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Isolation and Characterization of Mucinase Complex Secreted from *Vibrio parahaemolyticus*

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Abstract Mucinase complex from *V. parahaemolyticus* ATCC 17802 was purified 6-fold with 0.4% yield by two sequential steps of Q-Sepharose and Superdex 200HR column chromatographies. Partially purified mucinase complex showed at least 8 times higher mucin-degrading activity than the culture filtrates. The mucinase complex also showed gelatin- and casein-hydrolyzing activities, which demonstrates that the protein is a complex compound containing several proteases. The optimum pH and temperature of partially purified mucinase complex for mucin degradation was 8.0 and 35°C, respectively. The partially purified mucinase complex showed high cytotoxic activity on vero cells when examined by MTT assay and microscopic observations. Cytotoxicity was significantly increased in proportion to the concentration of the mucinase complex. Mouse experiments revealed that the jejunum, ileum, and large intestinal tissues were damaged by the injection of the mucinase complex. In particular, the reduction of the goblet cells in the large intestine was remarkable. Collectively, these data suggest that the mucinase complex partially purified from *V. parahaemolyticus* ATCC 17802 contributes to the adhesion and invasion of *V. parahaemolyticus* into the host intestinal tract.

Key words: Mucin, mucinase complex, cytotoxicity, *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a human pathogen primarily producing gastroenteritis and secondarily producing wound infections [14, 15, 21]. The major symptoms of *V. parahaemolyticus* food poisoning are diarrhea, abdominal cramps, and nausea [2, 12, 15, 24]. A few studies have demonstrated enterotoxic activities, epithlial cell adherence, or rabbit ligated ileal loop responses to *V. parahaemolyticus* [5, 6, 29]. No definitive virulence mechanism responsible for the clinical syndrome caused by *V. parahaemolyticus* has been proven [7, 12, 15]. The thermostable direct hemolysin (TDH) or Kanagawa hemolysin is well known, and is expected to play a major role in the pathogenesis of *V. parahaemolyticus* [2, 22, 23]. Although there is an excellent correlation between hemolysin and human disease, the mechanisms of pathogenicity are still being debated, since other virulence factors such as lethal toxins [25], enzymes [14], adhesins [6], lipopolysaccharides [27], and vascular permeability factors [13], have been demonstrated to be associated with the pathogenicity of this species. It has been reported that some correlation between the production of extracellular enzymes and the toxicity among various *Vibrio* species are being observed [16, 17, 19]. In a previous study, there appeared to be a strong correlation between mucin-degrading activity and pathogenicity [17]. The mucinase activity was most distinct among extracellular enzymes tested in addition to the proteases.

Mucinase is usually isolated as a complex and this complex contains neuraminidase, endo-β-N-acetylhexosaminidase, nicotinamide-adenine-dinucleotidase, and proteinases [28]. Numerous studies have suggested that the mucin is the critical factor in the initiation of pathogenic bacterial infection by degrading the epithelial mucous layers in the respiratory or intestinal tract of host cells. Schneider and Parker [26] suggested that the mucinase complex facilitates *V. cholerae* penetration of the mucous barrier and that it may attack the glycosidic linkage between N-acetyl galactosamine and D-galactose in the monosialoganglioside GM1 and increases the receptivity for *V. cholerae* enterotoxin molecules. Gascoyne and Van Heyningen [11] and Ackerman *et al.* [1] attributed the conversion of membrane gangliosides to enterotoxin-binding gangliosides to the action of neuraminidase. The mucinase has also been studied for the development of an acellular cholera vaccine in view of its potential use as an immunogen [10].

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Therefore, the mucinase could affect *Vibrio parahaemolyticus* pathogenesis by suppressing the normal intestinal flora, thus allowing *Vibrio* to initiate infection. The degradation of the intestinal mucin might assist *Vibrio* in penetrating the mucous barrier and reaching the intestinal epithelial cell surface to which they adhere soon after infection. The degradation of mucus might also help provide nutrients for the *Vibrio* that infect the intestine.

Previously, the detection of the mucinase activity from the culture filtrates of *V. parahaemolyticus* was reported [18]. In this study, the mucinase complex was partially purified, characterized, and its pathogenic properties were examined *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial Strain and Culture Medium

Vibrio parahaemolyticus ATCC 17802 was incubated with shaking for 30 h at 37°C in 5 l of brain heart infusion (Difco, Detroit, MI, U.S.A.) medium supplemented with 1.5% NaCl and 10% polypeptone.

