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Degradation of Collagens, Immunoglobulins, and Other Serum Proteins by Protease of *Salmonella schottmülleri* and its Toxicity to Cultured Cells

Byoung-Kuk Na, Moon-Bo Kim[1], and Chul-Yong Song*

Department of Biology, College of Natural Sciences, Chung-Ang University, Seoul 156-756, and [1]National Institute of Health, Seoul 122-020, Korea

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The effect of the extracellular protease of *Salmonella schottmülleri* on human serum constituents such as immunoglobulins, hemoglobin and lysozyme and tissue constituents such as fibronectin and collagens was investigated. This protease degraded collagens (type I and III), fibronectin and serum proteins such as human hemoglobin and lysozyme. Bovine serum albumin was degraded slightly. Thus, the present study suggested the possibility that this protease is not only played an important role in invasion of *S. schottmülleri* by degrading the constituent proteins such as collagens and fibronectin but also induced complications observed in septicemia and chronic infections by degrading the serum proteins. This protease is also capable of degrading defence-oriented humoral proteins, immunoglobulins (IgG and IgM). Furthermore, it is toxic to HEp-2 cells. These findings clarified the possible role of *Salmonella* protease as a virulence factor in the pathogenesis of *Salmonella* infections.

Key words: Degradation, *Salmonella schottmülleri*, protease, toxicity, HEp-2 cells

Many different species of bacteria produce extracellular proteases whose activities are often considerably related with pathogenicity (4, 5, 10). Bacterial proteases exhibit their pathogenicity through several molecular mechanisms (8). One of these mechanisms, destruction of structural matrices such as collagen and fibronectin is important in tissue invasion of bacteria and degradation of serum protein is important when bacteria infected in blood. *P. aeruginosa* produce elastase and alkaline protease which could be released into the microenvironment and degraded collagen molecules, the major structural elements of all connective tissues (13). This supported that these enzymes may be important for tisssue invasion and necrosis (12, 16, 17). *Serratia* 56 K protease degraded various serum constituents including IgG and IgA and play a role as virulence factor in the pathogenesis of serratial infections (10, 11). An essential feature of *Salmonella* infections is invasion of the gastrointestinal mucosa and most *Salmonella* spp. proceed through the surface intestinal epithelial cells into deeper tissue (3). A comprehensive electron microscophy study revealed that *Salmonella* penetrate the intestinal epithelium (15). After entry into epithelial cells, *Salmonella* spp. continue through the cell and penetrate (transcytose) to the opposite surface of the epithelial cells (2, 15). *S. schottmülleri* produces at least four extracellular proteases (14) and these proteases thought to be associated with the pathogenicity of *S. schottmülleri*. In the present communication, we report the effect of *S. schottmülleri* 73 kDa extracellular protease on various serum proteins such as immunoglobulins, hemoglobin, lysozyme and bovine serum albumin and tissue constituents such as collagens and fibronectin. Furthermore, the cytotoxic effect of the protease on cultured cells in vitro was tested. The results may provide some clues for understanding the mechanism of *Salmonella* pathogenesis.

* To whom correspondence should be addressed.

Materials and Methods

Bacteria and culture condition

The strain of *Salmonella schottmülleri* used in this study was clinically isolated and kindly provided by Dept. of Microbiology, College of Medicine, Chung-Ang Univ. in 1993 and maintained in our laboratory thereafter. For

production of proteases, the organism was cultured in trypticase soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% $CaCl_2$ at 37℃ with vigorous shaking.

Preparation of collagens and other serum proteins

Purified collagens, type I (from bovine achilles tendon) and type III (from calf skin), and fibronectin (from human plasma) were purchased from Sigma Chemical Co., St. Louis, Mo.. The purified collagens were dissolved in 0.1 M sodium acetate buffer (pH 5.0) at a concentration of 1 mg/ml. Purified hemoglobin (from human blood), bovine serum albumin and lysozyme (from human) were purchased from Sigma Co., St. Louis, Mo.. Immunoglobulin G and immunoglobulin M were purchased from Organon Teknika N.V. Cappel Products. These proteins were dissolved in 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 1 mg/ml. Protein concentration was determined by the method of Lowry *et al.* (7) with BSA (Sigma Chemical Co., St. Louis, Mo.) as a standard.

