SHORT COMMUNICATION

Extracellular Protease Activity of Enteropathogenic
Escherechia coli on Mucin Substrate

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Enteropathogenic *Escherichia coli* (EPEC) causes gastrointestinal infections in human. EPEC invasion was initiated by attachment and aggressive colonization on intestinal surface. Attachment of EPEC alter the intestine mucosal cells. Despite this, the pathogenic mechanism of EPEC infectior has not been fully understood. This research hypothesizes that extracellular proteolytic enzymes is necessary for EPEC colonization. The enzyme is secreted into gastrointestinal milieu and presumably destroy mucus layer cover the gastrointestinal tract. The objective of this study was to assay EPEC extracellular protease enzyme by using mucin substrate. The activity of EPEC extracellular proteolytic enzyme on 1% mucin substrate was investigated. Non-pathogenic *E. coli* was used as a negative control. Positive and tentative controls were *Yersinia enterocolitica* and *Salmonella*. Ten EPEC strains were assayed, seven of them were able to degrade mucin, and the highest activity was produced by K1.1 strain. Both positive and tentative controls also showed the ability to digest 0.20% mucin.

Key words: EPEC, protease, mucin, diarrhea

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Escherichia coli is normal flora in human gastrointestinal track, however, some strains may cause several diseases including diarrhea. Frankel *et al.* (1995) stated that the main etiology of diarrhea diseases in developing countries is *enteropathogenic E. coli* (EPEC) that alter cell structure of the absorptive intestine by disturbing water, electrolyte, and nutrition absorption. The damage enables EPEC to penetrate into intestine epithelial cells and block the absorption process (Salyers & Whitt 1994).

EPEC adhere to the outer epithelial or intestinal mucosa. Mucosa surface coats by mucus layer, mainly compose of mucin, a 2×10^6 kD glycoprotein polymer (Bell *et al.* 1985). The effectiveness of mucosa layer as *in vivo* natural shield depends on the structure and thickness of the layer.

Several pathogenic bacteria, such as *Clostridium* RS 42, *Bacteroides* RS 2, *Bacteroides* RS 13, *Yersinia enterocolitica* is able to degrade mucin (Stanley *et al.* 1986; Mantle & Rombough 1993). *Candida albicans* was reported by Colina *et al.* (1996) produce aspartil protease that degraded mucin as well. However, mucin degradation by EPEC protease has not been reported, as yet. The objective of this study was to assay EPEC extracellular protease enzyme by using mucin substrate.

EPEC strains used in this study were collection of Biomedic Laboratory, Center of Biotechnology and Biological Research, Bogor Agricultural University. EPEC RB1.2 produced extracellular protease (Priyanto 1997). *Yersinia enterocolitica*

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as a positive control was collected of PT. Bio Farma (Persero), Bandung. *Salmonella* sp. and non-pathogenic *E. coli* as tentative and a negative control, were collected from Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University.

EPEC RB1.2 and three bacterial controls were grown in skim milk broth minimal (SMB Min) media. This media contained of 2% skim milk, 2% NaCl, and 0.5% yeast extract. The inocula were incubated at 37 °C and were shaked at 100 rpm for 12 hours. A number of 1×10^9 cells/ml were inoculated to SMB Min media and were incubated at 37 °C (shaked 100 rpm for 15 hours). The culture was then centrifuged at 4000 x g in 4 °C for 10 minutes to obtain the crude extract of protease enzyme (Priyanto 1997).

Protease activity of crude extract RB1.2 and 3 from the bacterial controls were measured in 1% casein substrate by using modified method of Walter (1984). One unit enzyme activity (U/ml) was equal to the amount of enzyme that produced 1 μmol tyrosine product per one minute in optimum condition. Protein concentration was measured using Bradford method (1976) and used bovine serum albumin (BSA) as the standard. Specific activity was determined as protease activity unit divided by the protein concentration.

Protease activity of RB1.2 was measured to obtain optimum mucin substrate concentration which was ranged from 0.50 up to 1.50% (Sigma M-2378) and 0.25 up to 1.00% for casein substrate as the control. Protease activity of ten EPEC strains was assayed in optimum substrate concentration, each in three

replicates, and tested by Tukey's pairwise comparisons value at α = 0.05.

