Purification of a Toxic Metalloprotease Produced by the Pathogenic *Photobacterium damselae* subsp. *piscicida* Isolated from Cobia (*Rachycentron canadum*)

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The aim of the present study was to purify and characterize a toxic protease secreted by the pathogenic *Photobacterium damselae* subsp. *piscicida* strain CP1 originally isolated from diseased cobia (*Rachycentron canadum*). The toxin isolated by anion exchange chromatography, was a metalloprotease, inhibited by L-cysteine, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(*β*-aminoethyl ether)*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 1,10-phenanthroline, *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), and *N*-*α*-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK), and showed maximal activity at pH 6.0–8.0 and an apparent molecular mass of about 34.3 kDa. The toxin was also completely inhibited by $HgCl_2$, and partially by sodium dodecyl sulfate (SDS) and $CuCl_2$. The extracellular products and the partially purified protease were lethal to cobia with LD_{50} values of 1.26 and 6.8 μg protein/g body weight, respectively. The addition of EDTA completely inhibited the lethal toxicity of the purified protease, indicating that this metalloprotease was a lethal toxin produced by the bacterium.

Key words: Metalloprotease, *Rachycentron canadum, Photobacterium damselae* subsp. *piscicida*

Introduction

Fish photobacteriosis is a bacterial fish disease commonly called "pasteurellosis" or "pseudotuberculosis" due to infection by the halophilic *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*). The signs of the disease in chronic form are typical whitish tubercles in the internal organs which consist of bacterial colonies (Magariños *et al*., 1996). Snieszko and colleagues (1964) first described the disease in wild populations of white perch (*Roccus amercianus*) and striped bass (*Morone saxatilis*) in Chesapeake Bay, USA. Recently, the disease has also been found in hybrid striped bass (Hawke *et al*., 2003). Janssen and Suragalla (1968) suggested the name *Pasteurella piscicida* for the causative agent of this disease. Since then, a variety of marine fish have been reported as natural hosts for the bacterium (Kimura and Kitao, 1971; Muroga *et al*., 1977; Ohnishi *et al*., 1982; Ueki *et al*., 1990; Kusuda and Nishimura, 1991; Toranzo *et al*., 1991; Balebona *et al*., 1992; Nakai *et al*., 1992; Fukuda *et al*., 1996; Liu *et al*., 2003).

Extracellular virulence factors including proteases produced by various *Vibrio* species isolated from diseased fish and shellfish have been suggested to play a significant role in pathogenesis (Inamura *et al*., 1985; Nottage and Birkbeck, 1987a, b; Norqvist *et al*., 1990; Farrell and Crosa, 1991; Stensvag *et al*., 1993; Morita *et al*., 1994; Lee, 1995; Lee *et al*., 1997). The extracellular products (ECP) of *P. damselae* subsp. *piscicida* strains were shown to be lethal for various fish species including gilthead sea bream, seabass, turbot, and rainbow trout with LD_{50} values ranging from 1 to 4.6 μg protein/g fish (Magariños *et al*., 1992, 1996). These values are similar to those obtained for other fish pathogens such as *Aermonas* and *Vibrio* (Toranzo and Barja, 1993).

Abbreviations: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; ECP, extracellular products; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(*β*-aminoethyl ether)*N*,*N*,*N*',*N*'-tetraacetic acid; FPLC, fast-protein liquid chromatography; HPA, hide powder azure; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLCK, *N*-*α*-*p*-tosyl-L-lysine-chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine-chloromethyl ketone.

Although slight variations in ECP activities among strains were described, specifically for caseinase, gelatinase, and dermonecrotic activities, all *P. damselae* subsp. *piscicida* isolates possess strong phospholipase, cytotoxic, and haemolytic activities in their ECP (Magariños *et al*., 1992). Recently, do Vale and colleagues (2005) demonstrated that ECP from exponentially growing *P. damselae* subsp. *piscicida* contain a secreted exotoxin with a putative metalloprotease signature. However, no information is available concering the extracellular proteases produced by pathogenic *P. damselae* subsp. *piscicida* isolated from diseased cobia.

In the present paper, an exoprotease has been purified from the ECP of *P. damselae* subsp. *piscicida* strain CP1. The virulence of the ECP and role of extracellular metalloprotease produced by *P. damselae* subsp. *piscicida* in *Rachycentron canadum* has been investigated and is discussed.

