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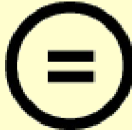
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석사학위 청구논문

Influence of temperature on the antigenic stability of influenza virus-like particles

2018

성신여자대학교 대학원

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Influence of the temperature on the antigenic stability of influenza virus-like particles

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논 문 개 요

인플루엔자 바이러스는 유전자 변이를 통해 전 세계에 걸쳐 매년 유행을 초래한다. 인플루엔자는 크게 사망에까지 이르게 할 수도 있어 인류에게 커다란 위협이 된다. 이러한 인플루엔자 바이러스 감염을 예방하기 위해서는 백신을 접종 받는 것이 가장 효과적인 방법이다. 인플루엔자 바이러스 백신으로는 바이러스 유사 입자 (Virus-like particle, VLP) 가 최근에 주목을 받고 있다. 바이러스 배양 방식으로 기존에 사용해오던 수정란을 통한 배양이 생산 시간과 생산량 등에 한계가 있어 세포를 통해 생산해낼 수 있는 VLP가 새로운 백신 플랫폼으로 떠오르고 있다. VLP는 바이러스와 유사한 구조를 가지고 바이러스의 항원 단백질을 발현하여 면역원성을 이끌어낸다. VLP의 면역원성을 이끌어내는 중요한 부위는 단백질로 구성되어 있는데 단백질은 열에 민감하여 이에 의해 구조적 변화가 일어나는 특징을 가진다. 따라서 이 연구에서는 인플루엔자 A 바이러스 H1N1 A/Puerto Rico/8/1934 (PR8) 를 이용하여 VLP를 만들고 항온항습기를 이용하여 VLP를 다양한 온도와 시간 조건으로 열에 노출 시킨 뒤, 그에 따른 VLP의 면역원성을 생체 외(in vitro), 생체 내(in vivo) 실험 방법을 적용하여 확인하였다. 바큘로바이러스 발현 시스템을 이용하여 생산해 낸 H1N1 PR8 VLP의 발현을 western blot analysis를 통해 확인하였고, 만들어낸 VLP를 항온항습기를 통해 4℃, 25℃, 42℃ 온도에 반응시켰다. 반응이 끝난 VLP의 면역원성 확인을 위해 혈구 응집 반응 (hemagglutination assay, HA assay) 을 실행하였고, 42℃에서 HA titer의 값이 4℃, 25℃에 비해 시간이 지남에 따라 크게 감소함을 확인하였다. 반응 시킨 VLP를 쥐에게 두 차례 접종한 후 채취한 혈액을 통해 효소면역분석법 (enzyme-linked immunospecific assay,

ELISA) 를 이용하여 항체 반응을 확인하였고, 야생형 바이러스 공격접종을 통해 백신으로써의 효능을 확인하였다. 4℃와 25℃ 그룹은 모든 시간 조건에 대해 유의미한 수준의 항체가가 유도되었으며, 42℃ 그룹은 시간이 지남에 따라 항체가가 크게 감소하였다. 그러나 2차 접종 후에는 4℃, 25℃, 42℃ 모두 항체가가 크게 향상됨을 보였고 공격접종 후에도 모든 그룹의 쥐가 모두 살아남았다. 이러한 결과를 통해 VLP가 4℃, 25℃, 42℃ 에서 한 달 간 노출 되었을 시 42℃ 에서는 면역원성에 영향을 받음을 확인하였고, 하지만 이러한 시료를 동물에게 두 번 접종했을 때에 충분한 면역원성을 이끌어 낼 수 있음을 확인하였다.

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INTRODUCTION

Influenza virus belongs to Orthomyxoviridae family which contains three genera of influenza viruses, influenza A, B, and C viruses (Fig. 1), (1). Influenza virus is made up of single stranded, segmented negative sense RNA. The RNA genome is segmented with eight genomic segments which are structural components containing polymerase protein (PB1, PB2, PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M1, M2), and non-structural protein (NS). The classification of influenza virus A, B, and C depends on viral protein differences between NP and M1 proteins (2-5).

Influenza A virus infects mammalian including humans and variety of avian species. Influenza B virus infects mainly humans and C virus infects pigs and humans in limited (6). Most of all, influenza A virus is more pandemic than B and C. Influenza A was emerged by an antigenic mutation of H1N1 and began in North America in April 2009 (7). The pandemic influenza A H1N1 2009 virus has affected more than 214 countries and caused more than 18,449 deaths, resulting in the first pandemic of the 21st century (8-9). Influenza A is the most dangerous because of its greater rate of mutation (10).

Vaccination has been a most effective way to control the infection of influenza (11). Several platform of vaccines have been developed including live attenuated vaccines, subunit vaccines, and nucleic acid

based vaccines, and recombinant virus-like particles (VLPs), (Table 1), (Fig. 2), (12-13).

Live attenuated vaccine is a less virulent form of native virus. Attenuation makes infectious agents to become less virulent (14). Subunit vaccines contain the antigenic parts of the pathogen resulting from virus or is grown in the lab using the virus DNA. Nucleic acid based vaccines contain an optimized mRNA which has an open reading frame that encodes the antigen of interest (15). VLP mimics the surface structure of native virus by presenting antigenic epitopes but has no viral genome, so that VLP is noninfectious, safe and promising vaccine candidates (16-18). But most of these platform was produced by traditional method used egg-based production. Using embryonated chicken eggs-based technology has limitation such as low yields and time consuming (19).

To overcome these limitation, VLP has been promised as an alternative because it can be produced in yeast, insect or mammalian cell, non-egg-based. VLPs are highly organized with viral protein and have an inherent ability for self-assembly (Fig. 3). VLPs are multiprotein structure which resembles intact virion and mimic conformation of native virus. These repetitive structure of antigen can be presented to dendritic cell, then, T and B lymphocytes are activated. This process elicits strong immunogenicity containing humoral and cellular immune response without adjuvants (20-25).

