulcers had been seen at endoscopy within seven days of study. Their mean age was 49 years (range 25–77 years). None took any drugs in the two days before the study.

Gastric juice was collected from the last of three 10 minute basal collections and after stimulation with pentagastrin 0·6 µg/kg per hour for at least 80 minutes. Juice (2 ml) was collected directly from the aspiration tube, and immediately neutralised by mixing with 3 ml 0·17 M sodium bicarbonate on ice. Samples were then frozen at −20°C before assay.

Gastric juice samples were analysed for bilirubin with a RA-1000 analyser (Technical Instrument Corporation), using Technicon method number SM-0179887. Tryptic activity was determined by the pH stat method using Nα-p-tosyl-L-arginine methyl ester (Sigma) as substrate.[13]

CHROMATOGRAPHY OF GASTROINTESTINAL PSTI

PSTI-LI in gastric juice and extracts of biopsy specimens taken from the colon and gastric antrum were analysed by reversed phase high pressure liquid chromatography on a 4·6× 250 mm, C-8 Dynamax column (12 µm, 150 Å, Rainin), eluted with a gradient of 16–30% acetonitrile in 0·1% triflouroacetic acid. Eluates were lyophilised before radioimmunoassay. The system had been previously calibrated with pancreatic PSTI.

STUDIES OF THE STABILITY OF PSTI IN GASTRIC JUICE

Pentagastrin stimulated gastric juice was obtained from two subjects with duodenal ulcers. Tris-HCl was added to a final concentration of 10 mM, to stabilise the pH during the study. Portions (10 ml) of each juice were adjusted to pH 2·0, pH 4·0, pH 6·0, and pH 7·4 by the addition of NaOH. Pure human PSTI was then incubated with each portion at an initial concentration of 60 ng/ml at 37°C. At the times shown in Figure 4, 250 µl samples were removed, immediately neutralised by addition of an equal volume of 0·17 M sodium bicarbonate, frozen on solid CO_2, and stored at −20°C until radioimmunoassay. In control studies PSTI was incubated as already described, but in Tris-HCl buffer at pH 2·0 and 4·0.

The stability of PSTI in unbuffered gastric

juice, pH 1·2, was also tested in the presence and absence of pepstatin (Sigma) 200 μg/ml with incubation for 1 hour at 37°C.

CONCENTRATION OF PSTI IN PANCREATIC JUICE

Pancreatic juice was collected from postoperative pancreatic drains from three patients (one man, two women), two of whom had undergone pancreatic surgery for pancreatic tumours and one of whom had chronic pancreatitis. The juice was frozen and stored at −20°C until the concentration of PSTI was determined by radioimmunoassay.

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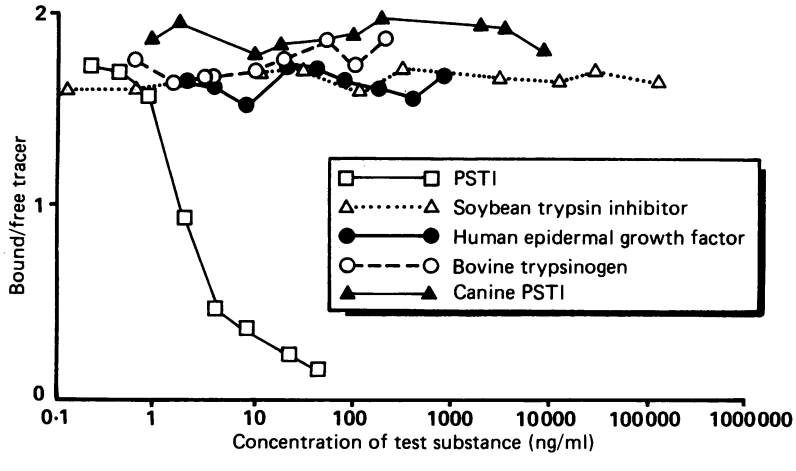
Sections 2 μm thick were cut from samples of normal oesophagus, stomach, and small and large intestine. Immunoperoxidase staining was performed using a routine peroxidase-antiperoxidase procedure.[14] Briefly, sections were dewaxed, rehydrated, and trypsinised at 37°C for 10 minutes to unmask antigenic sites.[15] Endogenous peroxidase was blocked using methanolic hydrogen peroxide for 30 minutes, and the sections rinsed in phosphate buffered saline and incubated with normal swine serum (Dako Ltd, High Wycombe, Bucks) for 15 minutes. They were then incubated with the primary antibody, T4, overnight at 4°C, rinsed, and incubated with swine antirabbit immunoglobulin (Dako) for 30 minutes at room temperature. After rinsing in phosphate buffered saline the sections were incubated with peroxidase-antiperoxidase complex (Dako) for 30 minutes at the recommended dilution and rinsed again. The sections were then developed with 3,3′-diaminobenzidine tetrahydrochloride (Aldrich Ltd, Gillingham, Dorset) for 5 minutes and then counterstained lightly with haematoxylin. Finally, the sections were dehydrated and mounted using pertex (Histolab and Cytolab, Hemel Hempstead, Herts). Negative controls were obtained by substituting normal rabbit serum for the PSTI specific antiserum.

