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CAMPYLOBACTER PYLORIDIS DEGRADES MUCIN AND UNDERMINES GASTRIC MUCOSAL INTEGRITY

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SUMMARY - The role of Campylobacter pyloridis, a spiral bacteria associated with gastritis and peptic ulcers in weakening the mucus component of gastric mucosal barrier was investigated. The colonies of bacteria, cultured from antral mucosal biopsies of patients undergoing gastroscopy, were washed with saline, passed through sterilization filter and the filtrate was examined for protease and glycosylhydrolase activities. The obtained results revealed that the filtrate exhibited a strong proteolytic activity not only towards the typical protein substrates such as albumin but also towards gastric mucin. Optimum enzymatic activity for degradation of mucin was attained at pH 7.0 and the protease activity was found in a low m.w. (<50K) protein fraction. The filtrate showed little glycosylhydrolase activity and did not cause the hydrolysis of mucin carbohydrates. The data suggest that C pyloridis infection weakens the gastric mucosal defense by causing proteolytic degradation of mucin component of the protective mucus layer.

INTRODUCTION - A unique property of gastric mucosa is its ability to withstand the insults by luminal contents and to resist the challenge by invading bacteria. Although the exact nature of the protective mechanism remains elusive, there are strong indications that the mucosal integrity is maintained by the surface layer of mucus and the cell membranes of gastric epithelium (1-3). Thus, the initial brunt of insult by a variety of injurious agents falls on the mucus layer which constitues the only identifiable physical barrier between gastric lumen and the surface epithelial cells of mucosa (1,3,4). Under normal physiological conditions the integrity of gastric mucus layer is successfully maintained by the factors controlling the synthesis, secretion and breakdown of its constituents (5,6). Disturbances in this delicate balance lead to the impairment of the protective function of mucus resulting in gastric disease.

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Among the aggressive factors deemed to play a role in the etiology of gastric disease is the recently recognized occurrence in gastric mucosa of a spiral bacteria,Campylobacter pyloridis (7-10). This organism, apparently dwells in close proximity to the surface epithelial cells where it is protected from noxious environment of gastric lumen by the mucus coating (9-11). Clinical data indicate the existence of a definite association of the bacteria with gastritis and peptic ulcer, the organism is rarely found in gastric mucosa of healthy individuals, and the patients treated with bacteriocidal antiulcer agents show improvement with the microorganism eradication (10). The nature of impairment evoked by this pathogen in gastric mucosal defense, however, is poorly understood. Here, we present evidence that Campylobacter pyloridis elaborates an extracellular protease which destroys the integrity of the protective mucus layer by causing degradation of its mucin constituent.

MATERIALS AND METHODS - The study was conducted with filtrates of C pyloridis cultured from gastric mucosal biopsies of patients undergoing routine gastroenterological tests for upper abdominal complaints. Three biopsy specimens were obtained from antrum of each patient. One tissue fragment from each patient was fixed in buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosine, and silver techniques (8,9). A second specimen was placed into Christensen urea broth and incubated at 37°C for up to 24h, while the third biopsy fragment was minced and inoculated onto Skirow's medium, trypticase soy blood agar, and chocolate agar (8,9). The cultures were incubated in a microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N_2) at 37°C for up to seven days. Isolates were considered to be C pyloridis if they grew microaerophilically, had characteristic tiny clear glistening colonial morphology in a Skirow's medium, stained easily with a spiral bacteria-sensitive silver stain, were catalase, urease and oxidase positive, showed resistance to nalidoxylic acid and were cephalothin-susceptible (8-10).

The plates with grown colonies of bacteria were gently washed with 0.15M NaCl and the solution filtered through Nalgene sterilization (0.20μm) filter to retain the bacteria. The filtrate was dialyzed at 4°C against distilled water and lyophilized. Such prepared powder was used as an enzyme source for the assays of proteolytic and glycosylhydrolase activities. The protease activity of dialyzed C pyloridis filtrate was determined using N-alkylated porcine serum albumin (12) and the undegraded gastric mucus glycoprotein isolated from pig gastric mucus (13). The purified mucus glycoprotein, p-nitrophenylglycosides and N-acetylneuramin-lactose (Sigma) served as substrates in the assays of exoglycosidase activities of the filtrate.

