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Characterization of extracellular proteases of *Aeromonas hydrophila* isolated from the intestine of carp(*Cyprinus carpio*)

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Aeromonas hydrophila isolated from the intestine of carp produced several kinds of proteases into the medium. Inhibitor assay with the culture supernatant of *A. hydrophila* showed that there were major metalloproteases and minor serine proteases. Gelatin SDS-PAGE showed two proteolytic bands. One broad protease band was inhibited by metalloprotease specific inhibitor, EDTA, indicating a metalloprotease. The other was inhibited by serine protease specific inhibitor, PMSF, suggesting a serine protease. The proteolytic activities of both extracellular proteases remained on Gelatin SDS-PAGE after heating at 70℃ for 30 min. However, the major metalloprotease was separated into two proteolytic bands on Gelatin PAGE by gel filtration chromatography on Sephadex G-75.

Key words : *Aeromonas hydrophila*, Carp intestine, Metalloprotease, Serine protease, Fish disease

Aeromonas hydrophila, a gram-negative facultative anaerobic rod, is an important pathogen of freshwater fishes and warm-blooded animals including human. In human it causes soft-tissue wound infections and diarrheal disease, and in fish it causes fatal hemorrhagic septicemia. *A. hydrophila* produces various kinds of toxic substances including hemolysins, cytotoxin, enterotoxin, aerolysin and proteases, some of which have been thought to be involved in pathogenicity of fish. But their roles in the pathogenesis of *A. hydrophila* are still unknown. It was reported that hemolysin was the active lethal factor in fish. Also it has been suggested that proteolytic enzymes excreted by *Aeromonas* spp. play an important role in invasiveness and establishment of infection.

Different strains of *A. hydrophila* produce proteases in various shapes of the number and nature. It was reported that there were two different kinds of proteases, one of which was heat-labile and the other heat-stable(Allan and Steven, 1981 : Thune *et al.*, 1982). In other case, it was also reported that only one heat-stable protease was produced in other strains of *A. hydrophila* although its specific protease activity was not characterized (Wakabayashi and Kanai, 1984). The existences of several kinds of proteases were demonstrated in *A. hydrophila* B_{51} by IEF analysis(Nieto and Ellis, 1986). Leung and Stevenson(1988) reported that 47 strains of *A. hydrophila* and 29 strains

of other aeromonads released two distinct types of proteases in extracellular products : a temperature-stable metalloprotease(TSMP) and a temperature-labile serine protease(TLSP).

In this study we report the existence and partial characterization of extracellular proteases, and this is the first report on the identification of heat-stable serine protease(TSSP) of *A. hydrophila* isolated from the intestine of *Cyprinus carpio*.

Materials and Methods

Bacterial strain

The several kinds of microorganisms were isolated from the intestine of *C. carpio* being bred in the aquaculture center of Pukyong National University. Among them one microorganism was identified as *A. hydrophila* by automatic microbial identification system(Hewlett packard HP 6890 Series GC System).

Cultivation

A. hydrophila was incubated in LB broth containing with ampicillin(80 μg/mℓ) at 30℃ with vigorous shaking.

Protease Assay

Azocasein was used to examine the proteolytic activity(Yanagida *et al.*, 1986). A 200 μℓ of culture supernatant was mixed with a 500 μℓ of 0.1% azocasein solution(pH 7.4) and was incubated at 37℃ for 1 hr. To terminate the reaction, the equal volume of 15% TCA was added and then cooled on ice bath for 30 min. After the mixture was centrifuged at 12,000 rpm for 10 min, the supernatant was transferred into a new tube and then its absorbance was measured at 390 nm.

Inhibitor Test

Before the azocasein solution was mixed, enzyme solution was incubated with each specific inhibitor at 37℃ for 30 min. Then, protease assay was performed as described above. Sample without adding inhibitor was used as positive control.

Gelatin-PAGE

SDS-PAGE was performed in 10% slab gel copolymerized with 0.1% gelatin according to the method of Dowdle and Heussen(1980). Samples were preincubated in the equal volume of 2× sample buffer(4% SDS, 20% glycerol, 0.001% BPB, 125 mM Tris-Cl, pH 6.8) at 37℃ for 30 min. After the electrophoresis, the gel was incubated in 10 mM Tris-Cl(pH 7.4) containing 2.5% Triton X-100 with gentle stirring at 4℃ for 1 hr to remove the SDS. The gel was incubated in 10 mM Tris-Cl buffer(pH 7.4) at 37℃ for 1 hr, stained for 10 min in the staining solution(0.18% amido black, 0.004% coomassie brilliant blue) and destained in the destaining solution(D.W. : methanol : glacial acetic acid=4.5 : 4.5 : 1). For inhibitor test, each specific inhibitor was added to Triton X-100 solution and incubation solution, respectively.

