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Purification, virulence and characterization of an extracellular peptidase produced by *Aeromonas salmonicida* ssp. *masoucida* isolated from cultured sea cucumber *Apostichopus japonicus*

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ABSTRACT: Strain H1, *Aeromonas salmonicida* ssp. *masoucida* isolated from an ulcer on the sea cucumber *Apostichopus japonicus* proved to be virulent toward this holothurian at 12°C. Extracellular products (ECP) of strain H1 were prepared, and an extracellular peptidase of 42 kDa was purified from the ECP using Sephadex G-100 gel filtration chromatography and diethylaminoethyl Sepharose ion exchange chromatography. Stepwise virulence tests on sea cucumbers indicated that the 42 kDa peptidase was the virulence factor of strain H1, and its LD_{50} for these holothurians was 1.12 µg g^{-1}. A peptidase activity test using azocasein as a substrate showed that optimum pH and temperature for the peptidase are 8.0 and 40°C, respectively; the peptidase was completely inactivated above 70°C. The peptidase was inhibited strongly by 5 mM phenylmethanesulphonyl fluoride, indicating that the enzyme is a serine peptidase.

KEY WORDS: Sea cucumber · Bacterial ulceration disease · *Aeromonas salmonicida* ssp. *masoucida* · Extracellular peptidase

INTRODUCTION

In recent times, the sea cucumber *Apostichopus japonicus* has become a major aquaculture product along the coasts of Shandong and Liaoning provinces in China, where 1 to 2 billion larvae may be produced and up to 90 000 t of sea cucumbers (live weight) are harvested annually (Wang et al. 2003). The rapid expansion and intensification of sea cucumber farming has led to the occurrence of various diseases, among which epidemic bacterial ulceration disease (which first appeared in 2004) is the most serious and responsible for great losses. In affected sea cucumbers ranging from 5 to 10 cm in length, the disease symptoms are wasting, excessive mucus production, gut disgorgement, serious ulceration on the body surface and finally a mortality rate up to 80% as the ulcers spread and autolysis sets in. In our early research, strain H1 isolated from diseased sea cucumbers and identified as *Aeromonas salmonicida* ssp. *masoucida* caused sea cucumber death at a LD_{50} concentration of 1.8×10^7 CFU per sea cucumber (using the injection challenge test at 16°C; Yang et al. 2007); but this LD_{50} dose did not completely explain the high mortalities in farming operations. Hence, in this study we re-tested the virulence of strain H1 at 12°C, and showed that it is highly lethal toward sea cucumbers at lower temperatures.

Aeromonas salmonicida is an important pathogen of many aquatic animals, including sea urchins (Gilles & Pearse 1986), Atlantic salmon (Bernoth 1997), eel (Kitao et al. 1984), goldfish (Dror et al. 2006) and carp (Evenberg et al. 1986, Austin & Austin 1993). Since diseases caused by *A. salmonicida* have caused severe

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losses in grow-out farms, it is essential to identify the main pathogenic mechanism of this bacterium in order to develop efficient strategies for preventing outbreaks of disease. Several potential virulence factors of *A. salmonicida* ssp. *salmonicida* and *A. salmonicida* ssp. *achromogenes* have been reported to date, including the surface A-layer protein (Chu et al. 1991), hemolysins (Hirono & Aoki 1993), H-lysin (Titball & Munn 1985) and peptidase (Whitby et al. 1992, Austin & Austin 1993, Gudmundsdóttir et al. 1995), but there is still no report on the causative agent of the disease caused by *A. salmonicida* ssp. *masoucida*. Our previous study showed that extracellular products (ECP) of strain H1 were lethal to sea cucumbers at a LD_{50} of 5.24 µg g^{-1}; the ECPs were inactivated by treatment at 70°C for 30 min, and the optimum temperature was 50°C (Yang et al. 2007). Here we show that an extracellular peptidase of 42 kDa purified from the ECP by Sephadex G-100 gel filtration chromatography and diethylaminoethyl (DEAE) Sepharose ion exchange chromatography is the virulence factor of strain H1.

MATERIALS AND METHODS

Microorganism and culture conditions. Strain H1 (*Aeromonas salmonicida* ssp. *masoucida*) was isolated from a sea cucumber ulcer (Yang et al. 2007); strains H2 and H3 were also isolated from sea cucumber ulcers but were non-pathogenic strains to holothurians. All strains were cultured on brain heart infusion agar at a salinity and temperature of 20 and 22°C, respectively.