The incubation mixtures for protease activity assays consisted of the following components: gastric mucus glycoprotein or N-alkylated porcine serum albumin substrate, 50-400μg; C pyloridis filtrate, containing 20-100μg protein; and 50mM sodium phosphate buffer, pH 7.0, in a final volume of 0.22 ml. The tubes containing the complete incubation mixtures were briefly sonicated (20s) and incubated at 37°C for 30 min. The reaction was terminated by the addition of 1ml of 0.1M borate buffer, pH 9.0. The protease activity of C pyloridis filtrate towards the mucus glycoprotein and albumine substrates

was measured by following the release of α-amino residues by the trinitrophenylation method (12,13). Tubes containing boiled enzyme and substrates, and the tubes with enzyme but devoid of substrates served as controls.

The assay mixtures for exoglycosidase activities of C pyloridis filtrate contained, in 0.12ml: phosphate buffer, pH 6.5, 50mM; p-nitrophenylglycoside, 0.2μM; bacteria filtrate, containing 50-100μg protein, in a final volume of 0.12ml. Reaction mixtures were incubated at 37°C for 1h and terminated by the addition of 0.5ml of 0.5M carbonate buffer, pH 10.8 (14). The α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-L-fucosidase, α-manosidase, β-N-acetylglucosaminidase, and α-N-acetylgalactosaminidase activities of C pyloridis filtrate were measured by following the release of p-nitrophenol (14), while the neuraminidase activity was assayed by measuring the release of neuraminic acid (15).

The protein content of samples was measured by the method of Lowry et al (16) with bovine serum albumin as standard. The content and composition of carbohydrates was determined by gas-liquid chromatography following methanolysis, re-N-acetylation and derivatization with silylating reagent (2,13), while sulfate was assayed turbidimetrically (17). Gel electrophoresis in 1% SDS was performed with 5% polyacrylamide gels (18). After electrophoresis, the gels were stained for protein with Coomassie briliant blue (18). The C pyloridis filtrate-degraded mucin was isolated from a scaled-up digestion mixture (pH 7.0) containing 10mg of mucus glycoprotein and 1mg of filtrate protein. After incubation at 37°C for 48h under a layer of thymol crystals, the digest was lyophilized, dissolved in 6M urea and chromatographed on a Bio-Gel A-50 column (2,19). The fractions containing the degraded mucin were pooled, dialyzed against distilled water, and lyophilized. The dry powder was then analyzed for protein and the content and composition of carbohydrates (2,13,16, 17).

RESULTS - Protease activity of C pyloridis filtrate was investigated using serum albumin and gastric mucus glycoprotein as substrates. By following the release of α-amino residues by the trinitrophenylation method (12,13), it was determined that with both substrates the protease attains its maximum activity at pH 7.0, after which a sharp drop in activity occurs. A 60% drop in protease activity was observed at pH 3.0, 50% at pH 5.0 and 45% at pH 8.0. Gel sieving chromatography of C pyloridis filtrate revealed one major peak of proteolytic activity that eluted from Bio-Gel A-0.5 column in a low molecular weight (<50K) protein fraction (Fig. 1).

Experiments on the effect of substrate concentration on the protease activity of C pyloridis filtrate established that under the assay conditions the rate of proteolysis was proportional to the albumin concentration up to 100μg and with mucus glycoprotein up to 150μg, and remained constant with time of incubation for at least 1h. The apparent Km values calculated from the double reciprocal plots of the data were 0.28g/l for albumin and 0.71g/l for

