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ABSTRACT

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.

32 **Keywords:** Hemagglutinin, Influenza virus, *Bombyx mori* nucleopolyhedrovirus,

33 Bacmid, Silkworm

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35

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 et al., 2001). Although there is no evidence showing that viral mutations enabling H5N1 to infect human cells have occurred in nature, some cases of human infection have been reported following close contact with the viruses. 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) 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

89 hemolymph of silkworm larvae, the native signal peptide sequence (amino acids 1-16)
90 and transmembrane domain sequence (amino acids 544-568) were deleted from the HA
91 gene, and the signal peptide sequence of bombyxin from *Bombyx mori* (bx signal) was
92 added at its N-terminus domain sequence. The bx signal peptide allows expressed
93 proteins to be efficiently secreted into the hemolymph of silkworm larvae (Park et al.,
94 2007). For purification of rHA, a 6 × His tag was added to the carboxyl-terminus of
95 rHA; also, a GS linker (GGGSGGGS) was designed between the HA region and the His
96 tag region. The bx signal gene was linked by 2 oligonucleotides (Bx-HA Primer-frw and
97 -rev) by a polymerase chain reaction (PCR). The PCR was performed as follows: 35
98 cycles at 98°C for 10 sec, 55°C for 30 sec, and 68°C for 2 min, after denaturation at
99 94°C for 2 min in a 50 μL reaction mixture containing 15 pmol of Bx-HA Primer-frw
100 and -rev (Table 1), 75 μmol of $MgSO_4$, 10 μmol of dNTPs, 1 unit of KOD-Plus-Neo
101 (Toyobo, Osaka, Japan), and a 10% volume of a 10 × reaction buffer. The PCR products
102 were separated by agarose electrophoresis using a 3% agarose gel, and the target DNA
103 fragments were excised and purified with an Illustra GFX PCR Gel Band Purification
104 kit (GE Healthcare, Piscataway, NJ, USA).
105 A fragment containing the GS linker and a His tag gene was synthesized as follows:
106 Three oligonucleotides, HA-GS-Frw, GSlinker, and His-rev, were added to 50 μL of a

107 reaction mixture similar to that described above, and PCR was also performed using
108 conditions similar to those previously described. To amplify the DNA fragment
109 bx-HA-His, the bx signal fragment, GS-His DNA fragment, and pBluescript II
110 SK(+)-pHA(H5N1) HA gene were added to a 50 μL reaction mixture containing 15
111 μmol of Bx-HA Primer-frw and His-rev, $MgSO_4$, dNTPs, and KOD-Plus-Neo. The
112 reaction was performed using similar condition as above but annealing at 65°C, and the
113 amplified bx-HA-His fragment was separated by agarose electrophoresis with a 1%
114 agarose gel, and purified using an Illustra GFX PCR Gel Band Purification kit. The
115 pFastBac1 fragment was amplified with primer 4120-frw and 4058-rev by PCR using
116 the protocol described above, but the extension time was 3 min and 30 sec. The
117 amplified DNA fragment was separated on a 0.5% agarose gel and purified. The
118 amplified bx-HA-His fragment and pFastBac1 fragment were ligated in a reaction
119 mixture containing 500 ng of bx-HA-His fragment, 500 ng of pFastBac1 fragment, 1 μL
120 of 5 × In-Fusion HD Mix (Takara, Shiga, Japan), followed by incubation at 50°C for 15
121 min. *E. coli* DH5α competent cells were transformed using 2.5 μL of the above reaction
122 mixture, and a heat shock treatment at 42°C for 45 sec. The cells were then plated on a
123 Luria Broth (LB) medium plate containing ampicillin (100 μg/mL). Following
124 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 silkworm larvae by cutting prolegs, and 1-phenyl-2-thiourea was added into hemolymph at 0.1 mM to prevent melanization of samples. Hemocytes and debris

were removed from hemolymph by centrifugation at 10000 × g for 15 min, and the supernatants were stored at -80°C for purification.

2.5. Purification of recombinant HA

The rHA in hemolymph was purified using fetuin-agarose chromatography followed by gel filtration chromatography, which was carried out as follows. A 20 mL volume of larval hemolymph was dialyzed overnight at 4°C with 50 mM sodium phosphate (pH 7.4) containing 150 mM NaCl (Buffer 1). The dialyzed hemolymph was then applied to a 2 mL fetuin-agarose (Sigma Aldrich Japan, Tokyo, Japan) column equilibrated with Buffer 1, and the column was washed with 20 mL of Buffer 1. The rHA was eluted with 100 mM boric acid (pH 10.0) and the pH values of elution fractions were adjusted to neutral. Then, a 10 mL of each elution fraction was concentrated to a volume of ~2.0 mL. The concentrated elution fractions were applied to a Superdex 200 10/300 GL column (1.0 × 24 cm, GE Healthcare) equilibrated with Buffer 1, and 0.5 mL volumes of column eluent were collected at a flow rate of 0.5 mL/min. Fractions containing the target protein were analyzed with SDS-PAGE and Western blot. Protein concentrations were determined using a BCA protein assay kit (Sigma, St. Louis, MO, USA).