Virulence test. Healthy sea cucumbers averaging 6 cm in length were provided by a sea cucumber farm (Shandong, China), and held at 12°C in flow-through seawater. The strains were suspended in sterile phosphate-buffered saline (PBS, 0.01 M $NaH_2PO_4 \cdot 2H_2O$, 0.01 M $Na_2HPO_4 \cdot 2H_2O$, 0.15 M NaCl, pH 7.2) and diluted serially after growing for 24 h on BHI agar at 22°C.

Virulence was tested in 2 ways: (1) sea cucumbers were slightly wounded (wound length: 2 mm) with a razor blade in suspensions of bacteria diluted in filtered seawater to concentrations of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU; (2) sea cucumbers were injected intramuscularly with 50 µl of bacterial suspensions at concentrations of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 CFU ind.$^{-1}$. In addition, strains H2 and H3 were injected at a concentration of 1×10^7 CFU ind.$^{-1}$ and acted as controls.

The virulence test lasted 2 wk, and mortalities of affected sea cucumbers were recorded daily. Re-isolation and identification of the bacteria from moribund sea cucumbers was conducted with BHI agar plates. LD_{50} was calculated by the Reed-Muench method (Reed & Muench 1938).

Transmission electron microscopy (TEM) observation. For TEM, a bacterial suspension of 10 µl was negatively stained with an equal volume of 1% phosphotungstic acid (PTA, pH 6.8) and applied to a Parlodion®-coated copper grid for 3 min, then dried for 10 min, and viewed with JEOL JEM-1200EX transmission electron microscope (Cheng et al. 2006).

Preparation of extracellular products (ECPs). The ECPs were extracted using the cellophane plate technique modified after Liu (1957). Exponential phase cultures of the strain grown in liquid BHI incubated for 18 h at 22°C were spread on sterile cellophane sheets overlying BHI plates (24 h, 22°C). The cells were rinsed from the plates with PBS (pH 7.2) and removed by centrifugation (8000 × *g*, 30 min, 4°C). The supernatant was sterilized by filtration through a 0.22 µm filter (Millipore), and then precipitated with 80% saturated ammonium sulphate; ECP produced from the precipitate were dialysed against PBS (pH 7.2) at 4°C for 24 h.

Peptidase purification. ***Gel filtration chromatography:*** The ECP were concentrated with stirred ultrafiltration cells (Millipore, cut-off 5 kDa) and loaded into a Sephadex G-100 gel filtration column. Equilibration and elution were carried out in 50 mM Tris-HCl buffer (pH 8.0). The fractions were collected at a flow rate of 0.5 ml min^{-1} and the protein concentration was monitored at an absorbance of 280 nm. Virulence (LD_{50} to sea cucumber) and enzyme activity of each peak were measured, and the purity of the protein was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme activity was determined by the method of Windle & Kelleher (1997), with azocasein (Sigma) as substrate. Azocasein was dissolved in Tris-HCl buffer (50 mM, pH 8.0) to a concentration of 5 mg ml^{-1}. A concentrated fraction (0.5 ml) of enzyme was incubated with 0.5 ml azocasein for 20 min at 37°C, and the reaction was terminated by adding 3.5 ml of 10% (w/v) trichloroacetic acid. After centrifugation (10 000 × *g*, 5 min), the supernatant was removed from the settled protein precipitate. The supernatant was mixed with an equal volume of NaOH (0.5M), and measured with a spectrophotometer at a wavelength of 440 nm. Controlled ECP were inactivated completely by heating at 100°C for 20 min. One unit of enzyme activity was defined as the amount of the enzyme giving an absorbance change of 0.01 in 1 min at 440 nm. Enzyme activities of the cultured supernatant and ECP were also measured as a control.

Ion exchange chromatography: Peak S2, which showed high virulence and enzyme activity according to the virulence and enzyme activity assays, was concentrated and fractionated with a DEAE Sepharose

Column (Amersham) using a technique developed by Nishida et al. (1998). Concentrated S2 was injected into the column after equilibration with buffer (20 mM Tris-HCl, pH 8.0) at a flow rate of 1 ml min^{-1}. The column was washed with the same equilibration buffer to eliminate unbound proteins. The bound proteins were then eluted using a stepwise procedure with a series of buffers containing NaCl at consecutive molarities of 0.05, 0.1, 0.15, 0.2 and 0.4 M. The protein concentration of the effluent was monitored continually at 280 nm. Virulence, enzyme activity and protein purity were measured for each peak.

