Original Article

Cloning and prokaryotic expression of the globular head domain of hemagglutinin antigen (HA1) of influenza A (H3N2) virus in Escherichia coli and Bacillus subtilis

Maryam Bidram[1], Farida Behzadian[1*], Fatemeh Fotouhi[2], Maryam Fazeli[3]

[1] Department of Biosciences and Biotechnology, Malek Ashtar University of Technology, Tehran, Iran
[2] Influenza Group, Pasteur Institute, Tehran, Iran
[3] WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran, Tehran, Iran

Received: 20 November, 2016, Accepted: 28 Febreury, 2017

Abstract

Background: The influenza virus hemagglutinin is the major surface protein of the influenza A virus which is composed of HA1 and HA2 subunits. HA1 has an important role in binding of virus to cells and designing neutralizing antibodies. Escherichia coli (*E. coli*) and Bacillus subtilis (*B. subtilis*) both are known as the most useful prokaryotic hosts to express recombinant proteins. The aim of this study was to clone and express recombinant HA1 protein in *E. coli* and B. subtilis bacteria.

Materials and Methods: HA1 gene was cloned into pET-28a vector and pHT43 shuttle vector and then, both transformed to *E. coli*. The recombinant plasmids were extracted and then transformed into the BL21 and WB600 as expressing hosts. After induction with isopropyl-β-d-thiogalactoside (IPTG), expressed recombinant protein was analyzed by SDS-PAGE. Finally, the expressed protein was confirmed by the Western blot.

Results: HA1 gene was cloned into pET-28a vector and pHT43 shuttle vector and then, both transformed to *E. coli*. The recombinant plasmids were extracted and then transformed into the BL21 and WB600 as expressing hosts. After induction with isopropyl-β-d-thiogalactoside (IPTG), expressed recombinant protein was analyzed by SDS-PAGE. Finally, the expressed protein was confirmed by the Western blot.

Conclusion: This study demonstrated a strategy for production and purification of recombinant protein in large scale to test as vaccine candidate against influenza and it's potentially immunogenicity be assessed in animal models.

Keywords: Influenza A Virus, H3N2 Subtype, Hemagglutinin

***Corresponding Author:** Farida Behzadian, PhD; Department of Biosciences and Biotechnology, Malek Ashtar University of Technology, Tehran, Iran. Email: fbehzadian@yahoo.com, Tel: (+98) 21 22 97 46 03

Please cite this article as: Bidram M, Behzadian F, Fotouhi F, Fazeli M. Cloning and prokaryotic expression of the globular head domain of hemagglutinin antigen (HA1) of influenza A (H3N2) virus in Escherichia coli and Bacillus subtilis Arch Med Lab Sci. 2017;3(1):1-6.

Introduction

Influenza virus is a member of the Orthomyxoviridae family and has segmented negative single strand RNA genome that encodes 11 proteins. Orthomyxoviridae family contains three genera of influenza viruses: influenza A, B and C. Influenza A (H3N2) virus is one of the human genotypes of influenza that causes seasonal epidemics (1). Historically, this virus has been responsible for 1968 and 1969 pandemic (Hong Kong Flu) that killed 34000 people just in USA (2). There are high rates of genetic shift and drift to generate various subtypes due to segmented viral genome of influenza and its RNA nature, and therefore, new strains are introduced each year which even may cause flu pandemics (3). This

fact makes the very important ability to produce vaccines, especially, when new vaccines shoud be produced in large scale, during short time. The outer surface of the virus has been covered with two antigenic glycoproteins: Haemagglutinin (HA) and Neuraminidase (NA) (4, 5). Haemagglutinin and Neuraminidase activities are critical for virus replication and influenza virus A can be categorize into subtypes based on their antigenic diversity, so that at present, there are 16 known HA subtypes and 9 known NA subtypes of influenza A virus (6). Haemagglutinin (HA) is the major surface glycoprotein in influenza A virus that is composed of two subunits: HA1 and HA2 (7). Since it is included the receptor binding sites, HA1 plays an important role in binding of virus to host cells (7). According to its presence in the community, Influenza A virus (H3N2) is always considered in the composition of the trivalent vaccines (8). Experimental evidence suggests that HA glycosylation may be important in folding and recognition of cell receptors, but it is not critical in immunogenicity and therefore, the immunogenic properties can be preserved even by its expression in prokaryotic systems (5). In other hand, prokaryotic system can be used to produce vaccines in large scale and during short times. Both E. coli and Bacillus subtilis are attractive hosts for the production of recombinant proteins (9).