2.6. SDS-PAGE and Western blot

159 The purified protein fractions were analyzed by SDS-PAGE as described by Laemmli
160 (Laemmli, 1970), and Western blot. Protein samples (10 μL) were separated by
161 SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad
162 Laboratories, Hercules, CA, USA). After blocking the PVDF membrane with PBS
163 (KH_2PO_4, 1.47 mM; Na_2HPO_4, 8.10 mM; NaCl, 136.89 mM; KCl, 2.68 mM) containing
164 2% skimmed milk (MPBS) at room temperature for 2 h, a 1 μg/mL concentration of
165 mouse anti-His antibody (GE healthcare) or rabbit polyclonal antibody to influenza A
166 virus H5N1 (avian flu) HA (Sino Biological Inc., Beijing, China) was added to the
167 membrane in an appropriate volume. After incubation for 1 h, the membrane was
168 washed 3 times with PBST (PBS containing 0.1% Tween 20) and anti-mouse IgG HRP
169 conjugate (Promega, Madison, WI, USA), or in other samples, goat anti-Rabbit
170 IgG-HRP (GE healthcare) was added prior to incubation for 1 h. After washing 3 times
171 with PBST, the bands were developed with ECL Plus reagents (GE Healthcare) and
172 detected using a VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA,
173 USA).

174 *2.7. Hemagglutination assay*

175 Samples of blood obtained from rabbits, sheep, and chickens were purchased from

176 Japan SLC Inc. (Hamamatsu, Japan). The blood was diluted with PBS (pH 7.4) and
177 centrifuged. The supernatant was then removed and erythrocytes were suspended with
178 PBS. This step was performed 4–6 times. Pelleted erythrocytes were suspended with
179 PBS to a final concentration of 4%. Two-fold dilutions of purified HA were prepared in
180 wells of a 96-well plate; then, 25 μL of the 4% erythrocyte suspension was added to
181 each well and incubated for 2 h at room temperature, followed by observation of
182 erythrocyte aggregation.

183 *2.8. Assay for direct binding of rHA to its receptor*

184 γ-Polyglutamic acid (γ-PGA; MW 990,000) from *Bacillus subtilis* was a kind gift from
185 Meiji Food Materia Co. Ltd. (Tokyo, Japan).
186 Poly{Neu5Acα2,3LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA} and
187 poly{Neu5Acα2,6LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA} were synthesized as
188 described previously described method (Ogata et al., 2009). The binding capacity of
189 purified rHA for these poly-sugar chains was tested using a previously described direct
190 binding assay (Ogata et al., 2009). γ-PGA and glycopolymers (0.5 nM, 50 μL/well) in
191 50 mM sodium acetate buffer (pH 4.0) were briefly immobilized on polystyrene
192 Universal-Bind microplates (Corning Incorporated, New York, NY, USA) using an

ultraviolet irradiation method.

Plates were blocked with 2% skim milk (Nakalai Tesque, Kyoto, Japan) in PBS (MPBS) at 25°C for 2 h, washed 3 times with PBST (0.2% Tween 20 in PBS), and then incubated with MPBS (50 μL/well) containing 0.2 mg/ml of purified proteins at 25°C for 1 h. The plates were then washed 3 times with PBST and incubated with 50 μL/well of 1000-fold diluted rabbit polyclonal antibody to influenza A virus H5N1 (avian flu) HA in MPBS at 25°C for 1 h. The wells were washed again and incubated with 50 μL/well of 5000-fold diluted goat anti-Rabbit IgG-HRP (GE healthcare) in MPBS. The plates were then washed 3 times with PBST and developed with TMBZ solution [100 μg/mL 3,3′,5,5′-tetramethylbenzidine (Sigma) and 0.04 μL/mL H_2O_2, in 100 mM NaOAc, pH 6.0; 50 μL/well]. After incubation for 5–30 min, the reaction was stopped by addition of 10% sulfuric acid (50 μL/well), and absorbance was read using a Model 680 microplate reader (Bio-Rad, Tokyo, Japan) at 450 nm, with 655 nm used as a control.