Optimal conditions for peptidase. To determine the optimal pH, solutions from pH 3.6 to 13 were prepared with the following buffers (50 mM) (Alam et al. 2005): sodium acetate (pH 3.0 to 5.0), potassium phosphate (pH 6 to 8), Tris-HCl (pH 7.0 to 9.0), sodium borate (pH 9.0 to 10.0), and glycine-NaOH (pH 11.0 to 13.0).

To determine optimal temperature profiles, enzyme assays were carried out at 4, 10, 20, 30, 40, 50, 60, 70 and 80°C using the method described in 'Peptidase purification'.

Thermal stability of peptidase. To determine the thermal stability of the peptidase, it was pre-incubated for 30 min at 4, 10, 20, 30, 40, 50, 60, 70 and 80°C, and activity was measured at 37°C.

Inhibition of peptidase. To investigate the effects of oxidizing agents, detergents, peptidase inhibitors and metal ions on peptidase activity, enzymatic assays were carried out after pre-incubation for 1 h with iodoacetic acid, sodium dodecyl sulphate (SDS), phenylmethanesulphonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), Ca^{2+}, Mg^{2+}, Mn^{2+} and Hg^{2+}, after which enzyme activity was monitored by the method described in 'Peptidase purification'.

RESULTS

Virulence tests and transmission electron microscopy observations

Three strains (H1, H2 and H3) were used in the virulence test. Strains H2 and H3 were not able to infect sea cucumber in either challenge method, while strain H1 was highly virulent to holothurians (Table 1). Strain H1 caused chronic infection at a LD_{50} of $1.0 \times 10^{5.5}$ CFU per sea cucumber through immersion challenge, and an acute infection at a concentration of $1.0 \times 10^{5.25}$ CFU per sea cucumber through injection challenge. The symptoms of moribund sea cucumbers in both challenge styles were similar to those observed in moribund, naturally infected individuals.

Pure cultures of the predominant strain of the bacterium were re-isolated from the ulcers of moribund sea cucumbers after bacterial challenge; these isolates were used to re-infect individuals, and similar symptoms were observed.

Strain H1 was a Gram negative, straight rod bacterium with rounded ends and no flagellum (Fig. 1). The cells were ca. 0.5 µm in diameter and 1.2 µm in length, and occurred in pairs.

Extracellular peptidase purification and virulence testing

Gel filtration chromatography was performed on ECP, and 2 peaks, S1 and S2, were collected (Fig. 2). Although the fraction at peak S2 was at a lower concentration than the fraction at peak S1, S2 had high enzyme activity and virulence (Table 2). Ion exchange chromatography was performed on the fraction from

Table 1. *Aeromonas salmonicida* ssp. *masoucida*, strain H1. Virulence to sea cucumbers. n = 10 sea cucumbers in each test group

Challenge style	Dose (CFU $ind.^{-1}$)	No. of dead sea cucumbers	Mortality (%)
Bath (wounded)	1×10^7	10	100
	1×10^6	7	70
	1×10^5	3	30
	1×10^4	0	0
Injection	1×10^7	10	100
	1×10^6	8	80
	1×10^5	4	40
	1×10^4	0	0

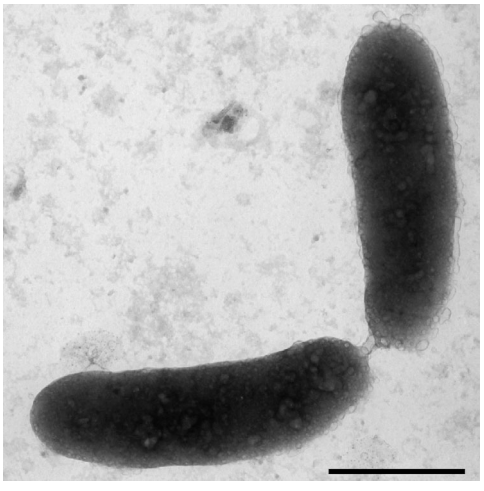

Fig. 1. *Aeromonas salmonicida* ssp. *masoucida*. Cell morphology (TEM) showing characteristic pairing (bar = 500 nm)