In this research, HA1 encoding sequence was cloned and expressed in *E. coli* and B. subtilis to assess produced proteins, a candidate vaccine against influenza A virus (H3N2).

Methods

Extraction and proliferation of HA1. The genome of influenza virus H3N2 (prepared by Pasteur Institute of IRAN) was extracted by PureLink Viral RNA/DNA mini kit (USA, Invitrogen). RNA was converted to cDNA by using RevertAiadTM H Minus M-MuLV reverse Transcriptase (Fermentas, Lithuania) and universal primers Uni12 according the following conditions: 10 min at 25°C and 60 min at 42°C.

The synthesized cDNA was used as a template and PCR reaction was performed by using High Fidelity Taq polymerase (Fermentase, Lithuania) to amplify HA1 gene with specific primers. Primers contained unique restriction sites for NheI (Forward) and SalI (Reverse) to facilitate cloning. Also, a stop codon was inserted at 5´ end of reverse primer. The sequences of primers were as follow:

Forward primer:
5'TACGCTAGCCAAAAACTTCCCGGAAATGACA AC 3'

Reverse primer:
5'TACGTCGACTTATCTAGTTTGTTTCTCTGGTA C 3'

The used conditions were: 95 Ċ for 3 min, 35 cycles of (95°Ċ 30s, 58°Ċ 30s, and 72°Ċ 1min) and final extension of 72 Ċ for 10min. Then, PCR product was examined by electrophoresis on a 1% w/v agarose gel.

Cloning of HA1 gene. First, the PCR product was ligated into pTZ57R/T vector (InsTAclone PCR Cloning kit, Fermentase, Lithuannia) based on TA cloning scheme and according manufacturer's instructions. Following blue/white colony screening, recombinant colonies were confirmed by colony PCR, using M13 universal primers. Then, DNA plasmid Mini extraction kit (Bioneer, Korea) was used to isolate related plasmids from bacterial cells. Because incorporated sites of XbaI and SmaI, which were designed in primers, were not compatible buffer to cause digestion with adequate efficiency, digestion was performed by EcoRI/SalI that their restriction sites presented in pTZ5R/T, at both sides of HA1 gene. Therefore, pTZ57R/T+HA1 was digested by using EcoRI/SalI to sub-clone in pET28a and was double digested by XbaI and SmaI in order to subcloning of HA1 in pHT43. Also, EcoRI and SalI were used to digest pET-28a and XbaI and SmaI were used to digest pHT43 shuttle vector. Following electrophoresis of digested products, extraction of the digested vector and insert from agarose gel was performed using a gel extraction kit (Invitrogen, USA). Then, ligation was performed with T4 DNA ligase enzyme (Fermentase, Lithuania) and ligation products were transformed into DH5-α strain by CaCl2 method. Finally, transformant cells, containing pET-28a, were plated on LB agar with kanamycin (30 µg/ml) and transformant cells, containing pHT43, were plated on LB agar with ampicillin (50 µg/ml) and incubated overnight at 37°Ċ. Then, colony PCR was

performed to identify recombinant colonies and finally recombination confirmed by enzymatic digestion. Resultant recombinant plasmids, pET-28a-HA1 and pHT43-HA1, were extracted and transformed to expression strains, E.coli (BL21) by chemical method and to Bacillus Subtilis (WB600) by electroporation respectively.

Expression. For protein expression, E. coli (BL21) cells containing pET-28a-HA1 were grown in 5ml LB broth supplemented with kanamycin (30 µg/ml) and Bacillus Subtilis (WB600) cells containing pHT43-HA1 were incubated in 5ml LB broth supplemented with ampicillin (50 µg/ml) overnight at 37°C. Then, 2ml of culture each were inoculated to 13 ml fresh mediums. When the OD600 reached 0.7, expression was induced by adding 0.1 mM IPTG to the cultures. No induced samples were harvested from the culture before induction (1 ml). After induction, sampling has continued until 4 hours (hourly 1 ml/h). The cell samples were precipitated by centrifugation (10000 ×g for 1 min), the supernatant was removed and protein preparation performed for each segment separately. The deposition procedure from the samples containing expressed protein in B. subtilis was performed by its saturation with NaCl and followed by centrifugation (10000 ×g for 10 min). Protein expression in each host was analyzed by SDS-PAGE and finally, western blot was performed to confirm expression of the intended protein in E. coli.