Partial Purification of Mucinase Complex

The culture medium was centrifuged at 10,000 rpm for 15 min at 4°C in a JA-10 rotor (Beckman) to harvest the cells. The supernatant was then brought to 70% saturation with ammonium sulfate and allowed to stand for 18 h at 4°C. The ammonium sulfate precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4°C, then resuspended in 20 mM Tris-HCl buffer (pH 7.5). The solution was dialyzed against the same buffer. The dialysate was loaded onto a Q Sepharose ion exchange column (2.5× 10 cm, Pharmacia Biotech., Uppsala, Sweden) equilibrated with the same buffer, and then eluted with a step gradient of 1 M NaCl. The fractions containing the mucin-degrading activity were pooled, concentrated, and applied again to the same column. The eluted proteins were further purified using a Superdex 200 gel filtration column (1.0×30 cm, Pharmacia Biotech., Uppsala, Sweden) by FPLC at a flow rate of 0.5 ml/min. The absorbance at 280 nm and protein concentration by Lowry's method were determined for each column fraction. Mucinase assays were also done on each fraction. The fractions with the enzyme activity were pooled, concentrated with ultrafiltration membrane YM10 (Amicon), and used as partially purified enzyme solution for further analysis.

Mucinase Assay

Bovine submaxillary mucin (0.5 g, Sigma) was dissolved in 50 ml of 20 mM Tris-HCl buffer (pH 7.5). The partially purified *V. parahaemolyticus* mucinase (1 ml) was added to 1 ml of the mucin solution and incubated at 37°C for 30 min. The reaction was stopped by adding 3 ml of 10% (w/v) trichloroacetic acid and incubated at 45°C for 15 min. Undigested mucin was removed by centrifugation at 1,200 ×g for 10 min and absorbance of the supernatants at 280 nm were recorded. The amount of N-acetylneuraminic acid (NANA, Roche, Indianapolis, IN, U.S.A.) released from the mucin was determined by reference to a standard curve of NANA. *V. cholerae* neuraminidase (Roche, Indianapolis, IN, U.S.A.) was used as a positive control. One unit of enzyme is defined as the amount to release 1 μmole of NANA from mucin per minute under the described conditions.

Analytical Methods

The protein concentration of the partially purified mucinase complex was determined by the Bradford method [4]. A dye reagent was purchased from Bio-Rad, and a bovine serum albumin (Sigma) was used as a standard protein. The prepared protein samples were analyzed by SDS-PAGE as described by Laemmli [20]. Electrophoresis was carried out with a Mini-PROTEAN 3 system (Bio-Rad, Hercules, U.S.A.). After running at 150 V, the gel was stained with a staining solution [1% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid] for at least 30 min with gentle shaking and then destained with a destaining solution [10% (v/v) methanol, 10% (v/v) glacial acetic acid] to visualize the protein.

Cytotoxicity Assay

Vero cells (Kidney of the Africa green monkey) were cultured at 37°C in a 5% CO_2 incubator using DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum. After removal of media, the cells were washed twice with phosphate-buffered saline and were treated with 2 ml of trypsin-versin solutions. The cells were harvested and 96-well tissue culture plates (Corning Costar Corp, Cambridge, MA, U.S.A.) were seeded with 2×10^5 cells/ml. Separated cells were cultivated for 18-24 h, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, St, Louis, U.S.A.) [7, 30] assay was conducted to detect the toxicity of the partially purified mucinase. Vero cells (2×10^4 cells/0.1 ml per well) were aliquoted on a 96-well tissue culture plate. Initial concentration of 10 μg/0.1 ml partially purified mucinase was sequencially diluted to 0.62 μg/0.1 ml, and 100 μl of mucinase was added to the cell, and cultured 5% CO_2 incubator at 37°C for 24 h. A 120 μl supernatant of the reaction mixture was removed and 100 μl of MTT solution, which was diluted in DMEM, was added and cultivated 5% CO_2 incubator at 37°C for 4 h. One hundred μl of dimethylsulfoxide (Sigma, St. Louis, U.S.A.) were added to the reaction mixture and agitated for 20 min. After the reaction was completed, conversion of MTT to formazan was quantified by measuring absorbance at 570 nm with subtraction of background absorbance at 650 nm. The relative cytotoxicity was

