Alimentary tract and pancreas

Mucus degradation by pepsin: comparison of mucolytic activity of human pepsin 1 and pepsin 3: implications in peptic ulceration

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SUMMARY The ability to digest mucus, mucolytic activity of isolated pepsins and samples of human gastric juice has been assayed by measuring the fall in viscosity when incubated with purified pig gastric mucus glycoprotein. Pure human pepsin 1, the peptic ulcer associated pepsin, digested gastric mucus glycoprotein at a faster rate than did pure human pepsin 3 (the principal human pepsin), or the equivalent pig pepsin (pepsin A). At pH 2.0 pepsin 1 had twice the mucolytic activity of pepsin 3. Above pH 3.8 this difference became more marked and whereas pepsin 1 caused substantial mucolysis up to and including pH 5.1, pepsin 3 had minimal activity. At pH 4.0 pepsin 1 had six times the mucolytic activity of pepsin 3. Gastric juices from patients with duodenal ulcer each exhibited substantial mucolytic activity between pH 2 to 5, similar to that of pepsin 1. In contrast, gastric juice from non-symptomatic volunteers exhibited little mucolytic activity above pH 4. Analysis of the mucus glycoprotein by gel filtration showed that an increase in lower molecular weight, pepsin degraded, glycoprotein was associated with the fall in mucus viscosity for all enzyme preparations. These results showed that pepsin 1 can digest the mucus more effectively than pepsin 3 and at higher pH values. The raised concentrations of pepsin 1 in the juice of peptic ulcer patients may thus promote the ulcerative process by increased erosion of the mucus barrier under conditions likely to pertain in the duodenal bulb as well as the stomach.

The mucus:bicarbonate barrier is considered to be an important component of the gastroduodenal mucosal defence mechanisms against autodigestion by the gastric juices.¹⁻³ To function as an effective barrier the adherent mucus must be present as a continuous layer, a state maintained by a balance between its secretion from the epithelial cells and its erosion by removal from the surface into the lumen.⁴ Factors which swing this balance in favour of degradation of the adherent mucus might therefore be expected to decrease gastroduodenal mucosal resistance and result in mucosal damage. Agents identified in such adherent mucus erosion *in vivo* are proteolytic degradation and mechanical sloughing. Extensive *in vitro* studies show that luminal pepsins are mucolytic and dissolve adherent mucus gel by breaking down the polymeric structure

of its component glycoproteins to give soluble, degraded mucus in the lumen.⁵⁻⁷ There is evidence for pepsin induced mucus degradation *in vivo*, thus after insulin stimulation of duodenal ulcer patients there is a concomitant rise in the gastric juice of both pepsin and lower molecular weight (degraded) glycoprotein and this increase of both components was absent in vagotomised patients.⁸ Proteolytic degradation of mucus in dog duodenal juice has been shown to occur over a wide range (pH 2.4-pH 8).⁹

The presence, in human gastric juice, of seven pepsins and one non-pepsin proteinase was first described in 1967.¹⁰ The gastric juice of patients with chronic gastric or duodenal ulceration has long been known to have a different pattern of protein hydrolysis relative to non-ulcer controls¹¹ and this is associated with a different composition of juice pepsins in these patients in that pepsin 1 is raised.¹² Pepsin 3 is the major pepsin in man and pepsin 1 accounts for 3-6% of total pepsin activity in non-ulcer controls, but in gastric and duodenal ulcer

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patients pepsin 1 accounts for 23% and 16.5% respectively of the total pepsin activity present.^{13 14} Pepsin 1 which is secreted by the fundic but not by the pyloric glands of the stomach¹⁵ has been shown to have a potent collagenolytic action, being five times more active in the digestion of collagen than pepsin 3.¹⁶ This observation may explain the faulty deposition of collagen and the chronic reaction seen histologically at the ulcer site in peptic ulcer patients. Seven pepsinogens the precursors of pepsins, have been identified in gastric mucosa by electrophoretic separation.¹⁷ Two groups pepsinogens PGI and PGII identified immunologically are also present in serum, and PGI concentrations are increased in duodenal ulcer patients compared with controls.¹⁸

Here we report that pepsin 1 has an increased mucolytic activity with respect to pepsin 3, and in particular will degrade gastric mucus at the higher pH values above pH 4.0. The possible implications of this finding for decreased mucosal resistance in peptic ulcer patients and the aetiology of the disease are discussed.

