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Short communication

Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain

Wei-wei Zhang [a,b], Yong-hua Hu [a,b], Hua-lei Wang [c], Li Sun [a,*]

[a] *Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, PR China*
[b] *Graduate University of the Chinese Academy of Sciences, Beijing, PR China*
[c] *Hang Zhou EnzySource Biotechnology Co., Ltd., Hangzhou, PR China*

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ABSTRACT

Pseudomonas fluorescens is an aquaculture pathogen that can infect a number of fish species. The virulence mechanisms of aquatic *P. fluorescens* remain largely unknown. Many *P. fluorescens* strains are able to secrete an extracellular protease called AprX, yet no AprX-like proteins have been identified in pathogenic *P. fluorescens* associated with aquaculture. In this study, a gene encoding an AprX homologue was cloned from TSS, a pathogenic *P. fluorescens* strain isolated from diseased fish. In TSS, AprX is secreted into the extracellular milieu, and the production of AprX is controlled by growth phase and calcium. Mutation of *aprX* has multiple effects, which include impaired abilities in interaction with cultured host cells, adherence to host mucus, modulation of host immune response, and dissemination and survival in host tissues and blood. Purified recombinant AprX exhibits apparent proteolytic activity, which is optimal at pH 8.0 and 50 °C. The protease activity of recombinant AprX is enhanced by Ca^{2+} and Zn^{2+} and reduced by Co^{2+}. Cytotoxicity analyses showed that purified recombinant AprX has profound toxic effect on cultured fish cells. These results demonstrate that AprX is an extracellular metalloprotease that is involved in bacterial virulence.

1. Introduction

Pseudomonas fluorescens is an aquaculture pathogen that can infect many fish species, including Indian major carps, black carp, common carp, and Japanese flounder (Geng et al., 2006; Swain et al., 2007). Infection of fish by *P. fluorescens* leads to the development of the so-called Red Skin Disease, which can occur all year round and especially in fish injured by, for example, inappropriate handling and transportation. Owing to the lack of effective means of control, the disease often leads to mortality, thus causing heavy economic losses. Currently, studies on the pathogenesis of aquaculture-associated *P. fluorescens* are scarce, and the virulence mechanisms of this bacterium remain largely obscure.

Many fluorescent Pseudomonads produce extracellular metalloproteases. Among the proteases that have been characterized on the genetic and biochemical level is AprX, which has been identified in several *P. fluorescens* strains (Ahn et al., 1999; Dufour et al., 2008; Kawai et al., 1999; Liao and McCallus, 1998). AprX is an extracellular alkaline metalloprotease of the serralysin family and presumably is involved in nutrient utilization by its ability to degrade proteins in the environment. Structurally, AprX possesses two conserved Zn^{2+}- and Ca^{2+}-binding domains that are typical for serralysin family proteases. The *P. fluorescens* AprX identified to date are from soil/plant isolates and from strains associated with spoilage of milk and dairy products. To our knowledge, no AprX from pathogenic *P. fluorescens* of aquaculture importance has been reported.

* Corresponding author. Tel.: +86 532 82898834; fax: +86 532 82898834.
E-mail address: lsun@ms.qdio.ac.cn (L. Sun).

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In this study, we describe the identification and characterization of an AprX homologue from TSS, a pathogenic *P. fluorescens* strain isolated from diseased fish. Our results showed that AprX is a calcium-dependent extracellular protease that is involved in bacterial virulence.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli DH5α (Takara, Dalian, China), S17-1λpir (Biomedal, Spain), and BL21(DE3) (Tiangen, China) were cultured in Luria-Bertani broth (LB) medium (Sambrook et al., 1989) at 37 °C. *P. fluorescens* TSS, a fish pathogen (Wang et al., in press), was cultured in LB medium at 28 °C. When appropriate, ampicillin (Ap) and tetracycline (Tc) were added at 100 and 15 μg/ml, respectively.

