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Quantitative analysis of the yield of avian H7 influenza virus haemagglutinin protein produced in silkworm pupae with the use of the codon-optimized DNA: A possible oral vaccine

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ABSTRACT

In this study, we aimed to quantitatively compare the increased production of three H7 influenza virus-like particle (VLP) haemagglutinin (HA) with the use of a codon-optimized single HA gene in silkworm pupae. Recombinant baculovirus (Korea H7-BmNPV) could produce 0.40 million HA units per pupa, corresponding to 1832 μg protein. The yield of the HA produced in larva was estimated to be approximately 0.31 million HA units per larva, and there were no significant differences between the three HA proteins. We could establish efficient recovery system of HA production in larvae and pupae with the use of three cycles sonication methods. Next, we compared yields of HA proteins from three different H7 and two H5 recombinant baculoviruses based on the amount of mRNA synthesized in BmN cells, suggesting that mRNA synthesis may be also a useful indicator for the production of HA. Based on HA titres from four recombinants, the yield of HA had a great influence on the codon-optimized effect and the characteristics of the viral *HA* gene. The recombinant containing codon optimized HA DNA of A/tufted duck/Fukushima/16/2011 (H5N1) did produce more than one million HA units, although another recombinant including of the wild H5N1 strain failed to show HA activity. Electron microscopy revealed the presence of large VLP and small HA particle in the heavy and light fractions. The purified VLPs reacted with the authentic anti-H7 antibodies and the antibodies prepared after immunization with the VLP H7 antigen. Also H5 and H7 VLPs could produce HI antibody in chickens and mice with oral immunization. The antibodies elicited with oral immunization were confirmed in fluorescent antibody analysis and western blotting in Korea H5-BmNPV and H7 HA-BmNPV recombinant infected BmN cells. Taken together, these findings provided important insights into future oral vaccine development.

1. Introduction

Since the first isolation of avian H5N1 influenza virus from a 5-year-old boy in Hong Kong in 1997 [1–3], subsequent circulation of the causative highly pathogenic H5 avian influenza (HPAI) virus in humans has posed a serious threat to public health [4]. Accordingly, scientists have recently developed an H5 vaccine [5,6], and many important advancements are being made in the development of vaccines targeting H5N1 virus. For example, in our laboratory, we successfully established large-scale production of the H5 VLP haemagglutinin vaccine in the silkworm using optimized synthetic chimeric HA DNA [7].

The H7N9 virus recently appeared in March 2013 in southern China as a second possible pandemic strain in humans [8–12]. During this outbreak, 440 human cases were reported, leading to 122 fatalities. Importantly, epidemiologists and epizootiologists are particularly interested in studying infection with avirulent viruses, unlike in cases of H5N1 HIPV infection in humans and chickens [4,13]. Accordingly, we have been studying avirulent avian H7N10 viruses isolated from wild ducks in Korea in 2010 [14], with the goal of developing an H7 influenza virus haemagglutinin (HA) vaccine in silkworms using HA DNA as a model. Avirulent viral DNA may be particularly useful because of its evolutionarily posi-

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tion and ease of handling [14]. Indeed, an avian H7 VLP vaccine was already developed using the Anhui/1/2013(H7N9) strain, and the immune responses induced by this vaccine were clearly confirmed in ferrets, supporting the potential efficacy of the vaccine [15]. Similarly, the above H7 vaccine prepared in insect cells has been evaluated in humans and has been shown to induce high seroconversion [16].

Recent epidemiological studies have revealed that human H7N9 isolates are distributed throughout different parts of China [17]. Therefore, in this study, we prepared recombinant baculovirus containing the *HA* gene of A/Anhui/1/2013 and A/Shanghai/1/2013 viruses and performed a comparative analysis of the yield of HA protein in order to improve vaccine development.

2. Materials and methods

2.1. Design and construction of three chimeric HA DNAs and the generation of three H7HA- BmNPVs

Codon-optimized chimeric DNA was designed on the basis of the *HA* gene of A/duck/Korea/A76/2010 (H7N7) virus by introduction of frequently used silkworm codons (Fig. 1). A total of 384 silkworm codons (22.9%) were designed to include 1295 (77.1%) viral codons. As a result, 1680 codon-optimized DNAs were then cloned in *Escherichia coli* for preparation of recombinant baculoviruses. Furthermore, Flag-tagged codons corresponding to eight amino acids (DYKDDDDK) were added to the end of the HA2 molecule. According to these procedures, chimeric DNAs containing *HA* genes of A/Shanghai/1/2013 (H7N9) and A/Anhui/1/2013 (Anhui H7) viruses were also constructed. The synthetic *H7HA* gene was prepared by Dragon Genomics (Shiga, Japan), and the resulting gene was inserted into the pBM-8 plasmid [18] using the In-Fusion technique (Clontech, Mountain View, CA, USA) to produce pBM-8-H7HA, as described previously [7].

2.2. Quantification of mRNA

RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After RNA extraction, RNA was treated with DNase 1 (Qiagen). RNA (1000 ng) was then reverse transcribed using SuperScript III reverse transcriptase (Life Technologies Japan Ltd. Tokyo, Japan) and random-hexamer primers (Life Technologies Japan Ltd.) according to the manufacturer's instructions. The cDNA concentrations were measured using SYBR Premix Ex-Taq II (Takara, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the following primer sets: influenza virus HA for Fukushima, TCAAACGAACAAGGTTCAGGT (forward) and TTGTTTGTCACACCGTCGAT (reverse); for Korea, CACAATCGACCTGGCTGACT (forward) and CGAAGCAACCTGTACCGTCT (reverse); for Anhui and Shanghai, TCACCTGGTGCTAGACCTCA (forward) and GAGCGATGAAAGCACCGTTG (reverse); and baculovirus GP64, CTGTAACGGCGGCTTGTT (forward) and GTCGCTTTCCAGCTGTACG (reverse). The amplification conditions were as follows: one cycle at 95 °C for 30 s and then 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Copy numbers were estimated from the standard curve obtained using serial 10-fold dilutions of the PCR product as the template.