STATISTICAL ANALYSIS

For statistical analysis Wilcoxon's rank sum test was used and results are expressed as median and range; $p<0·05$ was taken to be statistically significant.

Figure 2: The inhibition of binding of pancreatic secretory trypsin inhibitor (PSTI) tracer to antiserum T4 by human PSTI and other substances.



Results

PURIFICATION AND RADIOIMMUNOASSAY OF PSTI

The molecular masses of the four peaks, as determined by mass spectrometry, were I: 6242·5, II_1: 6241·8, II_2: 6241·6, and III: 6242·5, compared with the predicted molecular mass for protonated PSTI of 6242·1. Amino acid sequence analysis of peak I showed that the N-terminal tridecapeptide sequence of peak I was equal to that of human PSTI.

The interassay and intra-assay variabilities of the radioimmunoassay were 17% and 8% respectively. The detection limit of the assay was 0·05 ng/tube. The binding of tracer to antibody was not inhibited by bovine trypsinogen (Sigma), human epidermal growth factor (donated by H Gregory), soybean trypsin inhibitor (Sigma), or canine PSTI (purified by author) (Fig 2). The ratios of cross reactivity of the different forms to peak III were I: 0·70:1, II: 0·86:1.

CONCENTRATIONS OF PSTI-LI IN ENDOSCOPIC BIOPSY SPECIMENS

The concentrations of PSTI-LI in biopsy specimens taken from different regions of the human gastrointestinal tract, expressed as μg/g wet weight and μg/g protein in extracts, are shown in Table I. PSTI-LI was undetectable in specimens from the oesophagus, but the stomach contained the most PSTI-LI, the concentration being significantly greater in the antrum than in the body of the stomach ($p<0·01$). Concentrations of PSTI-LI were similar in the duodenum and colon. There was no significant difference between mucosal concentrations of PSTI-LI in the first and second parts of the duodenum, or between the regions of the colon. The median (range) concentration for each patient was 180 (80–210) μg/g protein in the duodenum and 160 (130–360) μg/g protein in the colon.

SECRETION AND STABILITY OF PSTI-LI IN GASTRIC JUICE

Trypsin was not detected in any sample of gastric juice. One sample which contained bilirubin was excluded from analysis. PSTI-LI was detected in gastric juice from all patients. The concentration

TABLE I *Tissue concentrations of pancreatic secretory trypsin inhibitor-like immunoreactivity in biopsy specimens taken from the upper (n=8) and lower (n=7) gastrointestinal tract*

	PSTI-LI/wet weight (μg/g)		*PSTI-LI/protein (μg/g)*	
Region	*Median*	*Range*	*Median*	*Range*
Oesophagus	<0·1	All <0·1		
Stomach:				
Body	19·9	11·0–40·2	370	350–570
Antrum	38·5	17·8–73·3	1240*	670–1700
Duodenum:				
First part	10·2	7·3–12·7	190	110–280
Second part	8·7	4·5–14·5	170	70–210
Colon:				
Ascending	12·9	7·1–13·3	230	150–240
Transverse	7·7	6·5–15·7	150	90–480
Descending	7·7	5·7–13·2	150	70–360
Sigmoid	9·7	7·1–15·5	210	110–430
Rectum	10·7	6·7–12·2	170	130–290

Statistical analysis compares tissue concentrations between the antrum and the body of the stomach. *p<0·01.