In previous studies, VLPs have been known as advanced vaccine platform with antigenic epitope that results in strong hummoral and

cellular immune responses (26-27). VLPs are made of protein that can be affected by temperature. Therefore, to use VLPs for vaccine, it is needed to confirm stability study of VLPs by temperature condition.

In this study, influenza H1N1 PR8 VLPs were produced using baculovirus-insect cell expressed system. For stability test of VLP by temperature condition, H1N1 PR8 VLPs were exposed to temperature & humidity chamber and evaluated immunogenicity.

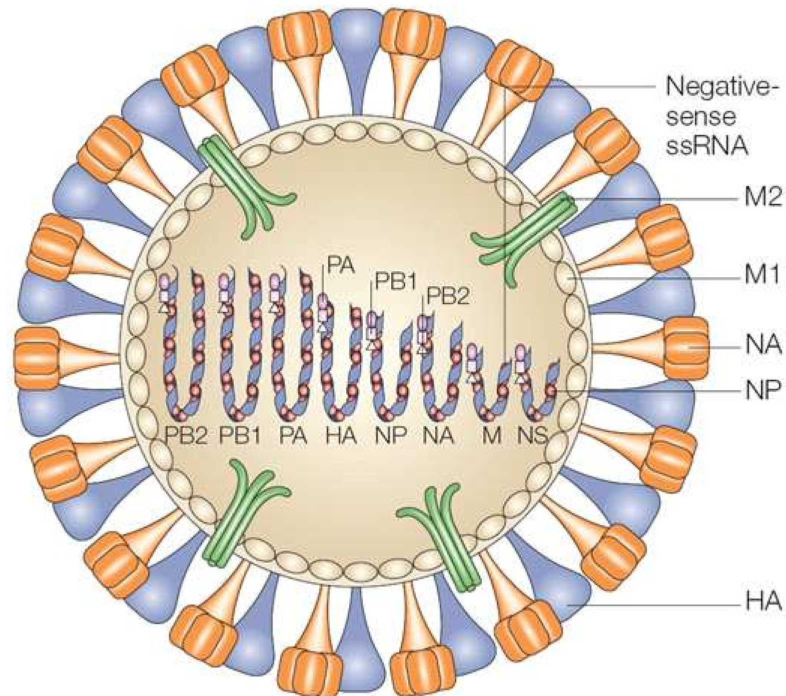


Figure. 1. Structure of influenza virus

The RNA genome is segmented with eight genomic segments that are polymerase protein (PB1, PB2, PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M1, M2), and non-structural protein (NS).

Table 1. Variety of influenza virus vaccines

Vaccine	Type of virus	Composition	Indications	Course/Route	Doses
Whole viruses	Inactivated	Whole inactivated viruses	Over 12 years	1 dose IM	0.5 mL
Split	Inactivated	Split viruses (HA, NA and capsule)	From 6 months	1 or 2 IM doses*	0.25 mL (6 to 36 months) 0.5 mL (>3 years)
Subunit	Inactivated	HA and NA in isolation	From 6 months	1 or 2 doses	-
Virosome	Inactivated	HA and NA absorbed into virosome particles	From 6 months	1 IM dose	0.25 mL (6 to 36 m) 0.5 mL (>3 years)
Cold-adapted	Attenuated	Whole attenuated cold-adapted viruses	Healthy 5 to 49-year-olds	1 or 2 nasal doses	0.5 mL

HA = hemagglutinin; IM = intramuscular; NA = neuraminidase.

*6 months to 9 years: two doses during the first year of immunization.

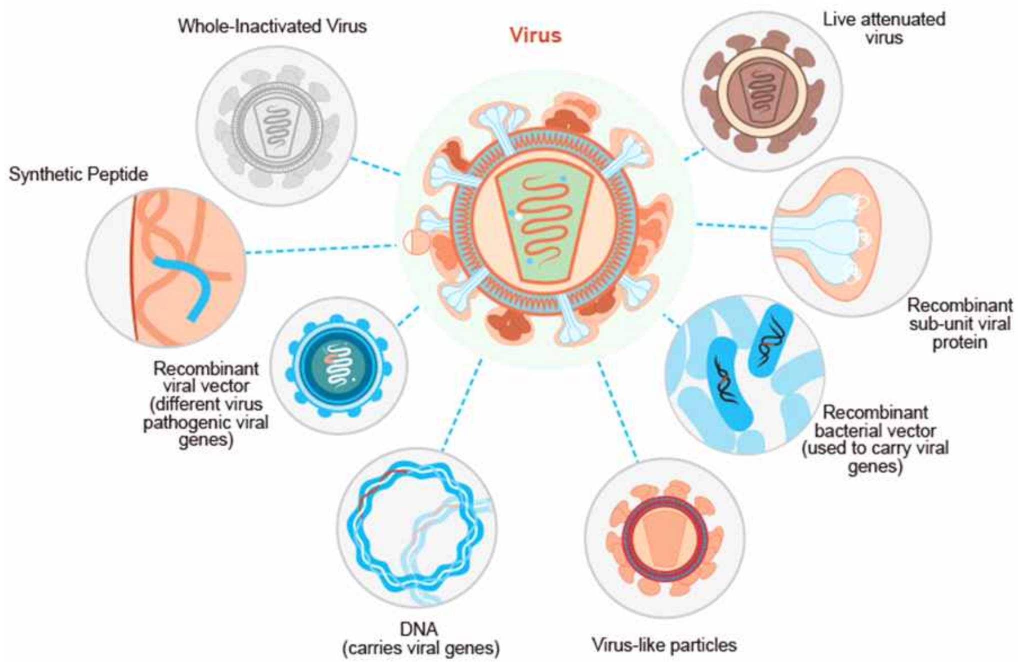


Figure. 2. Variety of influenza virus vaccine type

For prevention of influenza by vaccination, several platform of vaccines have been developed including whole-inactivated virus, synthetic peptide, recombinant viral vector, DNA, virus-like particles, recombinant bacterial vector, recombinant subunit viral protein, and live attenuated virus.

