Biologicals 44 (2016) 19–23

Contents lists available at ScienceDirect

Biologicals

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

Molecular cloning, expression and characterization of 100K gene of fowl adenovirus-4 for prevention and control of hydropericardium syndrome

M.S. Shah [a, b, *], A. Ashraf [c], M.I. Khan [a], M. Rahman [b], M. Habib [d], J.A. Qureshi [b, e]

[a] Department of Pathobiology and Veterinary Sciences, University of Connecticut, Storrs, CT 06269, USA
[b] Drug Discovery and Structural Biology Group, Health Biotechnology Division, NIBGE, Faisalabad, Pakistan
[c] Department of Zoology, Government College University, Faisalabad, Pakistan
[d] Vaccine Development Group, Animal Sciences Division, NIAB, Faisalabad, Pakistan
[e] Institute for Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan

ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form
22 September 2015
Accepted 6 October 2015
Available online 7 November 2015

Keywords:
FAdV-4
Hydropericardium syndrome
100K
Phylogenetic analysis
Non structural protein

ABSTRACT

Fowl adenovirus-4 is an infectious agent causing Hydropericardium syndrome in chickens. Adenovirus are non-enveloped virions having linear, double stranded DNA. Viral genome codes for few structural and non structural proteins. 100K is an important non-structural viral protein. Open reading frame for coding sequence of 100K protein was cloned with oligo histidine tag and expressed in *Escherichia coli* as a fusion protein. Nucleotide sequence of the gene revealed that 100K gene of FAdV-4 has high homology (98%) with the respective gene of FAdV-10. Recombinant 100K protein was expressed in *E. coli* and purified by nickel affinity chromatography. Immunization of chickens with recombinant 100K protein elicited significant serum antibody titers. However challenge protection test revealed that 100K protein conferred little protection (40%) to the immunized chicken against pathogenic viral challenge. So it was concluded that 100K gene has 2397 bp length and recombinant 100K protein has molecular weight of 95 kDa. It was also found that the recombinant protein has little capacity to affect the immune response because in-spite of having an important role in intracellular transport & folding of viral capsid proteins during viral replication, it is not exposed on the surface of the virus at any stage.

1. Introduction

Poultry has emerged as one of the fastest growing agricultural sector. Unfortunately this sector has major threat from infectious diseases both viral and bacterial. Among viral diseases, hydropericardium syndrome (HPS) is an important viral disease which is common in hot and humid season in some countries of the world [1] which is caused by Fowl adenovirus - 4 (FAdV-4) [2–4]. FAdV is non-enveloped virion, 70–90 nm in diameter size. Its genome consists of linear, double stranded DNA having approximate size of 43–46 kbp and codes for only a few structural and non structural proteins [5–7]. The capsid consists of three major structural proteins that include Hexon, Fiber and Penton base [8]. 100K is one of the important non structural proteins (NSP) of the virus as it acts as a molecular chaperone for folding of hexon and its assembly into capsomers. Nucleotide sequence of 100K region of the fowl adenovirus serotype 10 (FAdV-10) was found equivalent to the 100K gene of human adenovirus and it was found that 100K polypeptide of FAdV consists of 798 amino acids and its deduced molecular weight was 89.023 kDa [9]. It was found that Hexon alone was expressed as inclusion bodies in insect cells but when co-expressed with 100K protein it resulted in the formation of soluble trimeric hexon which were indistinguishable from native hexon capsomers isolated from partially disrupted adenovirions. Moreover, presence of 100K protein in complexes with hexon trimers further confirmed that 100K protein is a scaffold protein for hexon. Morphological characterization of purified 100K protein by Electron microscopic (EM) revealed that it was symmetrical, dumbbell shaped molecule consisting of two globular domains joined by a rod. EM characterization of Hexon-100K protein complexes showed that 100K- hexon interaction occurs through globular domains of

* Corresponding author. Department of Pathobiology and Veterinary Sciences, University of Connecticut, Storrs, CT 06269, USA. Tel.: +92 322 7748074
E-mail address: msalahuddin786@hotmail.com (M.S. Shah).

http://dx.doi.org/10.1016/j.biologicals.2015.10.002
1045-1056/

the 100K protein. Moreover, the hexon binding sites were found on globular domains of the 100K protein [10]. Non-structural proteins (100K and 33K) based enzyme linked immunosorbent assays (ELISAs) can differentiate FAdVs infected and vaccinated chickens, because antibodies specific to the 100K and 33K non-structural proteins were detected in chickens experimentally infected with FAdVs, but not in chickens vaccinated with inactivated FAdVs [11].

Considering the importance of the 100K protein in the viral life cycle, we therefore expressed and characterized the 100K protein of FAdV-4 in *Escherichia coli*.

2. Materials and methods

2.1. Viral strain

The viral isolate used in the present study, was previously isolated, characterized (as Fowl adenovirus-4) and maintained in Animal Sciences Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan [6].

2.2. Viral DNA extraction

DNA was extracted by phenol chloroform method [12]. Viral coat protein was digested at 37 °C for 45–60 min by lysis buffer having tris–HCl (25 mM), NaCl (100 mM), SDS (0.5%) and proteinase K (250 μg/ml). Phenol chloroform was added (100 μl) and DNA was extracted by centrifugation. Chilled ethanol was added in the supernatant. Then DNA was recovered by centrifugation and pellet was dissolved in 20 μl double distilled water.

