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Oral administration of protease inhibits enterotoxigenic *Escherichia coli* receptor activity in piglet small intestine

T L Mynott, R K J Luke, D S Chandler

Abstract

The virulence of enterotoxigenic *Escherichia coli* (ETEC) is attributed to their ability to adhere via fimbrial adhesins to specific receptors located on the intestinal mucosa. A novel approach to preventing ETEC induced diarrhoea would be to prevent attachment of ETEC to intestine by proteolytically modifying the receptor attachment sites. This study aimed to examine the effect of bromelain, a proteolytic extract obtained from pineapple stems, on ETEC receptor activity in porcine small intestine. Bromelain was administered orally to piglets and K88$^+$ ETEC attachment to small intestine was measured at 50 cm intervals using an enzyme immunoassay. K88$^+$ ETEC attachment to intestinal sections that were not treated with bromelain varied appreciably between sampling sites. Variability in receptor activity along the intestinal surface is thought to be caused by the localised effects of endogenous proteases. Oral administration of exogenous protease inhibited K88$^+$ ETEC attachment to pig small intestine in a dose dependent manner ($p<0.05$). Attachment of K88$^+$ ETEC was negligible after treatment, resembling the levels of attachment of K88 to piglets of the genetically determined non-adhesive phenotype, which are resistant to K88$^+$ ETEC infection. Serum biochemical analysis and histopathological examination of treated piglets showed no adverse effects of the bromelain treatment. It is concluded that administration of bromelain can inhibit ETEC receptor activity in vivo and may therefore be useful for prevention of K88$^+$ ETEC induced diarrhoea.

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Keywords: enterotoxigenic *Escherichia coli*, diarrhoea, K88 ETEC, pig intestine, protease.

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Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of disease in young children[1] and young animals.[2] The virulence of ETEC strains is attributed to their ability to adhere, via fimbrial adhesins, to highly specific receptors located on the intestinal mucosa.[3] These strains also liberate heat labile (LT) and/or heat stable (ST) enterotoxins which cause fluid secretion and diarrhoea. *E coli* strains which carry the K88 adhesin on their surface are a significant cause of diarrhoea in young piglets.[4] K88 occurs in several antigenic types or variants, all of which adhere to piglet enterocytes.[5] K88ab, K88ac, K88ad, and K88ad(e) variants have been characterised.[6 7]

In 1975, Rutter *et al*[8] showed that some pigs are resistant to colonisation and disease caused by K88-positive (K88$^+$) *E coli*. An adhesive phenotype (one susceptible to infection) is related directly to the ability of K88$^+$ bacteria to recognise intestinal receptors and attach to piglet intestinal brush border membranes. Intestine obtained from the non-adhesive phenotype (disease resistant pigs) do not bind K88$^+$ *E coli* and as a result such animals are resistant to K88$^+$ *E coli* infection.[9] The receptors on non-adhesive intestinal brush border cells may be absent or non-functional. A genetic basis for expression of the adhesive or non-adhesive phenotype exists.[8 10] Different phenotypes may also be distinguished, depending on the serological variant of the K88 antigen.[11] Also, in addition to genotype, physiological factors, particularly the level of intestinal proteolysis within the small intestine, may influence the ability of ETEC to attach to intestine.[12 13]

The ability of protease to prevent attachment of ETEC to small intestinal samples in vitro is well documented.[13–18] Presumably attachment ability is prevented because of proteolytic cleavage of ETEC receptor sites. This study aimed to investigate whether exogenous protease, administered orally, could inhibit porcine K88$^+$ ETEC receptor activity in vivo and therefore inhibit K88$^+$ ETEC attachment to small intestine. The use of protease, through its ability to modify intestinal receptor sites and reduce the binding properties of the intestinal mucosa, may be an important way of protecting the small intestine from microbial colonisation and disease.[12 13]