Purification procedure

All procedures were performed at 4℃. A 3 liter of culture solution of *A. hydrophila* grown at 30℃ for 20 hr was centrifuged at 8000 g for 15 min and the supernatant was filtered through 0.45 μm membrane filter. Ammonium sulfate was slowly added to the filtrate, with gentle stirring, up to

80% saturation. After standing overnight at 4℃, the precipitates were collected by centrifugation at 8000 g for 30 min and dissolved in about 20 mℓ of 10 mM Tris-Cl buffer(pH 7.4). A 1.2 mℓ of sample was applied to a Glass Econo-Column(2.5 × 80 cm) of Sephadex G-75 equilibrated with 10 mM Tris-Cl buffer(pH 7.4), eluted with the same buffer at the flow rate of 0.5 mℓ/min and collected in 2 mℓ of fraction volume.

Results

Inhibitor test

We observed the presence of serine and metalloprotease in the culture supernatant by inhibitor assay method. Serine protease specific inhibitor, PMSF, showed 75.3% of relative activity and metalloprotease specific inhibitors, EDTA, and o-phenanthrolin showed 53.4% and 47.3%, respectively. But cysteine protease specific inhibitor, leupeptin, and aspartic acid protease specific inhibitor, pepstatin, had little effect on the proteolytic activities of the culture supernatant of *A. hydrophila*. Therefore it was supposed that there might be at least two kinds of protease in the culture supernatant. To confirm the existence of two different kinds of proteases in culture supernatant, inhibitor assay was performed by adding the combination of serine protease and metalloprotease inhibitors. As shown in Table 1, the incubation of the culture supernatant with different kinds of specific inhibitors(PMSF+EDTA or PMSF+o-phenanthrolin) showed 28% of relative proteolytic activity but the pair of same metalloprotease specific inhibitor (EDTA+o-phenanthrolin) showed the similar inhibition effect(55.6% of relative activity) on that of the single treatment of metalloprotease inhibitors(53.4% and 47.3% of relative activity). It was assumed that in the culture supernatant metalloprotease had major activity and serine protease minor activity.

Table 1. Effect of inhibitors on protease activity

Inhibitors	Concentration	Relative activity(%)
Control		100
PMSF*	10 mM	75.3
EDTA**	50 mM	53.4
o-phenanthrolin	10 mM	47.3
leupeptin	2.3 mM	93.4
pepstatin	4.5 mM	98.4
Combination of two specific inhibitors		
[PMSF+	10 mM	28.1
EDTA]	50 mM	
[PMSF+	10 mM	28.1
o-phenanthrolin]	10 mM	
[EDTA+	50 mM	55.6
o-phenanthrolin]	10 mM	

*PMSF : Phenylmethanesulfonyl fluoride

**EDTA : Ethylenediaminetetraacetic acid

Inhibitor test by Gelatin-SDS PAGE

In order to identify the proteolytic activity and types of protease, Gelatin-SDS PAGE was performed as described in materials and methods with EDTA and PMSF inhibitors. Gelatin gel was electrophoresed and cut off along each lane. The gel slices were washed and incubated in different concentrations of inhibitors, PMSF(2, 3, 4 mM) and EDTA(10, 20, 30, 40 mM), respectively(Fig. 1 and Fig. 2). Control lane showed two proteolytic bands, which one was sharp and the other diffused. In the case of incubation with PMSF, the sharp small band was inhibited, suggesting as

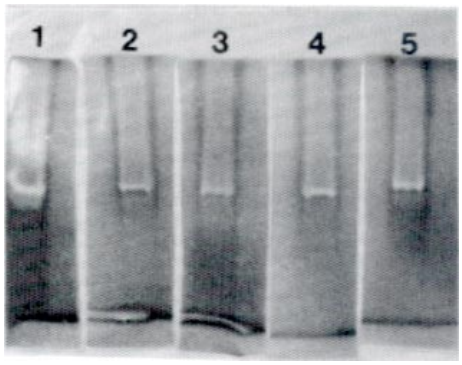

Fig. 1. Inhibitor test of the culture supernatant of *Aeromonas hydrophila* with EDTA, metalloprotease specific inhibitor.
1, Control ; 2, 10 mM ; 3, 20 mM ; 4, 30 mM ; 5, 40 mM.

