# MUCOSAL ENTEROKINASE ACTIVITY IN COW'S MILK PROTEIN SENSITIVE ENTEROPATHY

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# ABSTRACT

Enterokinase has a critical role in initiating proteolytic digestion by hydrolysing the conversion of pancreatic trypsinogen into trypsin. The enzyme is synthesised by enterocytes of the proximal small intestine and initially incorporated into the brush border from where it is released into the intestinal lumen by the action of pancreatic secretions. The aim of the study was to analyse enterokinase activity in the duodenal mucosa of infants with diarrhoeal disease including cow's milk protein-sensitive enteropathy. Our observations show that the mean depletion of enterokinase was only 17% compared to 60-80% for other brush border enzymes like disaccharidases, peptidases and alkaline phosphatases in infants with diarrhoea. This suggests that enterokinase activity in the small bowel enteropathies may be dependent not only on the degree of mucosal damage specifically but also on the extent of damage to the goblet cell population where the enzyme is synthesised. Thus the enterokinase activity was reduced in acute and chronic diarrhoea with marked mucosal damage where significant reduction of goblet cell population was evident but the enzyme was relatively little affected when the mucosa was damaged mildly.

Keywords: enterokinase, duodenal mucosa, diarrhoea, cow's milk allergy, enteropathy.

#### SINGAPORE MED J 1995; Vol 36: 393-396

## INTRODUCTION

Immunological and digestive functions of the intestine play a complementary and facilitary role in antigen exclusion by the gut. Secretory IgA facilitates the role of protein digestion by holding the protein molecule to the digestive site for action by the proteases. Tryptic hydrolysis then breaks down the complex antigenic protein into relatively nonimmunogenic peptides which are then hydrolysed into amino acids by the cellular peptidases. Thus tryptic hydrolysis is an important step in reducing antigenic proteins in reaching the immune system.

Trypsinogen is a pancreatic enzyme and its activation is dependent on mucosal enterokinase released into the lumen. Congenital deficiency of enterokinase has been shown to result in severe protein maldigestion<sup>(1,2)</sup>. Since enterokinase is a mucosal enzyme, acquired enterokinase deficiency could occur in clinical situations associated with widespread damage to the small bowel mucosa.

Previous studies had shown that cow's milk protein allergy and soy protein allergy could complicate acute gastroenteritis during the post-hydration period resulting in persistent villus atrophy and chronic diarrhoea<sup>(3,4)</sup>. Mucosal damage in acute gastroenteritis may result in depletion of mucosal enterokinase which in turn causes failure of activation of trypsinogen and

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tryptic hydrolysis of proteins. This could hamper efficient intraluminal phase of protein hydrolysis.

Intraluminal digestion of ingested proteins is important for two reasons: (1) to meet the nutritional needs of the infant, and (2) to reduce their antigenicity and render them harmless to the intestinal mucosa. Thus from a teleological point of view the depletion of enterokinase in small bowel enteropathies to the extent of impairing protein digestion will be detrimental to the survival and well being of the individual and may even lead to fatal consequences.

Presently, there is very little information on the activity of enterokinase in infants where the mucosal surface has been damaged by antigens as in cow's milk protein-sensitive enteropathy. The aim of the study was to determine the enterokinase activity in infants with acute gastroenteritis, chronic diarrhoea and cow's milk protein sensitive enteropathy. All these infants who develop diarrhoea show severe damage to the intestinal mucosal surface.

## PATIENTS AND METHODS

#### Patients

A series of 30 infants with diarrhoea were studied. Clinically, all the infants were intolerant to lactose. Lactose intolerance was confirmed by positive clinitest on the liquid stools in all patients. In some cases the diarrhoea was suspected to be due to milk protein sensitive enteropathy because of the family history of atopy and previous history of cow's milk allergy in older siblings. Investigations on admission included examination of the stools for *Giardia lamblia*, viral and bacterial enteropathogens as previously described<sup>(5)</sup>.

After appropriate correction of fluid and electrolyte imbalance, the infants were started on a lactose and cow's milk protein-free formula such as Pregestimil, Nutramigen, or Prosobee. If the infants did not tolerate the oral foods or if they were severely malnourished, they were given parenteral nutrition.

All infants remained in hospital until their stools were normal, they were feeding well, and were gaining weight. They were then discharged and the mothers of the infants instructed not to offer any other food without consulting the clinician beforehand. The infants were readmitted 6 to 8 weeks later for milk challenge studies.