Methods

SUBJECTS

Pepsins 1 and 3 were obtained from human gastric juice by a series of chromatographic procedures using DEAE-cellulose after the method of Roberts and Taylor.¹⁹

Insulin (0.2 units/kg iv) and pentagastrin (6 µg/kg im) stimulated gastric juice was assayed from duodenal ulcer patients undergoing routine gastric secretion tests.²⁰ Pentagastrin (3 µg/kg im) stimulated gastric juice from non-symptomatic volunteers (18–20 years) was also assayed. Gastric juice samples from each individual were analysed separately for H⁺, pepsin and mucolytic activity. The juice samples were collected as follows: basal period (30 min); pentagastrin period (60 min: after pentagastrin im); insulin control period (30 min: after iv insulin); insulin period (30 to 75 min: after iv insulin).

Purified pig gastric mucus glycoprotein was used for mucolytic studies.⁷ Mucus gel scraped from the mucosal surface was solubilised by homogenisation in 0.2 M NaCl containing 0.02% wt/vol sodium azide and the glycoprotein separated from contaminant protein by fractionation using Sepharose 4B gel filtration followed by equilibrium centrifugation in a CsCl density gradient.

Mucolytic activity of the isolated pepsins and gastric juice samples was assessed by measuring the fall in specific viscosity ($\eta_{sp} = \eta_{rel} - 1$) when incubated with 5 mg/ml solution of purified mucus

glycoprotein over 30 minutes at 37°C. The mucus solution was buffered, over pH range 1.2–3.5, by 0.1 M glycine/HCl and over 2.2–7.0, by 0.1 M citrate/sodium phosphate. The pepsins were incubated with the mucus solution as a 0.5% (wt/wt) enzyme protein:glycoprotein, and the fall in viscosity monitored as a function of time at 37°C. The small amount of endogenous mucolytic activity in the mucus samples was corrected for by incubation of the mixture without added pepsin. Viscosity was measured with a Contraves low shear cup and bob viscometer over a range of shear rates. Each viscosity value was calculated from the gradient of a straight line plot of readings taken for at least five different shear rates.

The proteolytic activity of the pepsin samples was assayed by measuring the hydrolysis of haemoglobin at pH 1.9 and estimating the peptides released into solution after precipitation of the undigested protein.²¹ The concentration of all pepsin and juice samples were related to the rate of hydrolysis of haemoglobin by standard pig pepsin (pepsin A). For assaying the proteolytic activity of pepsin as a function of pH the buffers were the same as for the mucus solutions above.

Fractionation of the isolated mucus glycoprotein into native and proteolytically degraded glycoprotein was achieved by gel filtration on a column (120×1.5 cm) of Sepharose 2B (Pharmacia Limited) eluted with 0.2 M NaCl; 0.2% sodium azide.⁶ Approximately 1 ml of neutralised, pepsin digested, glycoprotein solution (about 3 mg/ml) was applied to the column and the eluted fractions (3.5 ml) were assayed for glycoprotein by a PAS method.²²

Results

Incubation of gastric mucus glycoprotein with human pepsin 3 at pH 2.5 resulted in a rapid fall in the specific viscosity over the first 30 min followed by a slower fall during the subsequent five hours (Fig. 1). In the control, mucus glycoprotein incubated at pH 2.2 without pepsin showed a minimal fall in viscosity (about 5% of total viscosity). A similar hyperbolic pattern of falling viscosity was seen when mucus glycoprotein was incubated with human pepsin 1, with gastric juice aspirated from duodenal ulcer patients or with gastric juice from non-symptomatic medical students. For subsequent assays for mucolytic activity the fall in viscosity when samples were incubated for 30 min with gastric mucus was used. This resulted in a substantial drop by about 50% (0.5–1.0 units) in mucus specific viscosity over 30 min, providing a good range for a sensitive assay of mucolytic activity. After mucolytic

digestion the mucus solution still retains some viscosity reflecting the presence of the proteolytically degraded glycoprotein units. The gastric mucus glycoprotein used for this assay was purified by equilibrium centrifugation in a CsCl gradient and had previously been shown by polyacrylamide gel electrophoresis in sodium dodecyl sulphate²³ to be free of contaminant protein which might interfere with the mucolytic assay.