Material and Methods

Bacterial strains and extracellular products

Photobacterium damselae subsp. *piscicida* strains CP1, CP2, CP3, 9205, and 9304, originally isolated from diseased cobia (*Rachycentron canadum*) in Taiwan, were virulent to cobia (Liu *et al*., 2003) and were used in this study. Stock cultures of each strain were grown on brain heart infusion agar; BHIA (supplemented with 1.0% NaCl) (Difco, Becton Dickinson, le Pont de Claix, France) plates for 24 h at 30 °C. Two swabs of these bacteria suspended in 10 ml phosphate buffered saline (PBS), pH 7.2, were spread onto BHIA overlaid with sterile cellophane and grown for 48 h at 30 °C. The ECP were harvested by a procedure previously described (Lee and Ellis, 1990). Briefly, 5 ml of PBS were added to the surface of the cellophane-overlaying BHIA (+1% NaCl) and spread completely. The harvested bacterial suspension was then centrifuged at 25,000 x *g* for 60 min at 4 °C; the resulting pellet was discarded. The supernatant fluids were passed through a 0.22-μm filter (Millipore, Bedford, Ireland), and the ECP were stored in 1-ml aliquots at –20 °C. Total protein was measured by the method of Bradford (1976) with bovine serum albumin as a standard. Protease activity was measured by hide powder azure (HPA) digestion as previously described (Lee and Ellis, 1990). Briefly, enzyme solution (0.1 ml) was incubated with 25 mg HPA in 2.4 ml PBS at 37 °C for 15 min with shaking. On addition of 2.5 ml 10% trichloroacetic acid (TCA) and after centrifugation the absorbance of the supernatant was measured at 600 nm. Blanks were prepared by the addition of TCA to HPA in the absence of the enzyme. One unit of protease activity is an increase in absorbance of 0.01.

Purification of extracellular protease

To purify the extracellular protease, three consecutive steps of anion exchange chromatography were conducted. ECP were dialyzed against deionized distilled water overnight at 4 °C, lyophilized, and resuspended in 20 mM tris (hydroxymethyl) methylamine (Tris buffer, pH 7.0) prior to the fractionation by fast-protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden) with an anion exchange column (Q Sepharose High Performance; Pharmacia) equilibrated with 20 mM Tris buffer (pH 7.0). Fractions (3 ml) were eluted with a gradient of 0 to 1 M NaCl in 20 mM Tris buffer at a rate of 1 ml/min. The fractions possessing protease activity were pooled, concentrated with an Amicon (Beverly, MA, USA) ultrafilter (YM3 membrane) and dialyzed against distilled water overnight at 4 °C, and then equilibrated with 20 mM Tris buffer (pH 7.0) prior to the fractionation with an FPLC-anion exchange column (RESOURCE Q, Pharmacia). Fractions (2 ml) were eluted with a sodium chloride gradient (0–1 M) at a rate of 1 ml/min. The fractions possessing protease activity were pooled and processed as above prior to the fractionation with an FPLC-anion exchange column (Mono Q; Pharmacia). Fractions (1 ml) were eluted with a sodium chloride gradient (0–1 M) at a rate of 1 ml/min. Aliquots of 1 ml were stored at –20 °C in Eppendorf tubes.

Molecular mass determination and protease zymogram

Phastgel gradient (10–15%) polyacrylamide (Pharmacia) was employed in native PAGE or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and used to check the purity of fractions and determine the molecular mass of the protease. For SDS-PAGE, the SDS sample buffer was composed of 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% SDS, 0.5% bromophenol blue, and 20% glycerol; the pH value was adjusted to pH 8.0. Pharmacia high-molecular mass calibration kits

were used as marker proteins (myosin, 224 kDa; *β*-galactosidase, 122 kDa; bovine serum albumin, 96 kDa; ovalbumin, 51.5 kDa; carbonic anhydrase, 35.3 kDa; soybean trypsin inhibitor, 28.7 kDa; lysozyme, 21 kDa; aprotinin, 7.2 kDa). Electrophoresis was conducted on the PhastSystem (Pharmacia) according to the recommendations of the manufacturer. For native PAGE, protease was dissolved in 20 mM Tris buffer (pH 8.0), and electrophoresed in Phastgel native buffer strips (0.88 M L-alanine, 0.25 M Tris, pH 8.8; Pharmacia). After electrophoresis, the gels were stained with silver stain reagent (Pharmacia) for protein staining or overlaid with gels containing 1% agarose and 0.4% sodium caseinate in PBS for 2 h at 25 °C for a protease zymogram. Following removal of the overlay, the zymogram gels were stained with Coomassie brilliant blue R 250. The separation and development protocol followed the recommendation of the manufacturer.

