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Original Article

Bacillus subtilis as a Host for Recombinant Hemagglutinin Production of the Influenza A (H5N1) Virus

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Abstract

Background and Aims: Influenza A(H5N1) viruses circulating in animals might evolve and acquire the ability to spread from human to human and thus start a pandemic. Hemagglutinin (HA) has been shown to play a major role in binding of influenza virus to its target cell and the main neutralizing antibody responses elicit against this region. Recent studies have shown that glycosylation of HA is not necessary for its immunogenicity. *Bacillus subtilis* has been identified as a free endotoxin host for high expression and secretion of heterologous proteins with immunological activity. This bacterium is not capable of supporting glycosylation process. However, it could be an appropriate host to produce new recombinant HA1 for vaccine research purposes. In this study we constructed a recombinant B. subtilis that was able to express and secrete HA1 protein into cytoplasmic and extracellular medium.

Materials and Methods: HA1 gene was amplified and cloned into pGEM® 5Zf(–) vector. It was then subcloned into shuttle vector PHT43 and transferred to E.coli for propagation. Accuracy of PHT43-HA1 construct was confirmed by sequencing and restriction map. The recombinant plasmid was extracted from E.coli and used to transform of B.subtilis by electroporation. Following IPTG induction, the total cell protein and the protein secreted into media were analysed through a time course using SDS-PAGE.

Results: The accuracy of PHT43-HA1 construct was confirmed by sequencing and enzymatic digestion analysis. SDS-PAGE results showed that the recombinant HA1 protein was successfully expressed and secreted into medium.

Conclusion: The HA1 protein produced here could be considered and evaluated as a protective antigen which its immunogenicity potential needs to be assessed in animal models along with proper control groups.

Keywords: *Bacillus subtilis*, hemagglutinin, influenza, H5N1

Introduction

Influenza A viruses are zoonotic pathogens that continuously circulate and change in several animal hosts, including birds, pigs, horses and humans. Due to the segmented genome of these viruses, the emergence of novel virus strains that are capable of causing human epidemics or pandemics is a serious possibility (1). The pandemic potential of H5N1 strain for humans has been well documented (2). These viruses can cause a number of diseases along with organ failure and death among infected patients (3, 4). Hemagglutinin (HA) is the dominant glycoprotein on the surface of the influenza virus and a recognized key antigen in the host response to influenza virus in both natural infection and vaccination. The HA protein,

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encoded by segment 4 of the viral genome, is initially translated as a preprotein (HA0) that is then split into two subunits (HA1 and HA2), which subsequently assemble to form a trimeric structure (HA1-HA2). In the spatial conformation that the mature hemagglutinin takes, only the HA1 subunit becomes exposed where the most of its antigenic determinants are found (5). HA1 includes the site of binding to the target cell receptor (6).In the case of influenza viruses, there is experimental evidence to suggest that HA glycosylation might be important for proper folding and virus-host receptor recognition, but not for immunogenicity. Thus, if it is expressed in the prokaryotic system, it preserves the same antigenic properties. Besides this, expression of fragments of the HA protein in prokaryotic systems can potentially be the most efficacious strategy for the manufacture of large quantities of influenza vaccine in a short period of time (7). Bacillus subtilis, have been explored as a host for the expression of foreign proteins with pharmacological or immunological activities. In contrast to gram-negative E.coli, this bacterium contains no lipopolysaccharides (LPS) in the outer cell membrane. Additionally, it has a naturally high secretory capacity and exports proteins directly into the extracellular medium, which simplifies downstream purification and prevents the formation of inclusion bodies (8, 9).

Methods

Polymerase chain reaction (PCR) was carried out using Pfu polymerase (Fermentas, Lithuania). The forward primer with a BamHI cut site (5́GTTGGATCCGATCAGATTTGCATTGGT TAC3́) and reverse primer with a SmaI cut site, stop codon and a His-tag (5́TATCCCGGGTTAGTGATGGTGATGGTG ATGTCTCTTTTTTCTTCTGCTCTC3́), designed according to cloning strategy, were used to amplify of HA1 region from pFastBacIHNM1 (10).