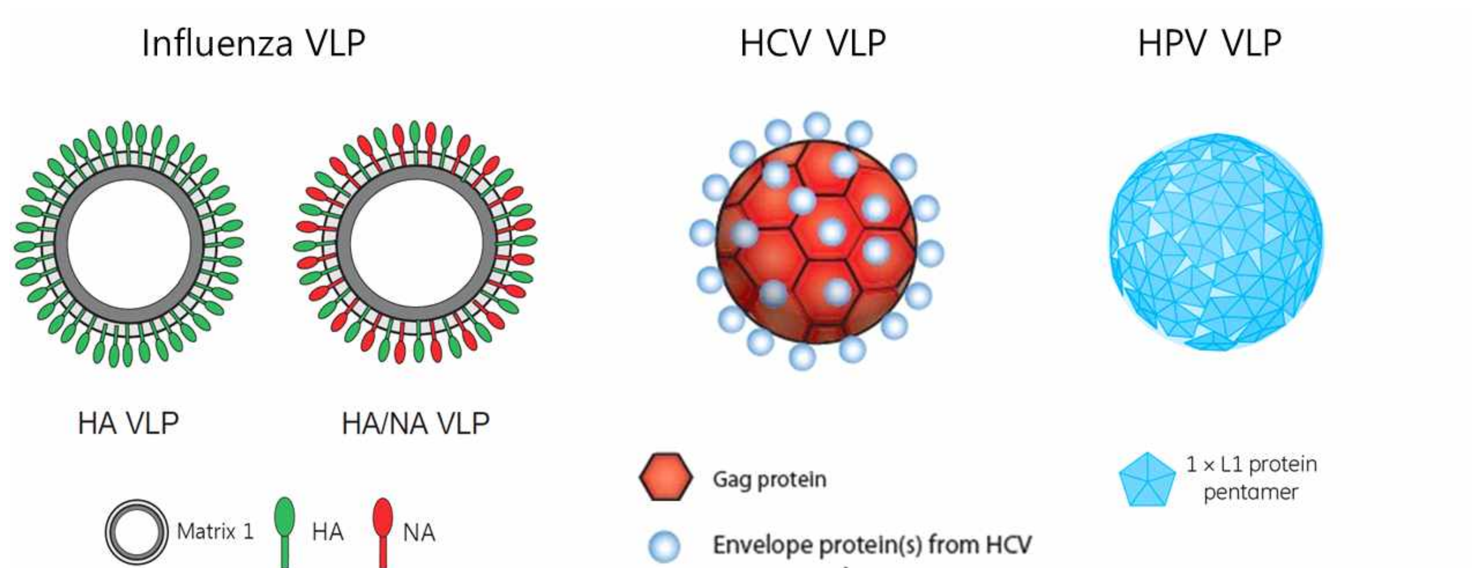


Figure. 3. Variety of VLP vaccines
VLP is promising vaccine candidates. VLP platform has been used in developing vaccine candidates on a variety of viruses including influenza virus, hepatitis c virus (HCV), and human papilloma virus (HPV).

MATERIALS AND METHODS

Virus and cells

For the producing VLPs, Influenza virus H1N1 A/Puerto Rico/8/1934 (PR8) was used. Virus was propagated in 10-day-old embryonated chicken eggs incubated for 3 day. Spodoptera frugiperda (Sf9) cells were maintained in SF900II medium (Gibco, San Diego, CA) containing 10% FBS, 100U/mL Penicillin, and 100ug/mL Streptomycin at 27℃ shaking incubator.

Cloning of HA and M1 genes

Viral RNA was extracted from PR8 virus using Viral Gene spin™ (intron, Seongnam-si, South Korea). Hemagglutinin (HA) and Matrix 1 (M1) gene (Table 2),(Table 3) were generated by RT-PCR (Table 4) using PrimeScript™ One Step RT-PCR kit ver2 (Takara, kusatsu, Japan) using specific primer (Table 5).

HA sequence specific primer forward: 5`AGGATGAAGGCAAACCTACTG,

reverse: 5`AGGAAGCTTTCAGATGCATATTCTGCAC

Matrix1 specific primer forward: 5`AggTCTAGAATGAGTCTTCTAACCGAGGT,

reverse : 5`AGGCTCGAGTCACTTGAACCGTTGCATC

After RT-PCR, HA and M1 gene was ligated with pFastBac1 vector between XbaⅠ and HindⅢ site and between XbaⅠ and XhoⅠ site, respectively (Fig. 4). Each ligation was transformed into DH5α. Colony

was selected and inoculated into liquid luria-bertani (LB) containing ampicillin. DNA was extracted using Viral Gene spin™ (iNtRON, Seongnam-si, South Korea). To generate recombinant bacmids, each recombinant plasmid pFastBac1 was transformed into DH10Bac. When streak on agar plate, the agar plate contains kanamycin, gentamicin, tetracycline, isopropyl β-D–1-thiogalactopyranoside (IPTG), and x-gal. After transformation, colonies which have recombinant bacmids containing gene of interest were selected by blue and white screening. Selected white colony was inoculated into liquid LB containing kanamycin, gentamicin, and tetracycline. Bacmid DNA was extracted using Plasmid Midi Kit (QIAGEN, Hilden, Germany). Concentration of extracted DNA was measured and then sequence was confirmed by sequencing.

Generation of recombinant baculoviruses expressing HA and M1

Each Recombinant bacmid encoding HA and M1 gene was transfected into Sf9 cells (Fig. 5). 2 mL of 1 x 10^6 insect cells were seeded in 6-well plate and incubated 1 hour at 27℃ for attaching. Mixture of lipofectamine (invitrogen, Carlsbad, USA) and SF900II medium, and mixture of plus reagent (invitrogen, Carlsbad, USA), SF900II medium and bacmid were prepared. These mixtures were incubated for 15 minutes at room temperature and each sample was mixed and incubated for 30 minutes at room temperature. After removed medium in 6-well plate, sample was added dropwise onto the cell and incubated at 27℃ for 3 days. Culture medium was harvested and clarified by centrifugation at 6000 rpm for 5

minutes at 4℃. To amplify volume, Sf9 cells were infected with recombinant baculovirus (rBV). 10 mL of 2 x 10^6 insect cells were seeded in T75 flask and incubated at 27℃ for 1 hour. After incubation, culture medium from 6-well plate was added onto the T75 flask and incubated at 27℃ for 3 days. Culture medium was harvested day 3 post-infection and clarified by centrifugation at 4000 rpm for 15 minutes at 4℃. For large culture, 100 mL of 2 x 10^6 insect cells were infected with culture medium from T75 in erlenmeyer flask and incubated at 27℃, 120 rpm shaking incubator for 3 days. When viability was 20~30%, culture medium was harvested and clarified by centrifugation at 4000 rpm for 15 minutes at 4℃.