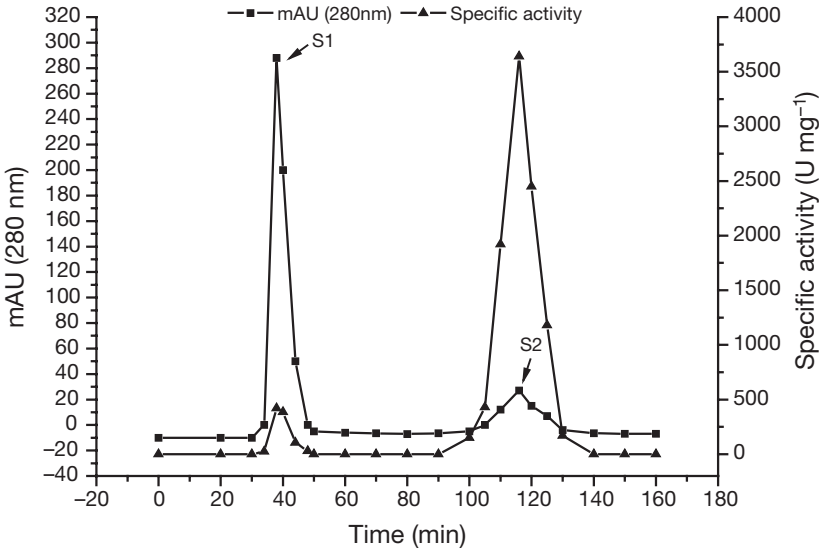

Fig. 2. *Aeromonas salmonicida* ssp. *masoucida*. Purification profile of peptidase from extracellular products (gel filtration on a Sephadex G-100 column). The concentrated extracellular products were injected into a 1.5 cm × 70 cm column, equilibrated and eluted with 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 ml min^{-1}. Two peaks (S1, S2) were collected, and enzyme activity of each peak measured at 37°C and pH 8.0

peak S2 (Fig. 2); peak D1 (among 5 peaks D1, D2, D3, D4 and D5 that separated) (Fig. 3) showed much higher enzyme activity and virulence (Table 2) than fraction S2, and was identified as the virulent factor of strain H1.

SDS-PAGE of the products at different stages of purification (Fig. 4) showed that the ECP consist of more than 13 protein bands; although the fraction from peak S2 (Fig. 2) had high enzyme activity and virulence, it was not a purified protein; the fraction from peak D1 (Fig. 3) consisted of a single protein band of 42 kDa, showing that the peptidase was purified.

The stepwise procedure purified the peptidase 21.9-fold, with a total yield of 1.7%. The enzyme was virulent (LD_{50}) to sea cucumbers at 1.12 µg g^{-1} body weight, which is 4.7-fold higher than the virulence of the ECP (Table 2).

Optimal conditions, thermal stability and inhibition of the peptidase

Measurements through a broad pH range revealed that the optimum working pH for the enzyme was 8.0 (Fig. 5). Enzyme activity was 70 to 80% of its maximal value at pH 5.0 and 10.0. Enzyme activity declined to a greater extent under acidic conditions than under alkaline conditions. The optimum temperature across the range 4 to 80°C was 40°C (at pH 8.0) (Fig. 6).

The peptidase was stable for 30 min when incubated at temperatures below 40°C, but activity declined strongly in temperature over 40°C. The enzyme was inactivated at temperatures >70°C (Fig. 6).

Effects of oxidizing agents, detergents, peptidase inhibitors and metal ions on enzyme activity were investigated by measuring residual activity after incubation with each of these potential inhibitors for a fixed length of time. Iodoacetic acid (oxidizing agent) and SDS (detergent) reduced enzyme activity to 58% and 84% of maximum, repectively. The enzyme was slightly inhibited by EDTA (inhibitor of metal-peptidase and chelating agent), with a loss of 18% of the original activity, but PMSF (the serine peptidase inhibitor) reduced enzyme activity by 98%; residual activity of the enzyme after incubation with Hg^{2+} and Mn^{2+} were 36% and 52% of maximum values, respec-