PCR was done under the following temperature profile: an initial denaturation at 93°C for 3 minutes, 35 cycles were run with denaturation at 93°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds, followed by a final extension at 72°C for 10 minutes. Subsequently, the PCR product was analyzed by agarose gel electrophoresis.

Cloning and subcloning of the HA1 amplicon: The PCR product was first cloned in the pGEM® 5Zf(–)vector (Promega, USA) through EcoRV site. The clones then were subjected bidirectionally to automatic sequencing using M13 universal primers. (Macrogen, Korea) .The target gene, HA1, excised by BamHI-SmaI double digestion and inserted into the E.coli-B. subtilis shuttle vector pHT43 (MoBiTec, Germany) linearized with the same restriction enzymes. T4 DNA ligase (Fermentas, Lithuania) was used for both ligation reactions. The recombinant colonies were selected by Colony PCR. The fidelity of PHT43-HA1 construct was confirmed by restriction analysis. Extracted PHT43-HA1 plasmid from the recombinant E.coli was used for transformation of Bacillus subtilis (WB600) by electroporation (Bio-Rad, USA). To ensure the accuracy of transformation after electroporation, appeared B.subtilis colonies on LB agar medium containing 5 μg/ml of Chloramphenicol as selective marker were verified by Colony PCR.

Gene expression in Bacillus subtilis (WB600): A colony of Bacillus subtilis (WB600) containing PHT43-HA1 plasmid was incubated at 37°C overnight in 5 ml of LB broth medium containing 5μg/ml of Chloramphenicol. Then, 0.5 ml of it was transferred to 15 ml of a new medium. When the optical density of the culture medium at a wavelength of 600 nm reached about 0.7, 0.1 mM of IPTG was added to induce expression. Sampling of the medium was carried out by taking 1 ml before induction and 4 ml (1 ml per hour) after that. The cell pellet of samples were isolated from its supernatant liquid by centrifugation (at g × 10000 rpm for 1 min), and the protein preparation process was done separately for each section. The procedure of protein precipitation from the supernatant liquid was performed during its saturation with NaCl, followed by centrifugation (at g × 10000

rpm for 10 min). Subsequently, protein expression was studied by analysis of samples in SDS-PAGE and western blotting (WB).

Results

Agarose gel electrophoresis of the HA1 PCR product showed correct weight of 1kb band. Analysis of sequencing results confirmed the fidelity of HA1 sequence fused with His-tag
The accuracy of the recombinant vector was rechecked by doing colony PCR and enzymatic digestion analysis. (fig. 1).
The results of SDS-PAGE for the samples after induction (up to 4 hours) indicate a gradual increase in the intensity of the 40 kDa band corresponding to HA1 protein. The existence

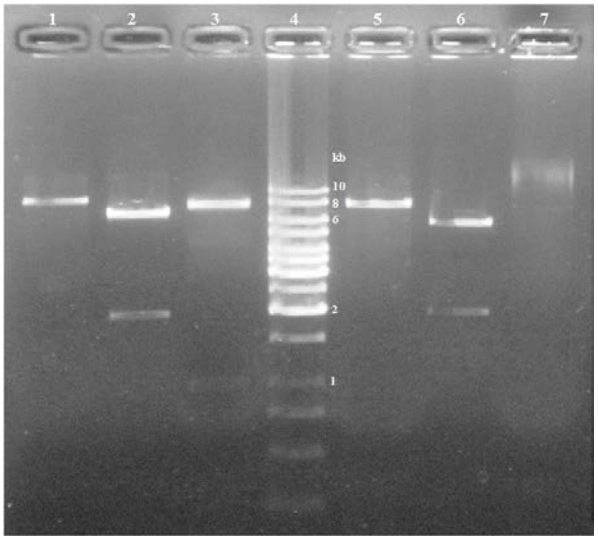

Fig. 1. Enzymatic digestion analysis of PHT43-HA1 construct (lanes 1 to 3) and PHT43 plasmid (lanes 5 and 6) in 1% agarose gel. Lane 1, digestion of recombinant vector (8 kb pHT43 vector +1 kb insert) by BamHI yielded fragment 9 kb in length; lane 2, digestion by HindIII (with two cut sites in PHT43 vector) yielded fragments 2 and 7 kb in length; lane 3, double digestion by BamHI and SmaI yielded fragments 1 and 8 kb in length; lane 4, GeneRuler™ 1 kb DNA ladder (Fermentas). Lane 5, double digestion of non-recombinant vector by BamHI and SmaI yielded fragment 8kb in length, Lane 6, digestion by HindIII yielded fragments 2 and 6kb in length; Lane 7, undigested PHT43 plasmid.

