Significance of Extracellular Protease for Growth of a Heterotrophic Bacterium, *Aeromonas salmonicida*

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The role of protease produced by a heterotrophic bacterium during growth was investigated with *Aeromonas salmonicida*, the pathogen of fish furunculosis, strain A-7301 and its protease-deficient mutant NTG-1 induced by mutagenesis. Strain A-7301 produced extracellular protease in a mixed amino acid medium (composed of Gly, Ala, Val, Ile, Leu, Thr, Ser, Cys, Met, Phe, Tyr, Lys, Arg, Pro, His, Try, Asp, Asn, Glu, and Gln at equal concentrations of 0.1 g/liter). Its multiplication rate was limited by the amounts of amino acids present, whereas strain NTG-1 showed no protease production despite considerable growth similar to that of A-7301. There was no difference between A-7301 and NTG-1 in amino acid requirements for growth, and seven amino acids (Gly, Ala, Val, Thr, Cys, Met, and His) were found to be indispensable. A defined level of the mixed amino acids (0.4 to 0.5 g/liter) was needed for A-7301 to initiate a large production of protease. Neither of the strains grew well in a casein medium, to which no amino acids were added. However, when a protease fraction obtained from extracellular products of A-7301 by DEAE-cellulose column chromatography was added, NTG-1 successfully reproduced in the casein medium. These results indicate that the extracellular protease plays an important role in supplying *A. salmonicida* cells with available amino acids as nutrients and that higher growth is closely associated with protease production which stimulates further reproduction.

Certain heterotrophic bacteria, including pathogenic bacteria, produce protease extracellularly (1, 13, 20, 23). It is believed that extracellular proteases aid in the reproduction of these bacteria by making available small peptides and amino acids as nutrients from digested proteins present in media and environments such as host tissues. However, this conventional understanding of the role of proteases, insofar as I know, is largely unsupported by experimental evidence.

Extracellular protease produced by *Aeromonas salmonicida* was suggested to be a major virulence factor by Shieh (18) and Sakai (15). Some properties of the protease were reported by Shieh and McLean (19), Mellergaard (9), and Sheeran and Smith (17). Ellis et al. (3) suggested that protease of *A. salmonicida* extracellular products was one of the components responsible for lethality to fish and furuncle formation, both of which are characteristics of fish furunculosis. Recently, Sakai (16) has reported that, while the protease-deficient mutant NTG-1 resembled the parent strain in all other characteristics, including those considered by others (4-7, 11, 12, 21, 22) to be involved in pathogenesis (autoagglutination, hemagglutination, adhesion to fish tissues, serum resistance, and the production of hemolysin and leukocytolysin), this mutant lacked the capacity to produce skin lesions (furuncles) and mortality. In the same report, NTG-1 proliferated poorly in fish, even though its capacity to persist in the tissues remained unchanged. This suggests that the extracellular protease is of importance in the growth as well as the pathogenicity of *A. salmonicida*. In this report, the role of protease in the growth of *A. salmonicida* was investigated by using a protease producer and a protease-deficient mutant.

MATERIALS AND METHODS

Animals. Healthy rainbow trout (*Salmo gairdneri*), weighing 25 to 35 g and 80 to 120 g, were used for the determination of 50% lethal doses (LD₅₀s) and for preparation

of normal serum, muscle extracts, and muscle homogenates. The fish were reared in laboratory aquaria with well water at 10°C and were fed daily with commercial dry pellet food until use.

Bacteria. *A. salmonicida* A-7301 (the strain number of the Hokkaido Fish Hatchery) was isolated from a natural outbreak of furunculosis in sockeye salmon (*Oncorhynchus nerka*). A protease-deficient mutant, NTG-1, was induced from A-7301 with *N*-methyl-*N'*-nitro-nitrosoguanidine as reported previously (16). Principal morphological, cultural, and biochemical characteristics were determined by routine methods (10). All characteristics tested, with the exception of protease production (which was negative in NTG-1), were identical as follows: shape, short rods; Gram stain, negative; motility, negative (no flagellum); no growth in nutrient broth at 37°C; catalase and oxidase, positive; arginine dehydrogenase, positive; Hugh-Leifson test, fermentative; Voges-Proskauer reaction, negative; indole production, positive; methyl red test, positive; nitrate reduction, positive; hydrogen sulfide production, negative; citrate utilization, negative; starch hydrolysis, positive; negative for acid from D-xylose and positive for acid from D-galactose, lactose, maltose, sucrose, and starch; autoagglutinative; and hemagglutinative against rainbow trout erythrocytes.