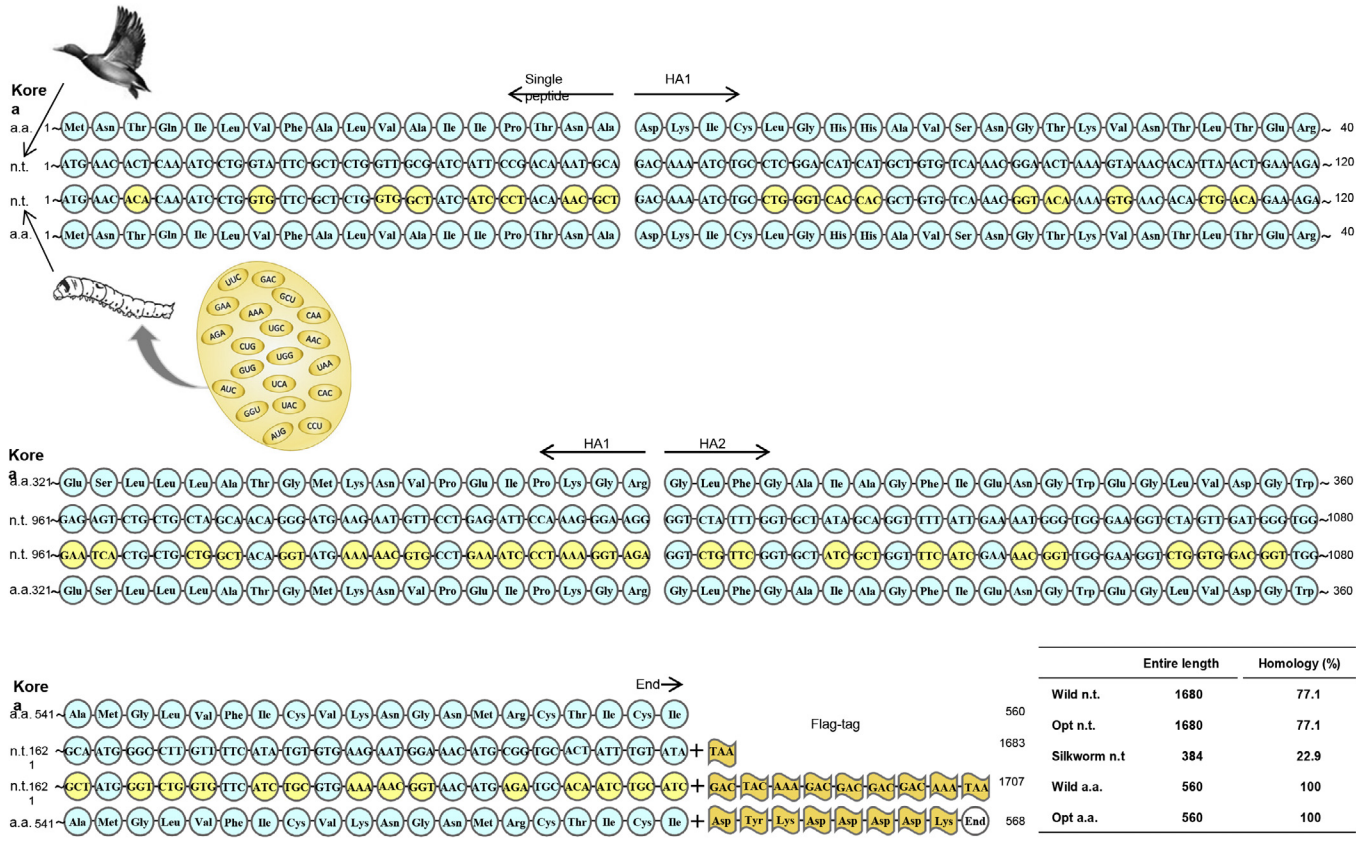


Fig. 1. Functional design of the target chimera DNA for the production of Korea H7 haemagglutinin protein by a combination of the authentic Korea H7 haemagglutinin gene (Wild n.t.) and silkworm-derived codons (Opt. n.t.). Because the authentic gene did not contain basic virulent sequences, the last portion of the *HA1* gene was not modified with the introduction of the further codons. However, the Flag tag (GACTACAAAGACGACGACGACAAATAA) was added to the C-terminus of the H2 peptide. For interpretation of the references to codons in this legend, the reader is referred to the web version of this article. Additionally, codons that are frequently used in the silkworm are shown to the left of the nucleotide and amino acid sequences.

2.3. Virus antigen, antibodies, and western blot analysis

The mouse monoclonal anti-FLAG antibody M2 and the anti-DYKDDDDK-tag mouse monoclonal antibody were purchased from Sigma (St. Louis, MO, USA) and Wako (Osaka, Japan), respectively. The fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody and the peroxidase-conjugated goat anti-mouse IgG antibody were purchased Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Western blot analysis was carried out as described previously [7]. Inactivated A/Barn Swallow/Hong Kong/1161/2010 (H5N1) and A/Anhui/1/2013 (H7N9) and their ferret antisera were kindly provided by Dr. R. G. Webster and Dr. R. Webby of St. Jude Children's Research Hospital (Memphis, USA).