3. **Results**

3.1. Construction of recombinant Bacmid, BmNPV-bx-HA-His

The bx signal peptide gene was amplified by PCR with 2 oligonucleotides,

210 Bx-HAPrimer-frw and -rev. A DNA fragment with a size of 102 bp was confirmed by
211 agarose electrophoresis, and another DNA fragment with a size of 106 bp and
212 containing the GS linker and 6 × His gene was also successfully synthesized. An
213 overlap PCR was performed by using a mixture containing the bx signal peptide gene,
214 the GS-His tag gene, a plasmid containing the HA gene as a template, and primers
215 Bx-HA Primer-frw and His-rev (Table 1). This reaction produced a DNA fragment with
216 a size of 1.7 kbp, which was amplified and purified, and confirmed to code for the gene
217 bx-HA-His. The pFastBac1 fragment was amplified with 4120-frw and 4058-rev. A
218 DNA fragment with size of ~ 4.7 kbp was observed following agarose electrophoresis
219 and considered to be the pFastBac1 fragment. The pFastBac1 fragment and bx-HA-His
220 were linked by an In-Fusion reaction to form pFastBac1-bx-HA-His. The presence of
221 the HA gene in the resulting plasmid was checked with a colony PCR, and a band of
222 ~1.7 kbp was confirmed in the PCR product, suggesting that the plasmid had been
223 successfully constructed. The pFastBac1-bx-HA-His plasmid was transformed in the *E.*
224 *coli* BmDH10Bac $CP^{-}Chi^{-}$ strain, which harbored the helper plasmid pMON7124 and
225 BmNPV bacmid DNA. In the *E. coli* BmDH10Bac strain, the bx-HA-His gene was
226 transposed into the BmNPV bacmid DNA by using the transposase derived from
227 pMON7124. After overnight culture on an LB Plate containing antibiotics, 5 colonies

228 were checked by PCR with the primers pUC/M13Frw and –Rev, and some clones were
229 confirmed to contain insert DNA of the right size. The inserts in these clones were
230 analyzed on a CEQ 8000 sequencer (Beckman Coulter Inc.) and confirmed to be the HA
231 gene, suggesting that the recombinant BmNPV bacmid, rBmNPV-bx-HA-His, was
232 successfully prepared.

233 *3.2. Expression of recombinant HA in silkworm larvae*

234 BmNPV-bx-HA-His was injected into silkworm larvae and these larvae were reared for
235 5–7 days, followed by collection of hemolymph. The presence of recombinant HA was
236 confirmed with Western blot (Figure 1). The presence of the split site (RRRKKRR), in
237 rHA (also rHA0) made it very easy to digest rHA into fragments of rHA1 and rHA2.
238 Based on the DNA construction used in this study, it was predicted that rHA0 would be
239 split at amino acid 366. The theoretical sizes of rHA0, rHA1, and rHA2 were calculated
240 to be 61.6 kDa, 37.5 kDa, and 24 kDa, respectively. As shown in Figure 1, when an
241 anti-His_6 polyclonal antibody was used to detect expressed protein, only 2 bands at 65
242 kDa, and 25 kDa were detected, because rHA1 does not have a His-tag sequence. rHA
243 was observed in hemolymph at both 6 and 7 days after injection of BmNPV bacmid
244 DNA, indicating rHA was preferentially secreted, rather than being accumulated in a fat

245 body. The detected band for rHA0 showed a molecular weight of ~ 65 kDa, which was
246 slightly larger than the theoretical molecular weight; this may be due to glycosylation of
247 the protein. These results suggest that recombinant HA was successfully expressed in
248 the silkworm larval hemolymph.

249 *3.3. Purification of recombinant HA*

250 Fetuin-agarose and gel filtration chromatography steps were used for purification of
251 rHA. In mammals, fetuin is an N-glycosylated protein and has both α2,3- and
252 α2,6-linked sialoside in its N-glycan residues (Baenziger et al., 1979). When functional
253 rHA is expressed, it can be purified using fetuin-agarose chromatography.
254 Fetuin-agarose from Sigma Aldrich has fetuin from fetal bovine serum. Hemolymph
255 was dialyzed with 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl
256 to remove low molecular weight contaminants, which can inhibit rHA binding to
257 fetuin-agarose. The dialyzed hemolymph was then subjected to fetuin-agarose
258 chromatography. Figure 2 shows SDS-PAGE and Western blot results produced by
259 analyzing various elution fractions obtained from fetuin-agarose chromatography. The
260 results showed that expressed rHA bound to fetuin from fetal bovine serum and that
261 functional rHA was purified using fetuin-agarose chromatography. Also, rHA0, rHA1,

and rHA2 were observed in all elution fractions. To confirm which form of rHA could bind to sialic acid, purified total rHA was subjected to size exclusion chromatography to separate bioactive form of rHA (Figure 3). This analysis produced 2 peaks, and all 3 types of rHA (rHA0, rHA1, and rHA2) were detected in the large first peak (Peak 1), observed in the void volume fractions, but no HA band was detected in the second small peak (Peak2) judging by SDS-PAGE analysis. However, by employing Western blot, all three rHA was detected in Peak 2, suggesting small amount of rHA exist in this peak as a monomer rather than oligomer. HA from influenza A virus normally forms trimers (~200 kDa); however, Figure 3 shows that the molecular weight of the first peak where all rHA bands were detected was > 669 kDa. This result suggests that rHAs expressed in silkworm larvae form oligomers, even if rHA0, rHA1, and rHA2 are present simultaneously. The proteins in each step were measured by BCA. Collected hymolymph of 30 silkworm larvae contained about 650 mg protein. After Fetuin-agarose chromatograph, 3.05 mg of proteins was purified partly. Finally, about 500 μg of oligomeric rHA with bioactivity was obtained in gel filtration chromatography.

