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Gene Cloning, Expression, and Characterization of Recombinant Aerolysin from *Aeromonas hydrophila*

Vijai Singh · Pallavi Somvanshi · Gaurav Rathore · D. Kapoor · B. N. Mishra

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Abstract Aerolysin is a significant virulent toxin protein secreted by *Aeromonas hydrophila*; it produces deep wound infections and hemorrhagic septicemia. The complete aerolysin gene (1,482 bp) was amplified from *A. hydrophila.* Furthermore, it was cloned and expressed into *Escherichia coli* BL21(DE3) codon plus RP cells using 0.5 mM IPTG for induction. The protein size was 54 kDa as estimated by SDS-PAGE, and it was purified by Ni–NTA affinity chromatography. Anti-His antibodies were used to characterize the expressed aerolysin by Western blotting and showed hemolytic activity with fish red blood cells. Aerolysin may be used as immunoassays for earlier control of *A. hydrophila* and is also compatible for vaccination.

Keywords *Aeromonas hydrophila* · Aerolysin · Expression · RBCs · Vaccine

Introduction

Aerolysin secreted by *Aeromonas hydrophila* binds to specific glycosylphosphatidyl inositol-anchored proteins of host red blood cells (RBCs) [1, 2] and forms pores in the cell membrane causing hemolysis [3, 4]. The disruption of aerolysin gene of *A. hydrophila* has been used as attenuated virulence [5], although it has been proposed that two hemolytic toxin models provide a more accurate prediction of *Aeromonas* virulence [6]. Purification and characterization of aerolysin from *A. hydrophila* is very arduous, and it was produced in low quantity. There is always a constant need of developing and thereby establishing immunoassays against *A. hydrophila* for diagnosis. The nucleotide sequence of *A. trota*

V. Singh (✉) · G. Rathore · D. Kapoor
National Bureau of Fish Genetic Resources, Canal Ring Road, PO-Dilkusha, Lucknow 226002, India
e-mail: vijaisingh15@gmail.com

P. Somvanshi
Bioinformatics Centre, Biotech Park, Lucknow 226021, India

B. N. Mishra
Department of Biotechnology, Institute of Engineering and Technology, Lucknow 226021, India

aerolysin gene has been determined by in vitro coupled transcription–translation analysis [7]. The hemolysin gene isolated from *A. hydrophila* A6 cosmid bank encodes a potential gene product. The virulence factors contribute to its pathogenicity and provide the ability to attach to host cells in the development of diseases. The second hemolysin gene encodes aerolysin, which is a pore-forming toxin, was partially amplified the C-terminal region of aerA of *A. hydrophila* [8]. The outer membrane protein of *A. hydrophila* was amplified by polymerase chain reaction (PCR); it was cloned, expressed; and confirmed by Western blotting. The Indian major carp *Labeo rohita* was immunized using purified protein, and antibodies, which are highly immunogenic in fish, were developed from the protein [9]. The present study was aimed to clone, express, and characterize the aerolysin of *A. hydrophila*.

Materials and Methods

Strains, Vectors, Enzymes, and Chemicals

Escherichia coli DH5α, *E. coli* BL21(DE3), and *E. coli* BL21(DE3)codon plus RP were preserved in laboratory; pTZ57R-T cloning vector was purchased from Fermentas; pET28a (+), proteinase K, and agarose were purchased from Bangalore Genei; Taq DNA polymerase, dNTPs, BamHI, NdeI, X-gal, IPTG, DNA marker were purchased from Fermentas; ampicillin, kanamycin, DTT, β-mercaptoethanol, PMSF, and RNaseA were purchased from Sigma Co. Nitrocellulose membrane was purchased from Millipore.

Construction and Identification of Aerolysin

Aerolysin gene of *A. hydrophila* AH14 has been amplified, cloned, and sequenced previously [10]. The primers were designed for aerolysin gene with *Nde*I (AHAF1 5′CATATGATG CAAAAACTAAAAATAACTGGC′3) and *Bam*HI (AHAR1 5′GGATCCTTGATTGG CAACTGGCGTCACG′3) restriction endonuclease sites. The subcloning was done using pET28a vector for expression of aerolysin. The aerolysin was ligated in pET28a expression vector with T4 DNA ligase and transformed into *E. coli* BL21(DE3) codon plusRP competent cells. The recombinant aerolysin gene was identified and characterized by restriction digestion with *Nde*I/*Bam*HI, PCR amplification, and DNA sequencing. Aerolysin protein sequence of *A. hydrophila* was used for identification of conserved domain using conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Expression and Characterization of Aerolysin