2.2. Cloning of aprX

An internal 620 bp DNA of *aprX* was generated by degenerate PCR using primers 2476F1/2476R1. The complete *aprX* sequence was obtained by genome walking using BD GenomeWalker Universal Kit (Clontech, USA).

2.3. Plasmid and strain construction

The primers used in this study are listed in Supplemental data Table 1. pEAX1 was constructed by inserting *aprX* (amplified by PCR with primers F6/R7) into pET258 (Zhang and Sun, 2007) between NdeI/XhoI sites. To construct TAM, markerless in-frame deletion of an internal 390 bp DNA (positions 475–864) of *aprX* was carried out as follows: overlapping extension PCR amplifications were performed with the primer pairs F11/R12 and F10/R13, respectively; the PCR products were used as templates for a fusion PCR that was performed with primers F11/R13. The PCR products were inserted into p7TS (Wang et al., in press) at the SmaI site, resulting in p7TAM. S17-1λpir was transformed with p7TAM, and the transformants were conjugated with TSS as described previously (Zhang et al., 2008a). The transconjugants were selected as described previously (Wang et al., in press) and analyzed by PCR with primers F13/R9. The PCR products were subjected to DNA sequencing to confirm the deletion.

2.4. Fish

Healthy Japanese flounder (*Paralichthys olivaceus*, ~12 g) were purchased from a commercial fish farm (Rizhao, Shandong, China) and maintained at 20–22 °C in aerated seawater that was changed twice daily. Sacrifice was performed by euthanizing the fish in MS-222 as described previously (Wang et al., in press).

2.5. Interaction of TSS and TAM with cultured fish cells

Japanese flounder gill cells (FG cells) were cultured and maintained as described previously (Tong et al., 1997). To determine interactions between FG cells and TSS/TAM, FG cells were cultured in 96-well plates to monolayer. 10^7 CFU of TSS or TAM was added per well. The plates were incubated at 30 °C for 1 h and washed 5× with PBS. The numbers of bacterial cells that became associated with FG cells were determined as described previously (Kolodziejek et al., 2007).

2.6. Mucus binding assay

Skin mucus was prepared by rubbing the mucus from the body surface of Japanese flounder. To prepare intestinal mucus, Japanese flounder were starved for 1 week to allow the food in the gut to be processed. Intestines were then removed from the fish under aseptic conditions and washed 3× with PBS. Intestines were split open with a scalpel, and mucus in the intestinal lumen was collected with a plastic spatula. The intestinal and skin mucus were suspended in PBS and filtered through 0.45-μm pore-size filter membranes. Binding of TSS and TAM to fish mucus was determined by the MTT method using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, China) as recommended by the manufacturer.

2.7. Bacterial dissemination in fish blood and tissues

TSS and TAM were cultured to an OD_{600} of 0.7 in LB medium and resuspended in PBS to 10^8 CFU/ml. Japanese flounder were divided randomly into two groups (five fish/group), and each group was injected intraperitoneally (i.p.) with 100 μl of TSS or TAM suspension. Blood, kidney, and liver were taken aseptically from the fish at 24 h post-infection. Bacterial recovery from the blood/tissues was determined as described previously (Wang et al., in press).

2.8. Respiratory burst assay

Two groups (10 fish/group) of Japanese flounder were i.p. injected with 10^7 CFU of TSS or TAM in PBS. Blood was colleted from the caudal veins of the fish at 12 h post-infection and used for respiratory burst assay, which was performed exactly according to the method of Kumar et al. (2008).

2.9. Purification of recombinant AprX

Recombinant AprX was purified from BL21(DE3) harboring pEAX1 as described previously (Zhang et al., 2008b). The protein was dialyzed overnight against PBS at 4 °C and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, USA).