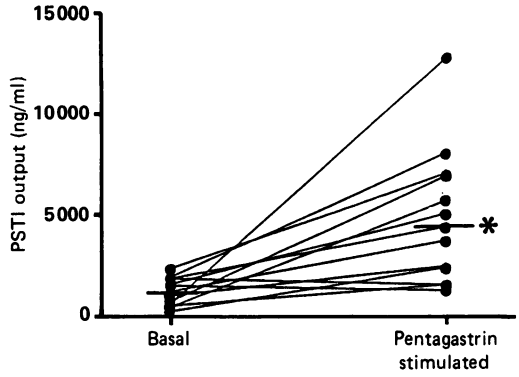


*Figure 3: Rates of gastric secretion of pancreatic secretory trypsin inhibitor (PSTI) before and after stimulation with pentagastrin. The horizontal lines indicate the medians. *p<0·05.*

TABLE II *Concentrations and secretion rates of pancreatic secretory trypsin inhibitor (n=13) in basal and pentagastrin stimulated gastric juice*

Concentration of PSTI-LI (μg/l):	*Median*	*Range*
Basal	9	3–21
Stimulated	12	3–21
Output of PSTI-LI (ng/h):		
Basal	1430	180–2810
Stimulated	4500*	1250–12770

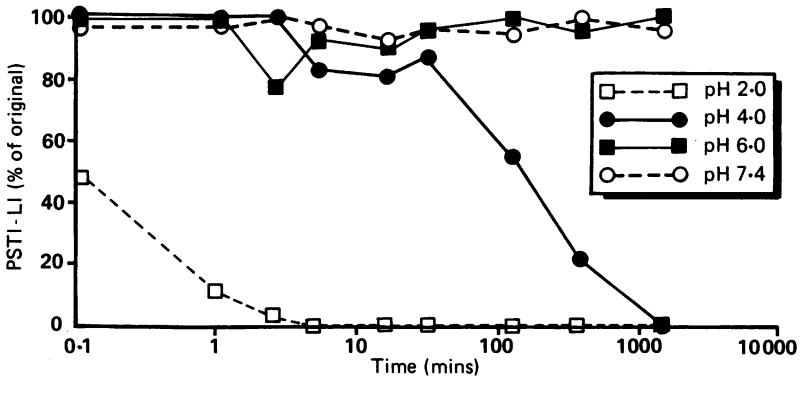
* p<0·05 *v* basal.

of PSTI-LI in basal juice was not significantly changed during stimulation with pentagastrin (Table II). Because of the increase in the volume of juice secreted, however, the output of PSTI-LI rose significantly (p<0·05) after stimulation with pentagastrin (Fig 3). PSTI-LI was labile in acidic gastric juice (Fig 4). The disappearance half times were less than 1 minute at pH 2·0, but about 2 hours at pH 4. There was no detectable loss of PSTI in gastric juice at pH 6·0 and 7·4 in 24 hours. There was no detectable loss of PSTI-LI after incubation in Tris buffer at pH 2·0 and 4·0 in the absence of gastric juice for 24 hours. After incubation of PSTI in unbuffered gastric juice, pH 1·4 for 1 hour in the presence of pepstatin, 81% of original immunoreactivity remained. No PSTI-LI was detectable in the control tube without pepstatin.

PSTI IN PANCREATIC JUICE

The median (range) concentration of PSTI in pancreatic juice, as measured by radioimmunoassay, was 12·4 (8·8–16·0) mg/l.

Figure 4: Effect of pH on the stability of pancreatic secretory trypsin inhibitor-like immunoreactivity (PSTI-LI) in gastric juice. The results are a mean of two experiments.



CHROMATOGRAPHY OF GASTROINTESTINAL PSTI-LI

PSTI-LI in gastric juice, gastric mucosa, and colonic mucosa eluted from reversed phase high performance liquid chromatography in the characteristic position of pure pancreatic PSTI (Fig 5).

