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Pancreatic secretory trypsin inhibitor in gastrointestinal mucosa and gastric juice

T C Freeman, R J Playford, C Quinn, K Beardshall, L Poulter, J Young, J Calam

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 immunohistochimistry was present in mucus secreting gastric foveolar cells, duodenal Paneth cells, and colonic non mucous 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 purified4 and cloned.3 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 gut4 and its isolation from the human stomach,3 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.

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. 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, Upsalsa, 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 run 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 II emerged as single peaks whereas peak II separated into 2 peaks – II, and II'. 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 I 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...
with $^{125}$I by the chloramine T method$^{11}$ and tracer, 1500 cpm/tube, was incubated with antisera 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 a second antibody (goat antirabbit antiserum, type R 0881, Sigma) diluted 1:5 in assay buffer, and 700 μl of 5% 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.

**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 biliarubin with a RA-1000 analyser (Technical Instrument Corporation), using Technicon method number SM-0179887. Trypsin activity was determined by the pH stat method using Nop-tosyl-L-arginine methyl ester (Sigma) as substrate.$^{11}$

**CHROMATOGRAPHY OF GASTROINTESTINAL PSTI**

PSTI-L1 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% trifluoroacetic 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 CO₂, 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 peptatin (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.

IMMUNOHISTOCHEMISTRY
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. Briefly, sections were dewaxed, rehydrated, and trypsinised at 37°C for 10 minutes to unmask antigenic sites. 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 pertx (Histolab, 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.

RESULTS
PURIFICATION AND RADIOIMMUNOASSAY OF PSTI
The molecular masses of the four peaks, as determined by mass spectrometry, were I: 6242.5, II: 6241.8, III: 6241.6, and III: 6242.5, compared with the predicted molecular mass for protonated PSTI of 6241.4. 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>
<thead>
<tr>
<th>TABLE 1</th>
<th>Tissue concentrations of pancreatic secretory trypsin inhibitor-like immunoreactivity in biopsy specimens taken from the upper (n=8) and lower (n=7) gastrointestinal tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td>PSTI-LI/wet weight (μg/g)</td>
</tr>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Stomach:</td>
<td>Body</td>
</tr>
<tr>
<td></td>
<td>Antrum</td>
</tr>
<tr>
<td></td>
<td>Duodenum:</td>
</tr>
<tr>
<td></td>
<td>Second part</td>
</tr>
<tr>
<td>Colon:</td>
<td>Ascending</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
</tr>
<tr>
<td></td>
<td>Descending</td>
</tr>
<tr>
<td></td>
<td>Sigmoid</td>
</tr>
<tr>
<td></td>
<td>Rectum</td>
</tr>
</tbody>
</table>

Statistical analysis compares tissue concentrations between the antrum and the body of the stomach. *p<0.01.
Pancreatic secretory trypsin inhibitor in gastrointestinal mucosa and gastric juice

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.

![Figure 3](image.png)

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

<table>
<thead>
<tr>
<th>Concentration of PSTI-LI (ng/l)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>9</td>
<td>3-21</td>
</tr>
<tr>
<td>Stimulated</td>
<td>12</td>
<td>3-21</td>
</tr>
<tr>
<td>Output of PSTI-LI (ng/l): Basal</td>
<td>1430</td>
<td>180-2810</td>
</tr>
<tr>
<td>Stimulated</td>
<td>4500*</td>
<td>1250-12770</td>
</tr>
</tbody>
</table>

*p<0.05 v basal.

The results indicate the medians. *p<0.005.

Figure 4: The 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.

![Figure 4](image.png)

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 x 250 mm, 12 μm, 150 Å). The arrow indicates the elution position of pure PSTI.

![Figure 5](image.png)

The results 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.

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).

Immunohistochemistry

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,
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.

The results of the present analytical studies are consistent with the results of others who have shown that pancreatic PSTI exists in multiple forms.1,2,3 These differ chiefly in the degree of deamination of the asparagine residues which are unusually abundant in PSTI. Fraction II1 and II2 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.1

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,10 occur more frequently in some diseases such as gastric ulcer,11 and occur more or less constantly after some forms of gastric surgery.12 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 others13 and approximately 1000 times higher than concentrations found in gastric juice. Concentrations of PSTI in the gastric mucobicarbonate 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.14 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.15 PSTI also inhibits elastase and chymotrypsin16 as well as trypsin.

Recent work has shown that PSTI is a growth factor affecting cell division and DNA synthesis in gastric and colonic epithelial cells in culture.17,18 PSTI may be a candidate tumour growth factor, although whether it is an autocrine or paracrine growth factor remains to be determined.
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. Others have shown that human PSTI stimulates growth of human fibroblasts and human endothelial cells. The growth stimulating effect of PSTI may be a consequence of its sequence homology with epidermal growth factor. Paired intragastric PSTI concentrations could contribute to trophic effects seen in the prolonged absence of gastric acid.

Gastrointestinal PSTI may provide an important and hitherto unrecognized 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.

We thank the Wellcome Trust for financial support; the Medical Research Council for funding RF as an MRC training fellow; and Dr S Iavi and Sister Francis-Reme and the nursing staff of the gastric clinic for help in the collection of biopsy samples and samples of gastric juice.