3.4. Binding assay of rHA to its receptor

279 A hemagglutination assay using erythrocytes obtained from sheep, rabbits and chickens,
280 was performed for functional analysis of purified HA. Erythrocytes from sheep and
281 chickens have numerous types of α2,3-sialylated glycans, but some α2,3-sialylated
282 glycans are not found on erythrocytes from rabbits (Takemae et al., 2010). Erythrocytes
283 from sheep and chickens were aggregated by purified HA, but erythrocytes from rabbits
284 were not aggregated (Figure 4A). These results indicated that purified rHA could bind to
285 α2,3-sialylated glycans, but not to α2,6-sialylated glycans.

286 The binding capacity of rHA in hemolymph and the binding of purified rHA to
287 poly-γ-glutamic acid (γPGA),
288 poly{Neu5Acα2,3LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA}, and
289 poly{Neu5Acα2,6LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA} were tested using
290 ELISA. γPGA bears no sugar chains, however,
291 poly{Neu5Acα2,3LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA} with an α2,3 linked
292 Neu5Ac mimics the avian type receptor of influenza virus, and
293 Neu5Acα2,6LN-LNnT/γPGA with a α2,6 linked Neu5Ac mimics the human type
294 receptor of influenza virus (Figure 4B). As show in Figure 4B, prior to purification,
295 hemolymph only slightly bound to α2,3-sialoglycopolypeptide, and purified rHA
296 showed significant binding to α2,3-sialoglycopolypeptide. However hemolymph and

297 purified rHA were not able to bind α2,6-sialoglycopolypeptide. This result corresponds
298 with the substrate specificity of HA obtained when using avian influenza A virus, where
299 binds to α 2,3-sialylated glycans, but not to α2,6-sialylated glycans. A hemolymph
300 sample from silkworm transfected with bacmid without HA gene was also tested, but it
301 did not bind to any of the above sugar chains (data not shown).

302 4. **Discussion**

303 HA is a very important protein in the study of influenza viruses, and exhibits very high
304 antigenicity. Therefore, when a host becomes infected with influenza virus, antibodies
305 will be produced in the body to neutralize viruses and enhance resistance of the host.
306 HA protein is a candidate for use in the production of vaccines, and has been expressed
307 in numerous hosts, including *E. coli*, mammalian cells, and insect cells. In this study,
308 HA from influenza A H5N1 virus (A/Vietnam/1194/2004), which is devoid of its
309 transmembrane domain, was expressed with a 6 × His tag at its C-terminus in silkworm
310 larvae, and we investigated its antigenicity and receptor binding capacity. During the
311 purification process, the binding of fractions from each purification step to a rabbit
312 polyclonal antibody to influenza A virus H5N1 (avian flu) HA was confirmed.
313 Therefore, rHA expressed in silkworm larvae retained a high degree of antigenicity,

314 similar to that of the native protein. This result indicates that rHA could be used in
315 vaccines to protect animals from infection with influenza viruses.
316 Western blot analyses of expressed rHA under reducing conditions showed a band at
317 ~ 65 kDa, which was ~ 4 kDa higher than the expected molecular weight. These results
318 are in agreement with a previous report showing that hemagglutinin was expressed at ca.
319 65 kDa in *Trichoplusia ni* larvae when using a baculovirus system (Gomez-Casado et al.,
320 2011), and also indicate that rHA expressed in silkworm larvae is modified with glycans
321 in a manner similar to modification of HA proteins produced in *Trichoplusia ni*.
322 The total rHA from influenza A H5N1 virus (A/Vietnam/1194/2004) was expressed
323 in silkworm larvae as 3 different forms: rHA0, rHA1, and rHA2. These forms were
324 found in purified fractions even after fetuin-agarose chromatography. In addition, these
325 HA proteins were observed in only the first peak (> 669 kDa) produced in size
326 exclusion chromatography. These results indicate that rHAs form oligomers with a
327 molecular weight bigger than 669 kDa. In a previous report (Santiago et al., 2012), HA
328 from influenza virus A/Brisbane/59/70 expressed in Sf-9 cells mainly formed high
329 molecular weight oligomers, and was suggested to be composed of at least 3 HA trimers.
330 Results of size exclusion chromatography in this study corresponded to results in a
331 previous report. However, while HA from influenza virus A/Brisbane/59/70 was mainly

expressed as HA0, in our study, HA from influenza A/Vietnam/1194/2004 was expressed and purified as rHA0, rHA1, and rHA2. This may be due to the fact that HA from influenza A/Vietnam/1194/2004 has a polybasic region between HA1 and HA2, but HA from influenza virus A/Brisbane/59/70 lacks this region. This polybasic region can be easily cleaved by a furin-like protease. Regarding the rHA in this study, some HAs were purified as cleaved forms (rHA1 and rHA2), while others remained as rHA0. This finding suggests that cleaved rHA (rHA1 and rHA2) and HA0 combined with each other to form oligomers. Also, in the case of an inactivated influenza H5N1 vaccine, most HA was processed to produce HA1 and HA2, which then combined to form oligomers (Santiago et al., 2012). In addition, inactivated influenza H5N1 vaccine was able to bind fetuin, and HA oligomers in our study bound to fetuin and α2,3-sialoglycopolypeptide, not α2,6-sialoglycopolypeptide. Purified HA also bound to erythrocytes from sheep and chickens, which have α2,3-sialylated glycans, but not to erythrocytes from rabbits, which have few α2,3-sialylated glycans (Takemae et al., 2010). These results indicate that functional HA oligomers can be expressed in silkworm larvae and be purified to near homogeneity using 2 chromatography steps. This HA could be used to develop product which may protect animals from influenza viruses by blocking the receptor with recombinant HA.