Media and culture. Bacterial strains were usually subcultured on nutrient agar (Eiken Chemical Co., Tokyo) slants at 20°C for 3 days and maintained at 15°C. Nutrient agar plates were used for viable counts. For the shake culture, Casamino Acids-tryptone (CT) broth contained the following (in grams per liter): Casamino Acids (Difco Laboratories, Detroit, Mich.), 2; tryptone, 2; NaCl, 5; KCl, 0.5; MgSO₄ · 7 H₂O, 0.01. CT broth was buffered at pH 7.2 with 40 mM Tris hydrochloride before it was autoclaved at 121°C for 20 min. Samples of cultures on slants were used to inoculate 250 ml of CT broth in 500-ml flasks, which were then incubated by shake culture at 15 to 25°C for 40 to 45 h. To determine the

FIG. 1. Comparison between A-7301 and NTG-1 in growth and protease production. The strains were incubated in CT broth with shaken culture. Growth (○) and extracellular protease (●) were assayed.

amino acid requirements of strains A-7301 and NTG-1 for growth, a mixed amino acid medium, made up of 20 amino acids (Gly, Ala, Val, Ile, Leu, Thr, Ser, Cys, Met, Phe, Tyr, Lys, Arg, Pro, His, Try, Asp, Asn, Glu, and Gln at equal concentrations of 0.1 g/liter), was used as a complete amino acid medium (to which no vitamin was added). Incomplete amino acid media, which lacked a single amino acid in the

composition of the complete amino acid medium, were used to determine indispensable amino acids required for growth. These media were prepared in hot saline containing NaCl (5 g/liter), KCl (0.5 g/liter), and $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.01 g/liter) and then adjusted to pH 7.4 with Tris hydrochloride before filtration (pore size, 0.45 μm). Bacterial cells were incubated with shake culture at 20°C for 60 h in 200 ml of those amino acid media. Hammarsten casein (Wako Pure Chemical Industries, Tokyo) media (adjusted to pH 7.4 with sodium hydroxide) supplemented with increasing concentrations of amino acids (0 to 1.0 g/liter of the complete amino acid composition) were also used. Fish serum was prepared from rainbow trout to compare growing properties between A-7301 and NTG-1. Blood was obtained by caudal peduncle amputation of five fish, and clots were removed by centrifugation at $1,000 \times g$ for 5 min at 4°C. Normal serum was threefold diluted with physiological saline (0.85% NaCl) and filtered (pore size, 0.45 μm).

Protease activity. Bacterial cells were removed from culture broth by centrifugation at $6,000 \times g$ for 10 min at 4°C, and culture supernatants were used as a crude enzyme preparation. Hammarsten casein dissolved at 1% (wt/vol) in 0.1 M glycine-sodium hydroxide buffer (pH 9.6) was used as a substrate for protease assay. Enzyme (1 ml) was added to 1.0 ml of substrate solution and incubated at 30°C for 10 min. The reaction was stopped by adding 1.0 ml of 1 M trichloroacetic acid. As a control, trichloroacetic acid was added at zero time. Reaction mixtures were allowed to stand several minutes and were then centrifuged at $6,000 \times g$ for 10 min at 4°C. Absorption of the supernatants was measured at 280 nm. One unit of protease activity was defined as an increase of 0.001 absorbance unit per min with casein as a substrate.

DEAE-cellulose column chromatography. After shake culture in CT broth at 20°C for 45 h, culture supernatants of strains A-7301 and NTG-1 were obtained by centrifugation at $6,000 \times g$ for 15 min at 4°C. Acetone (2.5 liters, 4°C) was gently added to 1 liter of the supernatant at 4°C. Acetone precipitates, collected by centrifugation at $10,000 \times g$ for 15 min at 4°C and evaporation of acetone, were dissolved in 2 ml of phosphate buffer and fractionated on a DEAE-cellulose column (1.6 by 20 cm), as previously described (16). Three protein peaks were obtained. As protease activity appeared exclusively in the second peak of A-7301 and no activity was found in any other peaks, the protease fraction (1 ml = 10 mg of protein possessing a protease activity of ca. 15,000 U) was added to 200 ml of filtered (pore size, 0.45 μm) casein (2 g/liter, pH 7.4) medium in 500-ml flasks. To investigate the role of extracellular protease in growth, NTG-1 was inoculated into the casein medium and incubated with shake culture at 20°C for 90 h. The second peak (1 ml =

