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Original Article

Prokaryotic Expression of Influenza A virus Nucleoprotein Fused to Mycobacterial Heat Shock Protein70

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Abstract

Background and Aims: The novel approaches in influenza vaccination have targeted more conserved viral proteins such as nucleoprotein (NP) to provide cross protection against all serotypes of influenza A viruses. Influenza specific cytotoxic T lymphocytes (CTL) are able to lyse influenza-infected cells by recognition of NP, the major target molecule in virus for CTL responses. On the other hand, studies suggest that fusing of molecular adjuvants such as Heat Shock Protein 70 (HSP70), member of intracellular chaperon super families, with an antigen can induce the cellular and humoral specific responses better than the same induced by antigen alone. It is shown that the C-Terminal of HSP70 (ctHSP70) is the main domain responsible for inducing immunity system.

Materials and Methods: In this study the open reading frame of NP gene from Influenza A virus (PR/8/34) and C-Terminal (359-625) domain of HSP70 gene from Mycobacterium tuberculosis were amplified and cloned into expression pET28a vector independently. Then the N-terminal of whole NP protein was fused to truncated HSP70 in same vector. The fidelity of cloned genes was confirmed by sequencing. All three types of clones were expressed in *E. coli* BL21 and confirmed by SDS-PAGE and Western blot analysis.

Results: Results showed the integrity of vector constructs and well expression of NP, ctHSP70 and fusion form of ctHSP70-NP recombinant proteins in BL21 host cells.

Conclusion: ctHSP70-NP fusion protein produced could be considered and evaluated as a universal influenza vaccine which its immunogenicity potential needs to be assessed in animal models along with proper control groups including recombinant NP and ctHSP70 proteins.

Keywords: Influenza virus; vaccine; Nucleoprotein; HSP70

Introduction

Influenza A viruses, members of Ortomyxoviridae family, are able to infect a large variety of animals as well as humans. Influenza A viruses with segmented negative strand genome are classified based on the antigenic properties of their two distinct surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which are located on the outer host-cell-derived envelope. Due to segmented nature of the genome composition and the wide range of host species, influenza A viruses continually undergo antigenic shift and /or reassortment, results in new emerged subtypes. Antigenic drift because of frequent mutations is the other biological feature of this virus leading to the gradual evolution of viral

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strains. Most of these mutations are nonsense as they do not lead to the serious conformational changes of the proteins (1). However, some mutations affect on the viral proteins such that the binding of host neutralizing antibodies is damaged. Currently, licensed influenza vaccines are inactivated or live attenuated viruses which their seeds annually introduce by World Health Organization according to the most prevalent strains in the upcoming influenza season. The major mechanism of these vaccines is based on induction of neutralizing antibodies against the viral surface hemagglutinin (HA) and neuraminidase (NA), two highly variable antigenic determinants. Hence, the development of a universal vaccine that provides cross-protection against variant subtypes of influenza A virus has been a main concern of the vaccinologist (2, 3).To establish a universal vaccine, the novel approaches have targeted more conserved sequences such as nucleoprotein (NP) (4). NP is an internal protein that showes more than 92% similarity in sequence among different serotypes isolated during the last two decades (5). Moreover, Influenza specific cytotoxic T lymphocyte (CTL) is able to lyse influenza-infected cells by recognition of NP, the major target in virus for CTL responses. Thus, the specific immunity induced against NP contributes to the clearance of the virus from the infected tissue and prohibits the spread of viral infection (6). However, one of the concerns in using NP as target for immunization is its limited potency to produce efficient immunity because of being small in size (7, 8). It has been shown that using molecular adjuvants such as Heat Shock Protein70 (HSP70) from much conserved intra cellular chaperons super families, called stress proteins, can activate innate and cellular immunity responses (9). Studies suggest that fusing of HSP70 with an antigen makes it an effective immunogen which can induce humoral and cellular responses, such as eliciting CD8 CTL, better than responses induced by specific antigen alone (10). It has been shown that the domain of HSP70 is responsible for inducing immunity system as molecular adjuvant with its 28 KDa C-Terminal (359-625) domain (ctHSP70) (11, 12).