350 The silkworm is an excellent living biofactory that has attributes of high
351 productivity, scalability, and cost efficiency. The silkworm can be used to produce
352 recombinant proteins of seasonal or pandemic influenza for use in vaccines and the
353 development of diagnostic methods or proteins for neutralization purposes.

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443 **Table 1. Primers used in this study**

Primer	Sequence (5'-3')
Bx-HA	GAAGCGCGCGGAATTATGAAGATACTCCTTGCTATTGCATTA
Primer-frw	ATGTTGTCAACAGTAATGTGGGTGTC
Bx-HA	GTTTGCATGGTAACCAATGCAAATCTGATCTGTTGACACCCA
Primer-rev	CATTACTGTTGAC
HAGS-frw	CCAAATATTGTCAATTTATTCTACAGTGGCGAGCTCCCTAGGT GGCGGTGGCTCT
GSlinker	GGTGGCGGTGGCTCTGGAGGCGGAGGCTCACATCATCACCA TCACCACTAA
His-rev	TACCGCATGCCTCGATTAGTGGTGATGGTGATGATGTG
4120-frw	TCGAGGCATGCGGTACCAAGCTTGTCGAG
4058-rev	AATTCCGCGCGCTTCGGACCGGGATC
4001-frw	GGATTATTCATACCGTCCCACCATCG
4185-rev	CAAATGTGGTATGGCTGATTATGATCC
pUC/M13 frw	CCCAGTCACGACGTTGTAAAACG
pUC/M13 rev	AGCGGATAACAATTTCACACAGG

444

445 **Figure legends**

446 **Figure 1.** Expression of rHA in silkworm larvae. Recombinant BmNPV bacmid DNA
447 mixture was injected into silkworm larvae and reared for 7 days. At 5, 6, and 7 days
448 after injection, silkworm larvae were removed and their hemolymph and fat bodies were
449 collected. Expressed rHA in each sample was detected by Western blot using mouse
450 anti-His tag antibody as a primary antibody. M: MagicMark XP Western Protein
451 Standard.

452 **Figure 2. SDS-PAGE (A) and Western blot analyses (B) of fractions from**
453 **fetuin-agarose chromatography.** Lanes M1, M2, H, FT, and W denote the Precision
454 Plus Dual Standard, MagicMark XP Western Protein Standard, hemolymph sample,
455 flow though fraction, and wash fraction, respectively. Lanes 1- 6, 7-8, and 9-10 denote
456 elution fractions at 150 mM, 500 mM, and 1 M NaCl, respectively. Rabbit polyclonal
457 antibody to influenza A virus H5N1 (avian flu) HA was used as the primary antibody,
458 and a goat anti-Rabbit IgG-HRP was used as the secondary antibody in Western blot
459 analysis.