Methods

ANIMALS

Approval for animal experiments was granted by the Victorian Institute for Animal Science Animal Experimentation Ethics Committee. Five pregnant sows (Large White X Landrace X Duroc) were purchased from a commercial farm where the incidence of pigs with the non-adhesive phenotype is low (1 in 10).[19] Piglets were born within three days of each other in the animal housing facility at the Victorian Institute of Animal Science (VIAS-Attwood, Victoria, Australia). Piglets were weaned at approximately 3 weeks of age (weight not less

K88$^+$ enterotoxigenic Escherichia coli (ETEC) attachment to piglet small intestine

Group*	Protease (mg)†	K88 receptor activity (mean (SD))‡			Non-adhesive (%)π
		Strong§	Moderate	Non-adhesive	
A	0	0·409 (0·244) 0·709 (0·090)	0·211 (0·178) 0·311 (0·108) 0·230 (0·095)	0·078 (0·029) 0·069 (0·045)	29
B	125	0·660 (0·096)	0·295 (0·139) 0·312 (0·187)	0·080 (0·099)$_{(2)}$ 0·119 (0·071)$_{(2)}$ 0·034 (0·014)$_{(2)}$ 0·055 (0·026)	57
C	250	nil	0·359 (0·225)$_{(2)}$	0·061 (0·027)$_{(2)}$ 0·079 (0·057)$_{(2)}$ 0·039 (0·013)$_{(2)}$ 0·191 (0·117) 0·045 (0·017) 0·044 (0·021)	86
D	625	nil	0·224 (0·195)$_{(2)}$	0·096 (0·036)$_{(2)}$ 0·176 (0·118) 0·033 (0·015)$_{(2)}$ 0·099 (0·115)$_{(2)}$ 0·034 (0·015) 0·064 (0·022)	86
E	1250	nil	0·238 (0·183)	0·036 (0·015)$_{(2)}$ 0·059 (0·015)$_{(2)}$ 0·032 (0·009)$_{(2)}$ 0·050 (0·040)$_{(2)}$ 0·033 (0·015) 0·182 (0·135)	86

*Number of pigs per group is 7. †Amount of bromelain administered per dose, three times a day for either two (2) days (n=4) or five days (n=3). There was no significant difference between duration of treatment and reduction in EIA activity (p=0·82). Ability of protease to reduce K88 receptor activity was significant (p<0·05; ANOVA). ‡Values represent the mean (SD) absorbance of the $A_{540\,nm}$ value of 19 sampling sites per pig. The large SD observed in some pigs reflect the variability of receptor activity along the length of the small intestine. §K88$^+$ ETEC attachment expressed as strongly adhesive (EIA activities >0·4); moderately adhesive (EIA activities 0·20 to 0·40); and non-adhesive (EIA activity <0·2). πNumber of K88$^+$ ETEC non-adhesive *versus* number tested expressed as a percentage. Of the total number of pigs treated with protease, 79% (22 of 28) were non-adhesive compared with 29% (2 of 7) pigs not treated with protease (p<0·02, Fisher's exact test).

than 6 kg), and housed in stables with straw bedding. Piglets were fed *ad libitum* a commercial starter diet (Gropower, Barastoc, Australia) for two weeks after weaning, followed by Grower 8 (Barastoc, Australia) in three divided meals (160 g/6 kg of body weight daily) until experimentation. Thirty five piglets of weight 20 to 25 kg (aged 10 to 16 weeks) were used for the study and randomly allocated between five treatment groups (Table).

PIGLET TREATMENT

Bromelain (E.C. 3.4.22.4) is a cysteine protease obtained from pineapple stems. Bromelain is a glycoprotein and active across a wide pH range, therefore it is ideally suited for the gastrointestinal environment. Different amounts of enteric protected bromelain (Detach, Cortecs Ltd, Middlesex UK; 1 g of Detach contains 125 mg of bromelain) was administered to pigs for two or five days to investigate the effect of duration of treatment on K88$^+$ ETEC receptor activity and to investigate the safety of treatment. The bromelain preparation was suspended in water (1g per 5 ml) and administered by mouth using a plastic syringe, three times a day 15 min before feeding (Table). Control piglets were untreated and administered 25 ml of water. All animals were monitored daily to observe any adverse clinical effects attributable to treatment. At the completion of the experiment animals were killed by barbiturate overdose, and autopsied.