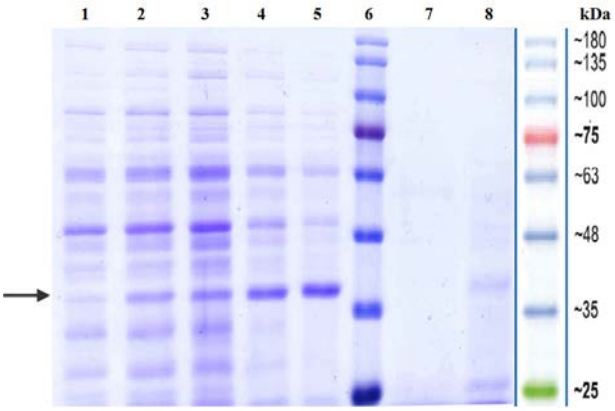

Fig. 2. Expression and secretion analysis of the recombinant HA1 protein (40 kDa) in cell pellet and the culture medium samples by 12% acrylamide gel. Lane 1, expression before induction; lanes 2 to 5, gradual increase of expression respectively from 1 to 4 hours after induction; lane 6, Prestained protein ladder (SinaClon); lane 7, lack of protein secretion in the medium before induction; lane 8, secretion of the recombinant protein in the medium 4 hours after induction.

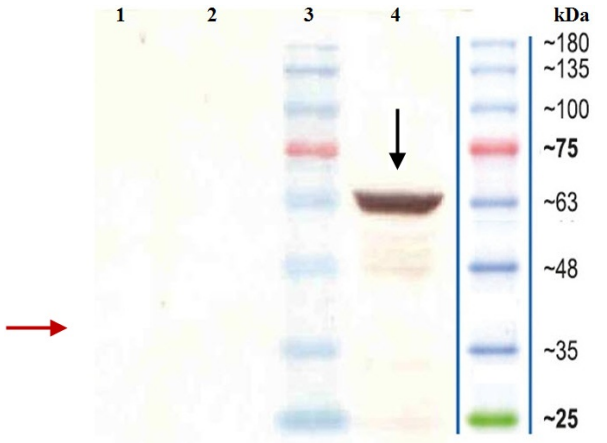

Fig. 3. The result of performing a modified Western blot to enhance sensitivity in the detection of membrane proteins. lane 1, lack of expression of HA1 protein before induction (red arrow); lane 2, lack of expression of HA1 protein 4 hours after induction; lane 3, Prestained protein ladder (SinaClon); lane 4, a positive result from the positive control protein containing His-tag weighing 57 KD (black arrow); lane without number (ladder), not including Western blot.