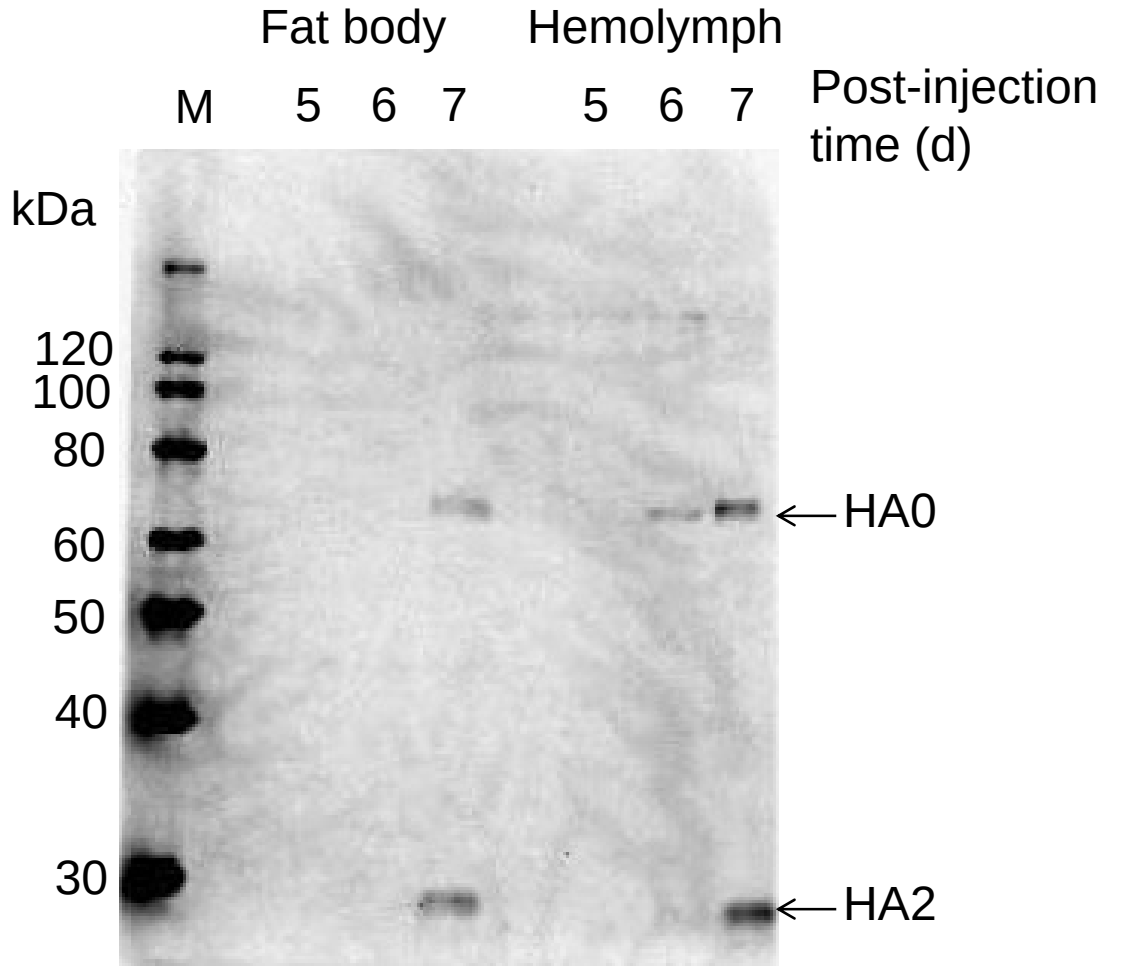
460 **Figure 3. Purification of recombinant hemagglutinin by gel filtration**
461 **chromatography.** (**A**) Purification profile of gel filtration chromatograph for pooled

462 elution fractions in affinity chromatography. Molecular weights of 669 kDa and 75 kDa
463 were indicated by arrows, respectively. P1 and P2 denote 2 large peaks. (**B**) SDS-PAGE
464 analysis of Peak 1 obtained by gel filtration chromatography. Lane M1 denotes
465 Precision Plus Dual Standard. Lane 1-7, collected fractions. (C) Western blot analysis of
466 peak 1. A rabbit polyclonal antibody to influenza A virus H5N1 (avian flu) HA was used
467 as the primary antibody, and a goat anti-Rabbit IgG-HRP was used as the secondary
468 antibody. Lane M2: MagicMark XP Western Protein Standard; Lane 1-7: collected
469 fractions.