Production of VLPs

To produce PR8 VLPs, Sf9 cells were co-infected with each rBV expressing HA and M1. Each culture medium of rBV containing HA and M1 was co-infected with 300 mL of 3~4 x 10^6 insect cells at a 0.5~1 multiplicity of infection in erlenmeyer flask and incubated at 27℃, 120 rpm shaking incubator for 3 days. When viability was 20%~30%, culture medium was harvested and clarified by centrifugation at 4000 rpm for 15 minutes at 4℃. The supernatants were concentrated by nitrocellulose membrane-based filtration system (NCFS) using stirred cell (Amicon, Darmstadt, Germany). Supernatants in stirred cell were given pressure by N_2 gas (Fig. 6). Through 100 kDa molecular weight cut-off (MWCO) membrane, other components less than 100 kDa were filtered and VLPs were remained in supernatants.

After concentration, PR8 HA VLPs were ultracentrifuged at 30000 rpm for 1 hour at 4℃ using 20%-60% sucrose gradient. Purified VLPs were carried between 20%-60% layers (Fig. 7)

Hemmagglutination assay

HA titers of PR8 HA VLPs were confirmed by Hemagglutination assay. A series of two-fold dilution of VLPs with phosphate-buffered saline (PBS) buffer were prepared in 96 well V-bottom plate and incubated at 4℃ for 1 hour with 0.5% chicken red blood cells (Fig. 8). HA titers of VLPs under each temperature and time condition was also confirmed in the same way.

Western blot analysis

Expression of HA in VLPs were determined by western blot analysis. VLPs were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gel and transferred onto polyvinylidene diflluoride (PVDF) membrane using semi-dry transfer system. Membrane was blocked with 5% skim milk and incubated with PR8 inactivated virus immunized serum followed detection by HRP-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, USA). Protein concentration of PR8 HA VLPs were measured by bicinchoninic acid (BCA) assay kit (Pierce, Rockford, USA).

Exposure of VLPs on variety temperature conditions

VLPs were diluted with PBS buffer and exposed to temperature using a Temperature & Humidity Chamber, TH-ME-025 (JEIO TECH, Daejeon, South Korea) (Table 6). For a control group, same procedure was performed using PR8 inactivated virus.

Immunization and virus challenge

For animal experiment, six weeks old female BALB/c mice (n=5) were immunized intramuscularly with 100 μL PBS containing 2.6 μg PR8 VLPs exposed to temperature condition. Group of mice immunized with pure PBS was used as naive. Mice were given 2 immunization at 6 weeks interval. Post 3 weeks after each first and second immunization, serum was collected by retro-orbital bleeding. At weeks 2 after the second immunization, mice were intranasally injected with 5 MLD_{50} of PR8 live virus in 30 μL of PBS buffer for challenge studies (Table 7), (Fig. 9). Challenged mice were monitored everyday for 2 weeks to record bodyweight and survival rate.

Quantification of antigen specific antibody

PR8 specific antibody response in serum was determined by enzyme-linked immunospecific assay (ELISA). 96-well plates were coated with coating buffer containing 1 μg/mL of PR8 inactivated virus at 4℃ over-night. Plates were blocked with 3% bovine serum albumin (BSA)

blocking buffer at 37℃ for 1.5 hours. Serum diluted with PBS buffer was added to plates and incubated at 37℃ for 1.5 hours. HRP conjugated Goat anti_mouse IgG diluted with PBS buffer (1:1000 dilution) was added to plates and incubated at 37℃ for 1 hour. For development, tetramethylbenzidine (TMB) substrate solution was added to plates and incubated at room temperature for 10 minutes. For stopping reaction, 0.5 M sulfuric acid was added to plates and incubated at room temperature for 3~5 minutes (Fig. 10). The optical density was measured at 450 nm using Multiskan™ FC Microplate Photometer (Thermo Scientific™, Waltham, USA).

Statistics

Statistical analyses were performed with GraphPad Prism software version 4. All data were presented as means ± SEM. For evaluating the statistical significance between control and treated group, Student’s t-test was performed. Values of $P < 0.05$ were considered to be significantly different.