2.10. Protease activity assay

The protease activities of the TSS/TAM supernatants, which were prepared as described previously (Zhang et al., 2008b), and the purified recombinant AprX were analyzed as described previously (Zhang et al., 2008b) using azocasein (0.5%; Sigma) as a substrate. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0.001 at 350 nm in 1 min. The effects of temperature, pH, and metal ions were determined as described previously (Zhang et al., 2008b).

2.11. Antisera and Western and immunoblotting analysis

Antisera to recombinant AprX was prepared by subcutaneously injecting adult rats with 60 μg of the purified AprX mixed in complete Freund's adjuvant, followed by three boosts with the same amount of protein in incomplete Freund's adjuvant at 20, 32, and 45 days after the initial immunization. The rats were bled 12 days after the last boost. Sera collection and Western and immunoblotting analysis were carried out as described previously (Sun et al., 2009).

2.12. Cytotoxic effect of AprX

This was performed according to the method of Alamuri and Mobley (2008). In brief, FG cells were cultured to confluence in 96-well plates. Purified AprX or PBS was added to the wells. After incubation at 20 °C for various times, the cells were used either for the determination of viability using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, China) or for microscopic observation. For the latter purpose, the cells were fixed with 4% paraformaldehyde and stained with Giemsa solution, followed by destaining with 10% methanol. The plate was dried and examined under an inverted microscope.

2.13. Statistical analysis

All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc., USA). Differences in binding to FG cells and cytotoxic effect were analyzed by one-way analysis of variance (ANOVA). All other differences were analyzed by Student's *t*-test. In all cases, the significance level was defined as $P < 0.05$.

2.14. Database search and in silico analysis

Database search was conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI). Structural analysis was performed using the NCBI Conserved Domain Search server.

3. Results

3.1. Sequence characterization of AprX

aprX (Genbank accession no. FJ687263) encodes a putative protein of 477 amino acids that shares the highest (98%) sequence identity with the AprX proteins of the *P. fluorescens* strains F and A506. Conserved domain search identified in AprX a zinc-dependent metalloprotease domain (residues 64–235) and a peptidase domain (residues 259–477). Within the peptidase domain is a calcium-binding domain that contains three tandem repeats of a motif with the pattern of GGxGxD, which is characteristic of the serralysin family protease.

3.2. Construction and characterization of an aprX null mutant

An *aprX* null mutant, TAM, was constructed by markerless in-frame deletion of an internal 390 bp segment of *aprX*. SDS-PAGE analysis of the supernatant proteins of TSS and TAM showed that they differed mainly in one protein, which appeared in the supernatant of TSS but was absent in that of TAM (Fig. 1a). This TSS-unique protein exhibits a molecular weight matching that of the processed AprX (47 kDa). Western immunoblot analysis showed that the 47 kDa protein reacted with anti-AprX antibodies but not with the preimmune serum (Fig. 1b). These results demonstrate that *aprX* encodes an extracellular protein.

3.3. Analysis of the extracellular protease activity of TSS and TAM

When cultured in standard LB medium, the extracellular protease activity of TSS increased with growth and reached maximum at OD_{600} of 3.5 (Fig. 2). The