2.4. Production of VLP-HA protein

A 0.2-mL aliquot of recombinant Korea H7-BmNPV virus was inoculated into silkworm pupae. Four 4 days later, all pupae that exhibited a colour change from brown to black were harvested. The skin was removed, and naked pupae were homogenized in PBS containing 0.01% formalin and phenylthiourea as an antioxidant; homogenization was performed on ice using six pulses in three cycles at 2-min intervals with a Handy Sonic (Model Tomy UR-20P). The resulting homogenates were centrifuged at 4200*g* for 30 min. The supernatant was further centrifuged at 30,000*g* for 30 min. This crude samples were sedimented in the presence of 20% sucrose (w/w) by centrifugation at 110,000*g* for 4 h. The supernatants were purified as described previously [7], but with centrifugation for 4 h.

2.5. Immunological investigation of the VLP vaccine

Four-week-old female ddY mice were separated into three groups (n = 5 mice per group). The first group was immunized orally without adjuvant at week 0 using H7 VLP containing 16,384 HA titres and then 1 month (4 weeks) later. The second group was immunized orally with two doses at weeks 0 and 1. The third group of mice were immunized orally three times at 1-week intervals with the same amount (16,384 HA titres) of H7 VLP antigen. Blood samples were collected from all mice at 4 weeks. Additionally, immune responses in mice immunized intraperitoneally with two doses of H7 VLP were determined.

Additionally, six-month-old chickens were also immunized three times orally with 16,384 HA titres of H7 VLP. The H5-Fukushima VLP vaccine was simultaneously administered orally at 16,384 HA titres, and the immune responses of the chickens were compared with those of the mice described above. Antiserum to Korea H7-VLP was also prepared in chickens via intramuscular immunization three times.

2.6. Haemagglutination and haemagglutination inhibition (HI) tests

Test samples were diluted with 50 μL phosphate-buffered saline (PBS) in 96-well microtitre plates, and haemagglutination and HI tests were carried out as described previously [7].

2.7. Haemadsorption tests

The culture medium from infected cells was removed and washed once with PBS. Next, a 0.5% chicken erythrocyte mixture was added to the infected cells. After a 30-min incubation, the test cells were washed through with PBS and observed under stereoscopic microscope (Olympus. Tokyo, Japan).

2.8. Sucrose density gradient centrifugation

VLP crude samples were prepared from concentrated pupae homogenates after centrifugation at 27,000 rpm for 4 h on a 20% (w/w) sucrose cushion in an SW28 swing rotor. Samples were then purified on a sucrose density gradient as described previously [7], and centrifugation was carried out for 4 h. For electron microscopic examination, sucrose was removed from the samples using column filtration (PD-10 Desalting Columns; GE Healthcare Bio-sciences AB, Sweden).

3. Results

3.1. Design of chimeric H7 HA DNA and the generation of the Korea H7 BmNPV recombinant strain

First, we aimed to design chimeric HA DNAs from A/duck/Korea/A76/2010 (H7N7), A/Anhui/1/2013 (H7N9), and A/Shanghai/1/2013 (H7N9) viruses. In addition to the above H7 HA genomes, as shown in Fig. 1, 20 codons (UUC, GAC, GUU, CAA, AAC, AAA, UGC, UAA, CAC, UAC, GAA, AGA, CUG, UGG, UCA, AUG, CCU, GUG, AUC, and GGC), which are frequently used in the physiological metabolism of silkworms, were selected from 60 codons and were replaced with the wild-type codons of the target viral gene at 1295 positions. The synthesized DNA was then inserted into a baculovirus transfer vector, pBM-8, to obtain Korea H7-BmNPV. The above three recombinant baculoviruses were then generated via cotransfection of the three H7-BmNPVs together with the linearized DNA from the wild-type baculovirus BmNPV, as described previously [7]. According to the same method, expression plasmid containing of a wild strain (A/tufted duck/Fukushima/16/2011 (H5N1)) was also constructed.

3.2. Expression of the synthetic Korea H7-BmNPV genes in cultured cells

As shown in Fig. 2A, infection with wild-type BmNPV was confirmed in cytopathic effect and western blot analysis specific for baculovirus. Additionally, the expression of Korea H7 recombinant was determined in haemadsorption, FA, and western blot analyses. Haemadsorption tests revealed the expression of H7 Anhui, H7 Shanghai, and H5 Fukushima recombinants. For comparison of HA yields and mRNA copy numbers, the above three recombinants were injected into BmN cells and pupae. The results showed that HA units and RNA synthesis of codon-optimized H5 Fukushima had the highest titres (Fig. 2B, C), followed by both of the Korea H7 recombinant (Fig. 2B, C). It was of interest to know that recombinant including HA-DNA of H5 virus showed HA activity more than one million, although a recombinant containing HA DNA of wild stain did not produce any HA protein. In contrast, mRNA and HA yields of H7 Anhui and H7 Shanghai (HA yield data not shown) were extremely low. Thus, titres obtained from mRNA and HA titration were consistent, suggesting that mRNA synthesis may be a useful marker for understanding the production level of HA molecules.

Notably, when interpreting these results to determine the effects of codon optimization, we must emphasize that the transfer vector for the H5 Fukushima recombinant was different from those of the three other H7 recombinants. In any case, the yields of HA protein for each recombinant were greatly influenced by the insertion of silkworm codons and by the genomic structures of viral HA. Interestingly, the insertion of the recombinant gene in the baculovirus did not influence the synthesis of baculovirus *Gp64* mRNA.