Table 2. HA gene whole sequence

Gene	NCBI gene reference	Sequence
HA	CY084182.1	ggaaaataaa aacaaccaaa atgaaggcaa acctactggt cctgttatgt gcacttgcag ctgcagatgc agacacaata tgtataggct accatgcgaa caattcaacc gacactgttg acacagtact cgagaagaat gtgacagtga cacactctgt taacctgctc gaagacagcc acaacggaaa actatgtaga ttaaaaggaa tagccccact acaattgggg aaatgtaaca tcgccggatg gctcttggga aacccagaat gcgacccact gcttccagtg agatcatggt cctacattgt agaaacacca aactctgaga atggaatatg ttatccagga gatttcatcg actatgagga gctgagggag caattgagct cagtgtcatc attcgaaaga ttcgaaatat ttcccaaaga aagctcatgg cccaaccaca acacaaacgg agtaacggca gcatgctccc atgaggggaa aagcagtttt tacagaaatt tgctatggct gacggagaag gagggctcat acccaaagct gaaaaattct tatgtgaaca aaaaagggaa agaagtcctt gtactgtggg gtattcatca cccgcctaac agtaaggaac aacagaatct ctatcagaat gaaaatgctt atgtctctgt agtgacttca aattataaca ggagatttac cccggaaata gcagaaagac ccaaagtaag agatcaagct gggaggatga actattactg gaccttgcta aaacccggag acacaataat atttgaggca aatggaaatc taatagcacc aatgtatgct ttcgcactga gtagaggctt tgggtccggc atcatcacct caaacgcatc aatgcatgag tgtaacacga agtgtcaaac acccctggga gctataaaca gcagtctccc ttaccagaat atacacccag tcacaatagg agagtgccca aaatacgtca ggagtgccaa attgaggatg gttacaggac taaggaacat tccgtccatt caatccagag gtctatttgg agccattgcc ggttttattg aaggggggatg gactggaatg atagatggat ggtatggtta tcatcatcag aatgaacagg gatcaggcta tgcagcggat caaaaaagca cacaaaatgc cattaacggg attacaaaca aggtgaacac tgttatcgag aaaatgaaca ttcaattcac agctgtgggt aaagaattca acaaattaga aaaaaggatg gaaaatttaa ataaaaaagt tgatgatgga tttctggaca tttggacata taatgcagaa ttgttagttc tactggaaaa tgaaaggact ctggatttcc atgactcaaa tgtgaagaat ctgtatgaga aagtaaaaag ccaattaaag aataatgcca aagaaatcgg aaatggatgt tttgagttct accacaagtg tgacaatgaa tgcatggaaa gtgtaagaaa tgggacttat gattatccca aatattcaga agagtcaaag ttgaacaggg aaaaggtaga tggagtgaaa ttggaatcaa tggggatcta tcagattctg gcgatctact caactgtcgc cagttcactg gtgcttttgg tctccctggg ggcaatcagt ttctggatgt gttctaatgg atctttgcag tgcagaatat gcatctgaga ttagaatttc agagatatga ggaaaaacac

Table 3. M1 gene whole sequence

Gene	NCBI gene reference	Sequence
M1	CY147535.1	atgagtc ttctaaccga ggtcgaaacg tacgtactct ctatcatccc gtcaggcccc ctcaaagccg agatcgcaca gagacttgaa gatgtctttg cagggaagaa caccgatctt gaggttctca tggaatggct aaagacaaga ccaatcctgt cacctctgac taaggggatt ttaggatttg tgttcacgct caccgtgccc agtgagcgag gactgcagcg tagacgcttt gtccaaaatg cccttaatgg gaacggggat ccaaataaca tggacaaagc agttaaactg tataggaagc tcaagaggga gataacattc catgggcca aagaaatctc actcagttat tctgctggtg cacttgccag ttgtatgggc ctcatataca acaggatggg ggctgtgacc actgaagtgg catttggcct ggtatgtgca acctgtgaac agattgctga ctcccagcat cggtctcata ggcaaatggt gacaacaacc aatccactaa tcagacatga gaacagaatg gttttagcca gcactacagc taaggctatg gagcaaatgg ctggatcgag tgagcaagca gcagaggcca tggaggttgc tagtcaggct agacaaatgg tgcaagcgat gagaaccatt gggactcatc ctagctccag tgctggtctg aaaaatgatc ttcttgaaaa tttgcaggcc tatcagaaac gaatgggggt gcagatgcaa cggttcaagt ga

Table 4. Thermal cycler schedule of RT-PCR for producing HA and M1 gene

Step		Temperature (℃)	Time
RT	RT	50	30 min
Hold	Hold	95	2 min
3 step PCR (40 cycle)	Denaturation	95	5 sec
	Annealing	60	15 sec
	Extension	70	15 sec
Hold		4	Indefinitely

Table 5. Sequence – specific of primer

Gene	NCBI gene reference		Primer sequence (5'-3')
HA	CY084182.1	S	AGGATGAAGGCAAACCTACTG
		AS	AGGAAGCTTTCAGATGCATATTCTGCAC
Matrix 1	CY147535.1	S	AGGTCTAGAATGAGTCTTCTAACCGAGGT
		AS	AGGCTCGAGTCACTTGAACCGTTGCATC

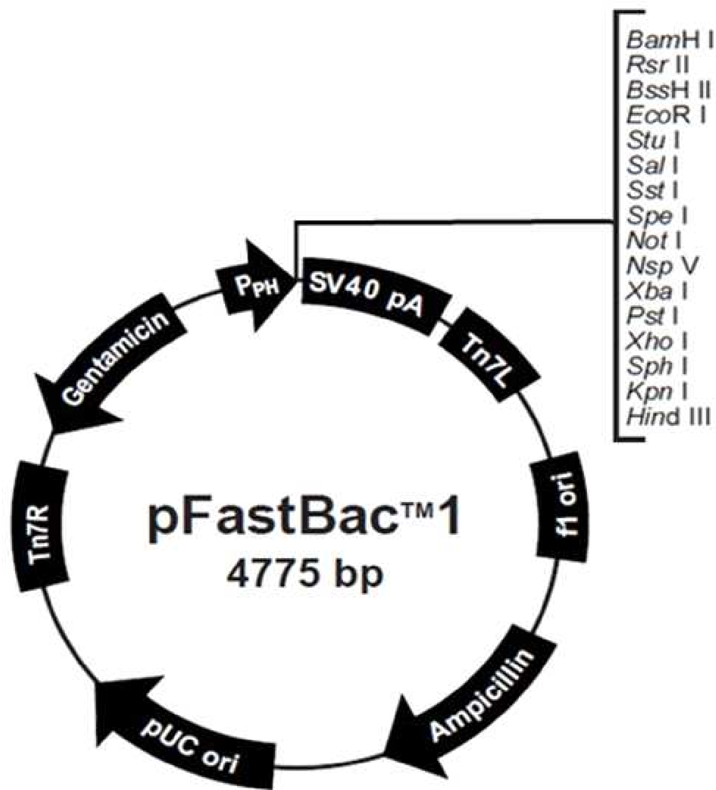


Figure. 4. Structure of pFastBac1 vector

HA and M1 gene was transformed into DH5α using pFastBac1 vector between XbaⅠ and HindⅢ site and between XbaⅠ and XhoⅠ site, respectively.