#### Milk challenge studies and biopsies

The infants were challenged with a low lactose cow's milk protein formula, Lactolac V (Cooperative Condens Fabriek, Firesland, Holland). The challenge studies were carried out as described previously<sup>(5)</sup>.

A proximal jejunal biopsy was performed immediately before and 20-24 hours after milk provocation test. The biopsy specimen was divided into 2 portions; one was fixed in gluteraldehyde for light and electron microscopical examination, the other was used for enzyme assay. The technical details of the biopsy procedure and the method of histological grading were as reported previously<sup>(6)</sup>.

#### Assay for enzymes and oligosaccharidases

Enterokinase activity was determined as originally described by Kunitz<sup>(7)</sup> in a micro modification of the method as reported by Hadorn et al<sup>(8)</sup>. The activation mixture consisted of 20 µl of homogenate (dilution, 1 mg of wet tissue per 100 µl of distilled water), 20 µl of 0.1 M sodium maleate pH 6.0, and 240 µl of distilled water. The mixture was preheated to 25°C, incubation temperature. The activation step of 30 minutes was initiated following the addition of 20 µl of trypsinogen solution (2 mg of trypsinogen per ml of 0.005 normal HCl) at 25°C. Trypsin formation was determined by the addition of 1.5 ml of 0,001 molar benzoyl-DL-arginine p-nitroanilide HCl as substrate (dissolved in dimethyl sulfoxide and diluted with 0.05 molar tris-(hydroxymethyl) aminomethane buffer, pH 8.2, and 0.02 M CaCl) to the reaction mixture incubated for 30 minutes at 25°C. The reaction was stopped by addition of 0.3 ml of 30% (v/v) acetic acid, mixed and read in a double-beam spectrophotometer (Hitachi 124 Japan) at 409 nm. A unit of enterokinase or trypsin activity was expressed as micromoles of substrate hydrolysed per gram of protein per minute at 25°C.

Alkaline phosphatase, oligosaccharidase and peptidase activities were measured as described by us previously<sup>(9,10)</sup>.

#### RESULTS

The clinical profile of the infants is summarised in Table I.

Table I - Clinical	features	of 20	infants	with	acute	and		
chronic diarrhoea.								

Clinical Parameters	Group 1	Group 2	Group 3
No. of patients	7	8	15
Age at initial admission (days)	70 ± 70	$88 \pm 40$	$180 \pm 72$
Sex ratio (M : F)	3:4	5:3	9:6
Ethnic group		•	
Malay	-	2	8
Chinese	6	2	3
Indian	1	4	4
Breast feed (< 30 days)	-	2	7
Birth weight (kg)	$2.9 \pm 0.4$	$3.0\pm0.5$	3±0.4
Duration of diarrhoea (days)	$12 \pm 3$	9.7 ± 3.8	4.9 ± 2.7

Group 1 consisted of 7 infants who had clinical and histological reaction to cow's milk protein provocation. The alkaline phosphatase, disaccharidase and peptidase were uniformly depressed in all 7 infants. However, the enterokinase activity was significantly depressed in 4 (24-70%), mildly depressed in 1 (12%) and elevated in 2. All the 4 infants who had significant depression of enterokinase also had histologically marked damage to the mucous villous surface with major reduction to goblet cell population. The one infant with mild depletion of enterokinase did not have damage to the goblet cell population.

Group 2 consisted of 8 infants who had histological changes but no clinical response to cow's milk protein challenge. The alkaline phosphatase activity was depressed in 4, unchanged in 1 (< 1%) and elevated in 2. The disaccharidases were significantly depressed in all 8 infants. The enterokinase activity was severely depressed in 1 (40%), mildly depressed in 3 (6-17%), not depressed in 1, and elevated in 2. The one infant who had severely depressed enterokinase activity in the biopsy also had histologically severe damage to the mucosa involving the goblet population.

Group 3 consisted of 15 infants who did not clinically or histologically react to cow's milk protein. Following cow's milk protein challenge, the alkaline phosphatase was depressed in 1, unchanged or elevated in 14; the disaccharidase activity was depressed in 4 and unchanged or elevated in 11; the dipeptidase was depressed in 2, unchanged or elevated in 14; the enterokinase activity was depressed in 3, unchanged or elevated in 12. There was no consistent pattern in change in enzyme activity of the different enzymes. In the three infants who had low mucosal enterokinase there were no histological alteration in the mucosa.