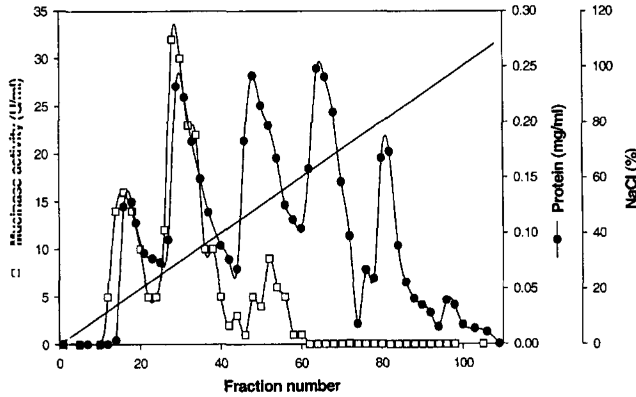

Fig. 1. Second anion exchange chromatography of mucinase complex from *V. parahaemolyticus* ATCC 17802.
Elution profile represents chromatography performed on a column of Q-Sepharose. A column (2.5×10 cm, Pharmacia Biotech., Uppsala, Sweden) was equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with 0–1.0 M NaCl linear gradient. Flow rate was 0.5 ml/min.

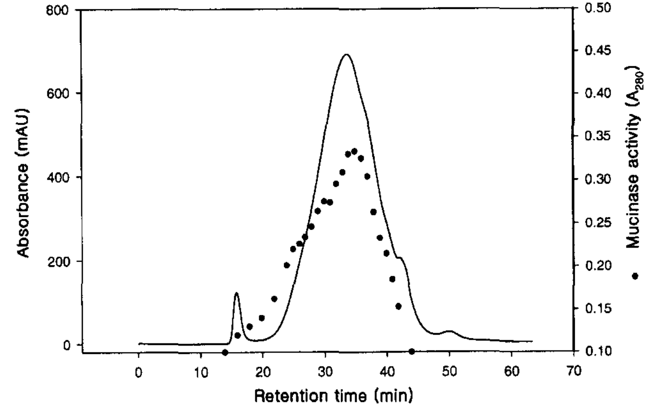

Fig. 2. Gel filtration chromatography of mucinase complex.
Elution profile represents chromatography performed on a column of Superdex 200. A column (1.0×30 cm, Pharmacia Biotech., Uppsala, Sweden) was equilibrated and eluted with 20 mM Tris-HCl buffer (pH 7.5). Flow rate was 0.5 ml/min.

calculated by subtracting from a value of 1.0 the fraction of total activity detected in a monolayer of cells treated with mucinase relative to control monolayer treated with DMEM.

Mouse Experiments

Six to seven week-old female BALB/C mice were used for the virulence test. The mice (BALB/C, n=3, 20±2 g) were given food and water in an environmentally controlled room with a 12 h cycle of light and dark. Mice were infected in the abdominal cavity by injecting 50 µg and 200 µg of the partially purified mucinase. After 7 days, mice were sacrificed and the entire jejunum, ileum, and large intestine were isolated, fixed in 10% buffered formaline and then embedded in paraffin. Longitudinal 4-µm sections were cut and stained with hematoxylin and eosin to visualize the cells. The control group was not injected with mucinase complex.

RESULTS AND DISCUSSION

Production and Partial Purification of Mucinase Complex from *Vibrio parahaemolyticus* ATCC 17802

The mucin-degrading activity was obtained from *Vibrio parahaemolyticus* ATCC 17802 with the brain heart infusion medium. The enzyme activity reached its maximum after 30 h incubation at 30°C. Because the purification method of mucinase complex was not established yet, this complex was partially purified by using Q-Sepharose and Superdex 200 column chromatographies. The enzyme activity was extremely unstable in culture filtrates. However, the precipitation of the proteins by 70% ammonium sulfate made the detection of mucinase activity stable. After overnight precipitation, the saturated protein was collected and resuspended in a total of 80 ml of 20 mM Tris-HCl (pH 7.5). The resuspended protein was concentrated (150 ml) and chromatographed on a Q-Sepharose Fast Flow. The enzyme activity was spread over the fractions. The second Q-Sepharose chromatography narrowed down the enzyme activity between 0.3 M and 0.4 M NaCl (Fig. 1). The broad distribution of the enzyme activity in more than one peak is due to the presence of the mucinase in various enzyme fractions such as neuraminidase and proteinases [28].