Results

Gel electrophoresis of the PCR products showed a 1 kDa band extracted from genome of influenza virus (H3N2) (similar to the HA sequence). Sequencing was confirmed accuracy of the HA1 sequence cloned into pTZ57R/T vector. Also, colony PCR and restriction enzyme digestion was performed to confirm accuracy of pET-28a-HA1 and PHT43-HA1. First, the restriction enzyme HindIII was used for simultaneous digestion of pET-28a and pET28a-HA1. Since, this enzyme has restriction sites on both plasmid backbone and HA1, restriction enzyme digestion of pET-28a-HA1using HindIII lead to the fragments about 600 bp and 5800 bp (Figure 1).

Restriction enzyme analysis using SmaI and XbaI enzymes was also performed for simultaneous digestion of pHT43-HA1 and pHT43. As it be expected, using two above restriction enzymes, the fragments about 1010 bp and 8075bp came out after digestion of the recombinant vector; whereas, an 8036 bp band was obtained after digestion of empty vector (Figure 2).

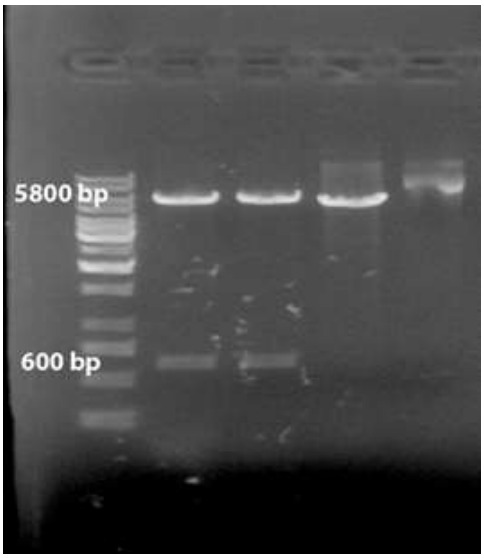

Figure 1. Restriction enzyme digestion of pET-28a-HA1using HindIII. Two DNA fragments about 600 and 5800 obtained has been shown in the figure.

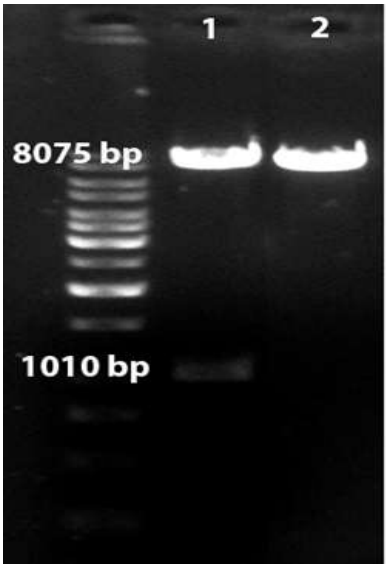

Figure 2. Restriction enzyme digestion of pHT43-HA1 and pHT43 using restriction enzymes SmaI and XbaI. Two DNA fragments about 1010 and 8075 obtained after pHT43-HA1 digestion (line 1) and only a DNA belt about 8075 gaind after digestion of pHT43 (line 2).