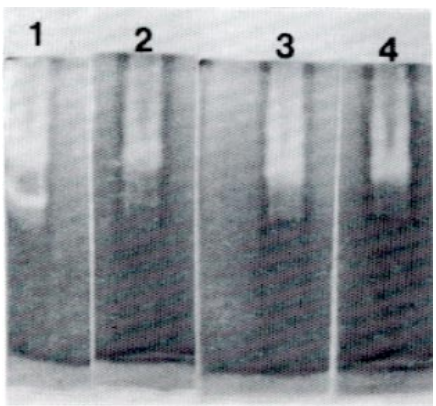

Fig. 2. Inhibitor test of the culture supernatant of *Aeromonas hydrophila* with PMSF, serine proteases specific inhibitor.
1, Control ; 2, 1 mM ; 3, 2 mM ; 4, 3 mM

a serine protease. In the case of incubation with EDTA, the upper broad band was disappeared, strongly suggesting that it is a metalloprotease.

Thermostability of proteases

Thermostability of proteases was examined by Gelatin-PAGE. The loading samples were incubated at 60℃, 70℃, 80℃ for 10, 20, 30 min, respectively. The proteolytic activity of large band still remained after incubation by far at 70℃ for 30 min, but reduced after incubation at 80℃ for over 10 min. The activity of small serine protease still remained by far at 70℃ for 30 min, but drastically disappeared from at 80℃ for 10 min(Fig. 3). These results strongly suggested that both proteases was heat-stable. Heat-stable serine proteases were not reported in Aeromonas sp. until yet. It is interesting that the serine protease produced by *A. hydrophila* in this study shows different thermostability with other serine proteases produced by different strains of *A. hydrophila*.

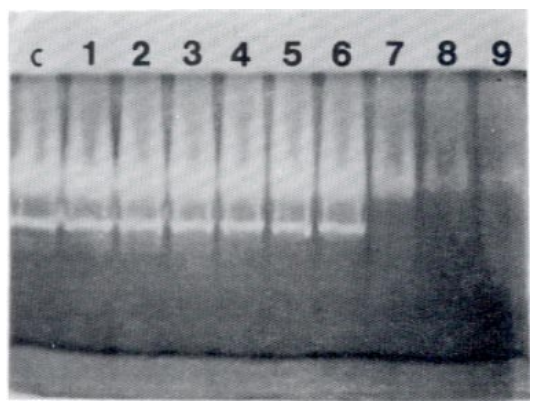

Fig. 3. Thermostability test of proteases of *Aeromonas hydrophila*.
C, Control ; 1, 2, 3 , at 60℃ for 10, 20, 30 min, respectively. 4, 5, 6 , at 70℃ for 10, 20, 30 min, respectively. 7, 8, 9 , at 80℃ for 10, 20, 30 min, respectively

Partial purification by gel filtration

A 1.2 ml of ammonium sulfate precipitates of culture supernatant was loaded onto Sephadex

G-75 column equilibrated with 10 mM Tris-Cl buffer(pH 7.4), eluted with the same buffer at the flow rate of 0.5 mℓ/min and collected into 2 mℓ fraction. Two protein peaks were observed as shown in Fig. 4. The proteolytic assay showed that a large activity peak existed from the beginning of the preceded protein peak to the middle of two protein peaks. However, a small proteolytic peak also existed at the start fraction of second protein peak. Each fraction was electrophoresed in 10% Gelatin-PAGE to see the activity. Each sample was preincubated with the same volume of 2× sample buffer(4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% BPB, 200 mM Tris-Cl (pH 6.8) at 37℃ for 30 min. As shown in Fig. 5, standard culture solution(nonfiltered) had three proteolytic bands, the far upper one newly appeared. From the fifth fraction, the clear bands appeared. However, the diffused band of metalloprotease in the standard was separated into two bands from fraction 7 to fraction 16. The lower band

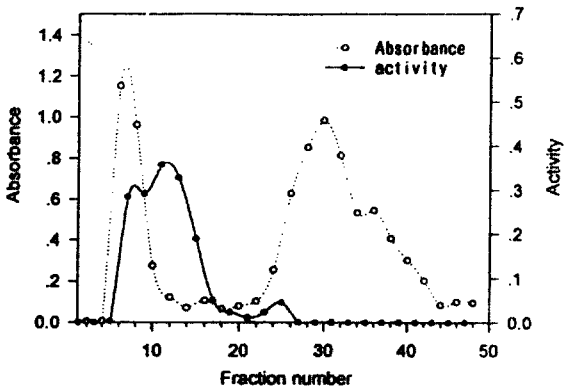

Fig. 4. Sephadex G-75 filtration chromatography of extracellular products of *Aeromonas hydrophila*. The column was packed and eluted with 0.01M Tris-Cl buffer, pH 7.4. Fraction volume was 2 mℓ. See text in detail.