Further purification by Superdex filtration removed a large amounts of low-molecular weight materials. The major peak of the mucinase complex was observed (Fig. 2). The complete separation of the protein containing mucinase activity from other extracellular proteins was difficult. The difficulty of the purification of the mucinase complex is most likely due to the special character of the complex structure. Further attempts to purify by chromatography produced

Table 1. The partially purification steps of mucinase from *V. parahaemolyticus* ATCC 17802.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrates	200	10,000	50	1	100
70% ammonium sulfate fractionation	45	4,500	100	2	45
1st Q-Sepharose	7.5	1,100	148	3	11
2nd Q-Sepharose	1	250	250	5	2.5
Superdex 200	0.13	39.3	302	6	0.4

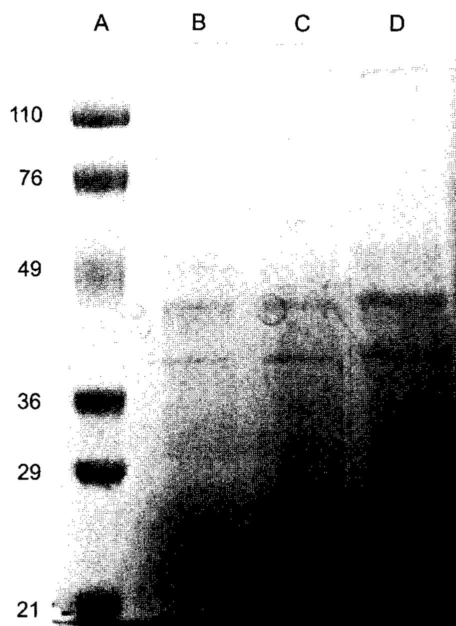

Fig. 3. SDS-PAGE of partially purified mucinase complex.
A. Standard protein mixture; B- D. Partially purified mucinase. Estimated molecular weights (10^3) shown against standard proteins.

fractions with much lower enzyme activity, therefore, were not further pursued. After the completion of four purification steps, the mucinase complex was purified 6-fold and the yield was 0.4% (Table 1). Active fractions on SDS-PAGE gels revealed two major bands which had the molecular weight of about 45,000 and 38,000 Da, respectively (Fig. 3). This molecular size is quite close to the reported value of mucinase isolated from *Vibrio cholerae* [26].

Enzymatic Characterization of the Partially Purified Mucinase Complex

The mucin-degrading activity of partially purified mucinase was examined and compared with that of culture filtrates. The enzyme activity of the partially purified mucinase (17 U/ml) was 8 times greater than that of the culture filtrates (2 U/ml). The enzyme was also active toward all of the protein substrates examined, including gelatin,

Table 2. Substrate specificity of the culture filtrates and partially purified mucinase from *V. parahaemolyticus* ATCC 17802.

Substrate	Culture filtrates (U/ml)	Partially purified mucinase (U/ml)
Mucin	2.0	17
Gelatin	1.2	15
Bovine serum albumin	1.0	12
Skim milk	0.9	10
Casein	0.5	8
Elastin	1.5	ND

ND: not detected.