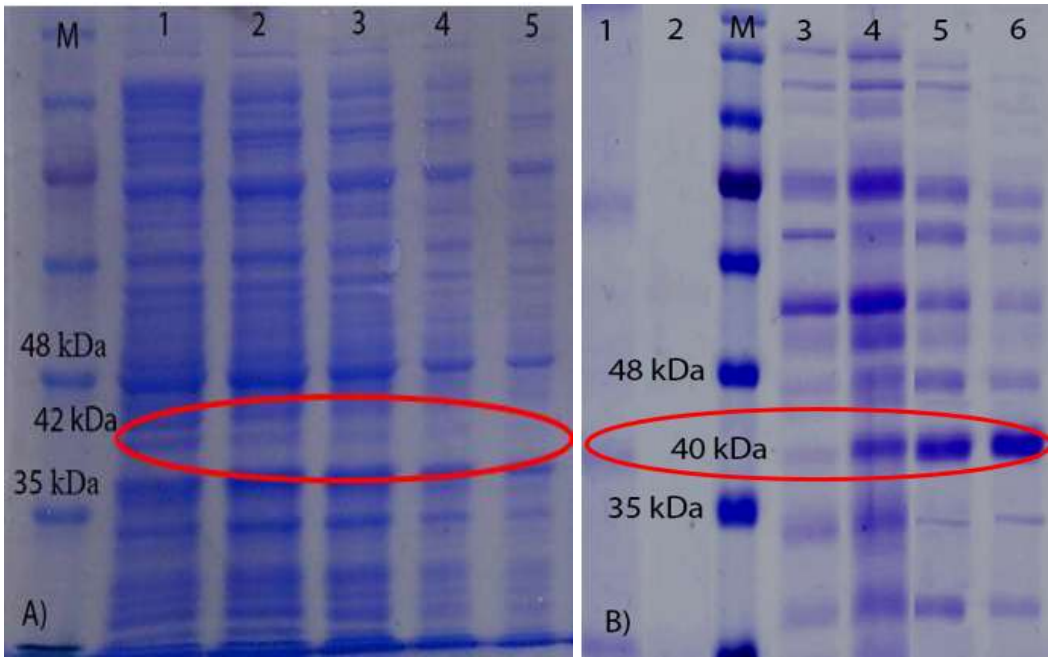

Figure 3. The SDS-PAGE results for evaluation of expression of recombinant HA1 protein in E.coli and Bacillus subtilis. A) line M: Prestained Protein Ladder (Sinaclon Cat. No. PR901641), line 1: Sample of fourth hour, line 2: Sample of third hour, line 3: Samples of second hour, line 4: Sample of first time, line 5: Sample before induction. B) line 1: Recombinant proteins secreted into the culture medium 16 hours after induction, line 2: Lack of protein secretion in culture medium before induction, line M: Prestained Protein Ladder (Sinaclon Cat. No. PR901641), line 3: Sample before induction, line 4: Samples of first hour, line 5: Samples of second hour, line 6: Sample of 16 hours after induction. The sharp band is seen in the last line.

The SDS-PAGE results showed that expression of recombinant HA1 protein gradually increased until 4 hours after induction (42 kDa expressed protein in E.coli and 40 kDa expressed protein in Bacillus subtilis). Also, a 40 kDa protein band has been presented in deposited samples that confirm secretion of HA1 recombinant protein. The results also showed that the expressed protein band in E. coli has approximately 42 kDa in western blotting.

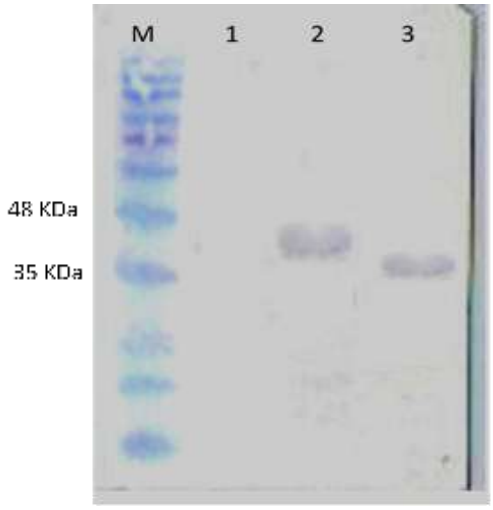

Figure 4. Detection of the recombinant HA1 protein by Western blot analysis using Anti-His6 monoclonal antibody; lane M, Prestained Protein Ladder (SinaClon Cat#PR901641) Lane 1, total cell protein sample of transformed E.Coli cells before IPTG induction; lane 2, the same sample 2h after IPTG induction, showing expected 42 kDa protein size; lane 3, A known His-tagged protein sample weighed 36 kDa, used as positive control for WB procedure.

Discussion

One of the most important demands is the improvement of influenza vaccine to reach a more widespread and stable immune responses against the viruses. Recombinant vaccine technologies as new methods make easier to produce seasonal and pervasive vaccines (10). In recent decades, researchers are trying to produce efficient and safe vaccine that can be replaced for old ones. Different prokaryotic and eukaryotic systems were also used

including insects and plants cells to express HA subtypes (11).

Recent studies on spatial structure of hemagglutinin suggested that its globular region (HA1) is an appropriate option in production of recombinant vaccine (12). Since, HA1 alone possess most antigenic hemagglutinin properties that cause induction of the antibodies major neutralization responses against this glycoprotein, hence, HA1 gene was used to development of modern vaccines such as single unit vaccine, DNA based vaccine and virus like particle (13). For designing an effective and safe HA1- based vaccine, it is essential to cover inducer of the production of neutralizing antibodies, the receptor binding domain (RBD) of H5N1 HA protein in HA1 subunit (14). RDB is the globular head of the HA1 subunit (1-320 amino acids). Khurana et al. (2011) in a study showed oligomers with defective function without addition of foreign oligomerization signal to the first five amino acids in the N terminus of HA1 in HA1 lacking amino acids 321 to 330 (15).

