Fish & Shellfish Immunology 68 (2017) 211–219

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A *Pacifastacus leniusculus* serine protease interacts with WSSV

Enen Guo [1, 2], Gül Gizem Korkut [2], Phattarunda Jaree, Irene Söderhäll, Kenneth Söderhäll*

Department of Comparative Physiology, Uppsala University, Uppsala, Sweden

ARTICLE INFO

Article history:
Received 21 April 2017
Received in revised form
6 July 2017
Accepted 9 July 2017
Available online 10 July 2017

Keywords:
Hematopoietic tissue
Invertebrate
Serine protease
Virus
WSSV

ABSTRACT

Serine proteases are involved in many critical physiological processes including virus spread and replication. In the present study, we identified a new clip-domain serine protease (*Plc*SP) in the crayfish *Pacifastacus leniusculus* hemocytes, which can interact with the White Spot Syndrome Virus (WSSV) envelope protein VP28. It was characterized by a classic clip domain with six strictly conserved Cys residues, and contained the conserved His-Asp-Ser (H-D-S) motif in the catalytic domain. Furthermore, signal peptide prediction revealed that it has a 16-residue secretion signal peptide. Tissue distribution showed that it was mainly located in *P. leniusculus* hemocytes, and its expression was increased in hemocytes upon WSSV challenge. *In vitro* knock down of *Plc*SP decreased both the expression of VP28 and the WSSV copy number in hematopoietic stem (HPT) cells. Accordingly, these data suggest that the new serine protease may be of importance for WSSV infection into hematopoietic cells.

1. Introduction

White spot syndrome virus (WSSV) is a major viral pathogen causing mortality in aquatic crustaceans, primarily shrimp. It is an enveloped, rod-shaped dsDNA virus with a 300kbp genome encoding 531 putative open reading frames. It has a nucleocapsid, a tegument and an outer envelope with over 30 envelope proteins [1] that aid virus entry as well as other infection processes. Of all WSSV structural proteins, VP26 and VP28 constitute 60% of the virion's envelope [2] and unsurprisingly, VP28 has been reported to be involved in cell attachment during virus infection [3,4]. Although more and more host proteins have been proven to participate in the WSSV infection process [5–9], the receptors besides Rab7 in *Penaeus monodon* [10] still remain to be identified. Therefore, details about how WSSV enters into the cell and successfully avoids the host innate immunity continues to be largely unknown, although it was recently shown that WSSV enters crayfish HPT cells via clathrin-mediated endocytosis in a pH dependent manner [11] and also other endocytic pathways seem to operate [12].

Serine proteases are characterized by three catalytic residues (H, D and S). These residues together form the catalytic triad [13], and function in extracellular, vesicular or granular locations. The first clip-domain to be identified in invertebrates was by disulfide determination of the proclotting enzyme in the horseshoe crab *Tachypleus tridentatus* [14]. Later, several clip-like domains were reported in serine proteases and homologues, such as in dorsoventral polarity in *Drosophila melanogaster* [15,16] and in innate immune systems [17], including the activation of proPO and production of antimicrobial peptides in insects [18,19]. In addition to its roles in digestion, blood clotting and the complement system, serine proteases also catalyze the processing of viral polyproteins or the processing of precapsids in order to promote the virus spread and replication in mammals [20].

Since crustaceans lack adaptive immunity, their defense mechanism against invading microorganisms exclusively relies on the innate immune processes. The circulating hemocytes carry on immune functions by participating in recognition, phagocytosis, melanization of pathogens and cytotoxicity [21]. These hemocytes fall into three groups: hyaline cells, semigranular cells (SGCs) and granular cells (GCs) [22]. In *Pacifastacus leniusculus*, the prophenoloxidase (proPO) activating system (proPO system) is an important immune response against xenobiotics. The proPO-system is mainly located in GCs, to a lesser degree in SGCs and not present in immature hemocytes in the hematopoietic tissue [23]. Normally, detection of xenobiotics will lead to activation of proPO. Active phenoloxidase will oxidize phenols into toxic quinones and eventually into melanin for the clearance of pathogens [24]. On the other hand, in WSSV-infected crayfish, not only WSSV

* Corresponding author. Dept. Comp. Physiology, Norbyv. 18A, 752 36, Uppsala, Sweden.
E-mail address: Kenneth.Soderhall@ebc.uu.se (K. Söderhäll).
[1] Present address: Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden.
[2] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2017.07.026
1050-4648/

could infect both SGCs and GCs successfully, but also the virus blocked release of the proPO system in an unknown way [25].

In this study, we have used 2-D electrophoresis and mass spectrum (MS) analysis to identify and characterize a new WSSV-interacting clip-domain serine protease in *P. leniusculus* by cross-linking the WSSV and crayfish hemocytes with the membrane-impermeable cross-linker sulfosuccinimidyl succinate (SEGS). This protease possesses the common features of the clip-domain serine protease family, with the clip domain at the N-terminal, and also a catalytic triad consisting of three conserved catalytic residues (H, D and S) at the C-terminal. Furthermore, it could interact with the WSSV envelope protein VP28, which is necessary for WSSV infection.

2. Materials and methods

2.1. Animals

Freshwater crayfish (*P. leniusculus*) from Lake Erken in Sweden, were maintained in tanks with aerated running water at 10 °C. Only intermolt crayfish were used in the experiments.

