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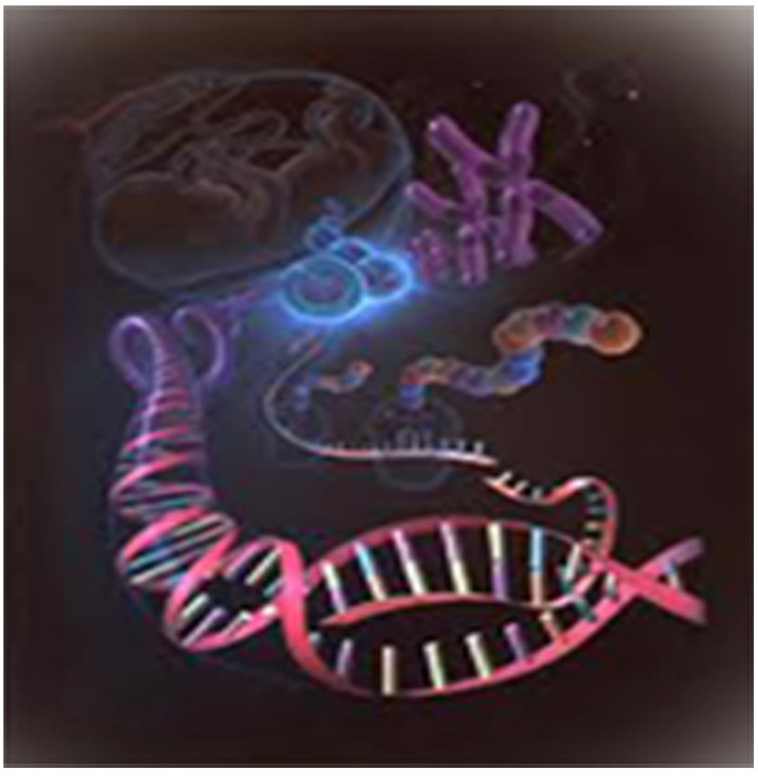

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Research Paper

HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS H5N1 NEURAMINIDASE EXPRESSED IN SF9 INSECT CELLS WITH A BACULOVIRUS FREE SYSTEM

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This article reports heterologous expression in Sf9 insect cells of N1 neuraminidase derived from avian influenza virus A/chicken/Iran/53-3/2008(H5N1). A gene encoding the neuraminidase N1 was fused directly in-frame with the adipokinetic hormone (AKH) secretion signal and 6xHis-Tag coding sequence upstream of the cloning site for expressing fusion recombinant N1 protein with N-terminal tags in pIEx-3 vector. Recombinant N1 neuraminidase was expressed in Sf9 insect cells as a 80 kDa secreted protein. This insect-based Baculovirus-Free heterologous expression system provided functionally recombinant N1 neuraminidase that should be useful in anti-influenza drug screening, detection and diagnostic tests and also as a potential protein-based vaccine.

Keywords: Highly Pathogenic Avian influenza, Neuraminidase (NA), H5N1, Heterologous expression, Sf9 insect cells, Baculovirus-Free

INTRODUCTION

Avian influenza (AI) is caused by type A strains of influenza virus, negative sense single strand RNA viruses, that belong to the genus influenza virus in the family Orthomyxoviridae. The type A viruses are classified into subtypes based on their surface glycoprotein antigens including haemagglutinin (HA) and neuraminidase (NA) (Swayne, 2000; Terajima Babon *et al.,* 2012).

The HA is categorized into sixteen subtypes while NA is categorized into nine subtypes. Various combinations have been detected in avian species (Webster and Hulse, 2004; Alexander, 2007). NA removes the terminal sialic acid

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residues from glycoconjugates, allowing release of viral progeny from infected cells and preventing viral aggregation. Inhibition of NA activity limits the spread of viral infection, thereby suppressing the onset of disease (Yin Liu *et al.,* 2013).

In biotechnology have made possible the production of recombinant proteins for human and animals (Young Britton *et al*., 2012). These biotechnology derived recombinant proteins form a new class of diagnostic assays and drugs for many diseases like genetic disorders, cancer, Influenza and AIDS (Zhao Bishop *et al.,* 2011). Use of DNA and proteins in diagnostics is increasing exponentially. The growing need of applications of recombinant proteins could be met by heterologous synthesis of them (Paakkonen and Tjaderhane, 2010).