Optimum pH value and thermostability

For determination of the optimum pH value, the purified protease preparation was added to PBS buffers with pH values adjusted from 4.0 to 12.0, using 1 M HCl and/or 1 M NaOH, and then incubated with HPA for the protease activity assay (in duplicate) as described above. The highest protease activity was used as control (100% of relative activity). For determination of the thermostability, aliquots of the purified protease were incubated for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80 °C, separately , and then directly cooled on ice prior to the protease activity assay. The protease activity of the sample incubated at 4 °C was used as control (100% of relative activity).

Enzyme inhibition

The effects of ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, USA), ethylene glycol-bis(*β*-aminoethyl ether)*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA; Sigma), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; Boehringer Mannheim, Germany), iodoacetamide (Serva, Heidelberg, Germany), 1,10-phenanthroline (Merck, Frankfurt, Germany), phenylmethanesulfonyl fluoride (PMSF; Sigma), sodium dodecyl sulfate (SDS; Serva), *N*-*α*-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK; Boehringer Mannheim), *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK; Sigma), $CaCl_2$ (Serva), $CuCl_2$ (Merck), $FeCl_2$ (Riedel-de Haën S. A., Seelze, Germany), $MnCl_2$ (Serva), $ZnCl_2$ (Merck), $HgCl_2$ (Merck), *N*-ethylmaleimide (Serva), and L-cysteine (Sigma) on the protease activity were examined. PMSF and TPCK were dissolved in isopropanol (Riedel-de Haën S. A.), and 1,10-phenanthroline was dissolved in methanol, all others were dissolved in distilled water. The concentration of each reagent was 5 or 10 mM as indicated in Table I. After incubation at 37 °C for 60 min, the changes in protease activity were determined using the HPA assay as described above.

Fish toxicity and toxicity inhibition tests

Cobia weighing approximately 5–6 g was separately held in tanks (2,500 l) supplied with air-lifted 3% salinity sea water at 25–28 °C. The LD_{50} tests, with batches of 10 fish per dose in duplicate, were conducted by intraperitoneal (i.p.) injection of 0.1-ml samples into each fish (Trevors and Lusty, 1985; Vera *et al.*, 1992). Sterile PBS was used in controls. Protease inhibitor (EDTA) was added to purified protease (2-fold LD_{50} value) and the mixture incubated overnight at 4 °C prior to the investigation of the inhibition of its toxicity in cobia; EDTA only was used as control. The protocol of toxicity inhibition tests was the same as described above in the LD_{50} tests. The mortalities of all tested animals were recorded for 2 weeks post i.p. injection of the sample. Experiments with the fish (cobia) were performed according to "The Ethical Guideline for Using Vertebrates as Experimental Animals in Taiwan" and have been approved by the "Ethical Committee for Using Vertebrates as Experimental Animals of National Taiwan Ocean University".

Results

ECP of P. damselae subsp. piscicida strains

The ECP of strains CP1, CP2, CP3, 9205, and 9304, respectively, were harvested after 48 h of incubation of the culture at 30 °C. The protease activities (HPA digestibility) of the ECP ranged from 400 to 2286 units/mg protein, the highest activity being produced by strain CP1 which was used for further purification.