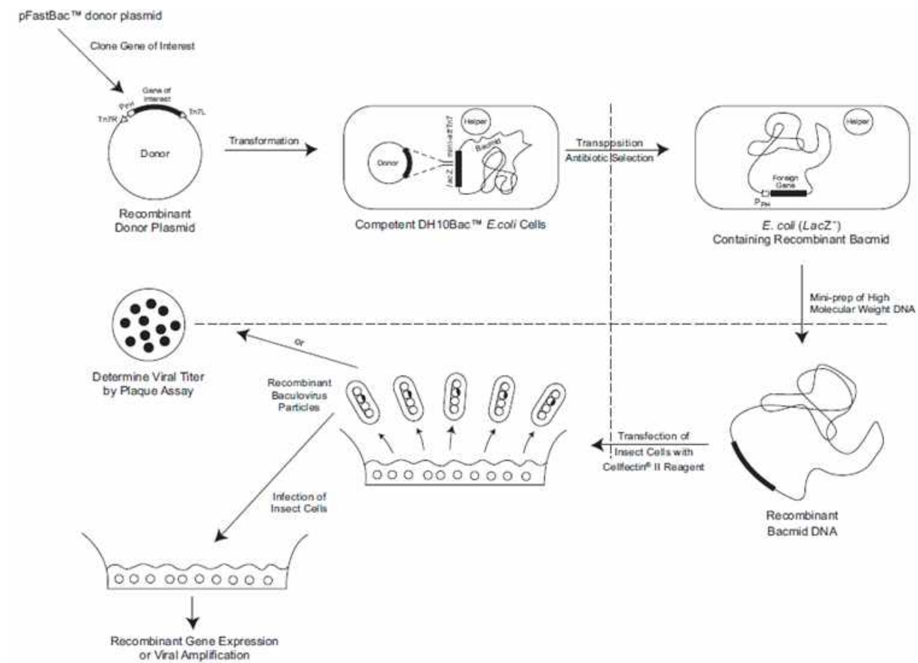


Figure. 5. Production of recombinant baculovirus expressing HA and M1

Each recombinant bacmid encoding HA and M1 gene was transfected into sf9 cells and sf9 cells were infected with recombinant baculovirus for amplifying volume.

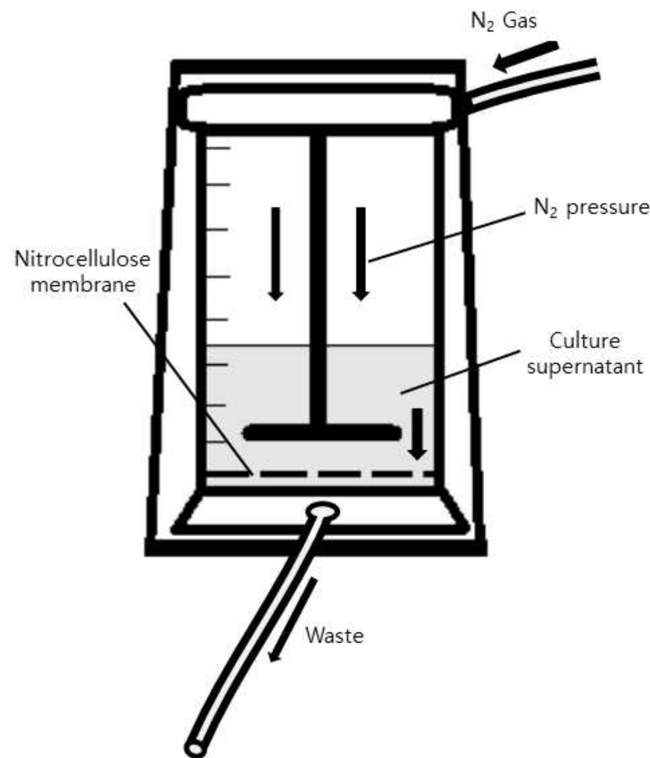


Figure. 6. Concentration of VLPs by NCFS using stirred cell

VLPs in supernatants were given pressure by N_2 gas so that concentrated by NCFS using stirred cell. Because VLPs are complex containing several of HA and matrix protein, VLPs are bigger than 100 kDa. So through 100 kDa MWCO membrane, other components less than 100 kDa were filtered.

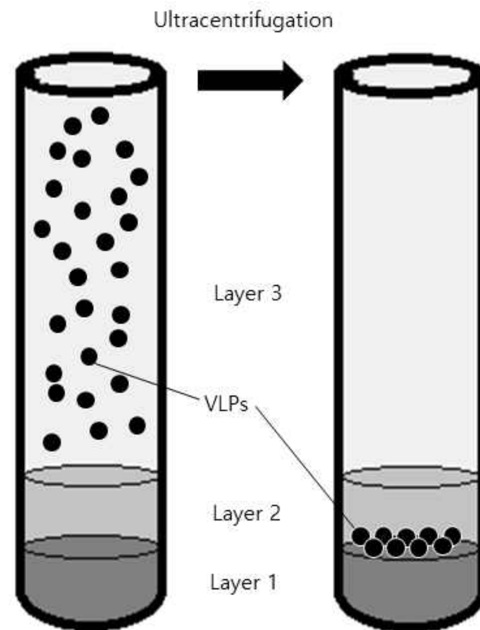


Figure. 7. Purification of VLPs by sucrose gradient
After concentration, VLPs were ultracentrifuged using 20%-60% sucrose gradient. Purified VLPs were carried between 20%-60% layers. Layer 1: 60% sucreose gradient; Layer 2: 20% sucrose gradient; Layer 3: supernatants.