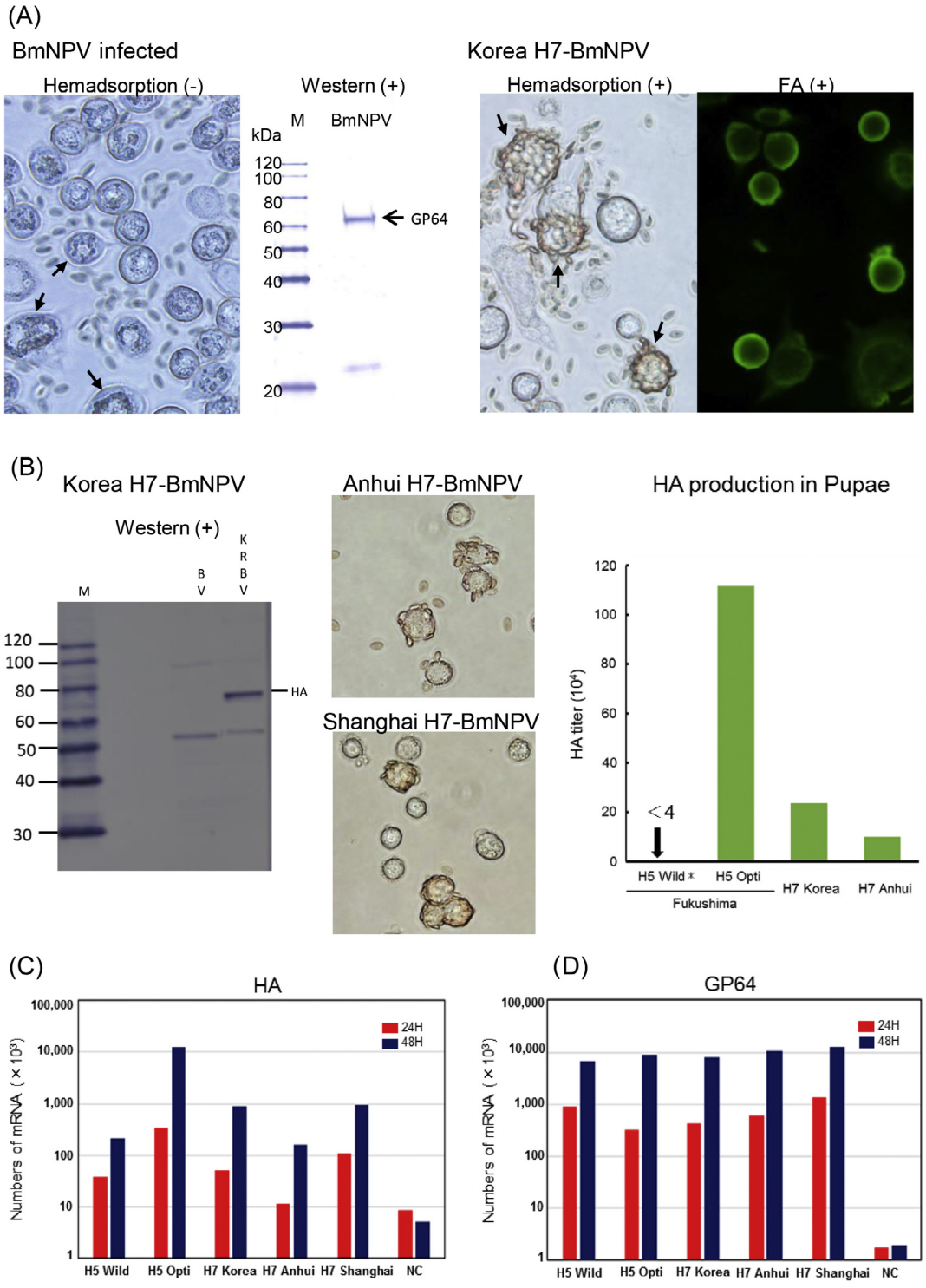
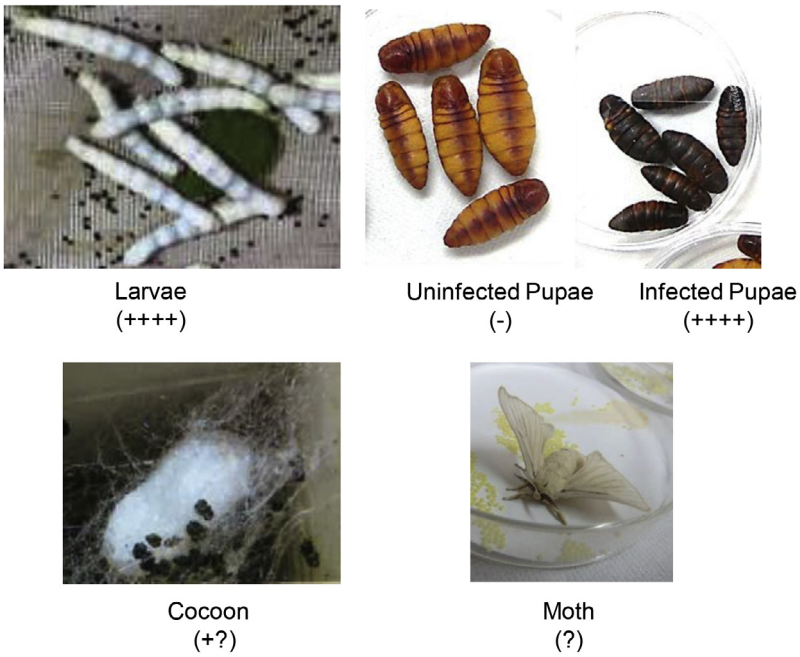


Fig. 2. Examination of protein expression on the recombinant baculovirus (Korea H7-BmNPV) using western blotting of baculovirus (negative control), haemadsorption, and fluorescent antibody tests (A). The expression of Korea H7-BmNPV, Anhui H7-BmNPV, and Shanghai was also examined using western blotting (Korea H7-BmNPV), haemadsorption and haemagglutinin tests. HA production of H7 recombinant, H5-wild strain and H5-codon optimized HA recombinant (B). (C) Comparative analysis of mRNA yield based on codon optimization and differences in the specific genome structure of the virus. (D) Number of mRNA copies following codon optimization, and mRNA expression of baculovirus-specific genes including HA DNA of H5 wild type Fukushima strain.