Purification and properties of the extracellular protease

ECP proteins of strain CP1 eluted as three major peaks from the FPLC-Q Sepharose high-per-

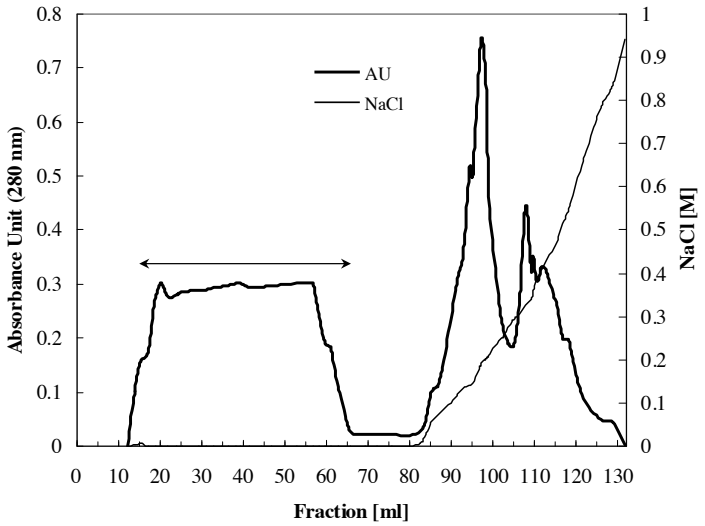

Fig. 1. Profile of CP1 ECP separated by FPLC-Q Sepharose high-performance chromatography (FPLC-HP). "↔" indicates the fractions exhibiting protease activities.

formance column, with protease activity eluted at 0 M NaCl (Fig. 1). Fractions (13–66 ml) possessing protease activity from eighteen similar collections (4,100 HPA units/mg protein) were pooled, ultrafiltrated, dialyzed, and eluted from the FPLC-RESOURCE Q column, with protease activity eluted at 0 M NaCl (Fig. 2). Fractions (1–34 ml) possessing protease activity from seventeen similar collections (4,300 HPA units/mg protein) were pooled, ultrafiltrated, dialyzed, and eluted from the FPLC-Mono Q column, with protease activity eluted at 0 M NaCl (Fig. 3). Fractions (1–27 ml) possessing protease activity (4,200 HPA units/mg protein) were pooled, ultrafiltrated, and dialyzed. Fractions collected from the first major peak (faction 1–27 ml) produced two polypeptide bands of 7.2 kDa and 34.3 kDa, respectively, in SDS-PAGE (Fig. 4), with only the 34.3-kDa polypeptide band of ECP exhibiting some caseinase activity in the protease zymogram. However, these fractions produced one single (34.3-kDa) polypeptide band in native PAGE (Fig. 5a), which showed strong caseinase activity in the protease zymogram (Fig. 5b).

pH optimum and thermostability

The purified protease showed maximal enzyme activity between pH 6 and 9. In the ther-

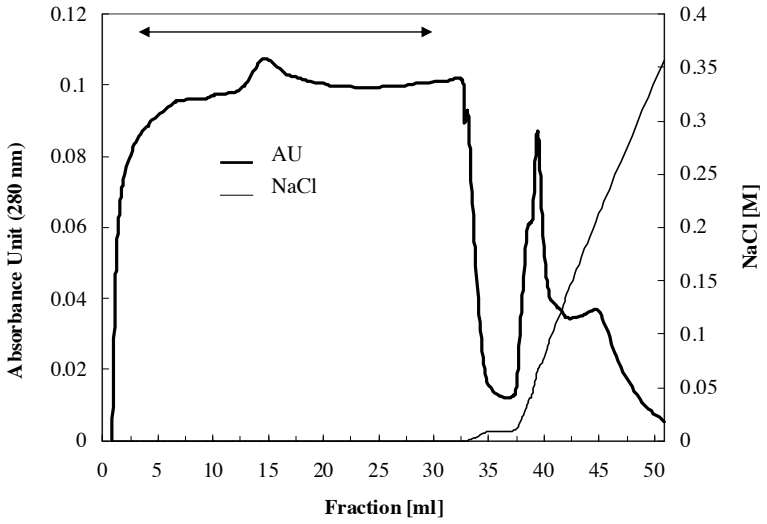

Fig. 2. Profile of FPLC-anion exchange chromatography (FPLC-RESOURCE Q) of pool of 13–66 ml from FPLC-HP. "↔" indicates the fractions exhibiting protease activities.