In this study, we have constructed a vector expressing a fusion protein of NP-ctHSP70 in a way that the N terminal of NP protein from Influenza A virus (PR/8/34) was fused in frame to ctHSP70 originated from Mycobacterium tuberculosis. The other new recombinant proteins, NP and ctHSP70, were the byproducts of this work which their prokaryotic expression have also been shown independently.

Methods

Primer designing, PCR and RT-PCR

Two sets of primers were designed to amplify genes of interest, ctHSP70 from *Mycobacterium tuberculosis* (H37Rv) genome and NP open reading frame (ORF) from influenza A virus (Puerto Rico/8/34(H1N1)) by Gene Runner 3.05 (Hastings software, Inc) software. According to the cloning scheme the proper restriction cut sites were created at 5' end of each primer. To amplify ctHSP70 region PCR was performed on DNA *extracted from Mycobacterium tuberculosis* using High fidelity Taq polymerase (Fermentas-Lithuania) and specific primers as below: 5' A CATATG GAG GTG AAA GAC GTT CTG C 3' as forward primer and 5'CCT GCTAGC CTT GGC CTC CCG G 3' as reverse primer. The underlined bases represent the NdeI and NheI restriction enzyme sites, respectively. Amplification reaction was carried out under the following profile: 3 min at 95°C followed by 31 cycles at 95°C for 30sec, 58°C for 30sec and 72°C for 60sec, with a final extension step at 72°C for 10 min.

To amplify NP ORF, total RNA was extracted from 250µl of the supernatant of infected MDCK cells with influenza A virus (Puerto Rico/8/34 (H1N1)), equal to 512 HAU/ml (Hemagglution Unite) using the RNX^{TM} solution (CinnaClone, Iran). 50µl of the extracted RNA was first converted into cDNA using the "RevertAid First Strand cDNA Synthesis" Kit (Fermantas-Lithuania) and random hexamer according to manufacturer's instructions. Then PCR was performed using

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High fidelity Taq polymerase and specific primers designed as below:

5' AGCTAGC ATG GCG TCT CAA GGCAC C 3' as forward and 5' AGT GTCGAC TTA ATTG TCA TAC TCC TCT GCA TTG 3' as reverse primer. The underlined bases represent the *NheI* and *SalI* restriction enzyme sites, respectively. Amplification reaction was done under the following profile: 3 min at 95°C followed by 30 cycles at 95°C for 30sec, 58°C for 30sec and 72°C for 90sec, with a final extension step at 72°C for 10 min.

PCR products were analyzed by 1% (w/v) agarose gel electrophoresis and purified by Quick Gel Extraction Kit (Invitrogen- USA).

Cloning

The amplicones were first cloned through A overhangs into pTZ57R/T vector (Fermentas-Lithaunia). The clones then were subjected to automatic sequencing bidirectionally using M13 universal primers.

The ctHSP70 and NP genes were exited from the corresponded clones by NdeI/NheI and NheI/NdeI double digestions respectively and cloned separately into linearized pET28a (Novagen) with the same digestions to construct pET28a+ ctHSP70 and pET28a+ NP (Fig.1)

NP ORF was exited from pET28a+NP and its 5' end fused to 3' end of truncated HSP70 into pET28a+ctHSP70 through NdeI/NheI sites to construct "pET28a+ctHSP70NP" (Fig. 1). Colony PCR, restriction map analysis and sequencing by three types of primers; forward primer of ct-HSP70, forward primer of NP and T7 terminator primer were used to confirm the fidelity of "pET28a+ctHSP70NP".