Prokaryotic and eukaryotic systems are the two general categories of expression systems (Hunt, 2005). There is no universal expression system for heterologous proteins. All expression systems have some advantages and disadvantages (Baldi Hacker *et al*., 2007). Choosing the best system requires evaluating the options from yield to glycosylation, to suitable folding to economics of scale-up (Geisse Jordan *et al.,* 2005).

Use the insect cells have emerged as a popular system for over producing recombinant proteins in eukaryotic cells (Paakkonen and Tjaderhane, 2010). Several factors have contributed to their popularity. Being insect cells, they utilize many of the protein processing, modifications and transport systems present in higher eukaryotic cells (Young Britton *et al.,* 2012). Insect cells adapted for growth in suspension cultures, making it possible to obtain large amounts of recombinant proteins easily (Chambers Austen *et al*., 2004; Nwe He *et al.,* 2006).

The NA protein has been expressed in a number of expression systems. Expression of this glycoprotein in some expression systems such as prokaryotic system has different result in glycosylation from that in native virus (Lee Sung *et al.,* 2007; Sylte, Hubby *et al*., 2007; Yongkiettrakul, Boonyapakron *et al*., 2009; Guo Yao *et al.,* 2011; Wang, Qin *et al.,* 2011). Proper glycosylation is important for the processing, the functional integrity, and the antigenicity of Neuraminidase (Nwe He *et al*., 2006; Li, Zhang *et al.,* 2008; Hayashi Chaichoune *et al.,* 2011; Khurana Verma *et al*., 2011). We expressed the NA protein in an insect expression system based on the Sf9 cells and baculovirus free approach that is called the InsectDirect™ System (novagen) (Loomis Yaeger *et al.,* 2005).

MATERIALS AND METHODS

Expression of Recombinant N1

The NA gene of H5N1 viruse from clade 2.2, (A/chicken/Iran/53-3/2008(H5N1)), codon optimized for insect cells and synthesized by GenScript based on the sequences from the NCBI influenza database. By GenScript, the synthesized NA gene cloned into a vector belong to InsectDirect™ System (Novagen). The pIEx-3 vector encode a secretion signal peptide of adipokinetic hormone (AKH) and 6xHis-Tag coding sequence upstream of the cloning site for expressing fusion recombinant NA protein with N-terminal tags. For influenza NA expression, plasmid DNA containing NA used to transfect *Spodoptera frugiperda (Sf9)* insect cells in serum-free medium by using *Insect GeneJuice*® Transfection Reagent (Novagen) and incubated at 28°C, following the instructions of the manufacturer. At 72 h post transfection, culture

medium from the transfected cells was collected.

Purification of Recombinant N1

Several batches of H5-His protein were purified from the supernatant of Sf9-cell cultures using the Insect RoboPop™ Ni-NTA His Bind Purification Kit (Novagen), following the procedure provided by the manufacturer. Briefly, Insect PopCulture® Reagent and Benzonase® Nuclease were added directly to each culture to mediate cell lysis and viscosity reduction, respectively. Ni-NTA His-Bind resin was then added to the lysate to bind the fusion protein. The affinity resin was captured, washed, and eluted using a 96-well filter plate.

Analysis of Recombinant N1

Purified rNA was analyzed with a sodium dodecyl sulfate and 10% polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred onto nitrocellulose membranes (Sigma). Expression of recombinant H5-His was analyzed by western blot using a His-Tag AP Western Reagents kit (Novagen). Briefly, the membrane was blocked one hour in a Tris-Buffered Saline (TBS) containing 3% Bovine Serum Albumin (BSA) at room temperature. Subsequently, the membrane was incubated at RT for 1 h with mouse anti-6xHis monoclonal antibody (Novagen) at a 1:1000 dilution in a 3% BSA-TBS buffer. The membrane was washed tow times with TBS-0.05% Tween 20. After washes, the blot was incubated for 1 h with a 1:5000 dilution of Goat Anti-Mouse IgG AP Conjugate antibody. After washing, the membrane-bound antibodies were visualized by AP-Buffer containing BCIP and NBT, following the manufacturer's recommendations.

RESULTS AND DISCUSSION

Highly Pathogenic Avian Influenza (HPAI) virus of the H5N1 subtype, has spread through domestic poultry or wild birds in more than 60 countries. The epidemic of HPAI resulted in tremendous economic losses and affected human health (Webster Peiris *et al*., 2006; Yin, Liu *et al*., 2013). Since 2003, clade 2 viruses circulated in birds in China and Indonesia and spread westward during 2005-2006, to the Middle East, Europe, and Africa (Alexander, 2007). Clade 2 viruses have been the main cause of human infections since late 2005 (Alexander, 2007; Yee, Carpenter *et al.,* 2009; Ceyhan Yildirim *et al.,* 2010).