TABLE 1. Effects of the addition of protease to muscle homogenates on the growth of NTG-1

Strain inoculated into muscle homogenate ^a	No. of viable cells/g; protease activity (U/g) after the following day		
	0	2	5
A-7301	8.5×10^5 ; ND ^b	4.4×10^6 ; 11.7	1.3×10^9 ; 67.8
A-7301 + protein fraction of NTG-1 ^c	5.9×10^5 ; ND	7.2×10^6 ; 20.4	7.8×10^8 ; 75.4
NTG-1	3.1×10^5 ; ND	5.8×10^5 ; ND	7.7×10^4 ; ND
NTG-1 + protease fraction of A-7301 ^d	5.0×10^5 ; 130.5	2.1×10^7 ; 108.3	8.3×10^8 ; 102.6

^a Homogenate prepared from muscle of rainbow trout.

^b ND, Not detected.

^c This protein fraction (having no protease activity) was obtained from extracellular products of NTG-1 by DEAE-cellulose column chromatography. This was comparable with a protease fraction of A-7301.

^d This protease fraction was obtained from extracellular products of A-7301 by DEAE-cellulose column chromatography.

FIG. 2. Effects of reaction temperature and preservation temperature on the activity of protease produced by A-7301. Panel A, Reaction temperature; panel B, preservation temperature.

10 mg of protein possessing no protease activity) of NTG-1 was also used. Exact protein content was measured by the method of Lowry et al. (8).

Bacterial growth in homogenates of fish muscle and muscle extracts. The surfaces of rainbow trout were disinfected with 70% ethyl alcohol, and muscle was isolated and pooled from five fish. The muscle was homogenized in nine times (for homogenates) or four times (for muscle extracts) its weight

of sterile saline (0.85% NaCl). One ml (containing ca. 10^6 viable cells) of bacterial cell suspension in saline was inoculated into 9 g of the homogenates in tubes, which was then incubated at 20°C for 5 days. Viable counts and the numbers of viable cells per muscle were calculated. Culture supernatants, obtained by centrifugation at $6,000 \times g$ for 10 min at 4°C, were assayed for protease activity. For the preparation of muscle extracts, extracts were collected from the homogenates by centrifugation at $6,000 \times g$ for 10 min at 4°C, and the supernatants were filtered (pore size, 0.45 μ m). Bacterial suspension was inoculated into tubes containing 6 ml of muscle extracts and incubated at 20°C for 4 days without shaking.

LD₅₀ determination. Groups consisting of five rainbow trout received 0.1 ml of viable cell suspensions which were serially diluted 10-fold with saline (0.85% NaCl). Intraperitoneally injected fish were maintained for 2 weeks, and mortality was recorded. LD₅₀s were calculated by the method of Reed and Muench (14).

RESULTS

LD₅₀s. Strain A-7301 was pathogenic to rainbow trout (the LD₅₀ dose was 1.3×10^5 viable cells per fish), and strain NTG-1 was nonpathogenic (LD₅₀ was more than 10^8 cells per fish).

Protease production. In shaken cultures in CT broth, the optimum temperature for growth of strains A-7301 and NTG-1 and for protease production in A-7301 was 20°C (Fig. 1). A-7301 initiated protease production at a mid-exponential growth phase (at 10 to 20°C), but the production was abrogated at 25°C despite considerable growth. Although the growth of NTG-1 was similar to that of A-7301, no protease activity was detected in the culture supernatants at 10 to 25°C. Mutant NTG-1 induced from A-7301 was confirmed to be a nonproducer of extracellular protease.

Extracellular protease. The effects of temperature on the protease of A-7301 are shown in Fig. 2. The optimal reaction temperature was 50°C at pH 9.6 (Fig. 2A; this pH was optimal for this protease). At 50°C, protease activity decreased by 35% after 3 h and at 60°C disappeared within 1 h (Fig. 2B). Preservation at 0 to 40°C resulted in no change in protease activity. The protease proved to be markedly stable; in fact, the activity remained completely intact in prolonged preservation at 20°C for 1 week.