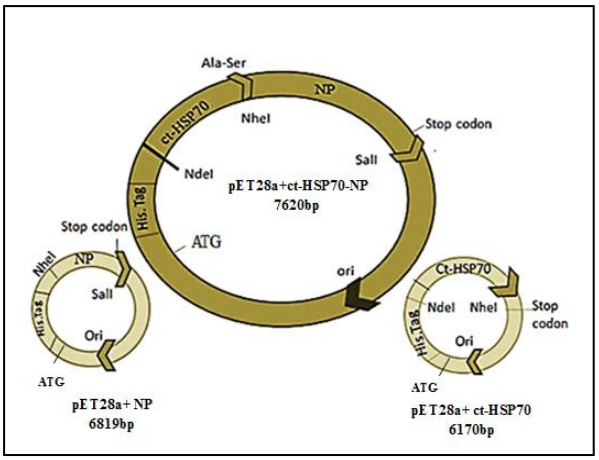


Fig. 1. Schematic picture for cloning of NP, ct-HSP70 and fusion form of ct-HSP70-NP in pET28a.

All DNA manipulations including restriction digestion, T4 ligation, and agarose gel electrophoresis were carried out as described by Sambrook and Russell (13).

Epitope mapping of ctHSP70, NP and ctHSP70NP proteins were determined with epitope prediction softwares; CTLPred online software for cytotoxic T lymphocyte epitope prediction and Bcepred online software for prediction of linear B-cell epitopes.

Expression of recombinant proteins in prokaryotic system

Three kinds of expression plasmids (pET28a+NP, pET+ctHSP70 and pET+ctHSP70-NP) were extracted from DH5α bacteria by plasmid extraction kit (Bioneer-Korea) and transformed into the *E.Coli* BL21 (λDE3) competent host cells.

After culturing transformed cells on LB-agar plates containing 50µg/ml kanamycin at 37°C during overnight, one colony from each type was inoculated in 15ml of LB broth containing 50µg/ml kanamycin and incubated until reaching to 0.5 to 1 optical density at 600nm. Then for expression of specific proteins, cultures were induced by adding IPTG (β-D-galactosidase) in final concentration of 1mM.Time course fractionating from cultures started before inducing and continued for 4 hours at stable 37°C temperature.

SDS-PAGE and Western blotting

Total cell protein (TCP) extracted Fusion protein (ct-HSP70-NP) and NP protein were analyzed on 10% SDS-PAGE and ct-HSP70 was analyzed on 12% SDS-PAGE beside of protein ladder (Fermantas). Acrylamide gel staining was followed by Coomassie brilliant blue R-250.

For further characterization, the separated proteins on SDS-PAGE were electrotransferred to nitrocellulose membrane and blocked with TBS/Tween20+1% BSA the blotted proteins were reacted with Anti-His_6 (Roche) diluted 1:6000 in TBS-T for 1h at room temperature. The appropriate secondary antibody, anti-mouse IgG Peroxidase conjugate (Sigma) diluted 1:5000 in TBS-T, was used and

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incubated for 2 h at room temperature. The specific protein bands were developed by DAB (diaminobenzidine) substrate (Roche).

Results

Predicting residues of epitopes in NP, ctHSP70 and the fusion form of ctHSP70-NP antigens showed that the most CTLs and B-cells epitopes were the same between fusion form and each antigen alone (results not shown).

PCR amplification of NP from influenza A virus and c-terminal domain of HSP70 from Mycobacterium tuberculosis was done successfully and on 1% agarose gel the results are shown in fig. 2 (A and B) demonstrating the size of 1513bp and 817bp for NP ORF and ct-HSP70 region respectively, indicating the flanked restriction sites incorporated into each amplicons.

Colony PCR with specific primer sets for each fragment, plasmid extraction and restriction enzyme analysis were confirmatory evidences for all clones constructed in this work. Analysis of sequencing results confirmed the fidelity of the inserted genes as well as fusion form of ctHSP70-NP.

Three types of clones (pET28a+NP, pET+ctHSP70 and pET+ctHSP70-NP) were expressed in BL21 strain and induced with IPTG.

SDS-PAGE of total cell proteins for NP protein on 10% gel acrylamide had exactly 57KDa size, ct-HSP70 on 12% gel acrylamide had 36KDa size and fusion protein (ct-HSP70-NP) on 10% gel acrylamide had exactly 87KDa size (shown on Fig. 3 A, B and C). Results showed that before induction, target proteins had not expressed but with IPTG induction they expressed perfectly. In the SDS-PAGE picture, collected fractions of fusion protein expression before induction and 4 hours after induction are shown.