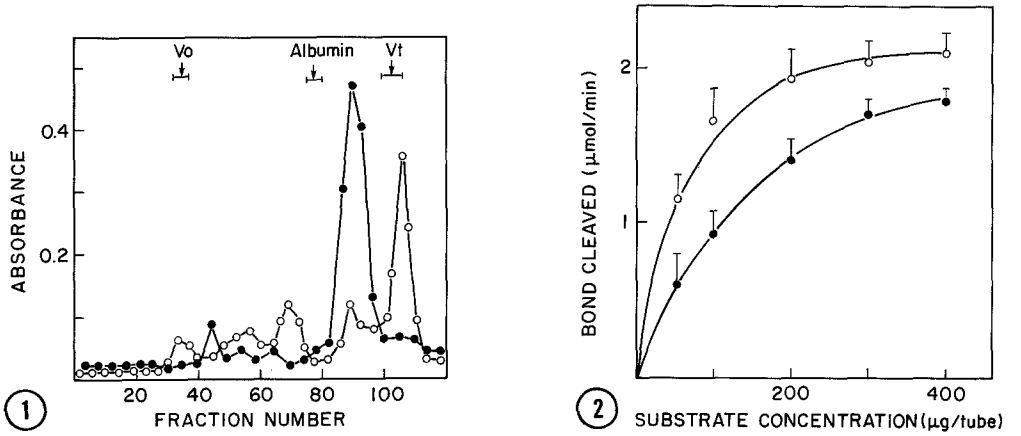


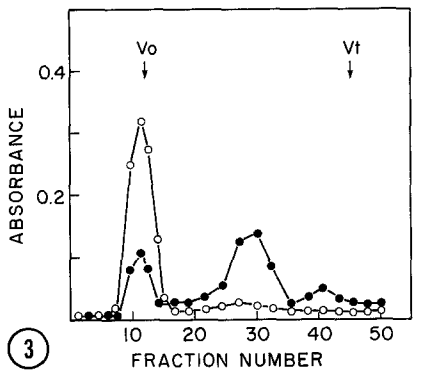
Fig. 1. Bio-Gel A-0.5 column chromatography of C pyloridis filtrate. The sample (20mg) dissolved in 2ml of 0.10M NaCl/0.05M sodium phosphate buffer, pH 7.0,was applied to a column (1.5 x 160cm) equilibrated with buffered 0.10M NaCl. Fractions of 2.8ml were collected and screened for protein (absorbance at 280nm, ○) and protease activity by the trinitrophenylation method (absorbance at 340nm, ●).

Fig. 2. Effect of albumin(○) and mucus glycoprotein(●) concentration on the protease activity of C pyloridis filtrate. The composition of the incubation mixtures was the same as described in the text, except that various concentrations (50-400µg) of albumin and mucin were used. The data show the means ± SD of four separate experiments performed in duplicate.

mucus glycoprotein. The effect of albumin and mucus glycoprotein concentration on the proteolytic activity of C pyloridis filtrate is shown in Figure 2.

Gel filtration of the C pyloridis protease-degraded mucus glycoprotein on Bio-Gel A-50 column yielded a glycoprotein peak in the included volume which separated clearly from the undegraded mucus glycoprotein which emerged in the excluded volume. The elution pattern of the purified undegraded pig gastric mucus glycoprotein and its protease digest is presented in Figure 3. The C pyloridis protease-degraded mucin, in comparison to intact mucus glycoprotein, contained about 22% less protein and showed 5.1% increase in the carbohydrate content, but the ratios of the individual sugars essentially remained unchanged (Table I). The fragmentation pattern of N-alkylated porcine serum albumin exposed to the action of C pyloridis filtrate protease is shown in Figure 4. Based on the size and distribution of the degradation products on SDS-polyacrylamide gel, it appears that the protease is of endopeptidase type.

Assays of C pyloridis filtrate for exoglycosidase enzymes, conducted with a battery of synthetic substrates, revealed the presence of a weak (0.12µmol



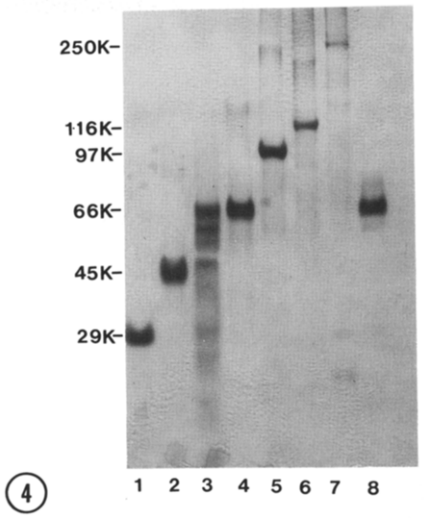


Fig. 3. Bio-Gel A-50 column chromatography in 6M urea/0.05M sodium phosphate buffer, pH 7.0, of purified pig gastric mucus glycoprotein before (○) and after (●) incubation with C pyloridis filtrate. The samples, 10mg each were dissolved in 1ml of 6M urea and applied to a column (0.9 x 120cm). Fractions of 1.8ml were collected and monitored for carbohydrate by phenol/H_2SO_4 method.

