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Expression and purification of bioactive hemagglutinin protein of highly pathogenic avian influenza A (H5N1) in silkworm larvae

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A B S T R A C T

The hemagglutinin (HA) of avian influenza viruses plays a very important role in the infection of host cells. In this study, the HA gene of the highly pathogenic avian influenza H5N1 virus was cloned and expressed in silkworm larvae. The expressed recombinant HA (rHA) was purified using fetuin-agarose chromatography and Superdex 200 10/300 GL gel filtration chromatography, and the identity of purified rHA was confirmed by SDS-PAGE and Western blot. Approximately 500 μg of purified rHA was obtained from a total of 30 silkworm larvae, suggesting the high efficiency of the silkworm expression system. The purified rHA bound to a rabbit polyclonal antibody against influenza A virus H5N1 (avian flu) HA, suggesting its antigenicity and potential application in vaccine development. Gel filtration chromatography showed that purified HA was present in the void volume fractions, indicating that rHA may form an oligomer. The rHA bound to poly{Neu5Acα2,3LacNAcβ-O[$(CH_2)_5$NHCO]$_2$($CH_2)_5$NH-/γ-PGA}, which mimics an avian type receptor, but did not bind to γ-polyglutamic acid or human type receptor mimic, poly{Neu5Acα2,6LacNAcβ-O[$(CH_2)_5$NHCO]$_2$($CH_2)_5$NH-/γ-PGA}, suggesting that it could be utilized as a blocking agent against infection by highly pathogenic influenza viruses.

1. Introduction

Influenza is an infectious disease caused by influenza viruses, and may cause nausea and vomiting (Eccles, 2005). Currently, 3 types of influenza viruses (A, B, and C) have been identified, and the type A virus is the strain most likely to cause epidemics and pandemics, because it can undergo antigenic shift and present a new immune target to susceptible individuals. Influenza A viruses have been isolated from many species, including humans, pigs, horses, minks, felids, marine mammals, and a wide variety of domestic birds; however, wild fowl and shorebirds are thought to form the virus reservoir in nature (Olsen et al., 2006). Influenza viruses are classified on the basis of 2 proteins present on the surface of virus particles – the hemagglutinin (HA) and neuraminidase (NA) (Webster et al., 1992); and currently, influenza viruses representing 16 HA and 9 NA subtypes have been identified (Fouchier et al., 2005). HA is a glycoprotein responsible for binding to a cell's surface and mediating fusion of the viral and cellular membranes after endocytosis (Martin et al., 1998; Wiley and Skehel, 1987). HA is initially secreted as a precursor, which is called HA0, and displayed on the surface of viruses during virus assembly (Kido et al., 1993); it is then cleaved into HA1 and HA2 submits by furin-like protease. The cleavage site of HA and the proteases in the host determine the pathogenicity of the virus. The cleavage site of HA (RERRRKKRG) is conserved in H5N1 viruses, consists of polybasic amino acids, and has a positive charge. These characteristics make it easy to cleave and facilitates the binding of virus particles to host cells.

H5N1 influenza A viruses have spread to numerous countries in Asia, Europe, and Africa, where they not only infect large numbers of poultry, but also increasing numbers of humans, often with a lethal effect (Enserink, 2006; Webster et al., 2006). Generally, human and avian influenza A viruses differ regarding their recognition of host cell receptors: the former preferentially recognize receptors with saccharides terminating in α2,6-sialylgalactose (SAα2,6Gal), whereas the latter prefer receptors ending in α2,3-sialylgalactose (SAα2,3Gal) (Matrosovich et al., 2000; Rogers et al., 1983; Rogers and Paulson, 1983; Zambon, 2001). Although there is no evidence showing that viral mutations enabling H5N1 to infect human cells have occurred in nature, some cases of human

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Table 1
Primers used in this study.

Primer	Sequence (5′–3′)
Bx-HA Primer-frw	GAAGCGCGCGGAATTATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTC
Bx-HA Primer-rev	GTTTGCATGGTAACCAATGCAAATCTGATCTGTTGACACCCACATTACTGTTGAC
HAGS-frw	CCAAATATTGTCAATTTATTCTACAGTGGCGAGCTCCCTAGGTGGCGGTGGCTCT
GSlinker	GGTGGCGGTGGCTCTGGAGGCGGAGGCTCACATCATCACCATCACCACTAA
His-rev	TACCGCATGCCTCGATTAGTGGTGATGGTGATGATGTG
4120-frw	TCGAGGCATGCGGTACCAAGCTTGTCGAG
4058-rev	AATTCCGCGCGCTTCGGACCGGGATC
4001-frw	GGATTATTCATACCGTCCCACCATCG
4185-rev	CAAATGTGGTATGGCTGATTATGATCC
pUC/M13 frw	CCCAGTCACGACGTTGTAAAACG
pUC/M13 rev	AGCGGATAACAATTTCACACAGG

infection have been reported following close contact with the viruses (Yamada et al., 2006). Also, some experimental adaptation studies of the influenza H5 virus showed that H5 HA can convert to an HA that supports efficient viral transmission in mammals (Imai et al., 2012). Humans lack immunity to influenza viruses possessing an H5 HA, and emergence of a transmissible H5N1 virus would probably cause a pandemic.

Escherichia coli, mammalian cells, and animals have all been used to develop antibodies for detection or neutralization of influenza A virus HA protein. Although recombinant HA (rHA) has been purified and used for developing antibodies, the sugar binding capacities of most rHAs have not been investigated (Liu et al., 2011; Yousefi et al., 2012). In this study, the HA of virus strain H5N1 was expressed in silkworm larvae and then purified. The specificity for binding of rHA to receptors was also investigated.