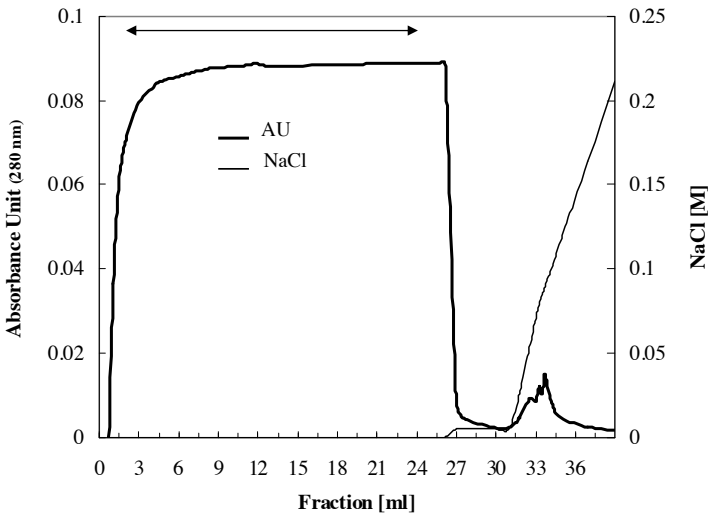

Fig. 3. Profile of FPLC-anion exchange chromatography (FPLC-Mono Q) of pool of 1–34 ml from FPLC-RESOURCE Q. "↔" indicates the fractions exhibiting protease activities.

mostability study, the relative protease activities of protease aliquots incubated at temperatures above 50 °C were lower by more than 25% when compared to the activity of the sample incubated at 4 °C, indicating that the purified protease was heat-labile (Fig. 6).

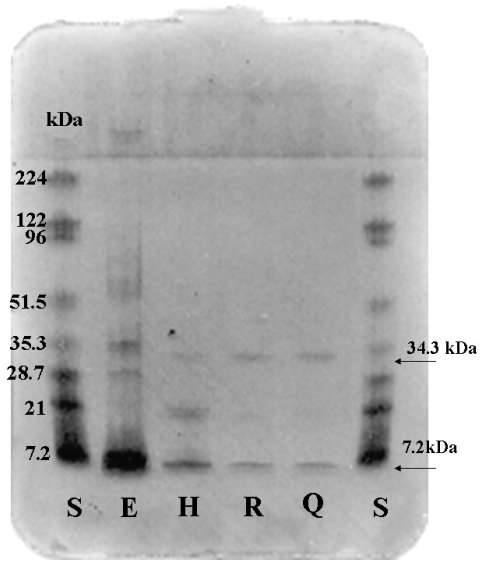

Fig. 4. SDS-PAGE and protease zymogram of purification step. Lane S, marker protein (1.0 μg); lane E, ECP (1.2 μg); lane H, FPLC-Q Sepharose high-performance partially purified protease (0.6 μg); lane R, FPLC-RESOURCE Q partially purified protease (0.4 μg); lane Q, FPLC-Mono Q partially purified protease (0.4 μg). SDS-PAGE Phastgel 10–15% gradient, silver stain.

Protease inhibition

As shown in Table I, the protease activity in crude ECP or in the partially purified preparation was completely inhibited by 5 mM L-cysteine, EDTA, EGTA, 1,10-phenanthroline, TLCK, and TPCK, respectively, indicating that the enzyme is a metalloprotease. In addition, the protease

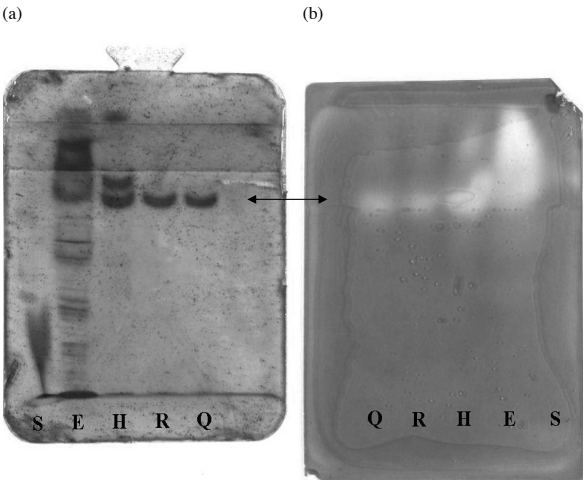

Fig. 5. Native PAGE and protease zymogram of proteins during the purification steps. (a) Lane S, marker protein (1.0 μg); lane E, ECP (2.2 μg); lane H, FPLC-Q Sepharose high-performance partially purified protease (0.6 μg); lane R, FPLC-RESOURCE Q partially purified protease (0.4 μg); lane Q, FPLC-Mono Q purified protease (0.4 μg). Phastgel 10–15% gradient, silver stain. (b) Zymogram of casein-agarose overlay of (a), stained with Coomassie brilliant blue R 250.