A single colony of *E. coli* BL21(DE3) codon plusRP positive clone having (pET28a-aerolysin) plasmid was inoculated into 5 ml Luria Bartani (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) broth containing 30 μg/ml kanamycin and 50 μg/ml chloramphenicol antibiotics. The culture was incubated at 37 °C overnight on shaker (200 RPM). Next day, 200 μl of overnight grown culture was inoculated into flasks each containing 100 ml of LB broth and again incubated until absorbance ($A_{600\ nm}$) of culture reached up to 0.6. Bacterial culture was induced by different concentration of IPTG (0.25 mM to 1.0 mM) and samples were taken at every hour up to 16 h. Similarly, the *E. coli* BL21(DE3) codon plusRP containing pET28a(+) was induced up to 16 h as a negative control. The 1.5-ml bacterial cultures were centrifuged at 13,000×*g* for 10 min at 4 °C. The bacteria were homogenized in 80 μl phosphate buffer saline (PBS pH7.2) and 20 μl of 5X SDS sample loading buffer

(0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT) was added. Mixed properly and boiled at 90 °C for 5 min and centrifuged at 13,000×*g* for 10 min. The 10-μl lysates were loaded directly onto 12% SDS-PAGE following the procedure earlier described [11]. The protein bands were visualized by staining with coomassie brilliant blue R250 dye.

Protein Purification Under Denaturing Conditions

The expression of recombinant aerolysin in *E. coli* can lead to the formation of insoluble inclusion body. The strong denaturants such as 8 M urea or 6 M guanidine-HCl were used to solubilize the inclusion bodies. The induced *E. coli* cell pellet was thawed for 15 min on ice and resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea pH 8.0) at 5 ml per gram weight. Cells were stirred for 60 min at 25 °C, and the lysate centrifuged at 10,000×*g* for 30 min at room temperature and the cellular debris was discarded. one milliliter of the 50% Ni-NTA slurry was added to 4 ml protein lysate and mixed gently by shaking 200 rpm for 60 minutes at room temperature. The lysate–resin mixture was loaded carefully in an empty chromatography column (Genei). Bottom cap was removed from the column and the protein flow-through was collected. The column was washed with 4 ml buffer C (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 6.3) and eluted the recombinant aerolysin four times with 0.5 ml buffer D (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 5.9), followed by four times with 0.5 ml buffer E (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 4.5).

Western Blot Analysis

The proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane (Millipore) by Amersham Pharmacia. Western blotting system followed the procedure as described by Sambrook et al. [11]. The membrane was checked by transferring of protein onto membrane with 1X Ponseau S solution. The membrane was properly washed with deionized distilled water and air dried. It was blocked by 3% bovine serum albumin (BSA) then dipped into 1:2000 BSA diluted Anti-His antibody (QIAGEN). The horseradish peroxidase conjugated rabbit antimouse antibody was diluted into 1:4000 and used as secondary antibody. The membrane was washed four times; and the band was detected by 3, 3′, 5, 5′-tetrametylbenzidine membrane peroxidase substrate until dark purple bands appeared. The reaction was stopped using distilled water, and then membrane was air-dried.

Hemolytic Assay

The determination of hemolysin activity of aerolysin was done using the disk diffusion method. The induced and purified aerolysin was used for demonstration of hemolytic activity with the fish RBCs. The 5% fish blood agar media was prepared, and a hole was made in blood agar. The 200 μl of these proteins were incubated at 25 °C for 24 h [12]. Hemolytic activity was elucidated with lysis of RBCs on blood agar. The clear zone surrounding the hole indicates the hemolytic activity and negative hemolytic activity indicates the no zone of clearance.

Results and Discussion

A. hydrophila causes several diseases in amphibians, reptiles, birds, and humans [13, 14]. It secretes extracellular enzymes such as proteases, DNase, RNase, elastase, lecithinase,

amylase, lipase, gelatinase, chitinase [15, 16], cytotoxic/ enterotoxins [17], and three hemolysins [18]. The conserved regions of aerolysin and hemolysin have been detected in *A. hydrophila* by PCR [19, 20]. In the pond cultured fish system of India, *A. hydrophila, A. sobria, A. caveii, E. tarda*, and *Flavobacterium* sp. are responsible for disease outbreaks. However, it is easy to diagnose and identify these pathogens based on clinical symptoms. There is a constant need to develop an appropriate molecular and immunological diagnosis of *A. hydrophila* infection in fishes to humans. A more practical approach in the development of diagnostic method is to clone, express, and purify the aerolysin protein from *A. hydrophila*. *A. trota* AK2 produced the extracellular pore-forming hemolytic toxin. Aerolysin was mutagenized with the transposon mini-Tn5Km1 to generate a hemolysin-deficient mutant (AK253) [7]. The nucleotide sequences of *aerA* gene located on the 1.8-kb ApaI-EcoRI fragment from genomic library was determined which consisted of ORF of aerolysin with 1479 bp. In vitro coupled transcription–translation analysis of the 1.8-kb region suggested that the *aerA* gene codes for a 54-kDa protein and was in agreement with nucleotide sequence data [7].