FIG. 3. Comparison between A-7301 and NTG-1 in growth and protease production in muscle extracts and normal serum prepared from rainbow trout. The strains were incubated at 20°C without shaking.

FIG. 4. Amino acid requirements of A-7301 for growth and protease production. The strain was incubated at 20°C for 60 h in mixed amino acid media with shaken culture. Growth (■) and extracellular protease (□) were assayed.

Growth in fish muscle extracts and serum. Viable counts of A-7301 increased from 2×10^6 cells per ml after inoculation to 2×10^8 to 5×10^8 cells per ml after 4 days in muscle extracts or normal serum media and produced extracellular protease in high activity (Fig. 3). NTG-1 showed no increase in viable counts, maintaining the initial levels of 10^6 cells per ml, and produced no protease (Fig. 3).

Amino acid requirements for growth. To determine the difference in amino acid requirements, the growth and protease production of A-7301 and NTG-1 were compared by using amino acid media of 24 and 20 combinations of amino acids, respectively. Seven amino acids (Gly, Ala, Val, Thr, Cys, Met, and His) were indispensable for the growth of both strains (Fig. 4 and 5). The amino acid media which

supported high growth also supported the higher protease production (Fig. 4). Strain NTG-1 never produced protease in any of the amino acid media tested, despite good growth (Fig. 5).

Correlation between growth and protease production. The correlation of extracellular protease with growth was investigated with amino acid media composed of increasing concentrations of an amino acid mixture (complete amino acids) and amino acid-casein media. In the amino acid media, the growth of either A-7301 or NTG-1 entirely depended on concentrations of amino acids present (Fig. 6A). There was no difference between the growth of A-7301 and NTG-1 in the amino acid requirements. Although A-7301 produced protease activity only when the mixed amino acid concen-

FIG. 5. Amino acid requirements of NTG-1 for growth. The strain was incubated at 20°C for 60 h in mixed amino acid media with shaken culture. Growth was determined. No protease activity was detected.

trations were above 0.6 g/liter, the protease had no effects on the growth when compared with that of NTG-1 showing no protease production.

Neither strain grew in casein alone (Fig. 6B). In the amino acid-casein media, the growth of NTG-1 was related to the amounts of amino acids, with the presence of casein having no effect. In contrast, A-7301 demonstrated higher growth in amino acid-casein media than that in media of equivalent amino acid concentration alone (Fig. 6B). This increased growth at more than 0.5 g of mixed amino acids per liter corresponded with higher levels of extracellular protease activity.

Role of protease in reproduction. Although A-7301 showed good growth and protease production in muscle homogenates, NTG-1 did not. However, when the protease fraction of A-7301 was added to the muscle homogenates, NTG-1 grew well (Table 1). The addition of the comparable protein fraction of NTG-1 was ineffective in promoting growth of A-7301 (Table 1). In the casein medium (supplemented with no amino acid), the growth of NTG-1 appeared within 30 h after the addition of the protease fraction of A-7301, although this strain could not grow in casein alone (see Fig. 7). The comparable fraction of NTG-1 was similarly

ineffective on the growth of NTG-1 (Fig. 7). Thus, the growth of NTG-1 on a whole protein substrate was stimulated by the protease produced by the parent strain.

DISCUSSION

A. salmonicida is a producer of extracellular protease which is related to furuncle formation, lethality, and other signs of fish furunculosis (3, 9, 15–19). In this study, seven amino acids (Gly, Ala, Val, Thr, Cys, Met, and His) were indispensable for growth and protease production by strain A-7301 (Fig. 4 and 6). These amino acids were also indispensable for growth of the protease-deficient mutant NTG-1. This implies that amino acid requirements are a considerably stable characteristic of *A. salmonicida*. A-7301 and NTG-1 showed very low growth in 0.2 g/liter of a complete amino acid mixture comprising 20 amino acids, and at increasing concentrations these strains grew at a rate proportional to the concentration of amino acids (Fig. 6). The minimal concentration of 0.6 g/liter was needed for A-7301 to produce protease (Fig. 6A). The growth of A-7301 at more than 0.6 g/liter was always accompanied by the production of extracellular protease. These results indicated that, although *A. salmonicida* usually reproduces and secretes protease, the