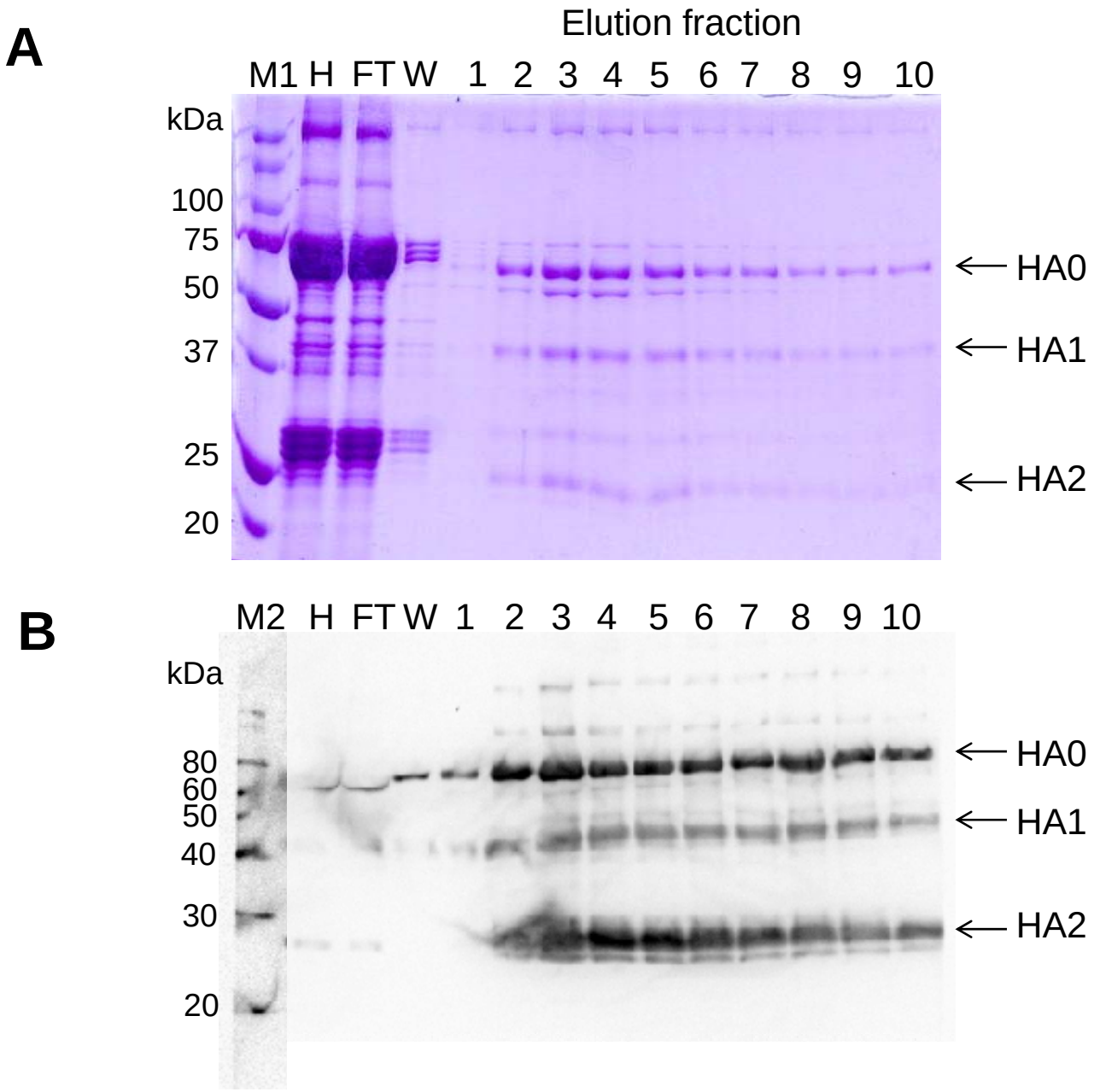
470 **Figure 4. Hemagglutination assay of rHA (A) and direct binding assay of rHA to its**
471 **receptor (B).** (A) Erythrocytes from sheep, rabbits and chickens were prepared and the
472 hemagglutination assay using rHA was performed according to the protocol described in
473 Materials and Methods. (B) γ-Polyglutamic acid (γPGA),
474 poly{Neu5Acα2,3LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA}(α2,3), and
475 poly{Neu5Acα2,6LacNAcβ-O[$(CH_2)_5$NHCO]$_2$$(CH_2)_5$NH-/γ-PGA}(α2,3) were
476 immobilized on microplates, and the protein solution containing hemolymph and
477 fractions obtained by affinity and gel filtration chromatography were added to wells of a
478 microplate. After washing, rabbit polyclonal antibody to influenza A virus H5N1 (avian
479 flu) HA was added as a primary antibody, and a goat anti-Rabbit IgG-HRP was added as

480 a secondary antibody. The signal was detected by addition of TMBZ substrate. The error

481 bars indicate the standard deviation of absorbance values (n = 3) and (*) $p < 0.01$.



Dong et al., Fig. 1.