Degradation of collagens and other serum proteins by purified protease

The extracellular protease was purified to homogeneity from culture filtrate of *S. schottmülleri* as described previously (14). In brief, the protease was purified using 0~75% ammoium sulfate precipitation, DEAE Sepharose Fast Flow ion exchange chromatography, Ultrogel HA chromatography and Sephacryl S-200 HR molecular sieve chromatography. Collagens and other serum proteins were incubated with purified protease at an enzyme : substrate ratio (1 : 150) for various time intervals (0 to 24 h) at 37℃. The reactions were stopped by adding an equal volume of denaturing sample buffer (0.125 M Tris-HCl (pH 6.8), 2% SDS, 2% sucrose, 0.1% β-mercaptoethanol) followed by boiling the sample for 2 min. SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (6). Nonreducing gel electrophoresis was carried out on 10% slab gels in 25 mM Tris-glycine buffer, pH 8.8 (1).

Effect on cells in culture

HEp-2 cells were cultured in tissue culture flasks (Corning Glass Works, Corning, N.Y.) with Eagle's minimum essential medium with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). After trypsinization, cells were suspended in the same medium, inoculated in Nunclon Δ-Multidishes (24 well) (A/S Nunc-Denmark) at 4×10^4 cells per well and allowed to grow in a humidified 5% CO_2 atmosphere at 37℃. After formation of monolayers, the medium was replaced by that containing the 73 kDa protease (9 μg/ml) plus 10% fetal bovine serum, and the culture was incubated. In addition, 73 kDa protease with EDTA was tested in the assay chamber. The toxicity of the 73 kDa protease to the cultured cells was evaluated by measuring cell growth or by examination of microscopic morphology with a phase-contrast inverted microscope after 24-h incubation.

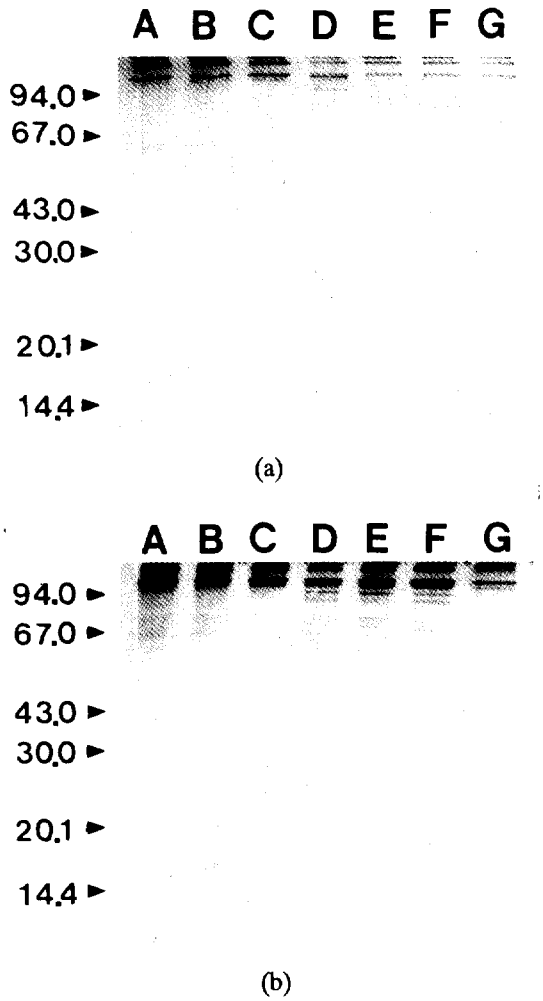

Fig. 1. Degradation of collagens by protease purified from culture filtrate of *S. schottmülleri*. (A) Collagen type I, (B) Collagen type III. Each reaction mixture contained protease was run under the condition described in materials and methods. Lane A-G, 10 μg of collagens incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

Results and Discussion

Degradation of collagens and fibronectin

Many pathologic changes observed in infected tissues may be related to degradation of extracellular matrix components by the extracellular proteases of bacteria (11). Collagens constitute a superfamily of extracellular