FIG. 6. Comparison between A-7301 and NTG-1 in growth and protease production in amino acid media and amino acid-casein media. The strains were incubated at 20°C for 60 h in increasing concentrations of the complete amino acid media (A) and amino acid-casein media (B) with shaken culture. Growth (○) and extracellular protease (●) were assayed.

low growth is not always associated with protease production in amino acid-poor environments. This low growth with an accompanying lack of protease production may have significance with respect to long-lasting persistence in hosts with no furuncle formation (2). This may be supported by evidence that protease nonproducer NTG-1 is able to retain its viability for a long time in media composed of muscle extracts, serum, and muscle homogenates (Fig. 3, Table 1). Thus, it is interesting in relation to asymptomatic or latent infection by *A. salmonicida* that a threshold level of amino acids is required for in vitro protease production. However, this level might be more narrow in vivo, as suggested by the protease production of A-7301 in low concentrations of amino acids such as muscle homogenates (Table 1).

In the amino acids-casein media, there was enhanced growth and protease production (Fig. 6B). When the growth of NTG-1 is compared with that of A-7301, it can be seen that protease aids the supply of amino acids from digested casein, allowing further growth of A-7301, although both strains had no difference in amino acid requirements for their

reproduction. This interpretation is supported by the effects of a protease fraction from A-7301 which stimulated an enhanced growth in NTG-1 in the casein medium (Fig. 7). As casein permitted no reproduction of NTG-1, the supply of amino acids required for growth must depend on the digesting function of the protease added. This role of protease is relevant to the finding that the reproduction of NTG-1 in the muscle homogenates is due to the protease fraction of A-7301 (Table 1). This implies that the protease might contain endo- and exoproteolytic activities, but the proteolytic activity of this enzyme is thought to be considerably nonspecific, and endo- and exoprotease activities are uncertain. However, the protease at least breaks down casein into oligopeptides. Since it is common knowledge that gram-negative bacteria retain aminopeptidases as well as hydro-

FIG. 7. Roles of protease in the growth of NTG-1. The protease fraction obtained from extracellular products of A-7301 was added to the casein medium (2 g of casein per liter) at 30-h intervals, and growth (○) and protease activity (●) were monitored. The protein fraction (showing no protease activity) obtained from extracellular products of NTG-1, which was comparable with the protease fraction of A-7301 in DEAE-cellulose column chromatography, was also added as a control, and growth (△) and protease activity (▲) were determined.

lytic enzymes in an intracellular gap between the peptidoglycan layer and cytoplasmic membrane (periplasmic space or periplasm), oligopeptides produced by the extracellular protease will ultimately be incorporated as available amino acids into bacterial cells by cleaving enzyme activities of aminopeptidases of *A. salmonicida*. The role of the extracellular protease, therefore, is considered to be important in the stage of the production of oligopeptides from environmental proteins.

Amino acid mixtures lacking Asp, Asn, Glu, and Gln or Asp, Asn, and Gln supported poor growth and poor protease production in A-7301 (Fig. 4). In particular, the lowest protease production was produced when Asn and Gln were absent. These results show that Asn and Gln are needed not only for growth but also for high protease production and, therefore, that transamination may concern the de novo synthesis of the protease (as an enzyme protein).

A possibility that extracellular protease is of significance in the reproduction of *A. salmonicida* is certain. In this bacterium, the protease is considered to be responsible for a supply of amino acids as nutrients, and the role is consistent with the conventional understanding of extracellular protease in heterotrophic bacteria. It is probable that proteases produced by other heterotrophs would play no small role in their growth properties and that extracellular proteases are not an irrelevant excretion from heterotrophs and *A. salmonicida*.

In addition, with respect to the pathogenesis of fish furunculosis, protease from A-7301 will effectively digest tissues in the proximity of infected sites, thereby suggesting that bacteria proliferate and damage the hosts further. The fortuitous lodging of bacteria in the derm may ultimately bring about dermal lesions containing many viable bacterial cells, high protease activities, and bloody, opaque fluids of digested tissues, as previously reported (16). The combination of enhanced growth with protease production in hosts is strongly suggested by the results of Table 1.

On the basis of the results of this study, the following is concluded. (i) *A. salmonicida* extracellular protease is a relevant excretion from bacteria to growth. (ii) The protease production is closely associated with the reproduction and growth of *A. salmonicida* in relation to a supply of amino acids. These findings concerning the role of extracellular protease would be of importance in the basic bacterial physiology of protease-producing heterotrophs as well as in the pathogenesis of diseases by pathogenic bacteria showing extracellular protease production.