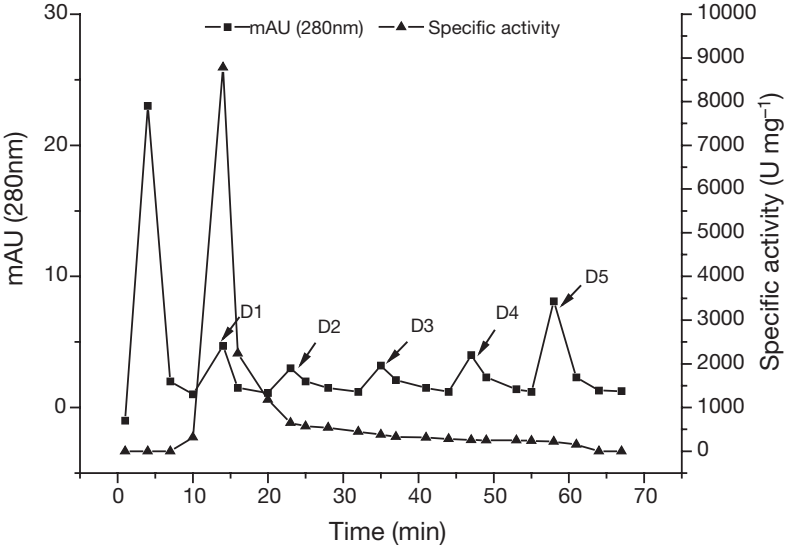

Fig. 3. *Aeromonas salmonicida* ssp. *masoucida*. Elution profile of peptidase from peak S2 (Fig. 2) by diethylaminoethyl Sepharose ion exchange chromatography. The concentrated S2 fraction was loaded into the column, which was then washed with 20 mM Tris-HCl (pH 8.0) and the bound proteins were eluted stepwise with the same buffer containing NaCl at 0.05, 0.1, 0.15, 0.2 and 0.4 M. Five peaks (D1, D2, D3, D4 and D5) were collected, and the enzyme activity of each was measured at 37°C and pH 8.0

tively. Ca^{2+} and Mg^{2+} increased activity by 9% and 3%, respectively (Table 3).

DISCUSSION

Since first reported by Morgan (2000), bacterial ulceration disease in sea cucumbers has been spreading widely and different pathogens causing severe loss have been isolated. *Vibrio harveyi* may be the pathogen of affected holothurians off Bribie Island, Australia (Morgan 2000). *Vibrio* spp., *Bacteroides* spp., and Alphaproteobacterium were isolated from lesions of sea cucumbers off Madagascar (Becker et al. 2004). In the present and our previous work, we demonstrated that *Aeromonas salmonicida* ssp. *masoucida* is the pathogen of sea cucumber ulceration disease in Shandong province, China; we also showed that strain H1 was more lethal at low temperatures (12°C), which might explain why sea cucumber ulceration disease prevails in winter in northern China.

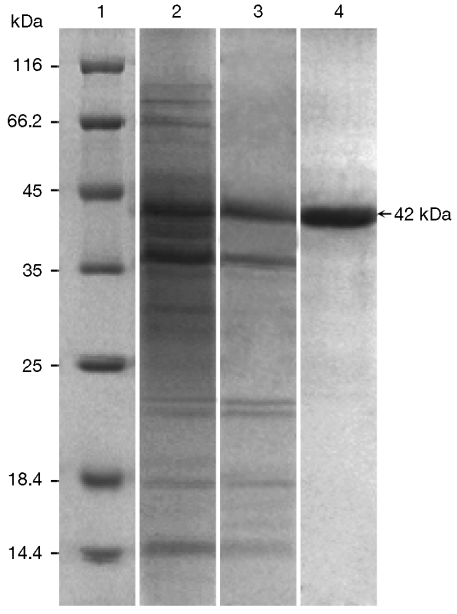

Fig. 4. *Aeromonas salmonicida* ssp. *masoucida*. SDS-PAGE of extracellular peptidase at different stages of purification. Lane 1: molecular weight marker; Lane 2: extracellular products; Lane 3: peak S2 from Sephadex G-100 gel filtration chromatography; Lane 4: purified peptidase (peak D1 from DEAE Sepharose ion exchange chromatography)

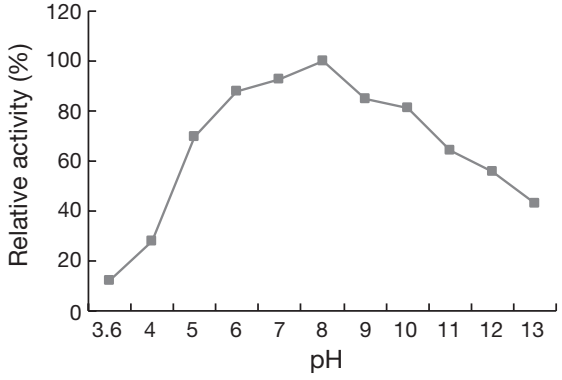

Fig. 5. *Aeromonas salmonicida* ssp. *masoucida*. Effects of pH on enzyme activity. Enzyme samples were incubated in respective buffers at 37°C and relative activity was calculated. Relative activity is a proportion (%) of maximum peptidase activity