ASSESSMENT OF K88 PHENOTYPE BY ENZYME IMMUNOASSAY (EIA)

The small intestine (length from pyloris to the terminal ileum was 8 to 10 m) of all pigs was removed immediately at death. Brush border material was obtained at 50 cm intervals (n=19 samples per pig) by gently scraping 1 cm sections of the villous surface with a sterile cotton-tipped swab. The swabs were then immersed in 3 ml of a working dilution buffer (WDB) consisting of phosphate buffered saline (PBS; 0·1 M, pH 7·4) to which bovine serum albumin (BSA; 0·25% w/v), EDTA (di-sodium salt; 1 mM), Tween 20 (0·05% v/v) and sodium azide (0·02% w/v) had been added. The swabs were vortexed to recover the epithelial cells. Brush border samples were assessed for their ability to attach to K88$^+$ ETEC (strain WG (0149:K91(B):K88ac:H10)[20] by enzyme immunoassay (EIA) as previously described.[13 21] The EIA gives 95% correlation with results obtained by traditional microscopic adhesion tests for assessing K88 phenotype[21] (piglets genetic propensity to produce K88 receptor on its intestinal surface). Briefly, K88$^+$ ETEC are immobilised to wells of a microtitre plate and incubated with mucosal samples. Mucosal material, bound to the bacteria is detected with antibody (rabbit IgG) raised against porcine intestine followed by urease-conjugated goat anti-rabbit IgG (Sigma) and urea substrate.

Disposable polystyrene microtitre plates (Nunc, Denmark) were used for all assays. K88$^+$ ETEC diluted to approximately 2×10^9 bacteria/ml in sodium bicarbonate buffer ($NaHCO_3/Na_2CO_3$; 0·1 M, pH 9·6), were adsorbed to wells (100 µl/well) by incubation overnight at 4°C. Active binding sites remaining on wells following coating procedures were routinely blocked by incubation (30 min at 37°C) with BSA (1% w/v) dissolved in PBS (200 µl/well). Mucosal material bound to K88$^+$ ETEC was detected using rabbit IgG raised against a Triton X-100 extract of K88-adhesive phenotype brush border vesicles, diluted in WDB.[21] Anti-rabbit urease-conjugated IgG (Sigma) was diluted in WDB containing ovalbumin (from hen egg, grade II, Sigma 1% w/v). All incubation steps, excluding the coating procedures, were performed at 37°C for 30 minutes. Between each of the incubation steps, supernatant liquid was removed from the wells which were then washed three times with washing buffer (0·1 M PBS; 0·05% v/v Tween 20). Before the incubation with substrate, wells were washed with distilled water to remove any effect of residual buffer on substrate solutions. Urea substrate [bromocresol purple (0·15 mM) urea (16 mM), EDTA (disodium salt, 1 mM); pH 4·8, 100 µl/well] was used to detect the presence of bound, conjugated enzyme. A positive reaction was indicated by a colour change from yellow to purple which was measured spectrophotometrically at A_{540nm}. All assays were standardised by developing the reaction until a positive control attained an EIA value of 0·6.

The K88 phenotype of the intestine scrapings was assessed and an adhesion pattern for the small intestine of each pig was established. To obtain an overall indication of the

K88-adhesiveness of a pig, the mean EIA value of all sampling sites (n=19 per pig) was determined. The adhesiveness of a particular pig could be designated as strongly adhesive (S; mean EIA>0·4); moderately adhesive (M; mean EIA 0·2 to 0·4) or non-adhesive (N; mean EIA<0·2). (See Table.)