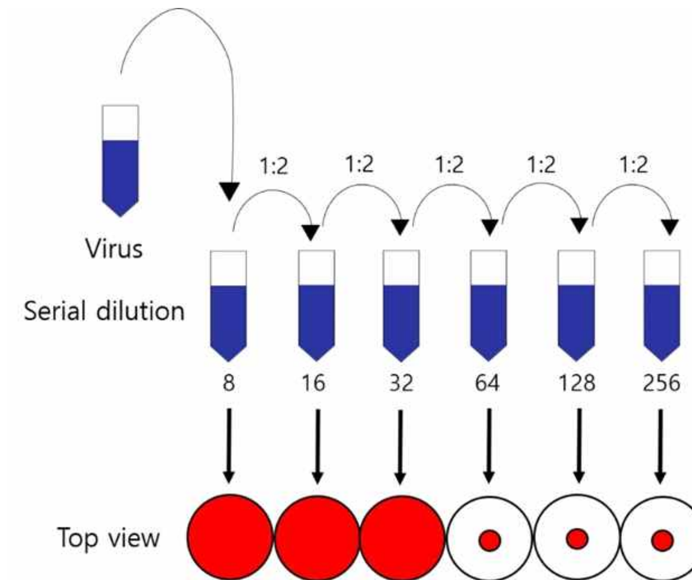


Figure. 8. Schematic diagram of Hemagglutination assay
HA titers of VLPs exposed to temperature was confirmed by hemagglutination assay. A series of two-fold dilution was performed with PBS buffer and incubated with 0.5% chicken red blood cells.

Table 6. Temperature condition for reaction with VLPs

Temperature (℃)	**Time**				
	1 Hour	6 Hours	1 Day	7 Day	1 Month
4	O	O	O	O	O
25	–	–	–	O	O
42	O	O	O	O	O

O: VLP was reacted in which condition; -: VLP was not reacted in which condition.

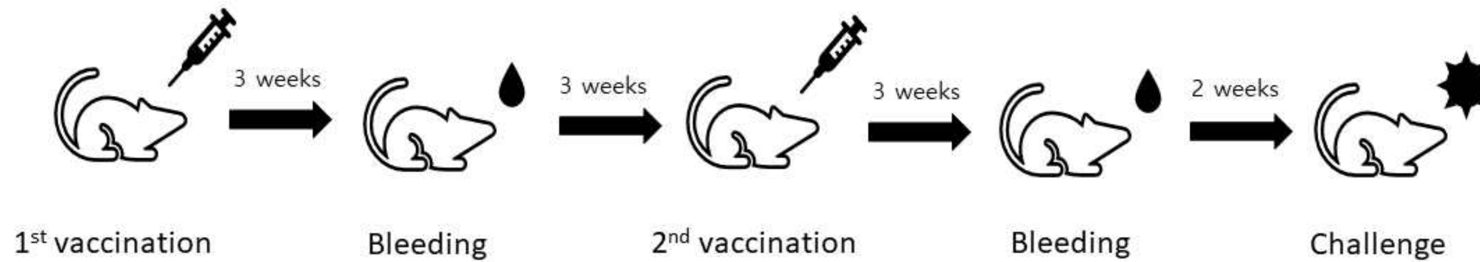


Figure. 9. Schematic diagram of immunization and challenge

Six weeks old female BALB/c mice (n=6) were given 2 immunization intramuscularly at 6 weeks interval with VLP exposed to temperature. Mice serum was collected by retro-orbital bleeding after 3 weeks from first and second vaccination. Post 2 weeks after second vaccination, mice were immunized with PR8 live virus for challenge studies.

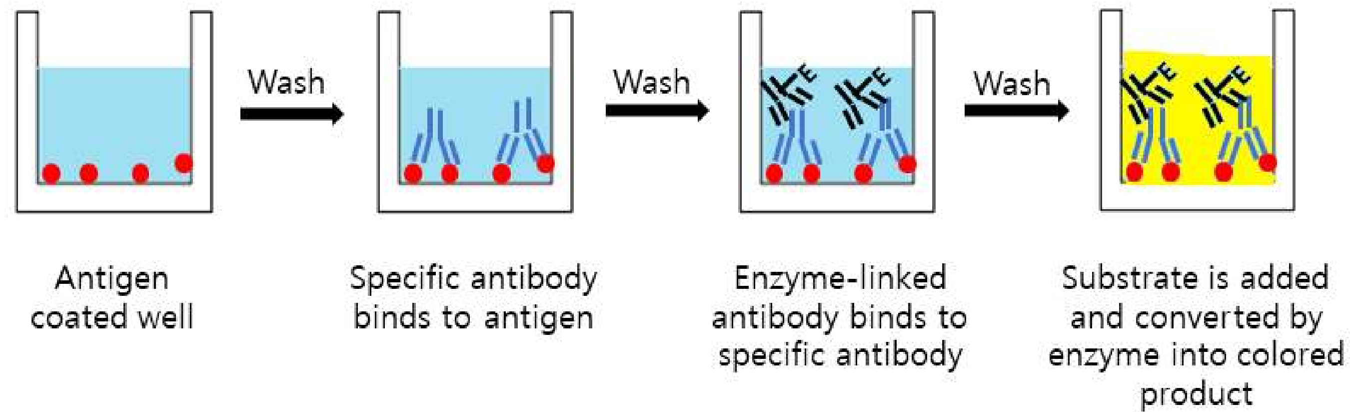


Figure. 10. Procedure of enzyme-linked immunospecific assay

PR8 specific antibody response in mice serum was confirmed by enzyme-linked immunospecific assay. 96-well plates were coated with PR8 inactivated virus as antigen and blocked with blocking buffer. After washing, mice serum was added to plates as specific antibody which binds to antigen. After washing, HRP conjugated goat anti_mouse IgG was added to plates. After washing, TMB substrate solution was added following stopping solution. The optical density was measured at 450 nm.

RESULTS

Characterization of PR8 VLPs

Influenza PR8 VLPs expressing HA and M1 were produced by baculovirus-insect cell expression system and concentrated by NCFS using stirred cell. After purification of VLPs by sucrose gradient with ultracentrifugation, total volume of extracted VLPs from sucrose was 2.9 mL, protein concentration was 20 mg/mL and HA titer was 4096 HAU/50 μL (Table 8). Expression of HA and M1 protein in PR8 VLP was confirmed by western blotting analysis and observed in size of 75 kDa and 25 kDa, respectively (Fig. 11). Theses results indicate that VLPs expressing PR8 influenza HA and M1 protein were effectively produced by baculovirus-insect cell expression system.