2.2. Cell culture and virus

Crayfish HPT cells were prepared and cultured as described previously [26], and 1/3 of the medium was changed at 48 h intervals. Crayfish SGCs and GCs were obtained as described in Söderhäll and Smith. (1983) [27]. Briefly, 1 mL hemolymph was carefully dropped into 1 mL cooled anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [27], and carefully loaded as a 2 mL mixture to the 8 mL continuous gradients of 70% Percoll (GE Healthcare) in 0.15 M NaCl, then centrifuged at 2900 × *g* for 20 min at 4 °C. The separated SGCs and GCs were collected subsequently.

WSSV used in this study was isolated from crayfish gills. WSSV purification was performed as described previously [28] and WSSV were suspended in sterile crayfish saline buffer (CPBS) at a concentration of 2×10^7 copies/ml.

The crayfish used in all experiments were originally WSSV-free.

2.3. Covalent cross-linking between WSSV and hemocytes

To analyze the interaction between WSSV and crayfish hemocytes, firstly SGCs and GCs were prepared and maintained separately in 0.15 M NaCl, and then cross-linked by adding membrane-impermeable cross-linker, ethylene glycol bis (sulfosuccinimidyl succinate) (SEGS) (Pierce) together with WSSV at 4 °C. After incubation for 30 min with gentle agitation, the reaction was stopped with 1 M Tris-buffered saline (pH 7.5) for 15 min, subsequently centrifuged at 1000 × *g* for 10 min at 4 °C to harvest the cells, and washed two times with CPBS [29]. The collected samples were used for 2-D electrophoresis and western blot.

Western blot was performed according to Lin et al. (2009) [29]. Briefly, the cross-linked samples were lysed and subjected to 12.5% SDS-PAGE under non-reducing conditions, and subsequently electrotransferred to Hybond-P extra membranes (Amersham Biosciences). After blocking with 10% skim milk, the membrane was blotted with VP28 polyclonal antibody, which was kindly provided by Professor Chu-Fang Lo, from Institute of Zoology, College of Life Sciences, National Taiwan University [1]. Actin (C-11: SC-1615) was used as an internal control and its antibody was obtained from Santa Cruz Biotechnology.

2.4. 2-D electrophoresis

The cross-linked samples were incubated in a lysis buffer (50 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS) containing the complete Mini-EDTA-free protease inhibitor (Roche). The samples were grinded by soaking up and down with a 23 G needle (BD Macrolance) connected to a syringe. Then the lysates were transferred to a clear tube and centrifuged at 18 000 × *g* for 30 min at 4 °C. The supernatant was transferred to a new tube and the protein concentration was measured with a 2-D Quant kit (GE Healthcare) and adjusted to 5 mg/mL with lysis buffer. Then, 50 μg of the lysed sample was taken out for 2-D electrophoresis. 0.625 μL 1 M DTT solution, 0.625 μL IPG buffer (pH 3–11 NL) (GE Healthcare) and rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue) were added to adjust the volume to 125 μL. The sample mixture was pipetted into the ImmobilineDryStrip Reswelling Tray. An ImmobilineDryStrip (7 cm, GE Healthcare) of pH interval 3–11 NL was gently placed onto the sample mixture with the gel-side down and kept overnight at room temperature, covered with the DryStrip Cover Fluid (GE Healthcare). The strip was transferred to an EttanIPGphor Isoelectric Focusing Unit (GE Healthcare) and the proteins were focused using the following program: i) set the voltage to 300 V for 0.3 h, ii) increase the voltage to 5500 V by a gradient for 0.3 h, and iii) hold the voltage at 5500 V until 22000 V hrs is reached. The strip was taken in a 15 mL Falcon tube and 5 mL fresh equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29% glycerol, 2% SDS, 0.002% bromophenol blue) supplemented with 0.04 mM DTT per 10 mL, was added and incubated for 15 min at room temperature with rotation. The solution was replaced with 5 mL equilibration buffer supplemented with 0.25 g iodoacetamide per 10 mL buffer and incubated for another 15 min with rotation. After equilibration, the IPG strips were applied onto a 7-cm acrylamide gel (12.5%) and the gel was sealed using the agarose sealing solution (0.5% agarose, 0.002% bromophenol blue stock solution in SDS running buffer). SDS-PAGE was performed at 20 mA/gel and electrophoresis was stopped before the bromophenol blue line migrated out of the gel. All electrophoretic procedures were performed at room temperature.

2.5. Silver staining and mass analysis

After 2-D electrophoresis, the gel was silver stained and the detailed process was performed according to Wu et al. (2008) [30]. A few changes in protocol are as follows; the gel was fixed in 50% v/v methanol and 12% v/v acetic acid containing 0.05% formaldehyde for 2 h or overnight, and washed with 35% ethanol four times for 20 min, incubated with 0.02% w/v sodium thiosulfate for 2 min, washed with water twice for 5 min and incubated for 30 min with 0.2% w/v silver nitrate. The gel was washed with water twice for 15 s, developed with 6% w/v sodium carbonate and 0.02% w/v sodium thiosulfate, 0.01% v/v formaldehyde mixture and the reaction was stopped with 60 mM disodium EDTA. The gel was eventually washed with water. The selected spots were excised from the gel and sent to SciLifeLab technology platforms for MS analysis. The peptide homology was deduced using BLAST on *Pacifastacus leniusculus* transcriptome database (GenBank BioProject Accession: PRJNA259594).

The silver stained band that we chose from SDS-PAGE was in-gel digested and analyzed by LC-Orbitrap MS/MS at the MS-platform at Science for Life Centre in Uppsala. The proteins were reduced, alkylated and in-gel digested by trypsin which eventually yielded peptides. These peptides were separated in reversed-phase on a C18-column and electrosprayed on-line to an LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was performed by applying CID MASCOT (Matrix