The morphological and enzymological changes are summarised in Table II.

# DISCUSSION

Enterokinase plays a key role in initiating proteolytic digestion by catalysing the conversion of the pancreatic trypsinogen into trypsin. Enterokinase is synthesised by the enterocytes in the proximal small intestine and is incorporated into the brush border membrane<sup>(11, 12)</sup>. Unlike most other brush border enzymes it is readily released into the intestinal lumen by the action of bile salts and pancreatic proteolytic enzyme<sup>(13, 14)</sup>. Deficiency of entcrokinase is associated with failure to activate pancreatic proteolytic enzymes and maldigestion of ingested protein. In situations of prolonged enterokinase deficiency, chronic diarrhoea associated with maldigestion and malabsorption of protein, with failure to thrive and hypoproteineamic edema occurs<sup>(1)</sup>.

In the present study, following cow's milk protein challenge, enterokinase activity was depressed in 9 of 15 infants with CMPSE (range 10% - 70%). As the mucosa recovered, the enzyme activity increased correspondingly although the relationship was not an absolute one in individual cases. However in comparison to the extent and severity of morphological changes, and depletion in the activities of the mucosal enzymes, disaccharidase, endopeptidases and alkaline phosphatase CMP challenge had much lesser damaging effect on enterokinase activity. This relative 'resistance' of enterokinase activity to CMP challenge, ensuring the uninterrupted intraluminal proteolysis of ingested proteins implies an important teleological advantage to the survival of the human young.

Enterokinase is a catalytic enzyme primarily involved in promoting the conversion of the inactive trypsinogen to its active component, trypsin. The quantum of enterokinase needed for this catalytic activity may be quite small compared to the amount of trypsin enzyme needed for its direct role in catalysing the hydrolysis of intraluminal proteins. Therefore it appears that enterokinase may not be a rate limiting factor in proteolytic hydrolysis. This has been borne out in clinical situations where chronic diarrhoea, maldigestion and malabsorption of protein, failure to thrive and hypo-proteinaemia were observed with almost complete absence of any enterokinase activity<sup>(1)</sup>.

 Table II - Changes in the mucosal anatomy and the levels of alkaline phosphatase, lactase, sucrase, amino peptidase A and enterokinase following cow's milk protein challenge in 30 infants.

Group No.	Histology <sup>(a)</sup>		Alkaline Phosphatase <sup>(b)</sup>		Lactase(c)		Sucrase <sup>(d)</sup>		Amino Peptidase <sup>(e)</sup>		Enterokinase <sup>(f)</sup>	
(No. of Patients)	Pre <sup>(h)</sup>	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Group 1	1.2	11.5	279	96.8	6.2	1.4	8.0	2.6	3.5	1.8	8.0	6.6
(7)	±1.8	±2,1	±85	±58	±2.9	±0.8	±3	±1.7	±1.6	±1.9	±4.3	±4.8
t-test	p<0.01		p<	0.01 p<0.01		p<0.01		p<0.01		NS		
Group 2	1.3	8.9	305	275	3.8	1.34	4.0	2,4	1.0	0.4	9.0	8,7
(8)	±1.5	±1.7	±123	±267	±2.5	±1.0	±1.6	±0.8	±0.2	±0.4	±4.2	±4,8
t-test	p<0.01		NS p<0.01		).01	p<0.05		p<	p<0.05		NS	
Group 3	2.1	2.7	250	283	4.7	4.6	7.3	8.4	2.0	2.5	9.5	9.8
(15)	±1.7	±2.1	<b>±1</b> 14	±114	±2.3	±3.1	±4.3	±5.3	±0.7	±0.9	±6.5	±5.3
t-test	N	Z(8)	j	NS	N	IS	N	IS	ſ	NS	Ν	IS

Group 1 : Clinical and histological reaction to cow's milk protein challenge.

Group 2 : Histological reaction without clinical symptoms to cow's milk protein challenge.

Group 3 : No histological or clinical reaction to cow's milk protein challenge.

(a) Mucosal surface graded in units, (6).

(b) Micromoles p-nitrophenyl phosphatase hydrolysed per minute per gram tissue protein.

(c) Micromoles per minute per gram wet weight.

(d) Micromoles per minute per gram wet weight.

(f) Micromoles substrate hydrolysed per gram protein per minute.

(g) Not significant.

(h) Pre- and post-challenge with cow's milk protein.