EPEC RB1.2, *Salmonella*, and *Y. enterocolitica* showed protease activity in 1% casein substrate at 0.005, 0.020, and 0.021 U/ml (Figure 1). However, there was no protease activity showed in non-pathogenic *E. coli* as a negative control. Protease activity of RB1.2 showed higher activity in 0.5% casein substrate compared to the 0.25 and 1% (Figure 2). Moreover, RB1.2 showed its highest protease activity in 1%
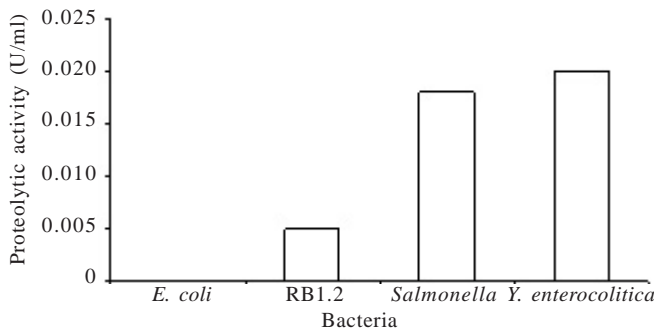

Figure 1. Protease activity of EPEC RB1.2 compare to non-pathogenic *E. coli* (negative control), *Salmonella* (tentative control), and *Y. enterocolitica* (positive control) in 1% casein substrate. The activity were assayed at 37 ℃, pH 8.
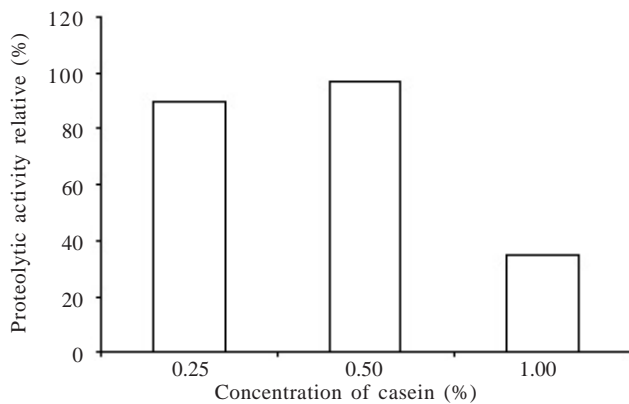

Figure 2. Protease activity of EPEC RB1.2 measured in several casein concentrations. The activity were assayed at 37 ℃, pH 8.
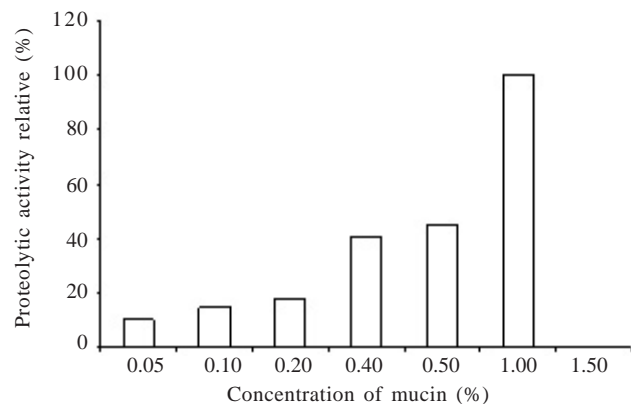

Figure 3. Protease activity of EPEC RB1.2 measured in several concentrations of mucin. The activity were assayed at 37 ℃, pH 8.

mucin substrate (Figure 3), and there was no protease activity in higher than 1% mucin.

The ability of *Y. enterocolitica* and *Salmonella* to hydrolyze mucin was compared to RB1.2 in several concentrations. The highest concentration of protease activity in mucin substrate of *Y. enterocolitica* and *Salmonella* was in 0.2% mucin on each 0.040 U/ml (Figure 4). There was no protease activity detected by using 0.5 and 1% mucin substrates. Isolate RB1.2 still showed its 20% protease activity relative on 0.2% mucin substrate.

Specific activity of ten EPEC strains and non-pathogenic *E. coli* as a negative control were assayed by using mucin and casein substrates. Different substrates produced specific activity (α = 0.05) (Table 1) as showed in seven strains of EPEC (K1.1, PC1.4, D4.4, I2.5, RB1.2, D14.5, and 0065). They showed different results compared to the negative control in 1% mucin substrate. Only PC1.4 and K1.1 showed a different response with control by using 0.5% casein substrate. EPEC K1.1 showed the highest specific activity (0.73 U/mg) in 0.5% mucin substrate, but PC 1.4 strain (0.667 U/mg) was the highest in 1% casein.