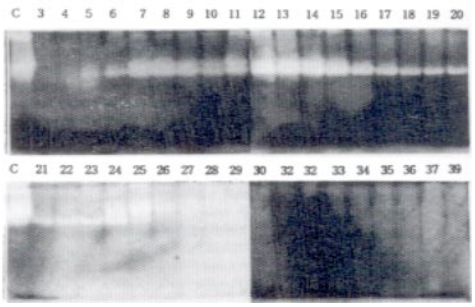

Fig. 5. Gelatin SDS-PAGE patterns of fractions of Sephadex G-75 filtrates.
C, standard(nonfiltered) ; 3-38, fraction number

of serine protease disappeared from fraction 25. From 24 to 26, the weakly diffused band appeared again. From fraction 27, none of proteases activity appeared.

Discussion

Several kinds of proteases produced by pathogenic bacteria play important roles in pathogenicity to host organisms. Especially, serine protease identified in *A. salmonicida*, which is a critical pathogen in salmonidal fish, is regarded as an essential pathogenic determinant. Although *A. hydrophila* is regarded as a conditional pathogen and widespreaded in aquatic environment, it is also included in the group of pathogenic bacteria. It is not known until yet the reason why *A. hydrophila* shows conditional pathogenicity while *A. salmonicida* does critical pathogenicity. We analyzed the characteristics of chromosome by Pulsed Field Gel Electrophoresis and determined the total size of *A. hydrophila* chromosome as 3.9 Mb(data not shown). *A. hydrophila* harbors one major chromosome and very large plasmids. The conditional pathogenicity of *A. hydrophila* could be resulted in the existence

of extrachromosomal state plasmids. By gel filtration chromatography with Sephadex G-75, we identified that *A. hydrophila* produced four kinds of different molecular weight of proteases and the amount of serine protease was very low as comparing with other metalloprotease. The amount of serine protease produced by A. salmonicida is much higher than that of *A. hydrophila* identified in this study. If the expression of serine protease is regulated by some environmental or biological factors and the amount of serine protease could be drastically increased by such factors, serine protease of *A. hydrophila* might function as essential pathogenic determinants. Therefore it is very interesting and important to identify the structure of serine protease gene and its expression in *A. hydrophila*. We will further characterize the characteristics of serine protease gene in *A. hydrophila*.

Acknowledgements

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References

Allan, B. J. and Stevenson, R. M. W. : Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. Can. J. Microbiol., 27 : 1114－1122, 1981.

Amborski, R. L., Borall, R. and Thune, R. L. : Effects of short-term cold storage on recovery of proteases from extracellular products of *Aeromonas hydrophila*. Appl. Environ. Microbiol., 48 : 456－458, 1984.

Bruke, V., Robinson, J., Cooper, M., Beaman, J., Partridge, K., Peterson, D. and Gracey, M. : Biotyping and virulence factros in clinical and environmental isolates of *Aeromonas species*., Appl. Environ. Microbiol., 47 : 1146－1149, 1984.

Charkraborty, T., Huhle, B., Hof, H., Bergbauer, H. and Goebel, W. : Marker exchange mutagenesis of the aerolysin determinant in Aeromonas hydrophila demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infection. Infect. Immun., 55 : 2274－2280, 1987.

Dowdle, E. B. and Heussen, C. : Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substances. Anal. Biochem., 102 : 196－202, 1980.

Graham, T. E., Riddle, L. M. and Amborski, R. L. : Extracellular proteases from *Aeromonas hydrophila* : Partial purification and effects on age-0 channel catfish. Trans. Am. Fish. Soc., 111 : 749－754, 1982.

Hsu, T. C., Waltman, W. D. and Shots, E. B. : Correlation of extracellular enzymatic activity and biochemical characteristics with regard to virulence of *Aeromonas hydrophila*. Dev. Biol. Stand., 49 : 101－111, 1981.

Janda, J. M., Guthertz, L. S., Kokka, R. P. and Shimada, T. : *Aeromonas species* in septicemia : laboratory characteristics and clinical observations. Clin. Infect. Dis., 19 : 77－83, 1994.

Janda, J. M., Oshiro, L. S., Abbot, S. L. and Duffey, P. S. : Virulence markers of mesophilic *aeromonads* : association of the autoagglutination phenomenon with mouse pathogenicity and the presence of a peripheral cell-associated layer. Infect. Immun., 55 : 3070－3077, 1987.