2. Materials and methods

2.1. Materials

E. coli DH5α was purchased from Agilent Technologies (La Jolla, CA, USA) and used for gene cloning. BmDH10Bac *CP*$^{-}$*Chi*$^{-}$ (Park et al., 2008) that was cysteine protease-deleted BmDH10Bac bacmid (Motohashi et al., 2005), was used for preparing recombinant bacmid for expression in silkworm larvae. Plasmid pFastBact1 was obtained from Invitrogen (Carlsberg, CA, USA).

2.2. Cloning of HA gene into a pFastBac1 plasmid

Plasmid pBluescript II SK(+)-pHA(H5N1) containing the HA gene (accession number: AY651333) of the avian influenza A H5N1 virus (A/Vietnam/1194/2004) was synthesized by Operon (Tokyo, Japan) and transformed into *E. coli* DH5α. The pBluescript II SK(+)-pHA(H5N1) was extracted from *E. coli* and used as a template for amplification of the HA gene. To enable secretion of expressed proteins into the hemolymph of silkworm larvae, the native signal peptide sequence (amino acids 1–16) and transmembrane domain sequence (amino acids 544–568) were deleted from the HA gene, and the signal peptide sequence of bombyxin from *Bombyx mori* (bx signal) was added at its N-terminus domain sequence. The bx signal peptide allows expressed proteins to be efficiently secreted into the hemolymph of silkworm larvae (Park et al., 2007). For purification of rHA, a 6 × His tag was added to the carboxyl-terminus of rHA; also, a GS linker (GGGSGGGS) was designed between the HA region and the His tag region. The bx signal gene was linked by 2 oligonucleotides (Bx-HA Primer-frw and -rev) by a polymerase chain reaction (PCR). The PCR was performed as follows: 35 cycles at 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 2 min, after denaturation at 94 °C for 2 min in a 50 μL reaction mixture containing 15 pmol of Bx-HA Primer-frw and -rev (Table 1), 75 μmol of $MgSO_4$, 10 μmol of dNTPs, 1 unit of KOD-Plus-Neo (Toyobo, Osaka, Japan), and a 10% volume of a 10× reaction buffer. The PCR products were separated by agarose electrophoresis using a 3% agarose gel, and the target DNA fragments were excised and purified with an Illustra GFX PCR Gel Band Purification kit (GE Healthcare, Piscataway, NJ, USA).

A fragment containing the GS linker and a His tag gene was synthesized as follows: Three oligonucleotides, HA-GS-Frw, GSlinker, and His-rev, were added to 50 μL of a reaction mixture similar to that described above, and PCR was also performed using conditions similar to those previously described. To amplify the DNA fragment bx-HA-His, the bx signal fragment, GS-His DNA fragment, and pBluescript II SK(+)-pHA(H5N1) HA gene were added to a 50 μL reaction mixture containing 15 μmol of Bx-HA Primer-frw and His-rev, $MgSO_4$, dNTPs, and KOD-Plus-Neo. The reaction was performed using similar condition as above but annealing at 65 °C, and the amplified bx-HA-His fragment was separated by agarose electrophoresis with a 1% agarose gel, and purified using an Illustra GFX PCR Gel Band Purification kit. The pFastBac1 fragment was amplified with primer 4120-frw and 4058-rev by PCR using the protocol described above, but the extension time was 3 min and 30 s. The amplified DNA fragment was separated on a 0.5% agarose gel and purified. The amplified bx-HA-His fragment and pFastBac1 fragment were ligated in a reaction mixture containing 500 ng of bx-HA-His fragment, 500 ng of pFastBac1 fragment, 1 μL of 5 × In-Fusion HD Mix (Takara, Shiga, Japan), followed by incubation at 50 °C for 15 min. *E. coli* DH5α competent cells were transformed using 2.5 μL of the above reaction mixture, and a heat shock treatment at 42 °C for 45 s. The cells were then plated on a Luria Broth (LB) medium plate containing ampicillin (100 μg/mL). Following overnight cultivation at 37 °C, colonies grown on the plate were checked by PCR with primers 4001-frw and 4185-rev (Table 1), to confirm that they harbored the HA gene. The plasmid pFastBac1-bx-HA-His was extracted from recombinant *E. coli*.

2.3. Construction of recombinant BmNPV bacmid

The resulting recombinant plasmid pFastBac1-bx-HA-His was transformed into *E. coli* strain BmDH10Bac *CP*$^{-}$*Chi*$^{-}$ (Park et al., 2008) and cultivated for 36 h at 37 °C, after which, and a PCR with primers pUC/M13Frw and -Rev (Table 1) was performed for white colonies which were thought to harbor the HA gene. The recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid DNA was extracted from confirmed *E. coli* cells, and designated as rBmNPV-bx-HA-His.

2.4. Expression of HA in silkworm larvae

Fifth instar silkworm larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were injected with 50 μL of a mixture containing 10 μg of rBmNPV-bx-HA-His and a one-tenth volume of DMRIE-C reagent (Life Technologies, Tokyo, Japan). The silkworm larvae were reared on an artificial diet (NOSAN Co., Yokohama, Japan) in a chamber (MLR-351H, Sanyo, Tokyo, Japan) at 27 °C and 65% humidity for 6–7 days. Larval hemolymph was collected from