In recent years, E. coli was applied widely in expression of recombinant proteins. In comparison with the other expression systems, E.coli is more suitable for continuous fermentation processes due to more rapid growth and its low cost (16). In other hand, expression system based on Bacillus subtilis possess some other suitable properties such as absence of codon biased, lack of mostly extracellular proteases and endotoxins, the ability to secrete soluble and active form of the protein into the medium and prevent to formation of inclusion bodies (17). In a similar study, it was confirmed that the globular region of hemagglutinin (binding region to receptor) which expressed in E. coli, as a recombinant protein, was concluded an immunogenic and protective response in mice (18). This protein can specifically bind to serum of infected individual with influenza virus. In the srudy by Farsad et al (2016), HA1-based vaccines was produced efficiently in bacterial systems with ability of large-scale production in response to a pandemic influenza virus threat (19).

In the study, data showed that the most production of recombinant HA1 protein in E. coli is four hours after induction and in Bacillus subtilis is 16 hours after induction. Although we observed high speed production of the target protein in E. coli, but its expression in comparison with Bacillus subtilis was lower and more expensive, because expression system by using Bacillus subtilis don't need to remove endotoxin and refolding process in order to obtain an active protein. According this research seems that the produced protein can be used for more evaluation of its immunogenicity in animal models as candidates for rapid and low-cost production of influenza vaccine against possible pandemic flu in the future.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors thank all the Virology Research Center staffs, Baqiyatallah hospital, for their kind advices and technical supports.

References

1. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses: Academic Press; 2005.
2. Barry JM. Observations on past influenza pandemics. Disaster medicine and public health preparedness. 2009;3(S2):S95-S9.
3. Cox N, Subbarao K. Global epidemiology of influenza: past and present. Annual review of medicine. 2000;51(1):407-21.
4. Gürtler L. Virology of human influenza. Influenza Report Kamps BS, Hoffmann C, Preiser W Flying Publisher, Wuppertal. 2006.
5. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual review of biochemistry. 2000;69(1):531-69.
6. Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. Science. 2004;305(5682):371-6.
7. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen L-m, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nature structural & molecular biology. 2009;16(3):265-73.
8. Belshe RB, Gruber WC, Mendelman PM, Cho I, Reisinger K, Block SL, et al. Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. The Journal of pediatrics. 2000;136(2):168-75.
9. Jana S, Deb J. RETRACTED ARTICLE: Strategies for efficient production of heterologous proteins in Escherichia coli. Applied microbiology and biotechnology. 2005;67(3):289-98.
10. Lee BY, McGlone SM. Pricing of new vaccines. Human vaccines. 2010;6(8):619-26.
11. Williams J, Crowe J. Recombinant Influenza H5 Hemagluttinin

Protein And Nucleic Acid Coding Therefor. Google Patents; 2007.
12. Chung KY, Coyle EM, Jani D, King LR, Bhardwaj R, Fries L, et al. ISCOMATRIX™ adjuvant promotes epitope spreading and antibody affinity maturation of influenza A H7N9 virus like particle vaccine that correlate with virus neutralization in humans. Vaccine. 2015;33(32):3953-62.
13. Margine I. Towards the development of a universal vaccine for influenza viruses: Induction of Broadly Protective Hemagglutinin Stalk Antibodies by Natural Infection and Novel Vaccination Strategies: Icahn School of Medicine at Mount Sinai; 2014.
14. Tsai HJ, Chi L-A, Alice LY. Monoclonal antibodies targeting the synthetic peptide corresponding to the polybasic cleavage site on H5N1 influenza hemagglutinin. Journal of biomedical science. 2012;19(1):37.
15. Khurana S, Larkin C, Verma S, Joshi MB, Fontana J, Steven AC, et al. Recombinant HA1 produced in E. coli forms functional oligomers and generates strain-specific SRID potency antibodies for pandemic influenza vaccines. Vaccine. 2011;29(34):5657-65.
16. Baneyx F. Recombinant protein expression in Escherichia coli. Current opinion in biotechnology. 1999;10(5):411-21.
17. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Applied microbiology and biotechnology. 2006;72(2):211.
18. Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, et al. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One. 2008;3(5):e2257.
19. Farsad A, Malekzadeh-Shafaroudi S, Moshtaghi N, Fotouhi F, Zibaee S. Expression of HA1 antigen of H5N1 influenza virus as a potent candidate for vaccine in bacterial system. Iranian Journal of Veterinary Research. 2016;17(4):237-42.