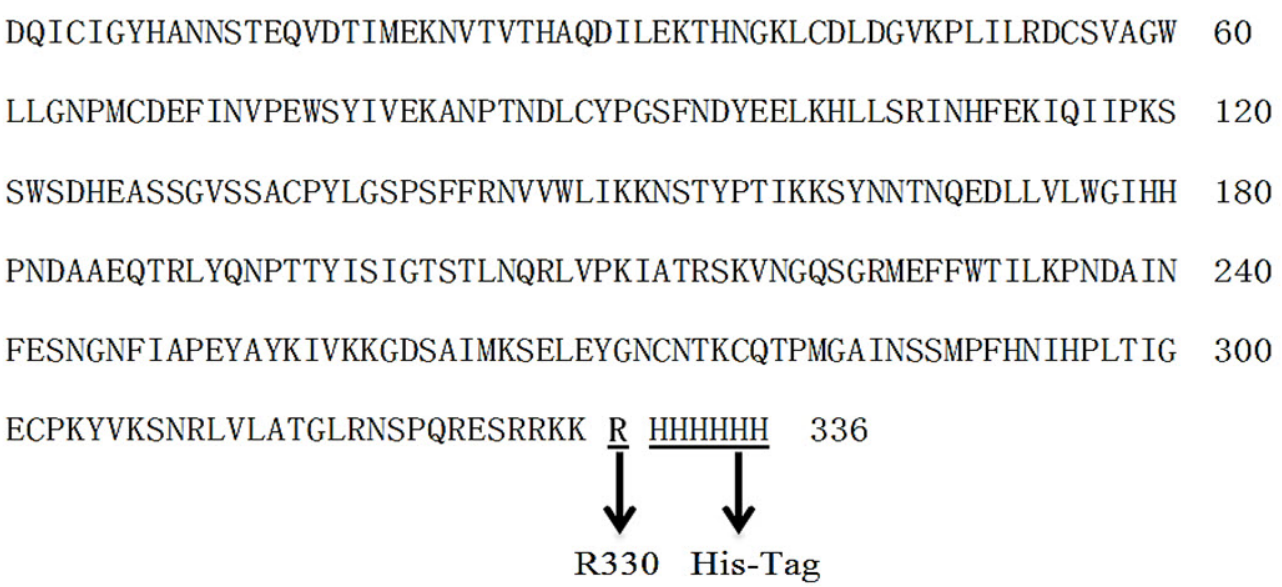

Fig. 4. HA1 protein sequence of type H5N1 influenza virus (A/Indonesia/5/2005) containing His-tag fusion. Position of cut site, arginine amino acid (R330) and His- tag that may has been removed by trypsin-like proteases of the host.

of the same band in a precipitated protein sample from one milliliter of the culture medium confirms the secretory expression of HA1 protein (fig. 2). Although SDS-PAGE analysis showed a typical result of new recombinant protein production increasing through the time, we could not verify it by WB using anti-His antibody. To check the materials and method used in WB test, the other new recombinant protein containing His-tag fusion applied as a positive control (fig.3). The first explanation was that HA1 is a hydrophobic and membrane protein (7) so the His-tag attached to the C-terminal of the HA1 protein may be hidden within its spatial structure and may be out of the reach of anti-histidine. However the result obtained from the proposed protocol of Kaur and Bachhawat, which is a modified WB protocol to enhance sensitivity in the detection of membrane proteins (11), did not confirm this supposition (fig. 3).

Discussion

A variety of subtypes of HA has been expressed in the prokaryotic expression system (12) and eukaryotic system including insect cells (13). HA gene has been used in the production of modern vaccines against influenza, such as subunit vaccines (14), viral vaccines (15), DNA-based vaccines (16), and virus-like particles (17).

By analyzing the results of Western blot in this study and investigations done on the HA of influenza virus, we can describe the cause of Western blot negative response to the expressed protein. After starting the activity of the trypsin-like protease, with the cutting site of R-X-R/K-R (X is not a basic amino acid), an arginine residue (R329) has been missing in where HA0 protein is cleaved into HA1 and HA2 subunits (18, 19). In addition, an important intracellular trypsin-like protease (protease II) has been recognized in Bacillus subtilis (20). As demonstrated in fig.4, in HA1 protein sequence of type H5N1 influenza virus (A/Indonesia/5/2005), the cutting site contains an arginine amino acid (R330). Considering that the designed reverse primer in this study adds His-Tag sequence to the HA1 protein exactly after this amino acid (R330), it seems that the His- tag attached to C-terminal of the protein has been removed by trypsin-like proteases of the host.

B. subtilis (WB600) is an attractive host for the production of heterologous secretory proteins for several reasons: absence of significant codon bias (21) deficiency of six extracellular

proteases (22) lack of endotoxin (LPS), and exist of high secretory capacity for exports proteins directly into the extracellular medium, (8, 23). Thus, the produced protein in this study could be considered as a candidate for easy and cost-effective production of influenza vaccine which its immunogenicity potential needs to be assessed in animal models along with proper control groups.

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