Aerolysin gene (1,482 bp) of *A. hydrophila* has been amplified, sequenced, and exhibit similarity with existing databases. It has been theoretically translated into protein sequences, and the expected protein size ~54 kDa was obtained [10]. There are several ambiguities between hemolysin, aerolysin, and cytolysin of *Aihydrophila*. Therefore, conserved domain database was used to solve the problem and aerolysin was present in the aerolysin superfamily (Fig. 1). A total of 492 amino acid residue present in aerolysin, and 120–492 regions containing the aerolysin domain was obtained. The pET28a-aerolysin clones were successfully constructed and used for expression in *E. coli* BL21 (DE3) codon plus RP cells. Constructs were confirmed by digestion with *Nde*I and *Bam*HI restriction enzymes. The two bands one of aerolysin (1,494 bp) and other pET28a expression (5,200 bp) were obtained (Fig. 2).

The cytolytic enterotoxin of *A. hydrophila* was cloned and its DNA sequence was determined. *N*-terminal amino acid residues of the *Aeromonas* cytolytic enterotoxin was used as a probe to screen a genomic library constructed in EMBL3. All biological activities associated with the cytolytic enterotoxin were neutralized by rabbit homologous polyclonal antibodies. The confirmation of protein on SDS-PAGE and Western blot of cell lysate of *E. coli* (lambda CH4) revealed a protein band of 52 kDa using antisera to the cytolytic enterotoxin. The analysis of ORF (1,479 bp) that encodes a protein of 54.5 kDa, a precursor form of the cytolytic enterotoxin, with a 23 amino acid leader peptide. The homology of cytolytic enterotoxin at nucleotide and protein sequences were coordinated with aerolysin of *Aeromonas* species but the restriction pattern was different [17].

Aerolysin has been expressed in *E. coli* under the control of bacteriophage T7 promoter. The coding region of the gene of *A. hydrophila* XS91-4-1 was cloned and expressed in *E. coli* BL21 cells excluding the signal peptide. The size of aerolysin was 68 kDa on SDS-PAGE and Western blot analysis. The recombinant aerolysin showed hemolytic activity in the agar diffusive hemolysis assay and in Western blot analysis demonstrated good

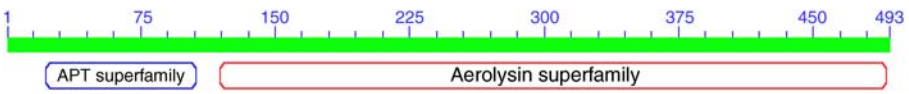

Fig. 1 Identification of conserved domain (aerolysin superfamily present in 120–492 amino acids) in aerolysin of *A. hydrophila*

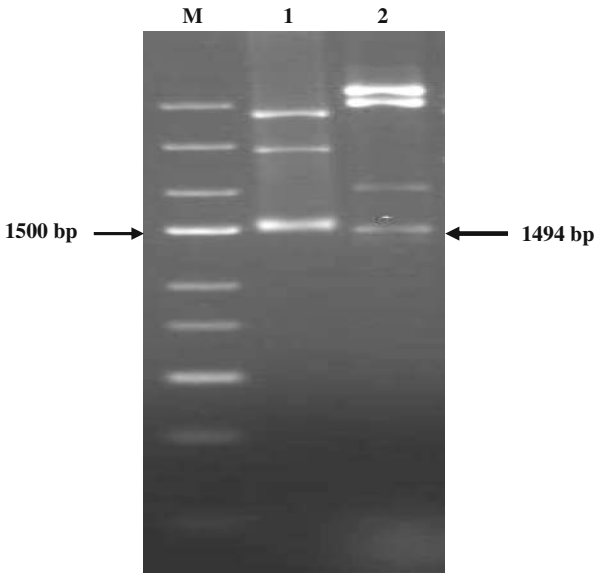

Fig. 2 Analysis of construct by double restriction digestion (NdeI and BamHI) of cloned aerolysin gene of *A. hydrophila* in pTZ57RT cloning and expression vector pET28a. Lane M: 100 bp expressed DNA ladder (Fermentas), Lane 1: Aerolysin gene in cloning vector (pTZ57R-T) and Lane 2: Aerolysin gene in expression vector (pET28a+)

antigenicity [12]. Hemolysin gene of *A. hydrophila* has been cloned, expressed by the induction with 1.0 mM of IPTG. The size of expressed hemolysin (68.0 kDa) had been estimated by SDS-PAGE and Western blot analysis [21].