Western blotting also confirmed the expected polypeptide bands which were His-tag fused N terminally with molecular weight of about 57 , 36 and 87KDa representing NP, ct-HSP70 and ct-HSP70-NP proteins respectively (Fig. 4A, B and C).

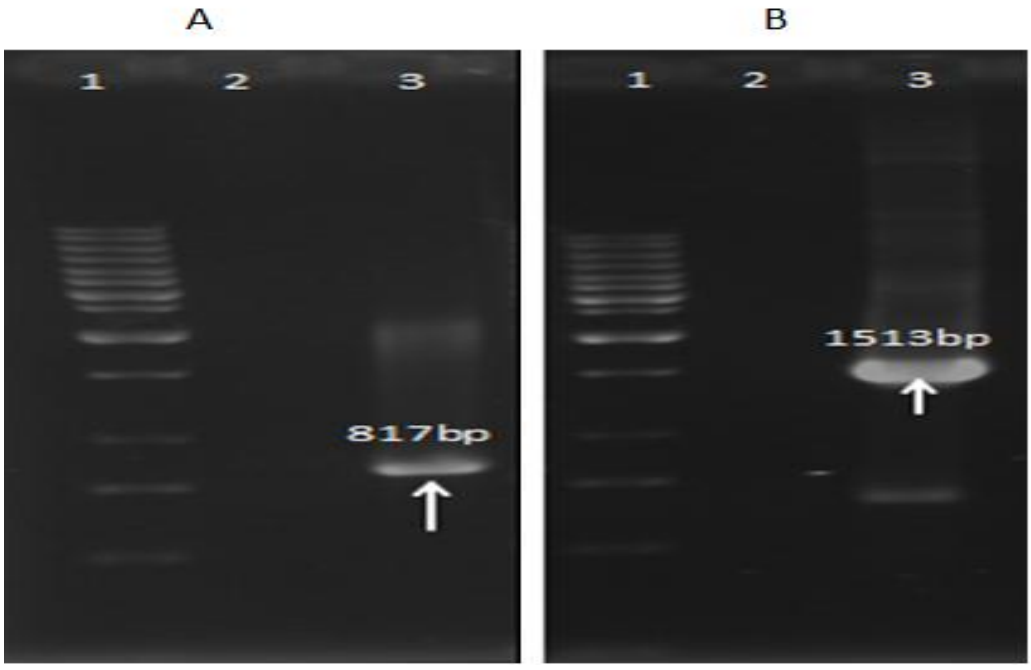


Fig. 2. A) 1% gel electrophoresis of PCR product for ct-HSP70 lane1. GeneRuler™ 1 kb DNA ladder (Fermentas) lane2.PCR product without template lane3. PCR product for ct-HSP70.
B) 1%% gel electrophoresis of PCR product for NP lane1. GeneRuler™ 1 kb DNA ladder (Fermentas) lane2.PCR product without template lane3. PCR product for NP.

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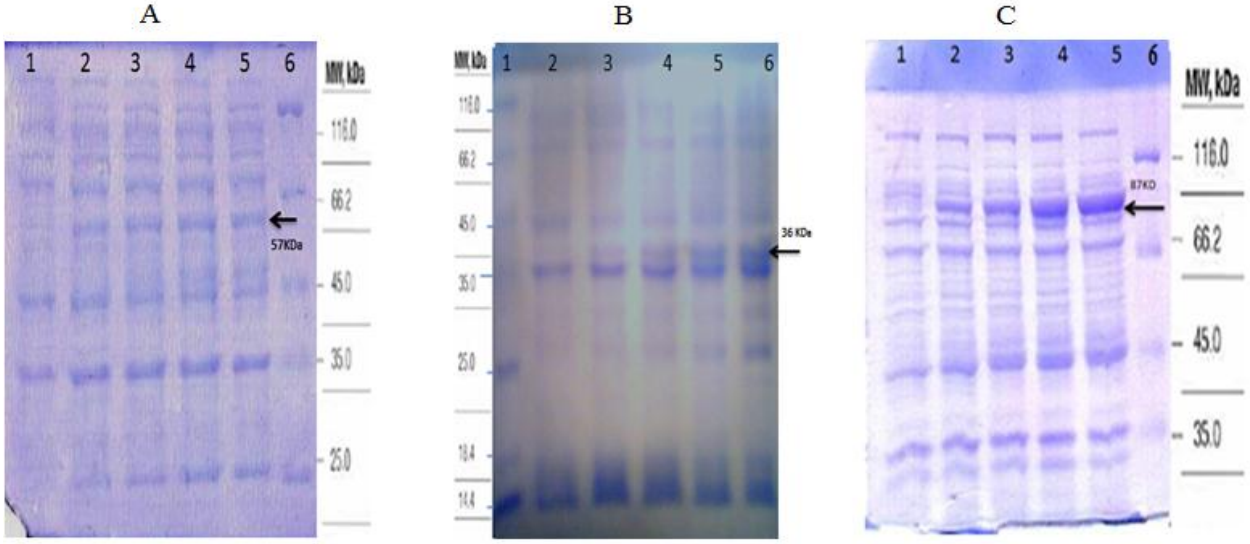