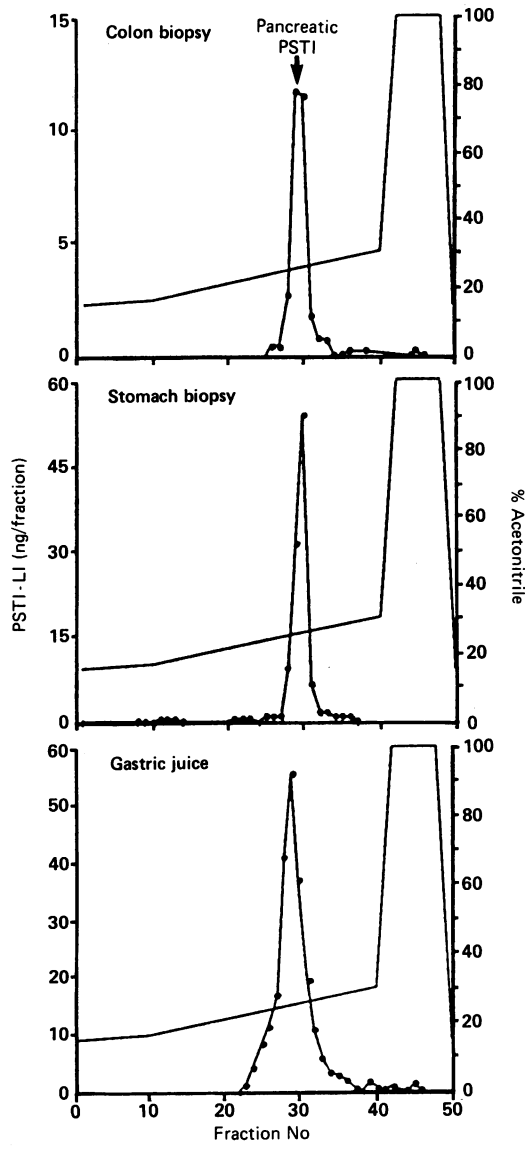


Figure 5: Elution of pancreatic secretory trypsin inhibitor-like immunoreactivity (PSTI-LI) from extracts of colon and gastric antrum and gastric juice from reverse phase high performance liquid chromatography (Dynamax, C-8, 4·6× 250 mm, 12 μm, 150 Å). The arrow indicates the elution position of pure PSTI.

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Cells containing PSTI-LI were seen in the stomach, duodenum, and colon but not in the oesophagus. In gastric mucosa from both the body and antrum, PSTI-LI was observed in the foveolar cells lining the gastric pits (Fig 6A) but was absent in the superficial epithelial cells. In the duodenum intense PSTI-LI was observed in the Paneth cells (Fig 6B) but was absent in other cell types. In colonic mucosa the non mucus secreting cells of the colonic crypts were positive (Fig 6C) but the goblet cells were negative.

Discussion

In this study we determined the concentrations of PSTI-LI for the first time in fresh tissue obtained from the gastrointestinal tract at endoscopy. PSTI-LI was present in the stomach,

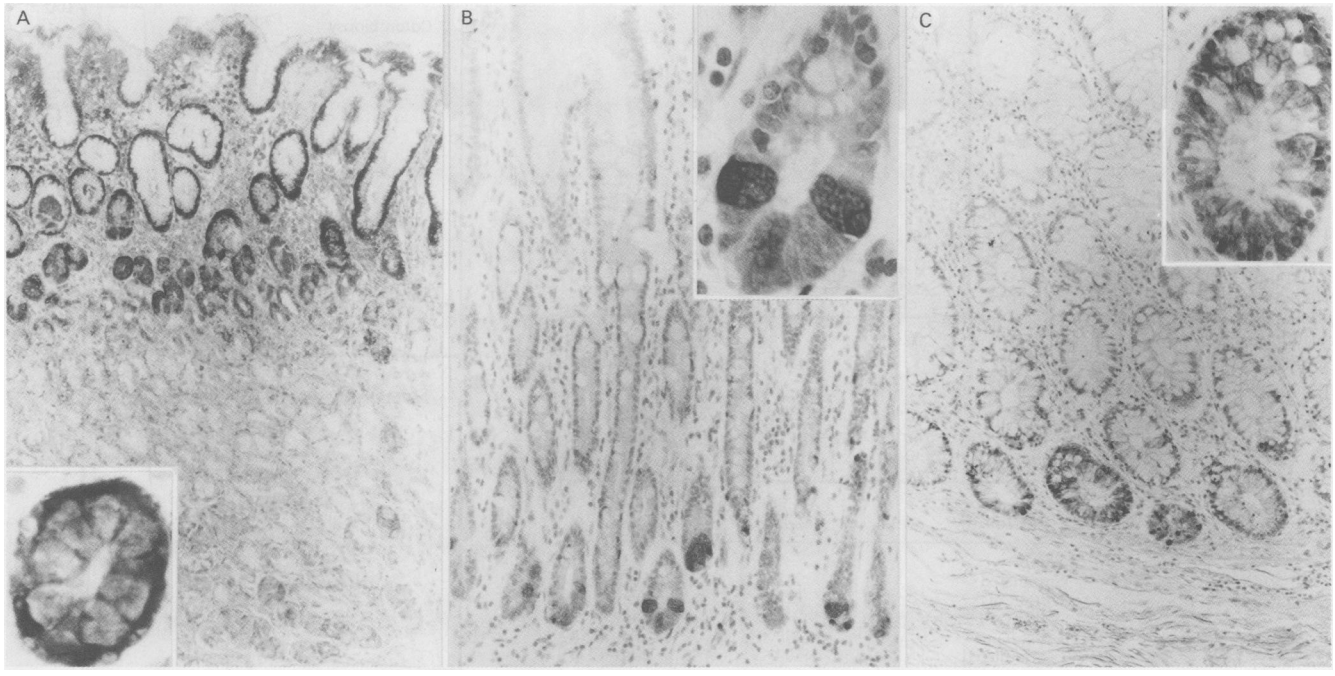

Figure 6: Paraffin sections of gastrointestinal mucosa stained with an indirect immunoperoxidase method using an anti-PSTI antibody, (A) normal gastric mucosa (original magnification ×31·5, inset ×236) showing foveolar cell positivity in gastric pits; (B) normal duodenal mucosa (original magnification ×50, inset ×193) showing Paneth cell positivity; (C) normal colonic mucosa (original magnification ×31·5, inset ×96) showing non mucus cell positivity in colonic crypts.