SERUM BIOCHEMISTRY

Blood samples (10 ml) were collected from piglets via the jugular vein one day before experimentation and again immediately before death. Serum samples were stored at −20°C until completion of the experiment, at which time they were submitted for biochemical analysis. Tests performed include a full biochemical profile, liver function tests, creatinine kinase, lipase, and amylase. As normal values for biochemical parameters cited in the literature vary because of factors such as differences in sample handling, assay technique, dietary influences or genetic differences between animals, pre-treatment values were taken as an indication of baseline normal values. These values were within the normal ranges cited in the literature.[22 23] Differences between serum samples before and after treatment were compared for each pig. In addition, the mean serum value for each parameter for the treated groups was compared with that for the non-treatment group.

HISTOPATHOLOGY

All animals were sacrificed by barbiturate overdose two or five days after beginning treatment. At autopsy, specimens were immediately processed for histological examination after fixation with neutral buffered formalin (0·65% (w/v) Na_2HPO_4, 0·45 (w/v) NaH_2PO_4, 10% (v/v) formalin). Sections of duodenum, midjejunum, and ileum, were stained with haematoxylin and eosin and examined by light microscopy for morphological changes. Sections of heart, kidney, liver, and mesenteric lymph nodes were also investigated.

STATISTICAL ANALYSIS

Differences in biochemical parameters, between serum samples, before and after treatment, and differences between treatment groups were assessed for clinical relevance by veterinary pathologists at VIAS-Attwood. Differences among mean serum biochemical values between treatment groups were subjected to one-way analysis of variance (ANOVA) using Microstat. Difference among mean EIA values for all treatment groups (0 mg to 1250 mg) representing mean $K88^+$ ETEC receptor activity was analysed by Genestat V for analysis of variance.

Results

$K88^+$ ETEC ATTACHMENT TO SMALL INTESTINE OF UNTREATED (CONTROL PIGS)

We first examined the ability of $K88^+$ ETEC to attach to the small intestine of pigs that were not treated with bromelain (group A). The Table summarises the results obtained from all untreated pigs. Two pigs were of the strongly adhesive phenotype, three pigs were moderately adhesive and two pigs were of non-adhesive phenotype. Figure 1 shows EIA values obtained from intestine of three pigs to show the three phenotypes obtained.

We next compared the binding of $K88^+$ ETEC to different sections of intestine in individual pigs. $K88^+$ ETEC attachment to intestine varied markedly between samples taken at 50 cm intervals (Fig 1). Multiple scrapings taken at the same site revealed similar results indicating that the variability between sites was a function of that section of intestine, not the assay. Earlier, Chandler *et al*[13] observed a similar variation in binding between sampling sites and related this variation to the state of distention or constriction of the small intestine at the sampling site. Despite the variation seen between individual scrapings, a consistent pattern was observed