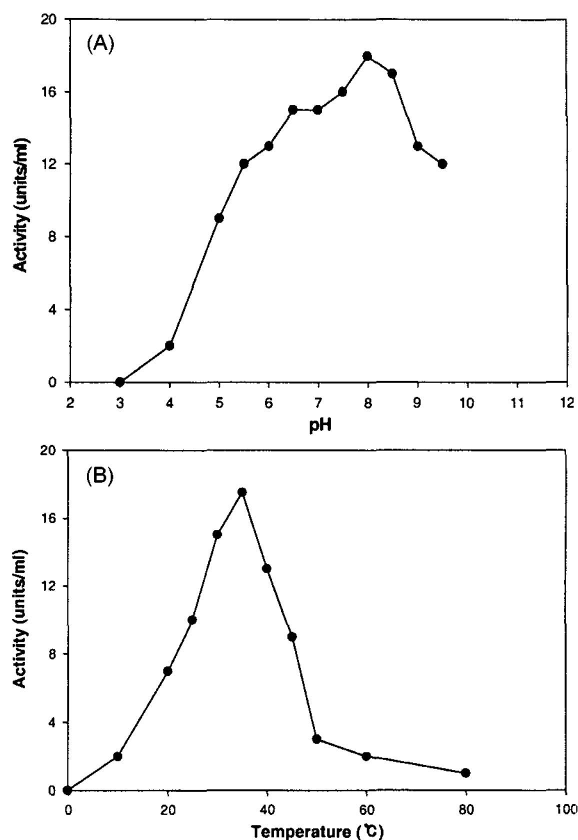

Fig. 4. Effect of pH and temperature on the mucin-degrading activity of the partially purified mucinase.
The mucinase activity for optimum pH (A) was examined using 20 mM buffers in the pH range 4.0-9.0. Buffers used are acetate buffer (pH 4-5.5), phosphate buffer (pH 6.0-7.0), Tri-HCl buffer (pH 7.5-8.5), boric acid/NaOH buffer (pH 9.0) at 37°C for 30 min. For the determination of the optimum temperature (B), the partially purified mucinase was incubated at various temperatures for 30 min. The enzyme activity was examined with mucin solution as described in Materials and Methods.

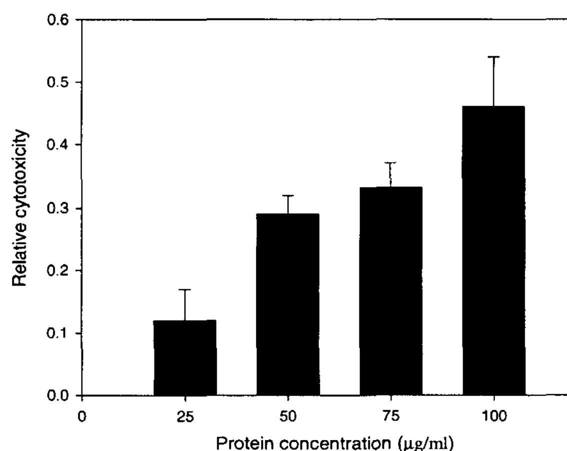

Fig. 5. Effect of the mucinase complex on the cytopathology of *V. parahaemolyticus* in Vero cells.
The relative cytotoxicity of each sample was determined versus control cells treated with an equal volume of DMEM, as described in Materials and Methods. The error bars indicate standard deviations.

bovine serum albumin, skim milk, and casein (Table 2). It was not surprising that the mucinase had proteolytic activity. The mucinase from *V. cholerae* also contained proteolytic activity and in fact was revealed as an alkaline protease [26]. The enzyme degraded both proteins and glycoproteins. Finkelstein *et al.* isolated a hemagglutinin from *V. cholerae* that showed protease activity [9]. It also occurred in several molecular forms. These previous results imply that the various proteolytic activities in purified mucinase are due to self-aggregation. Only the slight increase of specific activity after the serial purification steps may have resulted from autodigestion, a property that is common among protease. Autodigestion or self processing might have also been responsible for two active molecular weight forms that were found.

To examine the effect of pH and temperature stability of the mucinase complex, the partially purified mucinase complex was assayed for bovine submaxillary mucin degradation at various pH levels. The activity was determined using 100 mM of various buffers under the standard assay condition. The buffers used were acetate buffer (pH 3 to 5.5), phosphate buffer (pH 5 to 7), Tris-HCl buffer (pH 7 to 9),