The digestion of gastric mucus by human pepsin 1 and pepsin 3 were compared as a function of pH. At pH 2.2 and for the same proteolytic activity with respect to haemoglobin (25 µg/ml pig pepsin equivalents) pepsin 1 was nearly twice as active mucolytically as pepsin 3 (Fig. 2). At pH 4 the difference in the mucolytic activity was even more striking, with pepsin 1 having six times the mucolytic activity of pepsin 3 which had minimal activity at this pH. Pig pepsin, which is equivalent to human pepsin 3,²⁴ had

Fig. 1 Human pepsin digestion of pig gastric mucus glycoprotein at pH 2.5, 37°C. Pepsin 1 (▲) and Pepsin 3 (○), 0.5% enzyme/glycoprotein (wt/wt). The endogenous activity in the glycoprotein sample is indicated by (■).

Fig. 2 pH Profile of human pepsin 1 and 3 digestion of gastric mucus. Mucolytic activity of enzyme samples was assessed by measuring the percentage fall in specific viscosity (η_{sp}) over 30 min at 37°C. Fall was corrected for any endogenous enzyme activity in the mucus glycoprotein sample (□) Pepsin 3; (■) Pepsin 1.

a similar pH dependence of mucolytic activity to human pepsin 3. A linear relationship was observed between the fall in viscosity of the mucus glycoprotein and increased pepsin concentrations up to an enzyme concentration of 1.5% w/w enzyme/mucus glycoprotein ratio. This is three times the concentration of enzyme to substrate used in the assay of mycolytic activity and showed that the mucus glycoprotein substrate was in excess and was not rate limiting.

Gastric juice aspirated from duodenal ulcer patients after pentagastrin (6 µg/kg units im) and insulin (0.2 units/kg iv) stimulation contained pepsin activity at a concentration equivalent to 0.2–1 mg/ml pig pepsin. When these juice samples were incubated at 37°C with mucus glycoprotein there was substantial mucolytic activity over the pH range 1.5 to 5 (Fig. 3). Thus pentagastrin or insulin stimulated gastric juice from duodenal ulcer patients had a mucolytic activity similar to that of pepsin 1. In contrast pentagastrin stimulated gastric juice aspirated from non-symptomatic volunteers (range 0.2–0.25 mg/mg pepsin activity) showed minimal mucolytic activity above pH 3.8 (Fig. 3). In all mucolytic assays the juice was diluted to give the same pepsin activity of 25 µg/ml (measured using an albumin substrate). The relative mucolytic activity of juice samples over two different pH ranges, a low pH range (pH 1.5–3.8) and high pH range (pH 3.8–6.0) was assessed by measuring the areas under the plot of mucolytic activity versus pH (as in Fig. 3). There was a significant increase in the mucolytic activity above pH 3.8 for pentagastrin stimulated juice from DU patients compared with that from non-symptomatic volunteers ($n=3$ for each group $p<0.005$). Thus 33%, 38% and 28%

Fig. 3 Mucolytic activity of human gastric juice. Duodenal ulcer patient (○) non-symptomatic volunteer (▲) pentagastrin stimulated juice. Insulin stimulated DU patients' juice gave a similar pH profile to the pentagastrin stimulated juice.

Fig. 4 Proteolysis by duodenal ulcer patients gastric juice.

respectively of the total mucolytic activity in the juice samples from DU patients was in the higher pH group (3.8–6.0) compared with only 9%, 12% and 14% for the non-symptomatic controls.