The immunogenicity of the allergenic proteins is dependent on the protein molecule maintaining its integrity and configuration by resisting digestion. These molecules then by attaching to the appropriate surface receptor on the enterocyte, trigger a sequence of events which lead to mucosal damage. The luminal and mucosal capacity to hydrolyse proteins constitutes an important protective barrier for the intestinal mucosa against antigenic proteins. Since enterokinase plays a key role in initiating proteolytic hydrolysis by catalysing the conversion of trypsinogen to trypsin, then from a teleological viewpoint the integrity of the mucosal enterokinase apparatus is important for maintenance of a normal mucosa and the preservation of its vital digestive and absorptive functions.

Our observation that the depletion in enterokinase activity in infants with symptomatic cow's milk protein-sensitive enteropathy did not parallel mucosal damage and depletion of disaccharidases, peptidases and alkaline phosphatase lends support to the above hypothesis. Significant enterokinase depletion (> 20%) occurred in only 5 of 15 infants with significant mucosal damage and depletion of disaccharidases, peptidases and alkaline phosphatase. Furthermore, the mean decrease in value for enterokinase was 17% compared to 60 - 68% for the other enzymes.

Other workers have made similar observations. Thus in untreated coeliac patients enterokinase activity was found to be normal despite the severe morphological atrophy and marked decrease in enzymological activities<sup>(14-16)</sup>. The location of enterokinase enzyme in the mucosal villi and its mechanics of release and site of action may provide a basis for understanding this enigma.

Takano et al<sup>(17)</sup> showed by fluorescent antibody technique that enterokinase is present in the goblet cells and is diffusely localised on the surface of the intestinal epithelium. The goblet cells discharge the enterokinase which is adsorbed to the brush border membrane from where it is further released into the upper small bowel lumen. Thus, within the villus, enterokinase is present within the goblet cells and on the brush border membrane. The total enterokinase activity will be equivalent to that in goblet cells and also the brush border membrane. Takano's observation<sup>(17)</sup> suggests that enterokinase activity in small bowel enteropathies may not only be dependent on the degree of mucosal damage, but more on the extent to which the goblet cell population is reduced.

Severe mucosal damage in acute and chronic diarrhoea is often associated with significant reduction of goblet cell population and also enterokinase activity, but in coeliac disease the mucosal damage is not associated with reduction of goblet cell population and the enterokinase activity remains intact.

In cow's milk protein-sensitive enteropathy, two types of immune reactions are generally observed following cow's milkprotein provovation. The symptoms may be mediated by IgE antibody (Type I) where severe mucosal damage occurs with symptoms appearing within 24 hours, or mediated by immune complex (Type III) where mucosal damage is associated with a slower onset of symptoms developing in 2 - 30 days. In the present study, 4 of the 7 infants in Group 1 had rapid onset symptoms consistent with a Type I reaction. The mucosal damage in all 4 infants was associated with global depletion of all mucosal enzymes assayed including enterokinase. It is possible the mucosal damage in Type I reaction is associated with reduction of globlet cells as in acute gastroenteritis and chronic diarrhoea<sup>(16)</sup>.

The other 3 infants developed a Type III reaction. In the latter 3 infants who suffered from the mucosal damage and the disaccharidase, it was found that peptidase depletion were not associated with a corresponding depletion of enterokinase. It is possible the mucosal damage here is similar to that in coeliac disease where there is damage to the enterocyte without reduction of the goblet cell population.

There is no satisfactory explanation for an observation of depletion of enterokinase activity in the 4 infants from Group 3 who were non reactors to cow's milk protein provocations. This observation suggests that cow's milk protein can cause depletion of enterokinase activity without causing demonstrable histological alteration under the light microscope. In these four infants the ingestion of cow's milk may have evoked rapid discharge of enterokinase by the goblet cells in the mucosa into the lumen, as a protective response to speedily digest and thus remove the allergenic milk protein. This situation would result in a functional depletion of enterokinase in the biopsy obtained following the milk protein challenge. No doubt the simultaneous estimation of enterokinase activities in the intestinal lumen and mucosa before and after cow's milk-protein provocation may provide definitive information in regard the role of enterokinase in promoting antigenic exclusion in the gut. The present study nevertheless shows that the enterokinase system forms an effective mechanism of antigen exclusion in a subset of infants with diarrhoca.

# ACKNOWLEDGEMENTS

The research was funded (Grant No. 3/087/01) by the Ministry of Science, Technology and the Environment, Malaysia.

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