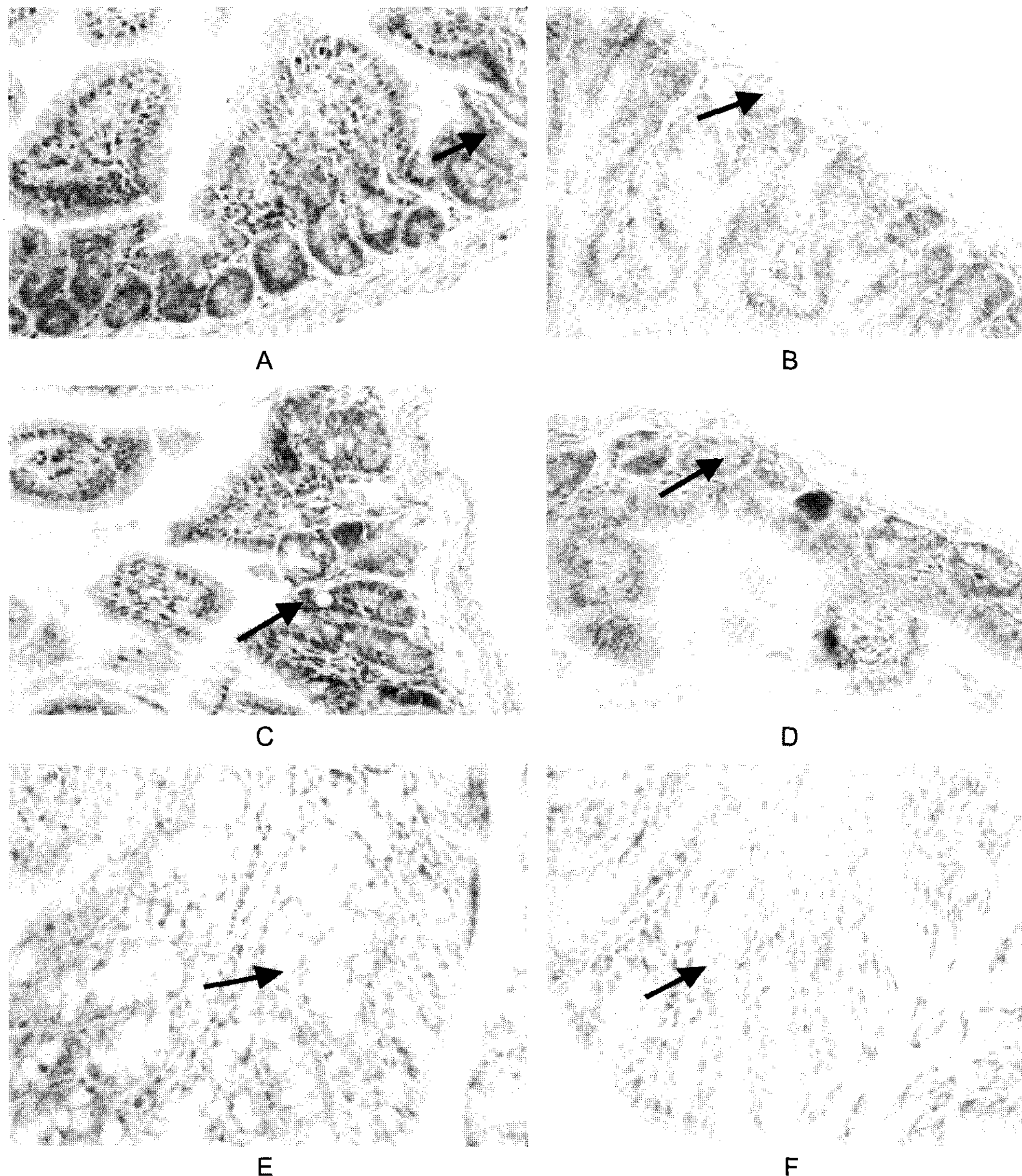

Fig. 6. Histopathology of goblet cells in an intestinal tissue of the mouse.
Arrows indicate the goblet cell of each tissue. (A) Normal jejunum tissue, (B) jejunum tissue after injection of mucinase, (C) normal ileum tissue, (D) ileum tissue after injection of mucinase, (E) normal large intestinal tissue, (F) large intestinal tissue after injection of mucinase.

boric acid/NaOH buffer (pH 9 to 10.5), and Na_2HPO_4/NaOH buffer (pH 10.5 to 12). The enzyme was most active at pH 8, although the preparation showed the activity over a pH range of 5.5-9.5 (Fig. 4A). The effect of temperature on enzyme activity was examined by the standard method, except that the temperature was changed between 0 to 80°C. The optimum temperature for the mucin degradation was 35°C (Fig. 4B).