The proteolytic activity in the juice from DU patients, using haemoglobin as substrate, was maximal at pH 2.3 and was also minimal above pH 4 (Fig. 4), this compares with the mucolytic activity maximum at about pH 2.0 (Fig. 3).

The structure of the mucus glycoprotein was investigated before and after proteolysis by gel filtration on Sepharose 2B. The undigested mucus glycoprotein eluted from the Sepharose 2B column predominantly in the excluded volume, evidence that it was the polymeric, gel-forming, non-proteolytically digested form (Fig. 5). After digestion for three hours with either pepsin 1 or pepsin 3 much of the glycoprotein was included on Sepharose 2B, showing that the drop in viscosity had been accompanied by fragmentation of the glycoprotein into lower sized, proteolytically degraded subunits. After further digestion with pepsin for 48 hours,

Fig. 5 Gel filtration on Sepharose 2B of purified pig gastric mucus glycoprotein (○). The glycoprotein before digestion with pepsin, (▲) after 3 hour digestion with pepsin 1, (---) position of completely degraded subunit (48 hours).

resulting in exhaustive proteolysis, the mucus glycoprotein eluted as a single included peak which was less broad than that obtained after only three hours digestion and nearer to the total volume of the column.

Discussion

The first stage in peptic erosion of the mucosa is penetration of the adherent mucus gel barrier. Studies *in vitro*⁵⁻⁷ and *in vivo*⁸ show pepsin will dissolve the mucus gel barrier to produce soluble degraded mucus glycoprotein in the lumen. In the work reported here we have quantitatively assayed *in vitro* the mucolytic potential of pepsins 1 and 3. Results show that pepsin 1 has greater mucolytic activity throughout the pH range 1.5–5 and this is particularly marked above pH 4. There is a similar marked increased mucolytic activity above pH 4 for gastric juice from a group of duodenal ulcer patients compared with juice from a group of non-symptomatic volunteers.

To assay the mucolytic activity of pepsin containing samples we have measured the fall in viscosity when incubated with a solution of purified gastric mucus glycoprotein. To obtain the relatively large amounts of mucus substrate for these mucolytic assays we used pig gastric mucus, which in terms of its gastric mucus secretion is a good animal model for man.^{5,6} Rheological studies on intact mucus gel have shown the same physical structure in human and pig stomach and pig duodenum and all three preparations are solubilised by proteolysis.²⁵ Detailed structural studies on the isolated human and pig gastric mucus glycoproteins have shown their similarity in structure and that mucolysis by pepsin can be explained by fragmentation of the gel forming polymeric glycoprotein into proteolytically degraded peptide units which are soluble.⁵⁻⁷ This mucolysis can be demonstrated by gel filtration on Sepharose 2B because the polymeric glycoprotein is excluded and the proteolytically degraded glycopeptide is included and therefore easily separated.⁶ This method was used here to confirm that concomitant proteolytic cleavage of the glycoprotein was accompanying the drop in viscosity of the mucus when incubated with the purified pepsins and the gastric juice samples (Fig. 5).

Over 30 min incubation there was a substantially greater drop in viscosity of the gastric mucus when incubated with human purified human pepsin 1 compared with purified pepsin 3 (human or pig) for the whole pH range 1.5–5. For the same degree of proteolytic activity (expressed as pig pepsin A equivalents on haemoglobin) pepsin 1 had a higher rate of mucolysis than pepsin 3 at the optimum pH