Joanne, M. R., Houston, C. W., Coppenhaver, D. H., Dixon, J. D. and Kurosky, A. : Purification

and chemical characterization of a cholera toxin-cross-reactive cytolytic enterotoxin produced by a human isolate of *Aeromonas hydrophila*. Infect. Immun., 57 : 1165－1169, 1989.

Joanne, M. R., Houston, C. W. and Kurosky, A. : Bioactivity and immunological characterization of a cholera toxin-cross-reactive cytolytic enterotoxin from *Aeromonas hydrophila*. Infect. Immun., 57 : 1170－1179, 1989.

Kindschuh, M., Pickering, L. K., Clearly, T. G. and Ruizpalacios, G. : Clinical and biochemical significance of toxin production by *Aeromonas hydrophila*. J. Clin. Microbiol., 25 : 916－921, 1987.

Lallier, R., Bernard, F. and Lalonde, G. : Difference in the extracellular products of two strains of *Aeromonas hydrophila* virulent and weakly virulent for fish. Can. J. Microbiol., 30 : 900－904, 1984.

Leung, K. Y. and Stevenson, R. M. W. : Charcteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. J. Gen. Microbiol., 134 : 151－160, 1988.

Loewy, A. G., Santer, U. V., Wieczorek, M., Blodgett, J. K., Jones, S. W. and Cheronis, J. C. : Purification and characterization of a novel zinc-proteinase from cultures of *Aeromonas hydrophila*. J. Biol. Chem., 268 : 9071－9078, 1993.

Mateos, D., Anguita, J., Naharro, G. and Paniagua, C. : Influence of growth temperature on the production of extracellular virulence factors and pathogenicity of environmental and human strains of *Aeromonas hydrophila*. J. Appl. Bacteriol., 74 : 111－118, 1993.

Nieto, T. P. and Ellis, A. E. : Characterization of extracellular metallo- and serine-proteases of *Aeromonas hydrophila* strain B51. J. Gen. Microbiol., 132 : 1975－1979, 1986.

Reilly, T. O. and Day, D. F. : Effects of cultural conditions on protease production by *Aeromonas hydrophila*. Appl. Environ. Microbiol., 45, No.3 : 1132－1135, 1983.

Rivero, O., Anguita, A., Paniagua, C. and Naharro, G. : Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. J. Bacteriol., 172 : 3905－3908, 1990.

Rivero, O., Anguita, J., Mateos, D., Paniagua, C. and Naharro, G. : Cloning and charaterization of an extracellular temperature-labile serine protease gene from *Aeromonas hydrophila*. FEMS Microbiol. Lett., 81 : 1－8, 1991.

Thune, R. L., Graham, T. E., Riddle, L. M. and Amborski, R. L. : Extracellular products and endotoxin from *Aeromonas hydrophila* : Effects on age-0 channel catfish. Trans. Am. Fish. Soc., 111 : 404－408, 1982.

Wakabayashi, H. and Kanai, K. : Purification and some properties of protease from *Aeromonas hydrophila*. Bull. Japan. Soc. Sci. Fish., 50(8) : 1367－1374, 1984.

Yanagida, N., Uozumi, T. and Beppu, T. : *Specific excretion* of Serratia marcescens protease through the outermembrane of Escherichia coli. J. Bacteriol., 166 : 937－944, 1986.

잉어(*Cyprinus carpio*)로부터 분리된 *Aeromonas hydrophila*의 extracelluar proteases 연구

이종규 · 김종필 · 최태진 · 송영환

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잉어로부터 분리한 *Aeromonas hydrophila*는 세포 밖으로 여러 종류의 proteases를 생산한다. *A. hydrophila*의 배양 상층액을 이용한 inhibitor assay를 통하여, 주된 활성을 나타내는 metalloprotease와 약한 활성을 나타내는 serine protease가 있음을 알게 되었다. Gelatin SDS-PAGE를 통하여 두 개의 활성 band가 관찰되었으며, 이 들 중 넓게 퍼진 band는 metalloprotease에 특이하게 작용하는 inhibitor인 EDTA에 의해 활성이 상실되었고 따라서 metalloprotease임을 알 수 있었다. 다른 하나는 serine protease에 특이하게 반응하는 inhibitor인 PMSF에 의해 저해되어 serine protease임을 알 수 있었다. 이러한 두 extracellular protease의 활성은 75℃에서 30 분간 열을 가한 후에도 Gelatin SDS-PAGE상에 남아있었다. 그런데, 주된 metalloprotease는 Sephadex G-75를 이용한 column chromatography를 거친 후 Gelatin Gel상에서 두 개의 band로 분리되었다.

Key words : *Aeromonas hydrophila*, Carp intestine, Metalloprotease, Serine protease, Fish disease