Pathogenicity of the Partially Purified Mucinase Complex

To test the effects of *V. parahaemolyticus*-derived mucinase on mammalian cells, the partially prepared enzymes were applied to Vero cells and incubated at 37°C for 24 h. The enzyme complex caused an overall morphological change of the Vero cells, observed by microscopy (data not shown). At lower concentrations (6 µg), the cells became markedly distended and elongated, while at higher concentrations (52 µg), significant rounding and burst of individual cells were observed. Cytotoxicity towards Vero cells was quantified using the well-established MTT assay, as described in Materials and Methods. Figure 5 shows that the mucinase complex demonstrated a dose-dependent cytotoxicity to Vero cells. In contrast, identical concentrations of culture filtrates and DMEM medium (negative control) did not induce morphological changes and were less cytotoxic towards Vero cells. This result indicates that the partially purified mucinase is strongly involved in *Vibrio* pathogenicity.

Although the mucinase complex showed the cytotoxic activity in Vero cells, *in vivo* experiment is necessary to verify the pathogenic effect of the mucinase complex. Therefore, a mouse model experiment was performed. After dilution with phosphate-buffered saline solution, 200 µg of partially purified mucinase was injected into the abdominal cavity of six-week-old mice. The movement of the mice injected with the mucinase complex was remarkedly weakened during one week of observation. After 7 days, the enzyme-treated mice were sacrificed, and jejunum, ileum, and large intestine were isolated and examined for any changes in the number and the size of the goblet cells in each intestinal tissue. The remarkable decrease of goblet cells was apparent in the large intestinal tissue (Fig. 6), and the shrinkage of the goblet cells was also observed. The viscous materials, which are composed of acidic glycoproteins secreted from the goblet cells, protect the epithelial surface from various pathogens by protecting barriers of microvilli of columnar cells. Therefore, the decrease of the goblet cells by mucinase may assist vibrios in penetrating the mucous barrier and reaching the intestinal epithelial cell surface to which they adhere soon after infection. Previous relevant study of *V. cholerae* mucinase suggests that the enzyme was important for induction of the disease. They demonstrated the protection against cholera-associated diarrhea in mice by antibody raised against purified mucinase [26]. The present results suggest that the mucinase may play an important role in the adhesion and invasion of vibrios into the host intestinal tract. Additional investigation for detailed biochemical and pathological studies of mucinase is necessary to assess the role of this mucinase complex in *V. parahaemolyticus* pathogenesis within the host.

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REFERENCES

1. Ackerman, G. A., K. W. Wolken, and F. B. Gelder. 1980. Surface distribution of monosialoganglioside GM1 on human blood cells and the effect of exogenous GM1 and neuraminidase on cholerae toxin surface labeling. A quantitative immunocytochemical study. *J. Histochem. Cytochem.* **28:** 1100-1112.
2. Baba, K., H. Shirai, A. Terai, Y. Takeda, and M. Nishibuchi. 1991. Analysis of the *tdh* gene cloned from a *tdh* gene- and *trh* gene-positive strain non-O1 and *Vibrio parahaemolyticus*. *Microbiol. Immunol.* **35:** 253-258.
3. Beuchat, L. R. 1982. *Vibrio parahaemolyticus*: Public health significance. *Food Technol.* **36:** 80-88.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
5. Carruthers, M. M. 1977. *In vitro* adherence of Kanagawa positive *Vibrio parahaemolyticus* to epithelial cells. *J. Infect. Dis.* **136:** 588-592.
6. Chakrabarti, M. K., A. K. Sinha, and T. Biswas. 1991. Adherence of *Vibrio parahaemolyticus* to rabbit intestinal epithelial cells *in vitro*. *FEMS Microbiol. Lett.* **84:** 113-118.
7. Chung, S. S., Y. U. Shin, H. J. Kim, G. H. Jin, and H. H. Lee. 2001. Transformation of an alkaline protease overproducer, *Vibrio metschnikovii* strain RH530, and improvement of plasmid stability by the *par* locus. *J. Microbiol. Biotechnol.* **11:** 222-228.
8. Denizot, F. and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89:** 271-277.
9. Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. C. Häse. 1992. *Vibrio cholerae* hemagglutinin/protease,

clonial variation, virulence, and detachment. *Infect. Immun.* **60**: 472-478.