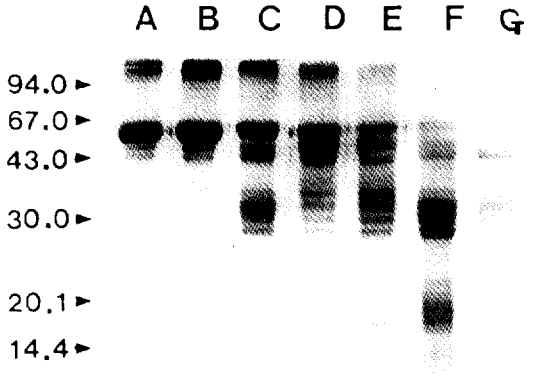

Fig. 2. Degradation of fibronectin by protease purified from culture filtrate of *S. schottmülleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. Lane A-G, 10 μg of fibronectin incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

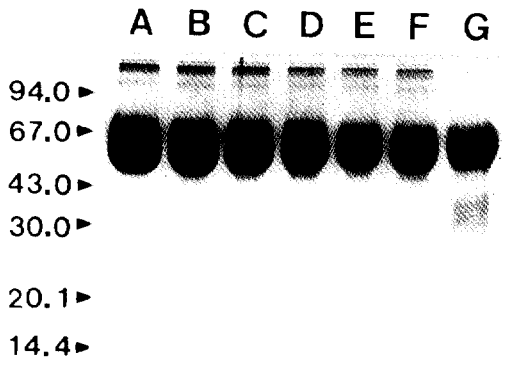

Fig. 3. Degradation of BSA by protease purified from culture filtrate of *S. schottmülleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. Lane A-G, 20 μg of BSA incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

matrix proteins with a structural role as their primary function. Particularly, type I and III collagens are abundantly found in dermis, tendon, bone and blood vessel walls and normally function as structural protein to maintain tissue integrity (9). They are participate in the formation of fibrils with molecules packed in quarter-staggered arrays and can interact with cells directly via specific cell surface receptors or indirectly via other extracellular matrix components.

Collagens, type I and III, were incubated with purified 73 kDa extracellular protease of *S. schottmülleri* at 37℃ and the degraded products were separated by SDS-PAGE under reducing conditions. The results demonstrate that extracellular protease of *S. schottmülleri* degrades type I and III collagens (Fig. 1). Proteolysis of this extracellular matrix protein is an end stage process, since no reparative mechanism exists to restore chain length and function. If degradation exceeds synthesis of these macromolecules, epithelial and vascular integrity cannot be maintained, which ultimately leads to tissue damage.

Fibronectin was also degraded to many fragments by this protease (Fig. 2). Fibronectins are high molecular weight glycoproteins found in many extracellular matrices and in blood plasma. They promote cell adhesion and affect cell morphology, migration, differentiation and cytoskeletal organization. The high susceptibility of fibronectin to the 73 kDa protease may be responsible for the pathogenesis of *Salmonella*, in which fibronectin plays a critical role in the maintenance of architecture by aiding the epitherium to adhere to the underlying connec- and analyzed by SDS-PAGE. Fig. 3 shows that BSA was degraded by this protease. Fig. 4 and Fig. 5 show that

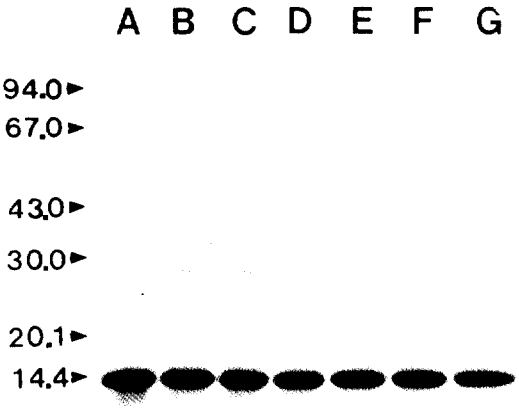

Fig. 4. Degradation of hemoglobin by protease purified from culture filtrate of *S. schottmülleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. Lane A-G, 10 μg of hemoglobin incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

tive tissue. Therefore, these suggested the possibility that 73 kDa extracelllar protease produced by *S. schottmülleri* is able to break down tissue constituents which normally function as structural protein to maintain tissue integrity before invasion of the organism.