3.3. Production of the H7 VLP vaccine in silkworm pupae

Life stages of silkworms are shown in Fig. 3A and B. The Korea H7 recombinant appeared to produce moderate levels of HA units/larva (314,026), which was judged as +4. After infection with the Korea H7 recombinant, the pupae changed colours from brown to black. Moreover, despite infection with the recombinant virus, some pupae grew up to the cocoon and moth stages. In order to confirm the increased yield of H7 Korea VLP, recombinant Korea H7-BmNPV virus was infected in 40 pupae. After infection, the skin colours of the pupae gradually changed, acquiring a deep black colour after day 4. These results suggested that the peak HA titre of

(A)



(B)

Yield of H7 hemagglutinin

Exp.	Elution (cycles)	HA titer	Volume (ml)	Protein (μg / ml)	Protein / HA (μg / HA)	HA production / head
Larvae I (30)#	1st.	294,912	5	n.d.	n.d.	
	2nd.	65,536	10	20,000	0.31	
	3rd.	16,384	10	8,600	0.52	
	Total	376,832	25	28,600	**m=0.41	314,026 (++++)##
Pupae I (40)#	1st.	262,144	35	44,210	0.16	
	2nd.	16,384	11	8,740	0.53	
	3rd.	8,192	11	2,740	0.33	
	Total	286,720	57	55,690	**m=0.34	408,576 (++++)##

* n.d; not determined.
**m; represents a mean protein concentration.
#; Numbers
##; Evaluation indicating from + to ++++ in the parenthesis shows the yield of HA proteins.

Fig. 3. Larva, pupae, moths, and cocoons of silkworms were used for the production of haemagglutinin (A). (B). Comparative analysis of the yield of haemagglutinin in the different sonication cycles. The HA yields in different silkworm groups and at the different elution cycles were evaluated on the basis of four scores as follow: +, >1024; ++, >10,000; +++, >100,000; and ++++, >200,000.

the recombinant virus was obtained on day 4. The homogenates were then subjected to sonication. The results of HA protein obtained from 40 pupae are shown in Fig. 3B. A total of 16,343,040 HA protein titres were recovered from three cycles of sonication per 40 pupae. The yield of haemagglutinin was estimated to be 408,576/pupa, showing a high level of haemagglutinin production (++++).

3.4. Purification and characterization of H7 haemagglutinin through sucrose density gradient centrifugation

In order to investigate the biological and structural characteristics of the recombinant protein, sucrose gradient samples were separated into about 39 fractions and subjected to sucrose concentration (Fig. 4A), HA activity (Fig. 4B), western blot (Fig. 4C), and protein content analyses (Fig. 4A). As shown in Fig. 4A and B, the major peaks of HA and protein concentration were separated into heavy (HF) and light fraction (LF) fractions, suggesting variations in the produced VLPs. Western blot analysis demonstrated that the HA fraction contained a 77-kDa HA molecule (Fig. 4C). Interestingly, large-VLPs and small-sized HA particles were present in the HF and LF (Fig. 4D–F and G), .respectively. On the basis of HA and protein analysis pattern, we obtained the attracted result as more than 90% of HA fraction was present in heavy fractions and furthermore, most of VLP was recovered in heavy fraction as purified sample.

3.5. Antigenic characterization of H7 Korea VLP

To investigate the immunogenicity of H7 haemagglutinin produced in silkworm pupae, H7 VLP vaccines were subjected to the HI reaction with antisera against authentic influenza viruses and silkworm-derived VLP antigens. As shown in Fig. 5A, antiserum against A/Anhui/1/2013 (H7N9) virus reacted with H7 Korea VLP