Table I. Effects of various concentrations of protease inhibitors, chelating agents, and metal ions on crude extracellular products (ECP) and the partially purified 34.3-kDa protease.

Reagent	Concentration [mM]	Relative activity (%)	
		ECP	Partially purified protease
None		100	100
Serine protease inhibitor			
PMSF	5	38	34
Cysteine protease inhibitor			
E-64	10	100	106
Iodoacetamide	10	120	117
N-Ethylmaleimide	10	111	86
L-Cysteine	5	102	0
Metalloprotease inhibitor			
EDTA	5	4	0
EGTA	5	3	0
1,10-Phenanthroline	5	15	2
Broad-spectrum protease inhibitor			
TLCK	5	89	0
TPCK	5	4	0
Other reagent			
SDS	10	99	31
Divalent metal ion			
$ZnCl_2$	5	98	94
$HgCl_2$	5	0	0
$CuCl_2$	5	87	8
$CaCl_2$	5	99	89

activity was also completely inhibited by $HgCl_2$, but only partially by PMSF, SDS, and $CuCl_2$. Furthermore, protease activities in both the ECP and partially purified protease were not inhibited by E-64, iodoacetamide, and *N*-ethylmaleimide.

Lethality tests and inhibition of the toxicity of the partially purified protease

The ECP and the partially purified protease were lethal to cobia (weighing 5–6 g) with LD_{50} values of 1.26 and 6.8 μg protein/g body weight, respectively. After injection of the toxic protease, no gross external signs (darkening of the body skin) (Liu *et al.*, 2003) were observed in the moribund and dead fish within 24 h. An amount of twice the LD_{50} value of the purified protease was used in the toxicity inhibition tests, and the toxicity of the enzyme was totally inhibited by the presence of EDTA. Neither mortality nor darkening of the body skin was observed in all controls injected with PBS or the solution (EDTA).

Discussion

Although *Photobacterium damselae* subsp. *piscicida* has been demonstrated to be a pathogen of a variety of marine fish, only a few pathogenesis studies concerning extracellular proteases produced by this species have been reported. All five virulent strains of *P. damselae* subsp. *piscicida*

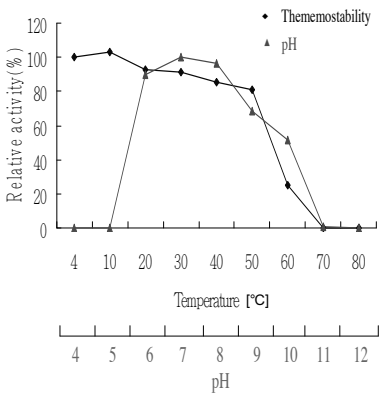

Fig. 6. Profiles of pH optimum and thermostability of the purified protease. For pH optimum (▲), the PBS buffer was in the pH range 4–12 and hide powder azure (HPA) was used as substrate. For thermostability (♦), aliquots of the enzyme were incubated for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80 °C, separately, and then directly put into an ice/water bath prior to the HPA assay.

studied here produced extracellular protease activity indicating that protease might be an important virulence factor.

In the present study, ECP of strain CP1 were lethal to cobia. A 34.3-kDa protease was partially purified by FPLC-HP, FPLC-RESOURCE Q, and FPLC-Mono Q (Fig. 4) and is suggested to be an exotoxin. Only a single band possessing protease activity was visible in zymograms of native PAGE separated ECP proteins (Figs. 5a, b). The low complexity of the electrophoretic profile of the ECP (Fig. 4) is in contrast to previous studies suggesting the secretion of a highly complex mixture of different proteins by *P. damselae* subsp. *piscicida* (Bakopoulos *et al*., 1997; Mazzolini *et al*., 1998). The larger protein complexicity of the ECP observed by those researchers could be explained by the fact that stationary phase cultures were used in those studies. In contrast, in the present study we used mid-exponential phase cultures. Since the bacteria were grown for 48 h, during the exponential phase, the protease described here was, in fact, an exoprotease and not an intracellular protease released by autolysis.