of about 2.5 (Fig. 2). Most striking, however, were the results for higher pH values (4–5) when pepsin 1 still showed substantial mucolytic activity, in contrast with pepsin 3 which had essentially no such mucolytic activity above pH 4. Gastric juice from duodenal ulcer patients after both pentagastrin and insulin stimulation showed more extensive mucolytic activity between pH 4–5, characteristic of pepsin 1 (Fig. 3). Pentagastrin stimulated gastric juice from non-symptomatic volunteers (age 18–20), however, did not show such increased mucolytic activity above pH 4. Direct comparison of the results from the DU patients and non-symptomatic volunteers is not possible as they were not age matched. What these results do show is that there are substantial changes in mucolytic activity between samples of human gastric juice from these two different groups of patients. Previous studies have shown that pepsin 1 concentrations are raised in gastric juice from DU patients compared with non-symptomatic controls^{12 13} and this correlates with the increased mucolytic activity of the former observed here (Figs 2 and 3). It is reasonable therefore to propose that this increased mucolytic activity of juice from DU patients could be because of increased pepsin 1. An alternative explanation is that the increased mucolytic activity in DU patient juice may be because of neutral proteases arising from reflux of duodenal contents. This possibility can be eliminated as proteolytic activity was minimal or absent in all samples of pH 6–pH 7 (Fig. 3) and also no bile was observed in the juice samples. Further work is in progress to investigate the exact relationship of these changes in mucolysis with the patterns of pepsins in peptic ulcer disease.

The thickness and structural integrity of the protective mucus layer covering the gastric mucosa is a balance between mucus erosion by pepsin or mechanical abrasion and the secretion of fresh mucus.⁵ Thus while luminal pepsin cannot diffuse through the adherent gastric mucus it will solubilise the barrier at its luminal aspect. Any factors that change the balance in favour of mucus erosion could lead to a breakdown of the protective mucus barrier and a deficiency in the mucosal protection mechanisms. The enhanced mucolytic activity of pepsin 1 described in this paper is therefore particularly interesting in view of the increased amount of this enzyme in gastric juice from peptic ulcer patients.¹² In gastric ulcer patients the output of total pepsin is unchanged from that of non-ulcer patients but the proportion of pepsin 1 relative to pepsin 3 is increased from an average of 3.6% to 23%.¹³ In duodenal ulcer patients, while the proportion of pepsin 1 increases to an average of 16.5% there is also an increase in the total amount of pepsin

activity.¹⁴ Perhaps the most significant factor of the increased pepsin 1 mucolytic activity is the extension of this activity above pH 4. This was not expected from the hydrolysis pattern of haemoglobin which for both pepsin 1 and 3 show minimal activity above pH 4 (Fig. 4). The pH of the stomach can frequently rise above pH 4 while that in duodenal lumen is usually above this value.^{26 27} Under these circumstances, in patients with peptic ulcer, in contrast with those without, more persistent erosion of the mucus barrier might be expected, even though the corrosive effect of the high acid will be absent. Duodenal mucus like gastric mucus has been shown to be digested by pepsin.²⁵ The digestion of mucus in dog duodenal juice at low pH values (pH 2.4)⁹ would indicate the presence of acid proteases in this juice. Increased proteolytic activity could, over a period of time, weaken and in places destroy the gastroduodenal mucus barrier with subsequent erosion of the underlying mucosa and hinder the mucosal repair processes. The increased collagenolytic action of pepsin 1¹⁶ would facilitate a higher rate of degradation of the underlying connective tissue network by the gastric juice in peptic ulcer patients.

The adherent gastric mucus barrier has been shown to be structurally weaker in peptic ulcer patients.²⁸ A large and significant decrease was observed in the amount of polymeric (gel-forming) glycoprotein in the mucus from the antrum of peptic ulcer patients compared with that from histologically normal gastric mucosa. Such a decrease in the proportion of polymeric material on the basis of rheological studies²⁵ would implicate a weaker gel structure in these peptic ulcer patients. The increased mucolytic activity due to the higher concentrations of pepsin 1 in these patients could well be a major factor in such weakening of the mucus barrier but there are other explanations. For example deficiencies at the mucosal level from increased lysosomal protease activity associated with the increased gastritis and cell turnover in these patients,²⁹ or possibly incomplete biosynthesis of the mucus glycoprotein. Certain areas of the stomach and duodenum are predisposed to ulceration and this may reflect pre-existing differences in protective capacity over the mucosa. If this was so then an over all weakening of the mucus barrier might expose such areas where intrinsic protection is weaker and lead to ulceration at specific sites.

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