10. Formal S. B., E. H. Labree, and H. Schneider. 1965. Pathogenesis of bacillary dysentery in laboratory animals. *Federation Proc.* **24:** 29-34.
11. Gascoyne, N. and W. E. van Heyningen. 1979. Unmasking of actual and potential receptor sites for cholerae toxin in intestinal mucosal homogenates. *J. Infect. Dis.* **142:** 235-236.
12. Hackney, C. R. and A. Dicharry. 1988. Seafood-borne bacterial pathogens of marine origin. *Food Technol.* **42:** 104-109.
13. Honda, T., M. Shimizu, Y. Takeda, and T. Minatani, 1976. Isolation of a factor causing morphological changes of Chinese hamster ovary cells from the culture filtrate of *Vibrio parahaemolyticus*. *Infect. Immun.* **14:** 1028-1033.
14. Janda, J. M., C. Povers, R. G. Bryant, and S. J. Abbott. 1988. Current perspecting on the epidemiology and pathogenesis of clinical significant *Vibrio* sp. *Clin. Microbiol. Rev.* **1:** 245-267.
15. Joseph, S. W., R. R. Cowell, and J. B. Karper. 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. *CRC Crit. Rev. Microbiol.* **19:** 77-124.
16. Kim, B. J., H.-J. Kim, S.-H. Hwang, S.-K. Bae, S.-D. Ha, J.-D. Kim, and J.-Y. Kong. 1998. Cloning and expression of a collagenase gene from the marine bacterium *Vibrio vulnificus* CHK279H. *J. Microbiol. Biotechnol.* **8:** 245-250.
17. Kim, Y., C. R. Jung, S. K. Kim, J. Y. Yang, and J. Cha. 2001. Distribution of extracellular proteases from *Vibrio* species. *J. Korean Soc. Food Sci. Nutr.* **30:** 222-227.
18. Kim, Y. H. and J. Cha. 2002. Development of a rapid spectrophotometric method for detecting bacterial mucinase complex. *J. Microbiol. Biotechnol.* **12:** 345-348.
19. Kregger, A. and D. Lockwood. 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. *Infect. Immun.* **49:** 533-590.
20. Laemmli, U. K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 630-685.
21. Lee, J. H., K.-H. Lee, and S. H. Choi. 2001. Enumeration of *Vibrio vulnificus* in natural samples by colony blot hybridization. *J. Microbiol. Biotechnol.* **11:** 302-309.
22. Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. *In vitro* hemolytic characteristic of *Vibrio parahaemolyticus*: Its close correlation with human pathogenicity. *J. Bacteriol.* **100:** 1147-1149.
23. Nichibuchi, M. and J. B. Karper. 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: A virulence gene acquired by a marine bacterium. *Infect. Immun.* **49:** 181-186.
24. Park, J.-H., J.-H. Lee, Y.-S. Kim, Y.-K. Hong, and I.-S. Kong. 2001. Molecular cloning and expression of a sodium-driven flagellar motor component gene (*motX*) from *Vibrio fluvialis*. *J. Microbiol. Biotechnol.* **11:** 973-978.
25. Sarkar, B. L., R. Kumar, S. P. De, and S. C. Pal. 1987. Hemolytic activity and lethal toxin production by environmental strains of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* **53:** 2696-2698.
26. Schneider, D. R. and C. D. Parker. 1982. Purification and characterization of the mucinase of *Vibrio cholerae*. *J. Infect. Dis.* **145:** 474-482.
27. Sochard, M. R. and R. R. Colwell. 1976. Tolerance and other biological properties of *Vibrio parahaemolyticus* endotoxins. *Jpn. J. Microbiol.* **20:** 309-319.
28. Stewart-tull, D. E. S., R. A. Ollar, and T. S. Scobie. 1986. Studies on the *Vibrio cholerae* mucinase complex. I. Enzymic activities associated with the complex. *J. Med. Microbiol.* **22:** 325-333.
29. Twedt, R. M., J. T. Peeler, and P. L. Spaulding. 1980. Effective ileal loop dose of Kanagawa-positive *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* **40:** 1012-1016.
30. Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Chrisity, W. D. Watkins, and J. G. Jr. Morris. 1993. Rapid identification of *Vibrio vulnificus* on non-selective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl. Environ. Microbiol.* **59:** 541-546.