As shown in Fig. 4, the protease fractions were resolved into two bands, one band was a 34.3-kDa protease and the other one a protein with a molecular mass lower than 7.2 kDa (non-protease activity). Whether this 7.2-kDa protein plays a role in the disease process is not clear.

The partially purified protease exhibited optimum enzyme activity at pH 6.0–9.0, therefore, it seems that the production of this protease by pathogenic *P. damselae* subsp. *piscicida* might play a certain important role in marine environment and/or in cobia, since the pH values of sea water and cobia tissues are normally around 8.4 and 7.2, respectively (unpublished obeservation). The partially purified protease was inactivated at temperatures above 50 °C (Fig. 6), suggesting that the enzyme is heat-labile.

In the present study, we were able to partially purify the 34.3-kDa protease, determined by SDS-PAGE (Fig. 4), from the ECP of the CP1 strain by FPLC-HP, FPLC-anion exchange (RESOURCE Q), and FPLC-anion exchange (Mono Q) chromatography (Figs. 1, 2 and 3). The purified protease revealed homogeneity on native PAGE (Fig. 5a), and its enzymatic activity was shown in the matching zymogram gels with sodium caseinate as the substrate (Fig. 5b). In enzyme inhibition examination, the partially purified protease was completely inhibited by the metalloprotease inhibitors EDTA, EGTA, and 1,10-phenanthroline, the cysteine protease inhibitor L-cysteine, the broad-spectrum inhibitors TLCK and TPCK, and only partially inhibited by other inhibitors, *i.e.* PMSF, SDS (Table I). It was also completely inhibited by $HgCl_2$ and $CuCl_2$, but only partially by $CaCl_2$. The differential inhibition of the protease activity in ECP and the purified preparation by $CuCl_2$ and SDS (Table I) may be due to other possible components present in ECP preferentially inhibited by $CuCl_2$ and SDS. Therefore, the enzyme is accordingly characterized to be a metalloprotease. Two extracellular metalloproteases (43 and 41 kDa) produced by the *Vibrio alginolyticus* NCMB 1339 strain have been reported to be toxins in larval *Ostrea edulis* (Nottage and Birkbeck, 1987a, b), and an extracellular metalloprotease (34 kDa) produced by the *Vibrio alginolyticus* S3y strain (Lee, 1995) is described to be a toxin in juvenile *Epinephelus malabaricus*. In addition, a 33-kDa alkaline serine protease produced by the *Vibrio alginolyticus* Swy strain has been suggested to be a toxin in *Penaeus japonicus* (Lee *et al*., 1997), however, the enzyme has only partially been purified.

There are some reports demonstrating that a metalloprotease is one of the virulence factors in vibriosis (Miyoshi *et al*., 1987a, b; Nottage and Birkbeck, 1987a, b; Norqvist *et al*., 1990; Farrell and Crosa, 1991). In addition, a few previous studies on toxic proteases in fish and shellfish have largely concentrated on Vibrionaceae, *i.e*., *Aeromonas* and *Vibrio* spp. (Nottage and Birkbeck, 1987a, b; Bejarno *et al*., 1989; Lee and Ellis, 1989; Gudmundsdottir *et al*., 1990; Ellis, 1991; Lee, 1995; Aguirre-Guzman *et al*., 2005). The present study, for the first time, describes a toxic protease of a *P. damselae* subsp. *piscicida* strain isolated from cultured cobia. The role of the 34.3-kDa protease in bacterial invasiveness is still not clear. In addition, whether the other four bacterial strains we isolated secrete the same 34.3-kDa protease or not is now under investigation.

As the toxicities of crude ECP and the partially purified protease were completely inhibited by the protease inhibitor EDTA in both the HPA digestion assay and the protease zymogram (data not shown, the SDS-PAGE buffer contained 1 mM EDTA) found in our present investigation, the protease was confirmed as a metalloprotease. Furthermore, there was apparent difference in

toxicity between ECP and the protease. The LD_{50} value of ECP (1.26 μg protein/g body weight) was lower than that of the partially purified protease (6.8 μg protein/g body weight) in cobia fish. It thus appears that the partially purified protease is not the major toxin in ECP of *P. damselae* subsp. *piscicida*. However, the present metalloprotease is a novel toxin puified from the bacterium isolated from cultured cobia. Another major toxin(s) produced by the bacterium is now under investigation.

Acknowledgement

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