Fig. 3. 10% acrylamide gel electrophoresis for Total Cell Protein A) NP protein expression. Lane 1. Expression before induction lane2.Expression 1 hour after IPTG induction lane3.Expression 2 hours after induction lane4.Expression 3 hours after induction lane5.Expression 4 hours after induction lane6.protein ladder (Fermentas) B) ct-HSP70 protein expression Lane 1. Expression before induction lane2.Expression 1 hour after IPTG induction lane3.Expression 2 hours after induction lane4.Expression 3 hours after induction lane5.Expression 4 hours after induction lane6.protein ladder (Fermentas) C) Fusion protein (ct-HSP70-NP) expression Lane 1. Expression before induction lane2.Expression 1 hour after IPTG induction lane3.Expression 2 hours after induction lane4.Expression 3 hours after induction lane5.Expression 4 hours after induction lane 6.protein ladder (Fermentas).

Discussion

Nucleoprotein of influenza virus, which has conservatory sequence and immunodominant epitopes being good CTL targets for releasing T-cell cross protections, is appropriate candidate for making universal vaccine (14).

NP was used in different types of vaccines such as DNA vaccines (15), viral vector vaccines (16), subunit protein vaccines (17) and multi gene vaccines (18) which all of them show virus clearance and effective immunization in virus challenge infections. Also efficacy of NP in vaccine has been compared with other viral proteins, HA & M2, against subtypes of influenza virus and the results show good capacity of this protein in immunization but it needs combination to another molecule to enhance its efficacy (7). Prokaryotic expression of NP, especially in *E. coli*, in both wiled type and optimized formation is achievable (19, 20). NP derived from *E. coli* can be good candidate for vaccination and elicits robust cross-protection in mice(5).Following this results we could express this protein perfectly in BL21 strain of *E. coli.*

For improving immunization responses for target antigen we used molecular chaperon, HSP70, as a fusion adjuvant. HSP70 can active Antigen-Presenting Cells (APCs) and stimulate maturation of Dendritic Cells (DCs) which can promoting Th1 responses (21). Studies on this stress protein, from mycobacterium tuberculosis, show its C-terminal domain (359-610 aa) capacity for inducing innate and cellular immunity through attaching to TLR2, TLR4, CD91 and CD40 receptors and inducing cells to release molecules such as Th1 cytokines, IL-12, TNF-α, C_C chemokine and Nitric Oxide (NO) (11, 22). studies showed that non-covalently bound between NP and Mycobacterial HSP70 can elicit specific T-cell responses in vivo (23). Also covalently fusion of specific antigen and C-terminal of HSP70 were reported by many researches and showed fully protection in many subtypes of influenza virus (24).

Considering type of target protein, method of cloning, ongoing application of expressed protein and way of extraction protein we

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choose pET28a as expressional cloning vector. pET system is one of powerful vector system for cloning and expression of recombinant proteins in *E. coli*. Expression of pET28a system under control of bacteriophage T7 lac promoter could be induced with IPTG for providing T7 RNA polymerase in host cell, BL21 and itself.