2.3. Construction of plasmids/vectors

Cloning strategy for recombinant vector/plasmid was devised and recombinant vector/plasmid (pSMJ-1) was constructed using Vector NTI soft ware. For this purpose available nucleotide sequences of target gene (100K) was retrieved from Genbank, DNA sequence was analyzed *insilico* and two restriction enzymes were selected among the multiple cloning site of the vector (pET28a). Restriction sites (*Nde* I and *EcoR* I) were incorporated at the 5′ and 3′ ends of amplified DNA to clone the fragments into respective sites of pET28a (Novagen) to generate pSMJ-1. The sequence was amplified by PCR using primers SMJ-1F (5′CAATTCCATATGGAA AGCACCGCCGACGGGGAT 3′) and SMJ-1R (5′GCCGGAATTCTCAGGT CGACCATTCTCTGG 3′).

2.4. Confirmation of the construct

The construct was initially verified by colony PCR and double digest method using corresponding restriction enzymes, then confirmed by DNA sequencing. DNA sequence of the construct was analyzed on an ABI 3100 capillary sequencer, using BigDye. DNA sequencing was performed at Department of Biotechnology and cell biology, University of Connecticut, Storrs, CT. USA. Nucleotide sequence was determined and Amino acid sequence was deduced and analyzed using BLAST (Basic local alignment search tool) program [13].

2.5. Phylogenetic analysis

Multiple sequence alignment and Phylogenetic analysis was done on observed divergence basis by “DNAMAN, Lynnon Biosoft” (Lynnon Corporation, Quebec, Canada). Pair-wise comparison of nucleotide sequences of 100K gene was done and Homology matrix and Distance matrix with other sequences of Adenovirus were calculated. Limited numbers of nucleotide sequences of 100K gene were available in public access databases GenBank and EMBL. Available sequences were used for multiple sequence alignments. The DNA sequence of 2397 bp 100K gene of our isolate (NIAB/NIBGE – 01) was submitted to the GenBank with Accession no. FR693741.

2.6. Expression of recombinant protein

Plasmid construct (pSMJ1) was transformed into *E. coli*, host BL21 (DE3) and Rosetta (DE3) to compare the expression level in these strains. Overnight culture of LB (Luria Britani) broth were used to inoculate the 100 ml LB broth and grown until OD_{600} reached 0.5. The culture was induced for 4 h in 1 mM concentration of isopropyl β-D-thiogalactoside (IPTG). The cells were harvested by centrifugation at 5000 rpm for 20 min at 4 °C and re-suspended in 5 ml PBS. The cells were lysed by sonication, the supernatant and pellet fractions were collected. The recombinant protein was detected by SDS-PAGE [14] and identified by immunoblot using anti polyhistidine antibody (Sigma). *E. coli* cells harboring wild type pET28a plasmid were processed likewise to serve as negative control [15].

For immunoblotting, proteins were transferred to PVDF membrane using semi-dry blotter (Trans blot SD – Biorad system) [16]. After successful transfer of Blot, the PVDF membrane was blocked for 1 h with 3% BSA & 0.05% Tween 20. Incubated the membrane for 1 h with mouse anti histidine antibody diluted 1:3000 in TBST having 3% BSA. The membrane was washed thrice with TBST and once with TBS. Membrane was incubated for 1 h with alkaline phosphatase conjugated with anti-mouse IgG (Sigma) used in 1:10,000 dilution in TBST with 3% BSA. Subsequently, the blot was washed 4 times with TBST. After washing, immune complexes were detected using bromo-chloro-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma).

2.7. Purification of recombinant protein

Cell lysates were loaded to the chromatographic columns having 300 μl of Ni NTA resin (Novagen) and allowed to pass through slowly to ensure maximum binding. Column was washed with 5.25 ml of the 1 × Ni-NTA wash buffer. Bounded fraction of proteins was eluted by 1 ml of 1 × Ni-NTA elute buffer and elute fractions were collected in clean microfuge tubes. The 10 μl of each washing and elute fractions were subjected to SDS-PAGE analysis.

2.8. Immunization and challenge studies

One day old 90 broiler chicks were purchased from a commercial hatchery and reared under standard managemental conditions. Experiment was conducted under the regulations of the Institutional Animal Care and Use Committee (IACUC) of Animal Sciences Division, NIAB, Faisalabad. Broiler chickens were divided into three groups having thirty chickens in each group at 14th day of age. Considering the immunopotentiator effect of Freund's complete adjuvant (FCA) the purified 100K protein was quantified and adjuvanted with FCA [17]. A total 200 μl dose (protein + adjuvant) was injected subcutaneously to each bird in two equal parts (100 μl at one site) to avoid/minimize the adverse effects of inflammation. Group A was immunized with recombinant 100K protein (25 μg/bird) by subcutaneous injection at 14th day of age [16]. Group B and C were injected with saline and they were treated as controls. Broiler chickens were kept under observation and blood samples were collected from wing veins pre-immunization and at 7th, 14th, 21st and 28th day after immunization for antibody titer determination by ELISA [18]. Four weeks post immunization chickens were challenged with 0.5 ml of semi-purified virus having 100 units of LD50 ($10^{5.5}$ units of LD50/ml). Virus was injected subcutaneously to