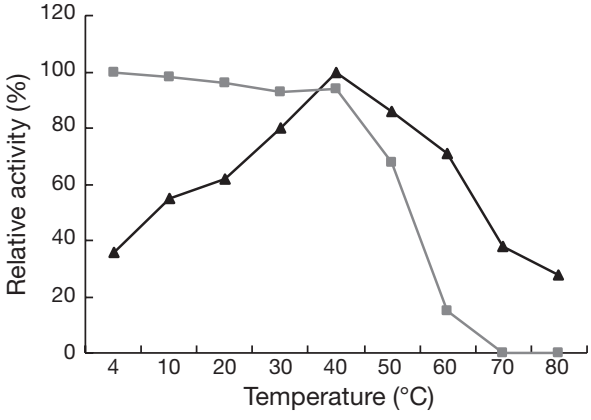

Fig. 6. *Aeromonas salmonicida* ssp. *masoucida*. Temperature optimum and thermal stability of the peptidase. –▲–: temperature optimum; enzyme activity was measured between 4 and 80°C at pH 8.0. –■–: thermal stability; peptidase was incubated for 30 min at temperatures shown and relative activity was calculated (proportion (%) of maximum peptidase activity)

Table 2. *Aeromonas salmonicida* ssp. *masoucida*. Purification of extracellular peptidase

Fraction	Total protein (mg)	Protein yield (%)	Specific activity (U mg^{-1})	Purification (fold: geometric mean increase)	Virulence (LD_{50}) (µg g^{-1} weight)	Purification of virulence (fold: geometric mean increase)
Culture supernatant	3250	100	400	1	—	—
Extracellular products	1850	57	588	1.5	5.24	1
Sephadex G-100	370	11.4	3640	9.1	2.16	2.4
DEAE Sepharose	56	1.7	8780	21.9	1.12	4.7

Table 3. *Aeromonas salmonicida* ssp. *masoucida*. Effect of various reagent concentrations on peptidase activity. SDS: sodium dodecyl sulphate; PMSF: phenylmethanesulphonyl fluoride; EDTA: ethylenediamine tetraacetic acid

Reagent	Concentration (mM)	Residual activity (%)
None	0	100
Iodoacetic acid	10	58
EDTA	5	82
PMSF	5	2
SDS	5	84
Hg^{2+}	5	36
Mn^{2+}	5	52
Ca^{2+}	5	109
Mg^{2+}	5	103

Although our work is the first to show that sea cucumber ulceration disease is caused by *Aeromonas salmonicida*, there is a report of sea urchin disease caused by *A. salmonicida* (Gilles & Pearse 1986). Thus, we have added to the list of hosts for this bacterium.

The fact that ECP of strain H1 contained a peptidase that was lethal to sea cucumbers whereas the inactivated ECP (100°C, 30 min) was not, shows that peptidase activity is an indispensable factor for strain H1 virulence (Yang et al. 2007). This conclusion was further corroborated by results of the virulence and enzyme activity analyses of different chromatogram peaks, which showed that virulence increased with increasing peptidase activity levels. A serine peptidase with a single band of 42 kDa molecular weight on the SDS-PAGE was purified and is considered to be the virulence factor of *Aeromonas salmonicida* ssp. *masoucida*. This is different from the 70 kDa serine peptidase of *A. salmonicida* ssp. *salmonicida* (Fyfe et al. 1987, Price et al.1990) and the 20 kDa toxic metallocaseinase of *A. salmonicida* ssp. *achromogenes* (Gudmundsdóttir et al. 1995).

The peptidase of strain H1 was active over broad pH (6.0 to 10.0) and temperature (30 to 60°C) ranges, with maximum activity at pH 8.0 and 40°C; the enzyme was not thermally stable and was completely inactivated over 70°C. The enzyme was strongly inhibited in the presence of 5 mM PMSF, a common inhibitor of serine peptidase, but was not very sensitive to other inhibitors, indicating that the peptidase of strain H1 is a serine peptidase.

The extracellular peptidase of *Aeromonas salmonicida* is a significant exotoxin that causes serious diseases and disorders in aquatic animals (Fyfe et al. 1987, Price et al. 1990, Gudmundsdóttir et al. 1995). We have shown that a 42 kDa serine extracellular peptidase is the main virulence factor of the *A. salmonicida* ssp. *masoucida*.

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