Following pET28a map we choose restriction sites for entrance of fusion genes. In fusion form (pET+ctHSP70-NP) two extra amino acids (Ala-Ser) from *NheI* site were fused two fragments. These extra amino acids could not change epitope mapping of our fusion protein because it was tested with CTL and B-cell epitope mapping software (in result was said).

Two sites of His.Tag and Thrombin from vector express with proteins they are necessary for extraction of recombinant protein but are removable after extraction.

We expressed successfully these recombinant proteins (NP, ctHSP70 and ctHSP70-NP) in BL21strain of *E. coli*.

Expression of proteins was done only in 37ºC and induced with 1mM IPTG. We can change temperature and concentration of inducer to receive the best improved condition for the most products in large scales.

In ongoing study, we are planning to purify these proteins and evaluate their potential to elicit protective immunity in animal models. Two types of new recombinant proteins (NP and ct-HSP70) prepared at the same conditions as the fusion form did, would be used as controls.

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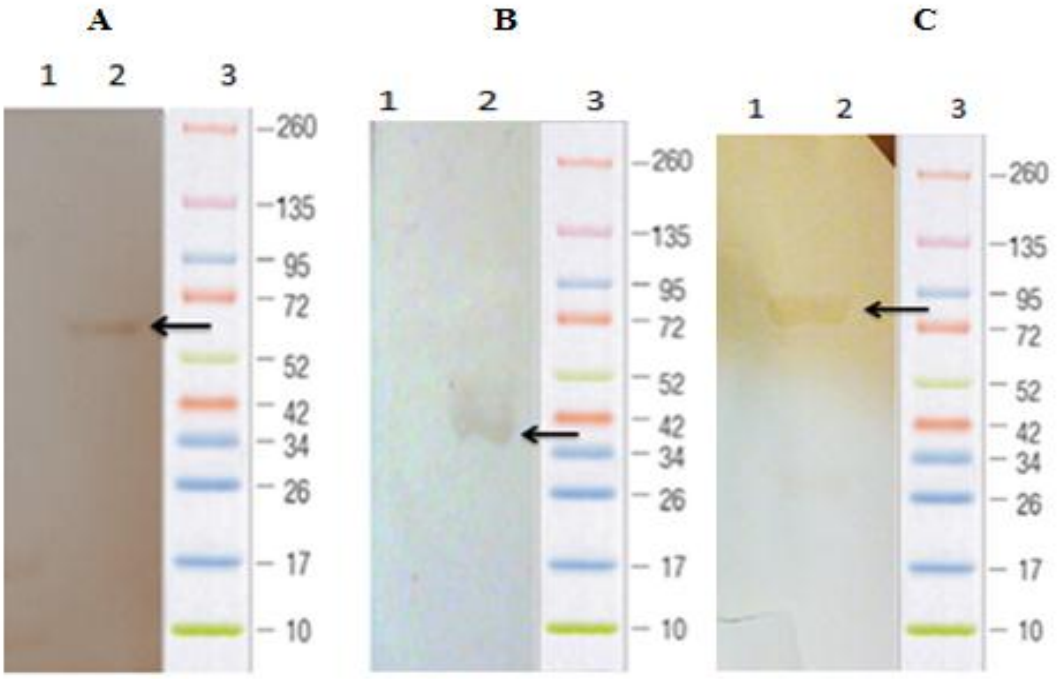


Fig. 4. A**)** Western blotting for NP protein lane1. Non expressed protein before induction lane2. NP protein expression 4 hours after inductionlane3.Spectra™ Multicolor Broad Range Protein Ladder B**)** Western blotting for ct-HSP70 protein lane1.Non expressed protein before induction lane2. ct-HSP70 protein expression 4 hours after inductionlane3.Spectra™ Multicolor Broad Range Protein Ladder C**)** Western blotting for fusion protein (ct-HSP70-NP) lane1. None expressed protein before induction lane2. Fusion protein expression 4 hours after inductionlane3.Spectra™ Multicolor Broad Range Protein Ladder.

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