Degradation of serum proteins

To investigate the proteolytic activity of the *S. schottmülleri* extracellular protease on serum proteins, including hemoglobin, BSA and lysozyme, the protease was added to the protein solutions and the mixture was incubated at 37℃ for various time intervals (0 to 24 h) hemoglobin and lysozyme were degraded slightly after treatment with this protease. Although the definite degradation products were not detected, the intensities of

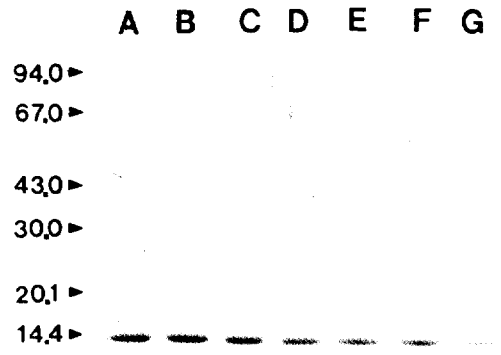

Fig. 5. Degradation of lysozyme by protease purified from culture filtrate of *S. schottmulleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. Lane A-G, 10 μg of lysozyme incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

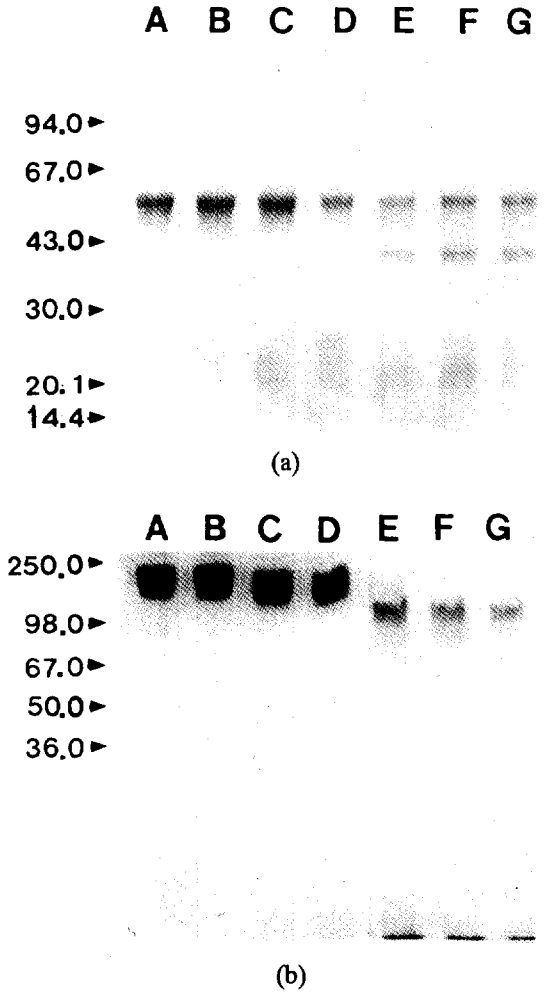

Fig. 6. Degradation of immunoglobulin G by protease purified from culture filtrate of *S. schottmülleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. (A) Reducing condition. (B) Nonreducing condition. Lane A-G, 10 μg of immunoglobulin G incubated with protease f or 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