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LITERATURE CITED

- Allen, B. J., and R. M. W. Stevenson. 1981. Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Can. J. Microbiol.* 27:1114-1122.
- Bullock, G. L., and H. M. Stuckey. 1975. *Aeromonas salmonicida*: detection of asymptotically infected trout.

- Prog. Fish-Cult. 37:237-239.
- Ellis, A. E., T. S. Hastings, and A. S. L. Munro. 1981. The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. *J. Fish Dis.* 4:41-51.
- Evenberg, D., R. V. Bostel, B. Lugtenberg, F. Schurer, J. Blommaert, and R. Bootsma. 1982. Cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*. I. Relationship between autoagglutination and the presence of a major cell envelope protein. *Biochim. Biophys. Acta* 684:241-248.
- Fuller, D. W., K. S. Pilcher, and J. L. Fryer. 1977. A leukocytolytic factor isolated from cultures of *Aeromonas salmonicida*. *J. Fish. Res. Board Can.* 34:1118-1125.
- Ishiguro, E. E., W. W. Kay, T. Ainsworth, J. B. Chamberlain, R. A. Austen, J. T. Buckley, and T. J. Trust. 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *J. Bacteriol.* 148:333-340.
- Kay, W. W., J. T. Buckley, E. E. Ishiguro, B. M. Phipps, J. P. L. Monette, and T. J. Trust. 1981. Purification and disposition of a surface protein associated with virulence of *Aeromonas salmonicida*. *J. Bacteriol.* 147:1077-1084.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mellergaard, S. 1983. Purification and characterization of new proteolytic enzyme produced by *Aeromonas salmonicida*. *J. Appl. Bacteriol.* 54:289-294.
- Mitruka, B. M. 1976. Methods and identification of bacteria, p. 13-82. CRC Press, Cleveland.
- Munn, C. B., E. E. Ishiguro, W. W. Kay, and T. J. Trust. 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infect. Immun.* 36:1069-1075.
- Nomura, S., and H. Saito. 1982. Production of the extracellular hemolytic toxin by an isolated strain of *Aeromonas salmonicida*. *Bull. Jpn. Soc. Sci. Fish.* 48:1589-1597.
- Pavlovskis, O. R., and B. Wretling. 1979. Assessment of protease (elastase) as a *Pseudomonas aeruginosa* virulence factor in experimental mouse burn infection. *Infect. Immun.* 24:181-187.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent end points. *Am. J. Hyg.* 27:493-497.
- Sakai, D. K. 1984. The non-specific activation of rainbow trout, *Salmo gairdneri* Richardson, complement by *Aeromonas salmonicida* extracellular products and correlation of complement activity with the inactivation of lethal toxicity products. *J. Fish Dis.* 7:329-338.
- Sakai, D. K. 1985. Loss of virulence in a protease-deficient mutant of *Aeromonas salmonicida*. *Infect. Immun.* 48:146-152.
- Sheeran, B., and P. R. Smith. 1981. A second extracellular proteolytic activity associated with the fish pathogen *Aeromonas salmonicida*. *FEMS Microbiol. Lett.* 11:73-76.
- Shieh, H. S. 1982. Lethal toxicity of *Aeromonas salmonicida* protease to Atlantic salmon. *Microbios Lett.* 20:137-139.
- Shieh, H. S., and J. R. McLean. 1975. Purification of properties of an extracellular protease of *Aeromonas salmonicida*, the causative agent of furunculosis. *Int. J. Biochem.* 6:653-656.
- Snell, K., I. A. Holder, S. A. Leppala, and C. B. Saelinger. 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with *Pseudomonas aeruginosa*. *Infect. Immun.* 19:839-845.
- Titball, R. W., and C. B. Munn. 1981. Evidence for two haemolytic activities from *Aeromonas salmonicida*. *FEMS Microbiol. Lett.* 12:27-30.
- Udey, L. R., and J. L. Fryer. 1978. Immunization of fish with bacterins of *Aeromonas salmonicida*. *Mar. Fish. Rev.* 40:12-17.
- Wretling, B., L. Heden, and T. Wadstrom. 1973. Formation of extracellular haemolysin by *Aeromonas hydrophila* in relation to protease and staphylolytic enzyme. *J. Gen. Microbiol.* 78:57-65.