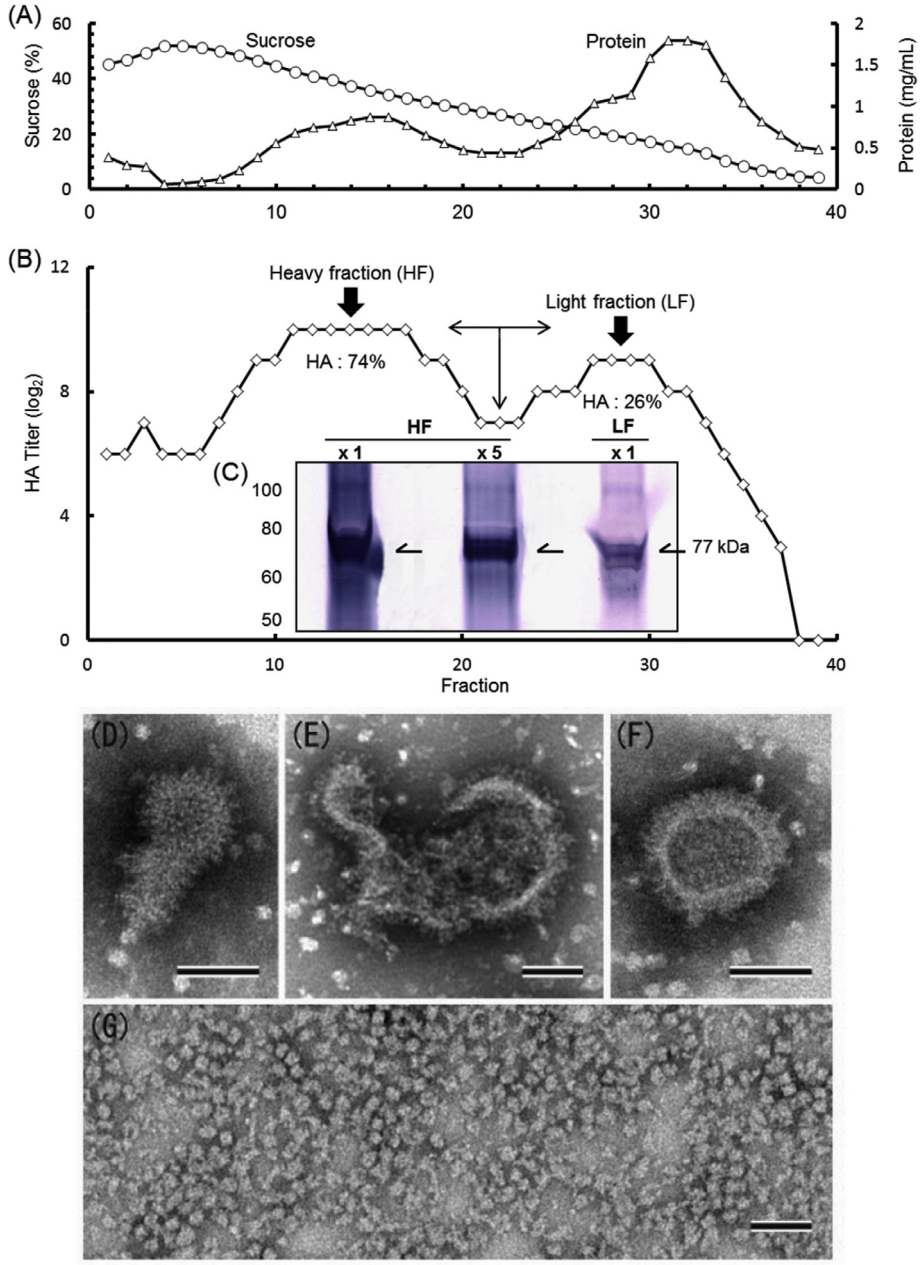


Fig. 4. Purification of Korea H7 haemagglutinin produced in pupae. Purification and characteristics were analysed on a 1–50% (w/w) sucrose density gradient centrifugation at 110,000*g* for 4 h at 5 °C. Sucrose and protein concentrations (A), HA activity (B), western blot analysis (C), respectively. In B, comparison of HA activity between Heavy and Light fractions were also showed together with western blot analysis. Electron micrography of heavy (D, E, F) and light fractions (G), respectively. Bar = 50 nm.

antigen at a level similar to that of authentic A/Anhui-H7 virus, yielding reaction titres of 80–160. Additionally, the reaction was increased when using potent IgG antibodies targeting silkworm-derived H7 VLP HA antigen prepared in chickens.

Consequently, H7 VLP antigen was injected into the muscles of chickens with up to three doses. As shown in Fig. 5B, the HI titres were approximately 240 in the single dose group. In contrast, higher HI antibody production was confirmed in chickens immunized with three doses of the vaccine, yielding HI titres of approximately 500.

3.6. Immune responses in chickens and mice following oral immunization with avian H5 and H7 VLP vaccine

Since most of avian influenza viruses infect bird species through the oral route, the immune responses to avian H5 and H7 VLP were examined in chickens and mice after oral immunization. Mice or chickens were orally administered 0.5 mL H5 Fukushima and H7 Korea HA antigens possessing 16,000 HA titres using a specillum, and immune doses were conducted three times (doses) at 1-week intervals (weeks 0, 1, and 2).

The results are shown in Fig. 5C and 5D together with HI patterns in 96-well plates (Fig. 5E). The HI titres elicited in mice tended to increase in accordance with the increase in the dose of H5 Fukushima and H7 Korea HA antigens (Fig. 5C–E). For example, after three doses of the vaccine in mice and chickens, HI titres reached approximately 450 against H5-HA VLP. To confirm the oral immune responses to the vaccination, fluorescent antibody analysis and western blotting were performed using the above antibodies produced in mice. As shown in Fig. 6A and B, H5HA-BmNPV-infected cells showed clear fluorescence after intraperitoneal injection or intra-oral inoculation with H5 HA VLP vaccines.

(A)

Antigens	Antisera to :			
	#①	*②	#③	*④
① A/Swallow HK (H5N1)	320	1,280	n.d.	[b]-
② A/Fukushima-H5VLP	1,280	4,096	-	-
③ A/Anhui (H7N9)	[a]n.d.	-	80	160
④ A/Korea-H7 VLP	-	-	40	2,048