Non-pathogenic *E. coli* as a negative control did not show protease activity by using 1% casein substrate. However, positive control bacteria (*Y. enterocolitica*) and tentative
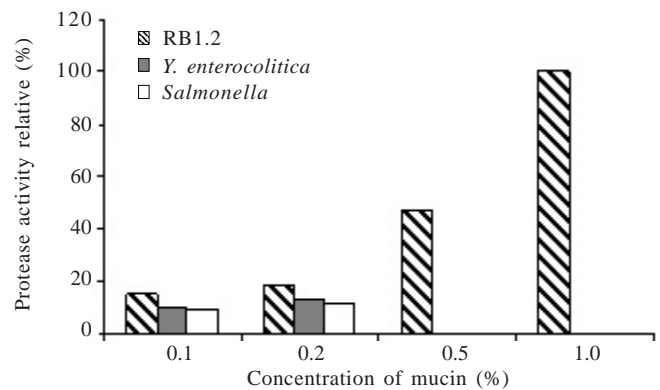

Figure 4. Protease activity of EPEC RB1.2 compared to *Salmonella* and *Y. enterocolitica* at several mucin concentrations. The activity were assayed at 37 ℃, pH 8.

Table 1. Specific activity of protease EPEC to 1% mucin substrat and 0.5% casein incubated in pH 8, 37 ℃

EPEC strain	Specific activity in	
	mucin substrate (U/mg)	casein substrate (U/mg)*
K1.1	0.703a	0.185b
PC1.4	0.582a	0.667a
D4.4	0.528ab	0.056c
I2.5	0.450abc	0.107bc
RB1.2	0.254bcd	0.070c
D14.5	0.176cd	0.065c
0065	0.162d	0.042c
D11.2	0.053de	0.079bc
E12.A	0f	0.066c
D14.10	0f	0.065c
E. coli non-patogenic (Control -)	0f	0.051c

*The same alphabet followed the number in the same column was referred to the value that has no significant difference on α 0.05 Tukey's Test

control (*Salmonella*) revealed higher protease activity than that of RB1.2. *Yersinia enterocolitica* is a motile gram negative rod that cause acute gastroenteritis in human. The highest activity of casein degradation of *Y. enterocolitica* showed specificity protease activity to hydrolyze casein substrate which is the highest compound in milk protein.

The data of mucin substrate degradation might showed that protease activity of EPEC in mucin substrate was higher than those of *Y. enterocolitica* and *Salmonella* (Figure 4). The capability of mucin degraded by EPEC *in vivo* presumably assist EPEC to perform an aggressive colonization to the intestine surface in the first step of pathogenesis. The EPEC extracellular protease enzyme was proposed to be one of EPEC virulence factors (Salyers & Whitt 1994). Further research need to be performed to answer the whole assumption, since *in vivo* condition of gastrointestinal lumen is much more complex than those of *in vitro*.

In several cases, *in vitro* condition is more conducive for bacteria growth, because it was treated in optimum pH and there is no nutrition competitor among pathogen bacteria as well as the normal flora. There is no mucosa activity such as peristaltic activity or cilia impulse that allows foreign material eliminate from mucosa support pathogen growth *in vitro,* as well.

Grange *et al.* (1998) studied the adherence mechanism of *Enterotoxigenic E. coli* (ETEC) to the intestine epithelium. The result showed that the receptor of host intestinal epithelium were *Intestinal Mucin-Type Glycoprotein* 1 (IMTGP 1) and IMTGP 2. *Entroaggregative E. coli* (EAEC) was known able to adhere to mucosa tissue by using *in vitro* organ culture method (Czeczulin *et al.* 1997). This adhesion caused hyper secretion mucus on mucosa layer.

Mucosa layer consists of covalent and noncovalent chains. The later consists of glycoprotein polymer structure which were degraded by protease enzyme. However, the non-covalent chain is relatively stable to protease, hypertonic effect, and solvent such as acid and ethanol (Bell *et al.* 1985).

Besides protease, glycosidase might degrade mucin as well. However, study on *Y. enterocolitica* by Mantle and Rombough (1993) showed that carbohydrates component of mucin, i.e. galactose and acetyl galactosamine labeled with radioisotope ^{3}H was not degraded into smaller molecules. This result supported the assumption that protease is the main enzyme that degraded mucin. Mantle and Rombough (1993) reported the ability of mucin degradation by *Y. enterocolitica* harbering plasmid was much higher than those without plasmid. Presumably, the enzyme involved in this process was supposed to be regulated by plasmid.

Protease activity of another EPEC RB1.2 (K1.1 and PC1.4 isolates) showed high hyolrolysis activity in casein and mucin. Hence, both isolates are interesting to be studied in molecular aspect, such as characterization of mucin encoding gene.

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