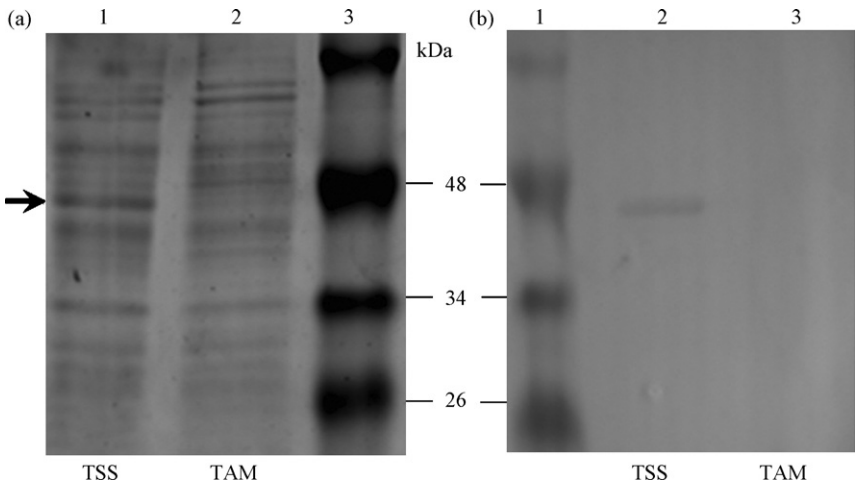

Fig. 1. SDS-PAGE (a) and Western immunoblotting (b) analysis of AprX in the supernatants of TSS and TAM. (a) Equal amounts of TSS (lane 1) and TAM (lane 2) supernatants were analyzed by SDS-PAGE. The TSS-unique protein is indicated by an arrow. (b) Equal amounts of TSS (lane 2) and TAM (lane 3) supernatants were resolved by SDS-PAGE; the proteins were transferred to a nitrocellulose membrane and blotted with anti-AprX antibodies.

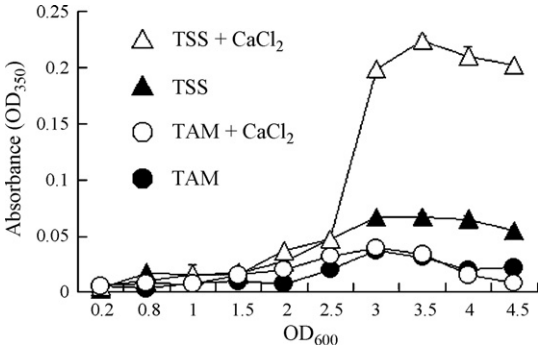

Fig. 2. Extracellular protease activity of TSS and TAM in the presence and absence of calcium. Supernatants were collected from TSS and TAM cultured in LB medium to different densities in the presence or absence of $CaCl_2$ and assayed for protease activity using azocasein as the substrate.

extracellular protease activity of TAM exhibited a similar trend, but was much lower than that of TSS in the measured value at each of the selected time point after OD_{600} 2.5. The extracellular protease activity of TSS, but not that of TAM, increased drastically in the presence of Ca^{2+} (1 mM) (Fig. 2). These results suggest that the production/activity of AprX is regulated by growth phase and Ca^{2+}.

3.4. Mutation of aprX has multiple effects

Since TSS is a fish pathogen, we compared TSS and TAM for the capacities that are known to be associated with bacterial virulence, which include interaction with host cells, mucus binding, dissemination in host blood and tissues, and modulation of host immune response. Cell binding assays showed that the numbers of TAM recovered from the intracellular of FG cells and those recovered from the entire FG cells were, respectively, 3.9- and 2.2-fold lower than those of TSS. Mucus binding assays showed that the numbers of TAM bound to fish skin and intestinal mucus were significantly lower (40 and 62% less) than those of TSS. Blood/tissue dissemination analyses showed that the amounts of TAM recovered from the blood, liver, and kidney were, respectively, 17-, 11-, and 14-fold lower than those of TSS. The respiratory burst activity of the neutrophils of the TAM-infected fish was ~2-fold higher than that of the TSS-infected fish.

3.5. Characterization of the protease activity of purified recombinant AprX

Enzymatic analyses showed that purified recombinant AprX exhibited apparent protease activity (15,000 U/ml), which was abolished in the presence of the serine protease inhibitor phenylmethanesulfonyl fluoride. AprX displayed maximum activity at pH 8.0 and 50 °C and was thermostable (Fig. 3). The proteolytic activity of AprX was increased 6.8- and 3.7-fold respectively, by Ca^{2+} and Zn^{2+} (10 mM), reduced 2.8-fold by Co^{2+} (10 mM), and unaffected by Na^{+}, K^{+}, and Mg^{2+}.