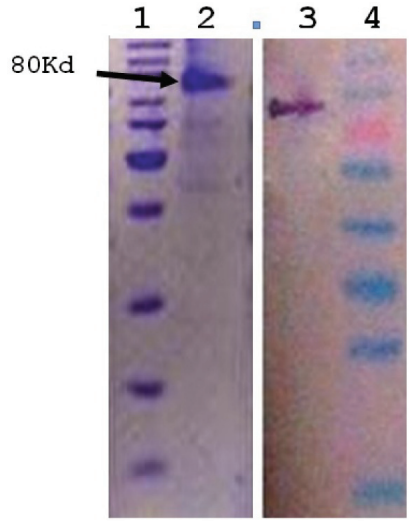
Control of H5N1 epidemics includes: effective vaccination, antiviral-drug treatment, using screening tests and also useful managements (Capua and Cattoli, 2013). It is also important to ensure that the vaccines currently in use are effective at protecting infected poultry from disease and decreasing viral load that is shed into the environment (Yee Carpenter *et al.,* 2009). In this way, Using recombinant proteins, has paved new ways for researchers (Kort Norton *et al.,* 2009).

In this study, The NA coding sequence gene of H5N1 viruse from clade 2.2, (*A/chicken/Iran/53-3/2008(H5N1)*), codon optimized for insect cells and synthesized by GenScript based on the sequences from the NCBI influenza database. By GenScript, the synthesized NA gene cloned into a vector belong to InsectDirect™ System (Novagen).

At 72 h post-transfection, the recombinant N1-His protein was secreted into the supernatant of Sf9 cultures and purified by the Insect RoboPop™ Ni-NTA His Bind Purification Kit (Novagen). The purified protein appeared as a single band with a relative molecular weight of approximately 80 kD, as estimated from migration in SDS-PAGE

relative to molecular weight standards (Figure 1, lane 1). Absence of other proteins besides purified N1-His in Coomassie-stained gels ensured proper purity of the preparation. In western blots, purified N1-His was detected by the anti-6xHis monoclonal antibady (Figure 1, lane 3).

Figure 1: Expression of N1 Gene (A/chicken/Iran/53-3/2008(H5N1)) in the Supernatant of SF9 Cells Transfected with N1-pIEx-3 vector by SDS-PAGE and Western Blot Analysis (Lanes 1-2) 10% SDS PAGE Gel. (Lane 1) Purified N1 Protein from Supernatant of SF9 cells Transfected with N1-pIEx-3 Vector. (Lane 2) Molecular Weight Marker. (Lanes 3-4) Western blot Analysis with (Lane 3) Purified N1 Protein Reacted with Monoclonal Antibody Specific to the 6-histidine Protein Tag, (Lanes 4) Molecular Weight Marker



The pIEx3™ vector and Insect GeneJuice Transfection Reagent facilitated rapid expression screening in insect cells because they enabled high efficiency transfection and high levels of target protein expression using a transient transfection protocol. Once a recombinant plasmid was isolated, expression results were obtained in 48-72 h. Furthermore, the yield of target protein was not negatively affected when the scale of the procedure was increased from 10 mL up to 0.1 or 1 L (Loomis Yaeger *et al*., 2005).

CONCLUSION

In conclusion, this study demonstrates the ability to perform rapid and small-scale expression of NA in Sf9 cells as a alternative to baculovirus-based production. The recombinant N1 neuraminidase, can be used in HPAIV anti-influenza drug screening, development of diagnostic tests and also as a potential protein-based vaccine.

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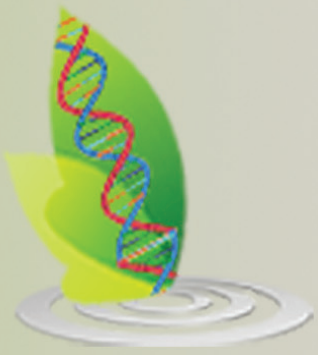
APPENDIX

Abbreviations

AI	Avian influenza
HPAIV	Highly Pathogenic Avian Influenza virus
NA	Neuraminidase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis



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