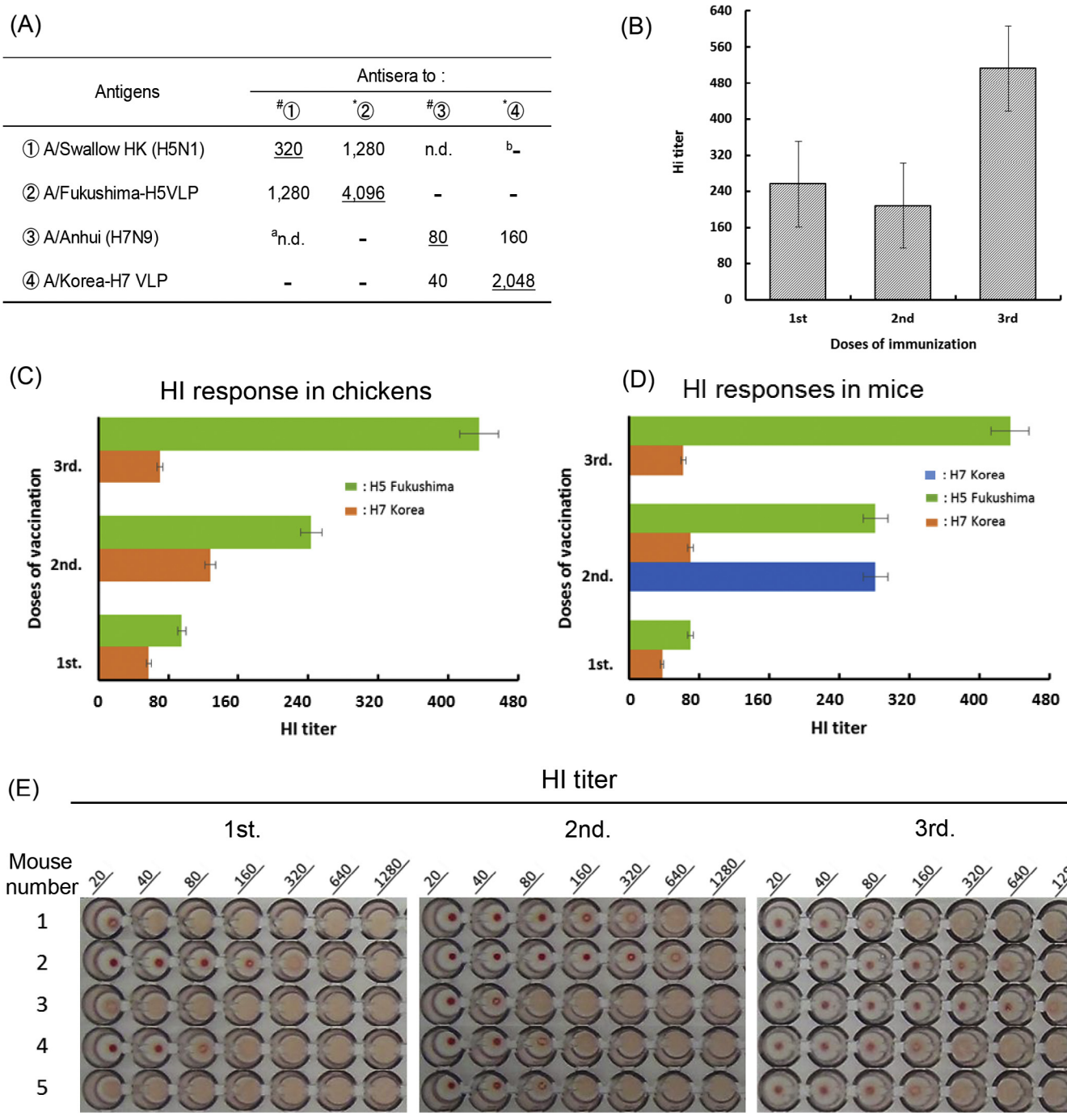


Fig. 5. Immune responses of H5 and H7 VLP haemagglutinin (A and B). (C and D) Antibody production of H5 and H7 VLP in pupae after oral immunization in chickens and mice.

Fluorescence was not observed in infected cells treated with antiserum to JEV (negative control). Interestingly, a 77-kDa peptide specific for HA was detected after treatment with antisera against FLAG monoclonal antibody, mouse antibody prepared with intraperitoneal immunization, and antibody obtained from oral immunization. Furthermore, H7-specific antibodies were identified in fluorescent antibodies produced by intraperitoneal and intra-oral immunization (Fig. 6E and F). In addition to FLAG antibodies, the above two antibodies were also used in western blot analysis (Fig. 6G). Our findings showed that one weak peptide was observed in anti-FLAG antibody-treated Korea H7 recombinant infected BmN cells. However, antibody administered by intraperitoneal injection revealed two peptides larger than the anti-FLAG antibody. In contrast, antibodies produced in mice with oral immunization yielded one sharp peptide Unlike normal HI antibodies produced by intraperitoneal immunization, two peptides larger than the normal HA peptide appeared in western blot analysis of samples from intra-oral antibody administration. Although the reason for this phenomenon is still unclear, the positive FA of antibody produced in mice increased the possibility that oral immunization with avian influenza virus HA could elicit antibodies specific to influenza virus.