3.6. Cytotoxicity analysis of the purified recombinant AprX

Cytotoxicity analyses showed that treatment with AprX significantly reduced the viability of FG cells in a dose-dependent manner (Fig. 4a). Consistently, microscopic observation showed that incubation with AprX had a profound damaging effect on FG cells and led to complete cell lysis at 60 min after the incubation (Fig. 4b). Together these results demonstrate that purified recombinant AprX can act as a cytotoxin upon FG cells.

4. Discussion

A previous study by Liao and McCallus (1998) showed that production of AprX in *P. fluorescens* CY091 was increased by $CaCl_2$. Similarly, we found that the extracellular protease activity of TSS, but not that of TAM, was enhanced by Ca^{2+}. The enhancing effect of Ca^{2+} was most apparent only after the cell density reached OD_{600} of 2.5, which is consistent with the report by Dufour et al. (2008), who showed that in *P. fluorescens* F, AprX-associated extracellular caseinolytic activity was detected at the entry into

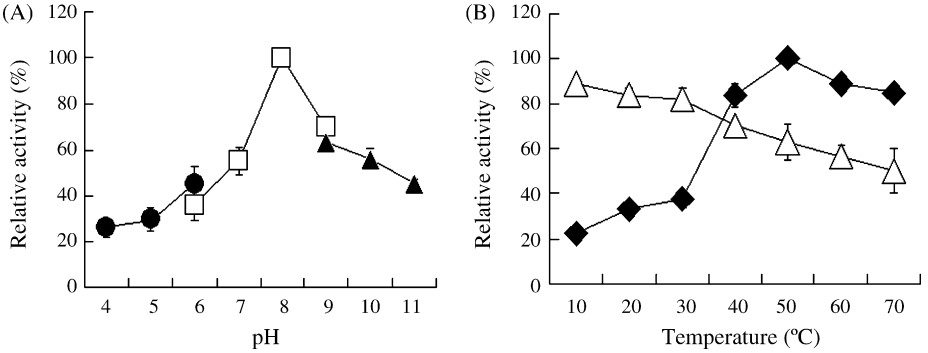

Fig. 3. Effects of pH (a) and temperature (b) on the activity of purified recombinant AprX. (a) The effect of pH was determined in three different buffers: 50 mM citric acid–sodium phosphate (pH 4–6; ●), 50 mM sodium phosphate (pH 6–9; □), and 50 mM glycine–NaOH (pH 9–11; ▲). (b) The effect of temperature (■) was determined in the assay buffer (50 mM potassium phosphate, pH 7.4) using azocasein as the substrate. Thermostability (△) was determined by preincubating the enzyme in the assay buffer at the indicated temperature for 1 h before initiating enzymatic reaction by the addition of azocasein. In each panel, the enzymatic activities were expressed as percentages of the maximum activity. Data are means for three independent assays and presented as the means ± S.E.