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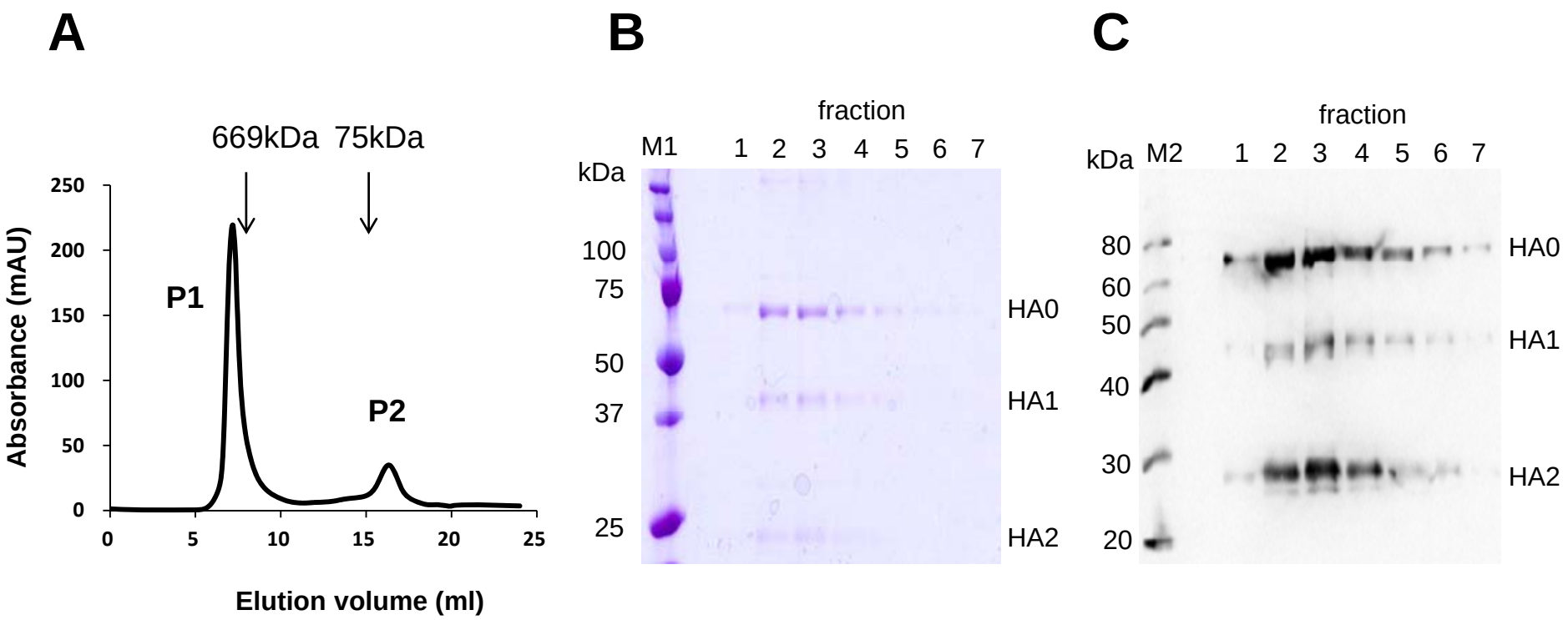
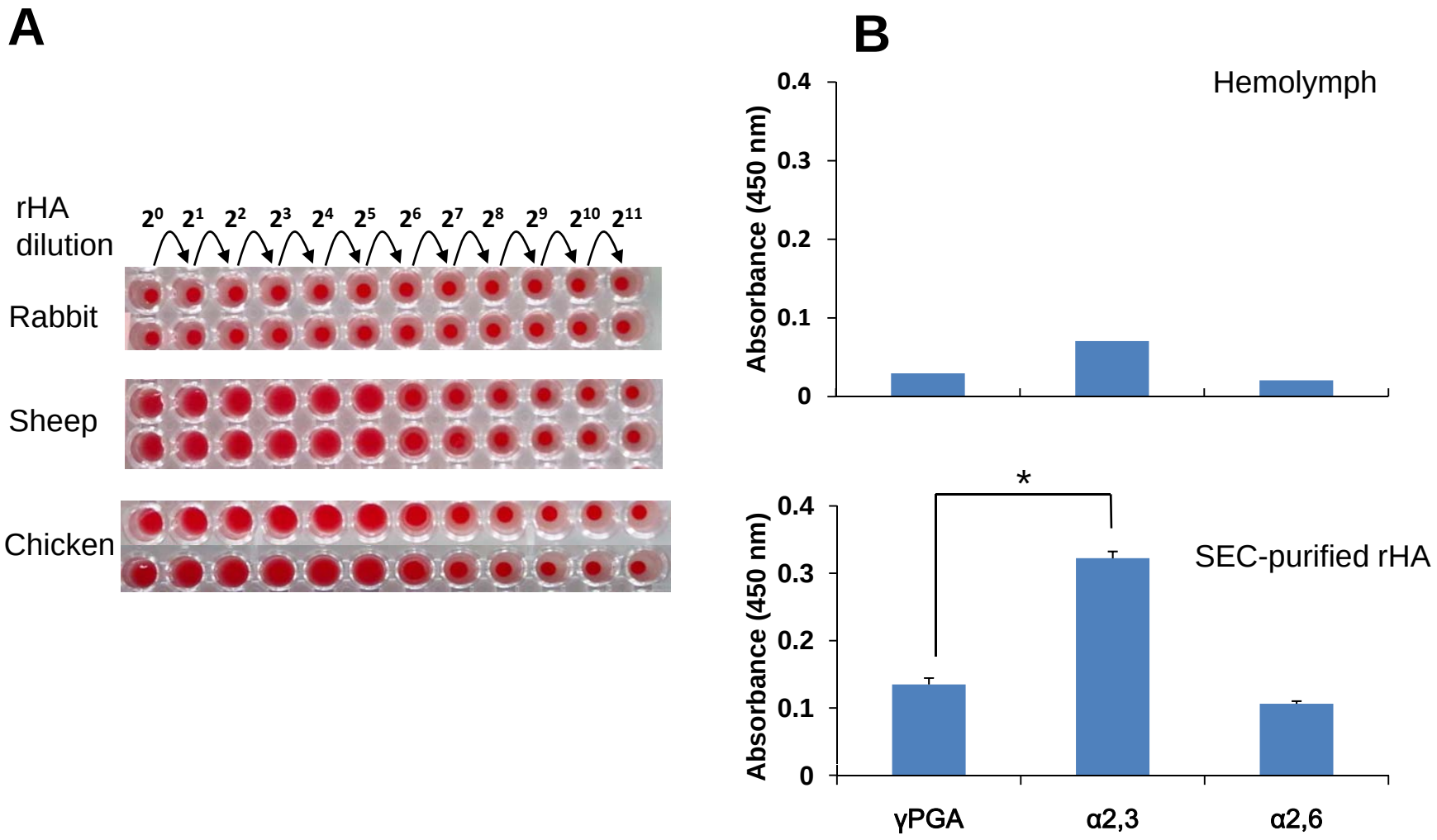


Dong et al., Fig. 3.



Dong et al., Fig. 4.