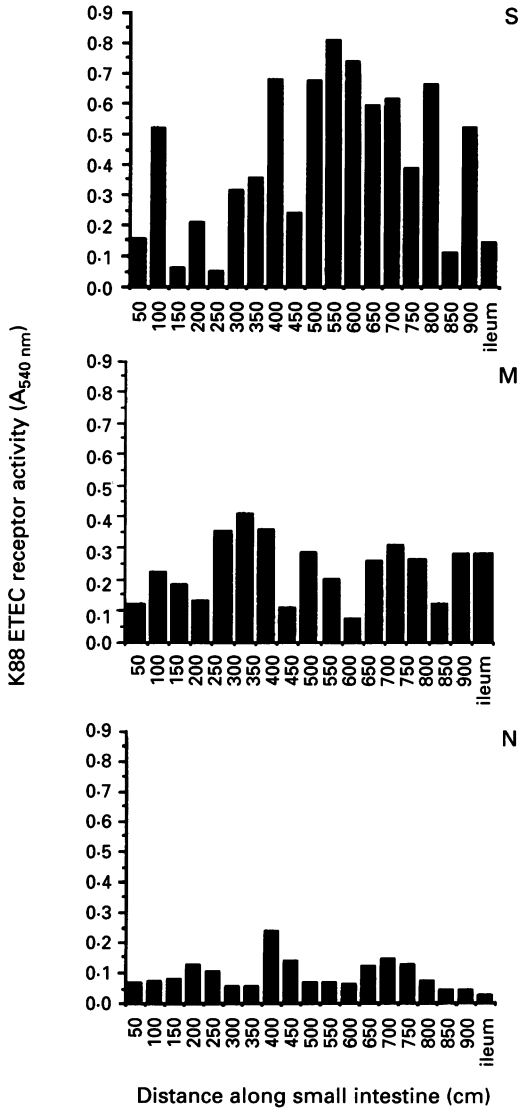

Figure 1: Use of enzyme immunoassay to demonstrate variability in K88⁺ enterotoxigenic Escherichia coli *(ETEC) receptor activity and attachment of ETEC to three untreated pigs. Columns represent the mean absorbance of the $A_{540\ nm}$ values of duplicate wells. S, strongly adhesive; M, moderately adhesive; N, non-adhesive.*

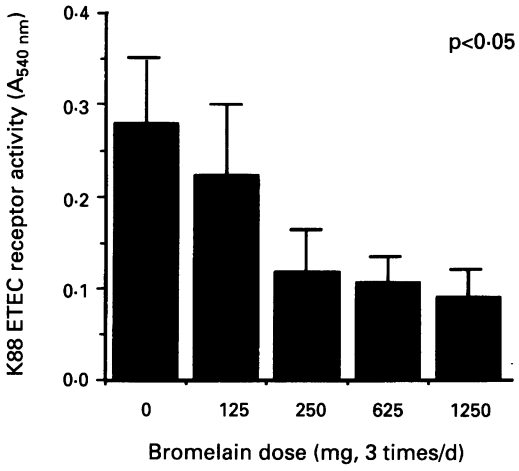

Figure 2: K88+ enterotoxigenic Escherichia coli *ETEC receptor activity of small intestine samples of pigs treated with bromelain or untreated. Columns with bars represent the mean (SEM) $A_{540\ nm}$ values of 19 samples taken from each pig (n=7 pigs per group). The reduction in enzyme immunoassay activity was significant (p<0·05).*

between segments. The mid-small intestine typically had high EIA values, indicative of high levels of K88 receptor activity and therefore K88+ ETEC attachment. In comparison, the duodenum and the ileum had lower attachment activity, as observed previously.[13]

K88+ ETEC ATTACHMENT ACTIVITY IN PROTEASE TREATED PIGS

To investigate whether exogenous bromelain could inhibit K88-attachment to piglet intestine, we orally administered different amounts of the protease and measured the ability of K88 to attach to intestine after treatment. Figure 2 shows mean EIA values for each treatment group. A dose-dependent inhibition of K88 attachment to pig intestine is observed. An analysis of variance revealed a significant treatment effect (p<0·05), confirming that the reduction in K88+ ETEC receptor binding activity was dependent on the protease and not a result of inherent variation. Bromelain administered in amounts of 250 mg or more were most effective in reducing K88+ ETEC attachment. In groups C, D and E, which received 250 mg, 625 mg or 1250 mg of protease per dose, six of seven (87%) pigs in each group were non-adhesive after treatment (p<0·05) in comparison with only two of seven (29%) in untreated pigs (Table). Of the 28 pigs receiving protease, 22 were non-adhesive (79%) in comparison with 29% in non-protease treated pigs (p<0·02, Fischer's exact test).