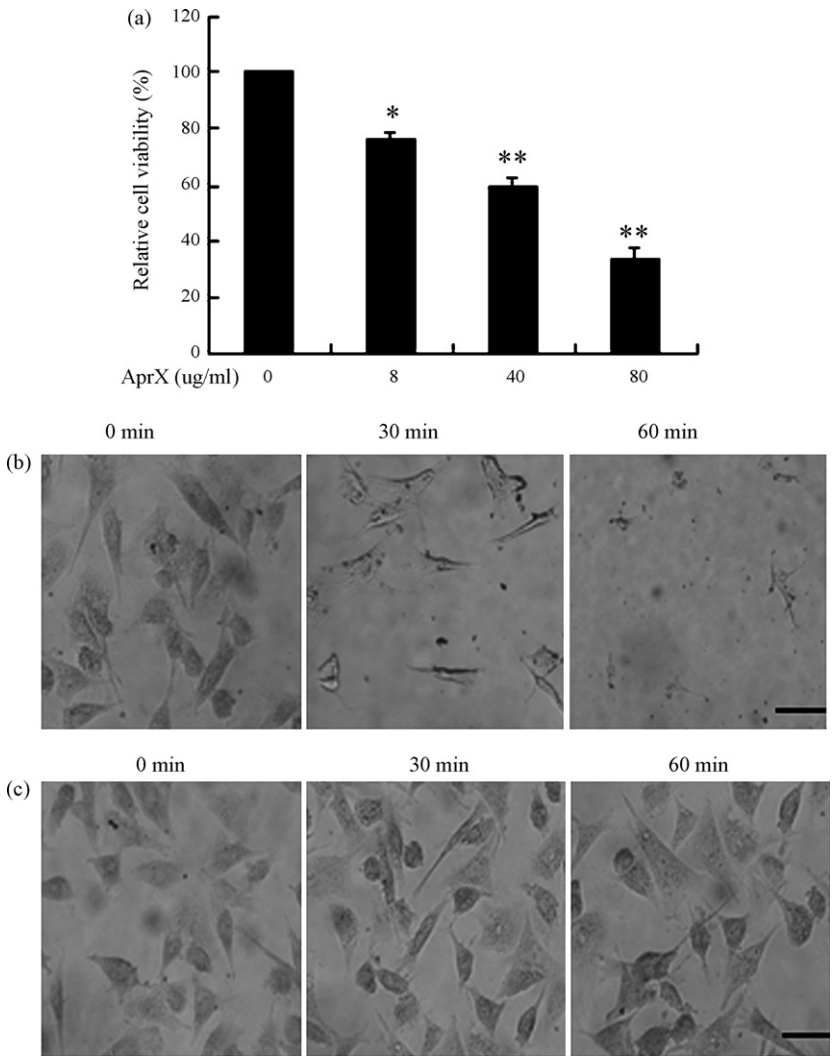

Fig. 4. Examination of the cytotoxic effect of purified recombinant AprX on cultured FG cells by cell viability analysis (a) and microscopy (b) and (c). (a) FG cells were treated for 30 min with different amounts of recombinant AprX. Viabilities of the cell after the treatment were determined by the MTT method. Data are the means of three independent assays and presented as the means ± S.E. $^{*}P < 0.05$; $^{**}P < 0.01$. (b and c) FG cells were treated with AprX (b) or PBS (c) for different times and fixed with paraformaldehyde. The cells were stained with Giemsa and observed under an inverted microscope. Images are taken at 120× magnification. Bar = 50 μm.

stationary phase. For the AprX of CY091, the optimal protease activity was observed at 40–45 °C and pH 7–8, while for the AprX of *P. fluorescens* F, the optimal pH and temperature are 8.5 and 45 °C. In our study, we found that the protease activity of recombinant AprX reached maximum at pH 8.0 and 50 °C and was increased by Ca^{2+}. These results indicate that the production and proteolytic activity of AprX, like those of other AprX, are regulated by Ca^{2+}.

For pathogenic bacteria, secreted proteases often play important roles during infection, as is in the cases of the aquaculture pathogens *Vibrio vulnificus* and *Vibrio anguillarum*, which utilize metalloproteases for mucosal colonization and interaction with hosts (Denkin and Nelson, 2004; Norqvist et al., 1990; Valiente et al., 2008). In our study, we found that mutation of *aprX* reduced the ability of TSS to interact with cultured host cells and to adhere to host mucus. Since AprX is an extracellular protease, it may facilitate the association of TSS with FG cells and host mucus by degradation of host proteins that are present on cell surface and in mucus. Consistent with these observations, the *aprX* mutant strain TAM exhibited significantly attenuated ability to disseminate and survive in host blood and tissues. These results, together with the observation that purified recombinant AprX was highly toxic on cultured FG cells, demonstrate that AprX is a virulence factor that contributes to bacterial infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.04.026.

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