Fig. 4. SDS-polyacrylamide gel electrophoretic pattern of porcine serum albumin following exposure to C pyloridis filtrate. The albumin samples were incubated at 37°C for 4h with the intact or boiled (3 min at 100°C) C pyloridis filtrate and aliquots applied to the gel for electrophoresis. Following electrophoresis, the gel was stained for protein. Lines 1,2,5-8, molecular weight markers. Line 3, albumin incubated with intact C pyloridis filtrate. Line 4, albumin incubated with boiled C pyloridis filtrate.

Table I. Protein and carbohydrate composition of the intact and C pyloridis filtrate-degraded pig gastric mucus glycoprotein

Component (mg/100mg)	Mucus glycoprotein	
	Intact	C pyloridis filtrate degraded
Fucose	9.7 ± 0.6	10.1 ± 0.6
Galactose	21.6 ± 2.9	22.6 ± 2.7
N-Acetylgalactosamine	13.1 ± 1.2	14.5 ± 1.3
N-Acetylglucosamine	16.4 ± 1.5	16.8 ± 1.7
Sialic acid	0.8 ± 0.1	0.9 ± 0.2
Sulfate	2.8 ± 0.4	2.8 ± 0.3
Protein	14.3 ± 1.1	11.2 ± 0.9

Values represent the means ± SD of triplicate analyses.

p-nitrophenol liberated/mg protein/1h) β-galactosidase activity, and showed the absence of neuraminidase, α-L-fucosidase, α-glucosidase, β-glucosidase, α-galactosidase, α-mannosidase, β-N-acetylglucosaminidase and α-N-acetylgalactosaminidase enzymes. Furthermore, the filtrate did not cause hydrolysis of the carbohydrate chains of gastric mucin.

DISCUSSION - Although the exact sequence of events involved in the pathogenesis of active chronic gastritis and peptic ulcer remains elusive, the consensus is that the injury to gastric mucosa occurs when the aggressive factors overcome those responsible for mucosal defense. Among the aggressive factors which appear to play a distinct role in gastrointestinal disease is an infection by Campylobacter pyloridis (7-11). While the available clinical data clearly implicate this bacterium in the causation of gastritis and peptic ulcers, the pathogenic mechanism remains poorly understood, although disturbances in the protective layer of mucus have been recently suggested (10). Since C pyloridis resides in proximity to the surface of gastric epithelium, deeply burried within the mucus gel and yet exhibits a free mobility in this viscous environment, the extracellular material elaborated by this microorganism was examined for the activities of enzymes capable of destruction of the integrity of mucus layer. Among the enzymes which are particularly detrimental to the integrity of gastric mucus are those exhibiting proteolytic and glycosylhydrolase activities.

The results obtained in this study demonstrate that C pyloridis filtrate, while devoid of glycosylhydrolase activity, contains a protease which is capable of rapid degradation not only such typical protein substrate as serum albumin but also of gastric mucin. Our data show that the protease elaborated by this bacterium is of endopeptidase type and that it attacks the regions of mucus glycoprotein molecule which are devoid of carbohydrates, leading to disintegration of the polymeric structure of this glycoprotein and formation of glycopeptides comparable in size (m.w. ≃ 500K) to those produced by the action of pepsin. Since such a proteolytically degraded mucin no longer poss-

esses the viscous and gel-forming properties (1,4,6), and its ability to retard the diffusion of hydrogen ion is severly impaired (20), the erosion of mucus gel by C pyloridis protease may indeed be of dire consequence to the mucosal integrity as it would compromise its luminal defense perimeter.

Our results, thus suggest that C pyloridis by affecting degradation of deeper levels of the mucus gel, which maintains neutral environment at the mucosal surface, drastically reduces the dimension and physicochemical characteristics of acid-bicarbonate mixing zone required for luminal acid dissipation. As a consequence the integrity of the mucus coat, which under normal physiological conditions so efficiently protect the gastric epithelium from corrosive action of acid and pepsin, becomes severly undermined and renders the epithelial surfaces of the stomach vulnerable to the damaging effects of luminal contents. Therefore, the degenerative changes induced in gastric mucosal defense by C pyloridis, may be the determining factor in the pathogenesis of gastritis and peptic ulcer.

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