duodenum, and colon but undetectable in the oesophagus. Tissue concentrations were greatest in the gastric antrum. PSTI-LI was found to be in a specific cell type in each region of the gut. We have shown for the first time that PSTI is secreted into the lumen of the stomach. PSTI is rapidly destroyed by acid pepsin but stable at the neutral pH found in the gastric mucus layer.

In a previous study Shibata *et al* measured PSTI-LI in cadaveric small intestine and surgically resected stomach,[6] and found concentrations over an order of magnitude lower than in the present study. The lower concentrations that they reported may have been due to loss of PSTI through hydrolysis by mucosal enzymes before extraction and a higher proportion of submucosal tissues in samples.

The cellular distribution of gastrointestinal PSTI-LI reported in the present study is consistent with the main findings of two other groups.[7,8] The weak PSTI-LI seen by others, however, in the goblet cells of the colon and in other gastric cell types, including chief cells, was not observed in the present study.

We are not aware of any previous report of the secretion of PSTI into gastric juice. The concentrations of PSTI-LI in gastric juice did not rise significantly during the infusion of pentagastrin, although there was a significant increase in the output of gastric PSTI. Pentagastrin has been reported to increase gastric secretion of carbohydrate from the same cell type in the cat.[16]

In the present study PSTI was shown to be rapidly destroyed by pepsin in gastric juice at acid pH, but stable if the juice was neutralised. PSTI probably exerts its protective effect in the gastric mucus layer which is kept at a neutral pH due to gastric secretion of bicarbonate.[17] Hydrolysis of PSTI by pepsin is probably responsible for the rapid loss of PSTI-LI in acidic extracts of gastric mucosa that was reported by Shibata *et al*.[6]

The results of the present analytical studies are consistent with the results of others who have shown that pancreatic PSTI exists in multiple forms.[2,3] These differ chiefly in the degree of deamination of the asparagine residues which are unusually abundant in PSTI. Fraction II_1 and II_2 had the predicted mass of the molecule whereas fractions I and III had a molecular mass consistent with monodeaminated PSTI. In addition, Kikuchi *et al* found a form of PSTI in pancreatic juice which had five amino acids missing from the N-terminal, which was not found here.[2]

The presence of PSTI in all regions of the gut, and its secretion into the lumen, suggest that PSTI may protect the whole gut from proteolytic enzymes. Gastric PSTI is presumably important during episodes of duodenogastric reflux which occur in health,[18] occur more frequently in some diseases such as gastric ulcer,[19] and occur more or less constantly after some forms of gastric surgery.[19] Reflux of duodenal juice may raise intragastric pH to levels at which pancreatic enzymes are active but PSTI is stable. Concentrations of PSTI measured in pancreatic juice in the present study were similar to those reported by others[3] and approximately 1000 times higher than concentrations found in gastric juice. Concentrations of PSTI in the gastric mucus-bicarbonate layer, however, may be considerably higher than those found in the lumen of the stomach. In addition, the concentration of trypsin entering the mucus layer may be diminished by dilution and by peptic destruction of trypsin in the lumen of the stomach.[20] It is interesting that gastric mucosal PSTI is most abundant in the antrum, which is most exposed to refluxed enzymes. Colonic PSTI may protect the colonic epithelium from pancreatic enzymes which remain active in colonic contents.[21] PSTI also inhibits elastase and chymotrypsin[4] as well as trypsin.

Recent work has shown that PSTI is a growth

factor as well as a protease inhibitor. We showed that human PSTI stimulates growth of AR4-2J cells derived from a rat pancreatic acinar cell tumour.[22] Others have shown that human PSTI stimulates growth of human fibroblasts[23] and human endothelial cells.[24] The growth stimulating effect of PSTI may be a consequence of its sequence homology with epidermal growth factor.[25] Raised intragastric PSTI concentrations could contribute to trophic effects seen in the prolonged absence of gastric acid.[26]

Gastrointestinal PSTI may provide an important and hitherto unrecognised protective mechanism. Further studies are required to determine the factors which control the secretion of gastrointestinal PSTI and its possible role in the control of gastrointestinal growth.

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