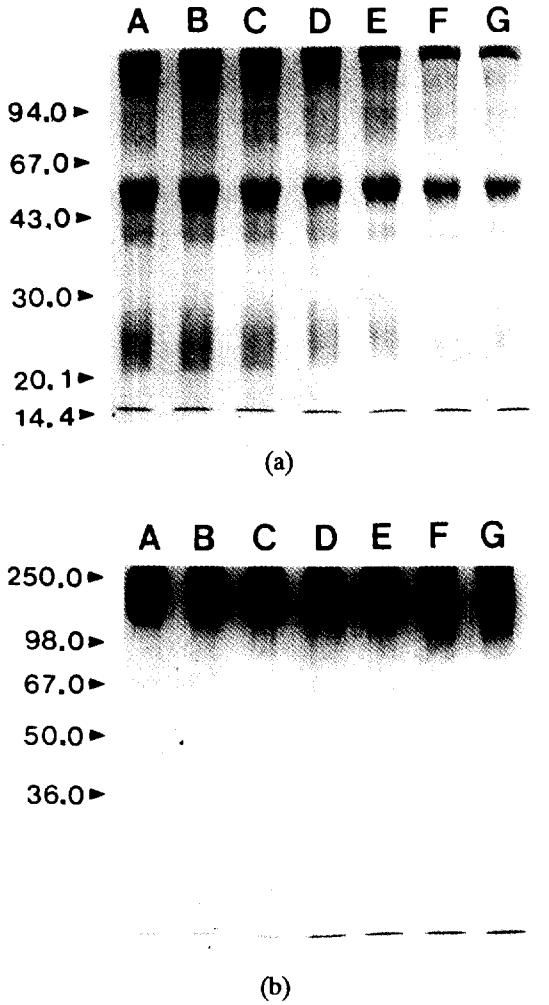

Fig. 7. Degradation of immunoglobulin M by protease purified from culture filtrate of S. *schottmülleri*. Each reaction mixture contained protease was run under the condition described in materials and methods. (A) Reducing condition. (B) Nonreducing condition. Lane A-G, 10 μg of immunoglobulin M incubated with protease for 0, 2, 4, 8, 12, 18 and 24 h at 37℃, respectively.

hemoglobin and lysozyme bands slightly decreased. This could be indicated indirectly hemoglobin and lysozyme were degraded by this protease. These data suggested the possibility that this protease may be involved in complications observed in septicemia and chronic infections which caused by this organism infected in blood stream by degrading the serum proteins.

Degradation of immunoglobulins

Samples of the reaction mixture containing the 73 kDa protease and IgG and IgM were analyzed by SDS-PAGE for proteolytic degradation after different incubation periods (Fig. 6 and 7). A reduced sample of undigested IgG migrated as two bands of approximately 55 (heavy-chain) and 23 (light-chain) kDa, as expected. Stepwise

Fig. 8. Cytopathic effect of 73 kDa protease on HEp-2 cells. Cells were cultured on 24 well multidishes in the presence or absence of 73 kDa protease with or without EDTA at 37℃ for 24 h. (A) Control cells, no protease. (B) HEp-2 cells were exposed to 73 kDa protease at 9 μg/ml, revealing extensive degenerative change. (C) Cells exposed to 9 μg/ml of 73 kDa protease with 5 mM EDTA. No protease toxicity can be seen.

increases in the incubation time of purified protease with IgG resulted in generation of a band of 41.5 kDa (Fig. 6A). This was thought to be the digested products of heavy chain. A nonreduced sample of undigested IgG migrated as one band of approximately 155 kDa, however, stepwise increases in the incubation time of purified protease with IgG resulted in generation of a bands of approximately 120 kDa which thought to be the degradation product of intact IgG (Fig. 6B). Therefore, this proposed that the protease cleaved specifically the Fc portion, not hinge region, of IgG. The protease also degraded IgM (Fig. 7). However, the degradation pattern of IgM by the protease was different to that of IgG. The intensities of heavy and light chains were gradually decreased with increasement of incubation time in reducing condition (Fig. 7A). This indicated that both of heavy and light chains of IgM were degraded by the protease. In nonreducing condition, the degradation aspect of intact IgM was observed, too (Fig. 7B). This study demonstrated that *S. schottmülleri* produces protease capable of cleaving IgG and IgM molecules. Immunoglobulin G and immunoglobulin M are involved in the complement system, opsonization and phagocytosis in bacterial infection. Therefore, degradation of IgG and IgM resulted in decrease of host immune activity to bacteria and could render hosts more vulnerable to subsequent opportunistic infections.

Effect of protease on cultured cells

Incubation of HEp-2 cells with the 73 kDa protease in the presence of 10% fetal bovine serum resulted in loss of more than 50% of cell viability, even at a low enzyme level of 9 μg/ml, within 24 h (data not shown). The morphological change of cells in culture after exposure to 73 kDa protease is shown in Fig. 8B. Cells were changed granularity around the nucleus, cytoplasmic vacuolation and round up of the cells with detachment from the substance by observed of microscopic morphology. However, with incubation of 73 kDa protease and EDTA in the culture system at 37℃ for 24 h, the toxicity of 73 kDa protease to cells was blocked completely (Fig. 8C). This confirmed the fact that this protease is closely related to the cytopathic aspect of HEp-2 cells obtained in this study. The tissue damage may be important in bacterial pathogenicity in that it can promote the invasion of invasive bacteria such as *Salmonella* into deeper tissue. Therefore, this represented the possibility that the protease promoted the invasion of *Salmonella* into deeper tissue by destructing the intestinal mucosal cells and involved in the tissue necrosis observed in late infection of *Salmonella*. The cytopathic effect obtained this study is not sufficient to deduce the definite mechanism of the protease in tissue destruction. However, degradation of tissue constitue proteins, such as collagen and fibronectin, as described above, may be suggested one