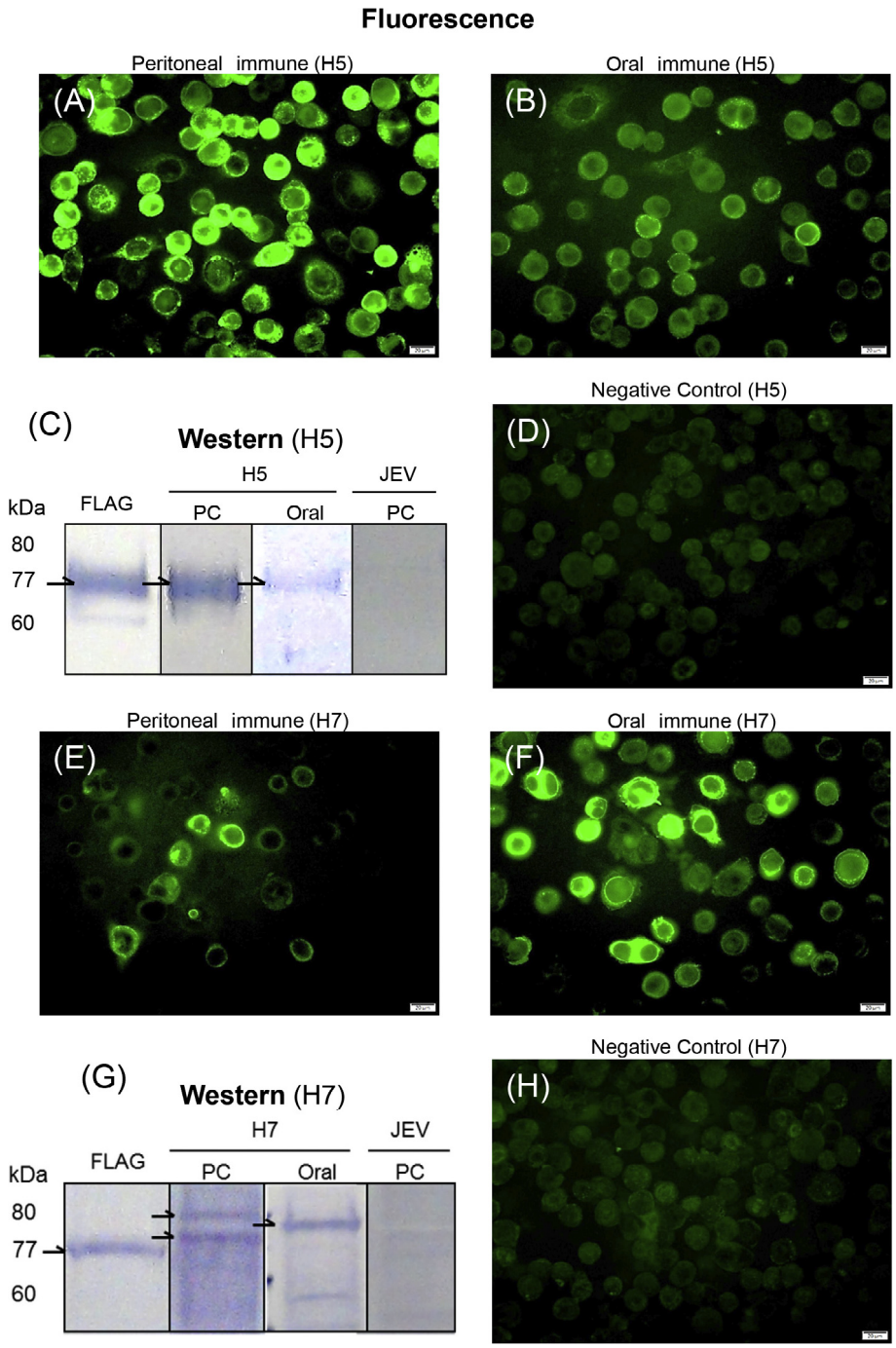


Fig. 6. Confirmation of anti-H5 antibody production in mice with intraperitoneal (A) or intra-oral immunization (B). (C) Detection of H5 Fukushima HA polypeptides with antibodies against FLAG. Peritoneal cavity (PC), oral immunization (Oral), negative control of JEV (JE). (D and H) Negative control of JEV (JE). (E and F) FA reaction in Korea H7 recombinant infected BmN cells treated with antibodies against intraperitoneal (E) and intra-oral (F) immunization. (G) Western blot analysis with antibodies to FLAG. Intraperitoneal immunization (PC), oral immunization (Oral), and JEV antigen (JEV) in the H7 Korea recombinant infected BmN cells.

4. Discussion

Through a series of recombinant baculoviruses expressing HA, including H5 Fukushima, H7 Korea, and H7 Anhui *HA* genes of avian influenza virus, we compared the yields of HA protein produced in silkworm systems on the basis of HA titration and mRNA synthesis. As a result, we found that the production level of HA proteins with each recombinant appeared to be influenced by not only the codon-optimized effect but also the characteristic structure of the *HA* gene. For example, despite the use of the same codon-optimized DNAs, the HA titre and number of mRNA copies of each recombinant were different from each other. Moreover, although remarkable advancements have been made in the field of vaccine development in recent years [7,15,16,19–24], it was interesting that the influenza VLP vaccine could elicit broader immune responses than the intact virion vaccine [21], suggesting potential applications as a future VLP vaccine. Since we don't have authentic H7 influenza virus, comparative immune responses between silkworm VLP and authentic virus vaccine may be done in near future. Furthermore, an influenza VLP vaccine of H7N9

virus containing the surface HA, neuraminidase, and internal M1 protein has been reported to produce protective immune responses in mice [22]. Recent VLP vaccine development is focused on H7 HA, and various HA VLP subtypes have been shown to protect a wide variety of epidemic or epizootic viruses [23,24].

Based on these previous works, the VLP vaccines developed herein may be obtained by large-scale production with a relatively low cost and rapid production, as is needed in the case of pandemic outbreaks. Therefore, a genomic system for the large scale production of vaccines should be established as soon as possible. Indeed, we could produce a high level of Korea H7 VLP in silkworm pupae, showing HA titres of 408,576 per pupa. However, the yields obtained in this study were still lower than that (approximately 1,000,000 HA titre) obtained for the H5 Fukushima recombinant [7]. In contrast, recombinant containing HA DNA of wild H5 Fukushima did not show meaningful HA activity. In any case, we were surprised to find that oral immunization with H5 and H7 VLP in chickens and mice resulted in significant increases in antibody titres according to the vaccine dose. For example, antibody titres in mice and chickens immunized with H5 VLP increased gradually with increasing doses of the vaccine. These antibodies were confirmed in further fluorescent antibody tests and western blot analyses. In order to elicit a strong antibody increase through broad mucomembranous stimulation, a large amount of VLP antigen may be necessary. When considering this immune condition, a large amount of VLP antigen may be produced in silkworms at a low cost.

In this study, we determined that avian HA vaccine protein elicited the HI antibody with oral immunization, suggesting further detail investigation. Additionally, it was also effect to establish a useful recovery system of HA vaccine from larvae and pupae of silkworm.

Conflict of interest

We are applying a patent based on the results described in the present report.

Acknowledgments

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