CLINICAL OBSERVATIONS

Because of the direct effect of an exogenous protease on the intestine, we conducted some preliminary serological and histopathological examination of samples obtained from pigs to investigate any adverse effects of treatment. We observed no abnormal morphological changes in tissue specimens, even at the highest protease treatment level. There was no clinical or statistical difference (p>0·2) in any of the parameters measured in samples before and after treatment (data not shown). Pre- and post treatment parameters measured remained within the normal range.[22 23]

Discussion

Attachment of K88+ enterotoxigenic *E coli* to specific receptors located on the intestinal brush border is an important initial factor in the establishment of diarrhoeal disease. The receptors for K88ac ETEC have been described as mucin-type sialoglycoproteins.[24] In vitro, protease treatment of intestinal brush border cells containing these receptors inhibits receptor activity and therefore prevents attachment of K88+ ETEC. Similarly, protease treatment of human and calf small intestine inhibits receptor activity and attachment of ETEC strains which carry the CFA/I and CFA/II, and K99 adhesins, isolated from human and calf diarrhoea, respectively.[16 25] Because of ETEC receptor sensitivity to protease, one possible way of preventing ETEC diarrhoea would be to prevent attachment of bacteria to intestine by proteolytically modifying receptor attachment sites.[12 13] In the present study, we investigated whether oral administration of bromelain, a cysteine protease, could inhibit K88+ ETEC receptor activity in vivo and therefore inhibit K88 attachment to porcine small intestine.

In pigs that were not treated with bromelain, K88 receptor activity varied appreciably along the length of the small intestine. This variability supports our earlier observations[13] and those of others investigating ETEC receptor activity in small intestine obtained from calves and lambs.[26] Variable patterns in receptor activity may reflect masking of receptor sites or release of receptors from the intestinal epithelium in vivo.[27] Alternatively, the fluctuating levels of receptor activity may reflect the action of endogenous enzymes in that part of the intestine. Several observations support a role for protease modification of intestinal receptors in vivo. ETEC receptor sites have been shown to be readily inactivated by trypsin[14] and by intestinal contents with high proteolytic activity.[13] Also, stabilisation of K88 receptor can be achieved by the addition of trypsin inhibitor to sample collection buffers.[13] Furthermore, pancreatic proteases are known to play a role in the final processing of microvillus proteins, and affect the release of some membrane-bound proteins into the lumen of the small intestine.[28 29] Some of the proteins that are released may be receptors for bacteria.

To confirm an effect of protease on ETEC receptor sites in vivo, we administered various amounts of protease orally to piglets. Exogenous protease, administered orally inhibited K88+ ETEC receptor activity and therefore ETEC attachment to small intestine. The effect of bromelain on K88 receptor activity was dose dependent, in that binding activity decreased with increased protease dose rate (p<0·05). Presumably, the protease modified K88+ ETEC enterocyte receptor sites, such that K88+ bacteria could no longer

recognise and attach to the small intestinal brush border.

The pattern of non-adhesiveness observed in protease treated pigs resembled that observed in pigs of the genetically determined, non-adhesive phenotype. Piglets of the non-adhesive phenotype are resistant to K88+ ETEC infection because they lack functional receptors for ETEC.[8 9] Therefore, the inability of K88+ bacteria to recognise receptor sites on the small intestine of bromelain treated piglets should render the animals resistant to colonisation by these bacteria and prevent diarrhoeal disease. We have previously shown that the oral administration of enteric-coated bromelain to rabbits inhibits colonisation of CFA/I+ ST+LT+ *E coli* (H10407) and protects against diarrhoea and diarrhoea induced death.[12] Further studies have demonstrated that bromelain significantly reduces diarrhoea in piglets challenged with K88+ ETEC (Chandler and Mynott, manuscript in preparation).

The data in this study support the view that increased proteolytic activity in the intestine favours low receptor activity, and hence resistance to ETEC colonisation. In addition to acidity in the stomach, local intestinal immunity, the flushing action of intestinal peristalsis, and competition with commensal organisms, intestinal proteolysis may be a previously undescribed host-defense mechanism. The novel concept of host receptor modification by oral administration of protease is a new approach to disease control that could provide broad spectrum protection and obviate the potential difficulty of antigenic variability of microbial virulence determinants.

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