In the present study, high level expression of aerolysin was obtained in 0.5 mM concentration of IPTG at 25 °C. The 54-kDa size of expressed protein was seen on 12% SDS-PAGE (Fig. 3). The monomer of aerolysin protein was eluted in the buffer E (pH 4.5). The eluted fraction of aerolysin, uninduced and induced protein was used for the characterization by Western blot analysis showing the 54-kDa band on the nitrocellulose membrane (Fig. 4). The induced and purified aerolysin showed the hemolytic activity with

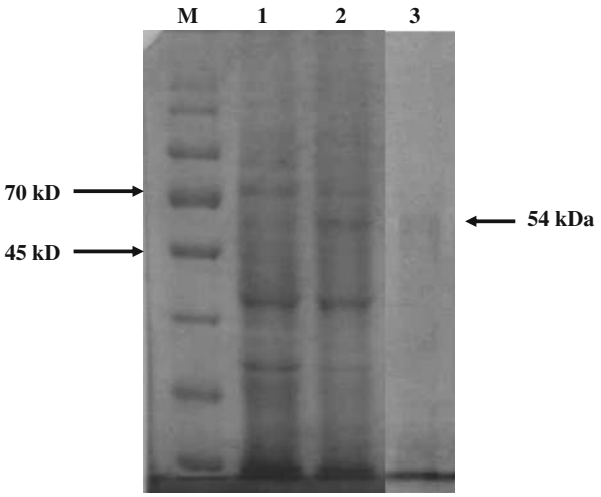

Fig. 3 12% SDS –PAGE analysis of expressed and purified aerolysin. Lane M: Prestain protein marker, Lane 1: Uninduced *E. coli* BL21(DE3) codon plus RP cells, Lane 2: Induced *E. coli* BL21(DE3) codon plus RP cells and Lane 3: purified aerolysin

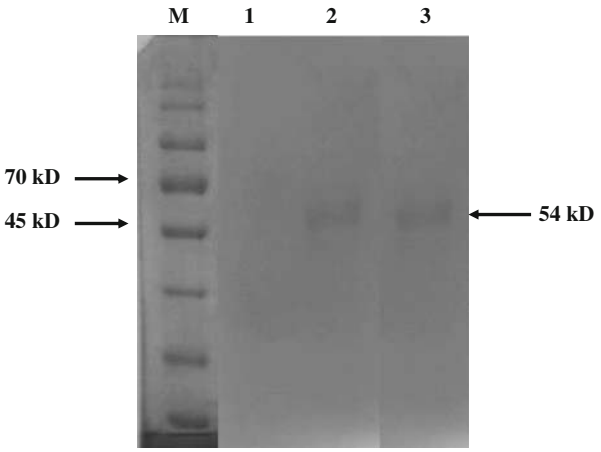

Fig. 4 Western blot analysis of recombinant aerolysin using the anti-His monoclonal antibodies. Lane M: Prestain protein marker, Lane 1: Uninduced *E. coli* BL21 (DE3) codon plus RP cells, Lane 2: Induced *E. coli* BL21 (DE3) codon plus RP cells and Lane 3: purified aerolysin

the fish RBCs and uninduced protein lysate did not show the hemolytic activity (Fig. 5). It indicates that the aerolysin may behave as a major virulent factor of *A. hydrophila.* The hemolytic activity with the fish RBCs was observed in this study and zone of clearance surrounding the hole of blood agar. Aerolysin positive and negative of *A. hydrophila* have been demonstrated with the fish RBCs [19]. Expressed aerolysin of *A. hydrophila* has been used for the hemolytic assay. The induced and purified aerolysin showed the hemolytic activity with RBCs [12].

Conclusions

The present study was done to clone, express, and characterize the aerolysin of *A. hydrophila* isolated from fish. The recombinant aerolysin of *A. hydrophila* could be produced and purified in adequate amount in the future and used as antigen to develop a convenient and economical diagnostic method which will reduce the cost, transport, and reproducibility problems associated with the present diagnostic tests which requires growth and purification. It can be used as diagnosis for *A. hydrophila* infection in fishes to control and prevent the spreading and mortality in aquaculture. The aerolysin can be used as a future vaccine candidate against *A. hydrophila.*

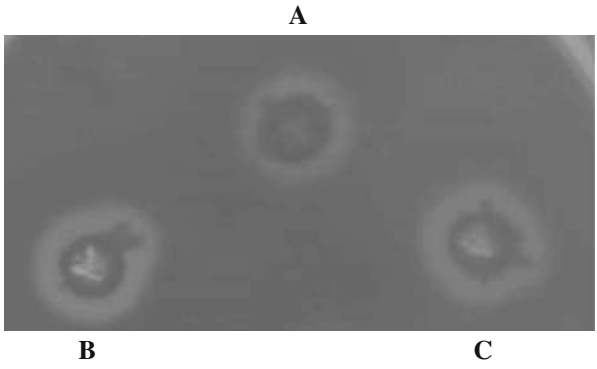

Fig. 5 Analysis of hemolytic activity of induced aerolysin (*A* and *B*) and purified aerolysin (*C*) on blood agar medium

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