possible mechanism of this protease. The protease may directly damage tissue structure by degrading the connective proteins or inhibit cell adhesion and locomotion by degrading endogenous pericellular fibronectin. Alternatively, diffusion of the protease as protease/α_2-macroglobulin (α_2M) into the cell via α_2M receptor may interfere with the intracellular signalling networks result in cell killing (8). To confirm more definitely the function of this protease as a virulance factor of *S. schottmülleri*, further detailed studies must be needed.

References

1. **Bollag, D.M. and S.J. Edelstein,** 1991. Protein method, p. 143-160. Wiley-Liss, Inc., New York.
2. **Finlay, B.B. and S. Falkow,** 1988. Virulence factors associated with *Salmonella* species. *Microbiol. Sci.* **5**, 324-328.
3. **Giannella, R.A., O. Washington, P. Gemski, and S.B. Formal,** 1973. Invasion of HeLa cells by *Salmonella typhimurium:* a model for study of invasiveness of *Salmonella*. *J. Infect. Dis.* **128**, 69-75.
4. **Heck, L.W., K. Morihara, W.B. McRae, and E.J. Miller,** 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. *Infect. Immun.* **51**, 115-118.
5. **Heck, L.K., K. Morihara, and D.L. Abrahamson,** 1986. Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. *Infect. Immun.* **54**, 149-153.
6. **Laemmli, D.K.,** 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
7. **Lowry, O.H., N.J. Rosebrugh, A.L. Farr, and R.J. Randall,** 1951. Protein measurement with the foline phenol reagent. *J. Biol. Chem.* **193**, 265-275.
8. **Maeda, H. and A. Molla,** 1989. Pathogenic potentials of bacterial protease. *Clin. Chem. Acta.* **185**, 357-368.
9. **Miller, E.J. and S. Gay,** 1982. Collagen: an overview. *Methods Enzymol.* **82**, 2-32.
10. **Molla, A., K. Matsumoto, I. Oyamada, T. Katski, and H. Maeda,** 1986. Degradation of protease inhibitors, immunoglobulins and other serum proteins by *Serratia* protease and its toxicity to fibroblasts in culture. *Infect. Immun.* **53**, 522-529.
11. **Molla, A., T. Kagimoto, and H. Maeda,** 1988. Cleavage of immunoglobulin G(IgG) and IgA around the hinge region by proteases from *Serratia marcescens. Infect. Immun.* **56**, 916-920.
12. **Morihara, K. and J.Y. Homma,** 1985, Pseudomonas proteases, p.41-75. In 1. Holder(ed.). Bacterial enzymes and virulence. CRC Press, Inc., Boca Raton, Fla.
13. **Mull. J.D. and W.S. Callahan,** 1965. The role of the elastase of *Pseudomonas aeruginosa* in experimental infection. *Exp. Mol. Pathol.* **4**, 567-575.
14. **Na, B.K. and C.Y. Song,** 1995. Purification and characterization of an extracellular protease from culture filtrate of *Salmonella schottmülleri. Jour. Microbiol.* **33**, 244-251.
15. **Takeuchi, A.,** 1967. Electron microscopic studies of experimental *Salmonella* infection: 1. Penetration into the intestinal epithelial by *Salmonella typhimurium. Am. J. Pathol.* **50**, 109-136.
16. **Woods, D.E. and B.H. Iglewski,** 1983. Toxins of *Pseudomonas aeruginosa*: new perspectives. *Rev. Infect. Dis.* **5**, S 715-722.
17. **Wretlind, B. and O. Pavlovski,** 1983. *Pseudomonas aeruginosa* elastase and its role in *Pseudomonas infections. Rev. Infect. Dis.* **5**, S998-1004.