Table 7. VLP sample profile after purification

	Volume (mL)	Protein concentration (mg/mL)	HA titer (HAU/50μL)
PR8 VLP	2.9	20	4096

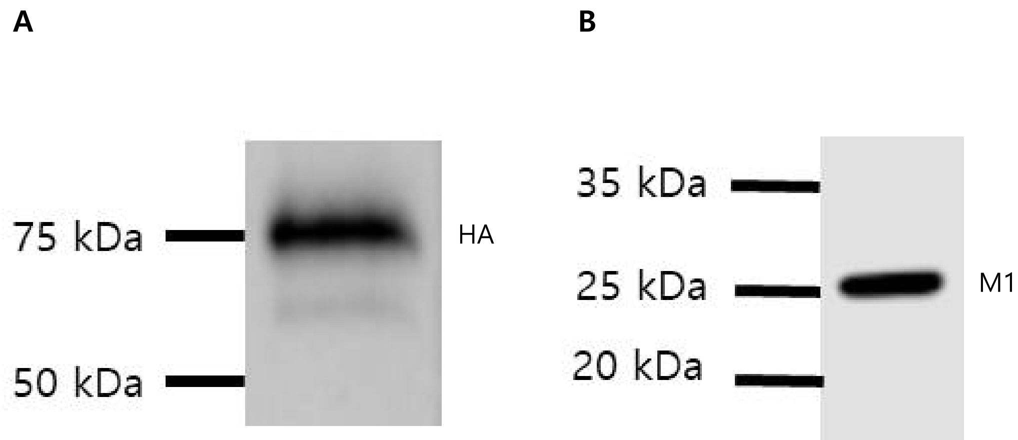


Figure. 11. Western blot analysis of PR8 VLPs
HA protein in PR8 VLPs was detected by western blotting using PR8 inactivated virus immunized serum followed by detection with HRP-conjugated IgG secondary antibody. The expected molecular weights of HA is 75 kDa (A). M1 protein in PR8 VLPs was detected by western blotting using mAb mouse anti_influenza M1 IgG followed by detection with HRP-conjugated IgG secondary antibody. The expected molecular weights of M1 is 25 kDa (B).

HA titer of VLPs after exposure to temperature

VLP exposed at 4℃ was used as positive control. At day 1, HA titer of all temperature condition showed 128 HAU/50 μL. At day 7, HA titer of 4℃ and 25℃ was similar without any significant difference and 42℃ showed huge decrease. At month 1, HA titer of 25℃ showed slightly decrease compared to 4℃ and 42℃ showed huge decrease (Fig. 12). It means that whenever VLPs were exposed to high temperature, HA titer was more decreased in vitro.

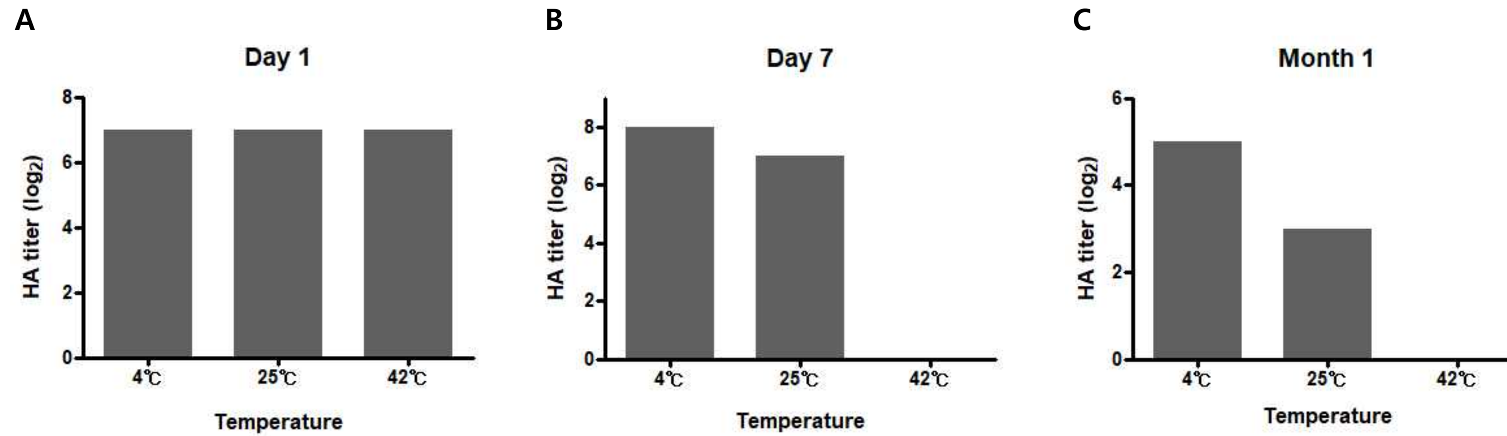


Figure. 12. HA titer of virus-like particles after exposed to temperature

VLPs were exposed at 4℃, 25℃ and 42℃ for day 1, day 7 and month 1 (A-C). Serial dilutions were made with 10^{11} to 10^{0} copies. At day 7 and month 1, titers in 42℃ groups were significantly decreased.

Immune response after first immunization

The group of mice immunized with VLPs exposed at 4℃ was used as positive control. Antibody response of mice immunized with VLPs exposed at 25℃ for month 1 and day 7 showed similar antibody response without significant difference. The group of mice immunized with VLPs exposed at 42℃ for month 1, day 7 and day 1 showed significant difference between each of days. Antibody response of 42℃ in month 1 especially showed significant decrease compared with day 7 and day 1. And antibody response of 25℃ group was two fold higher or nearly two fold than 42℃ group (Fig. 13). These results mean that the more VLP is exposed to high temperature, the more vaccine effectiveness of VLP is decreased.

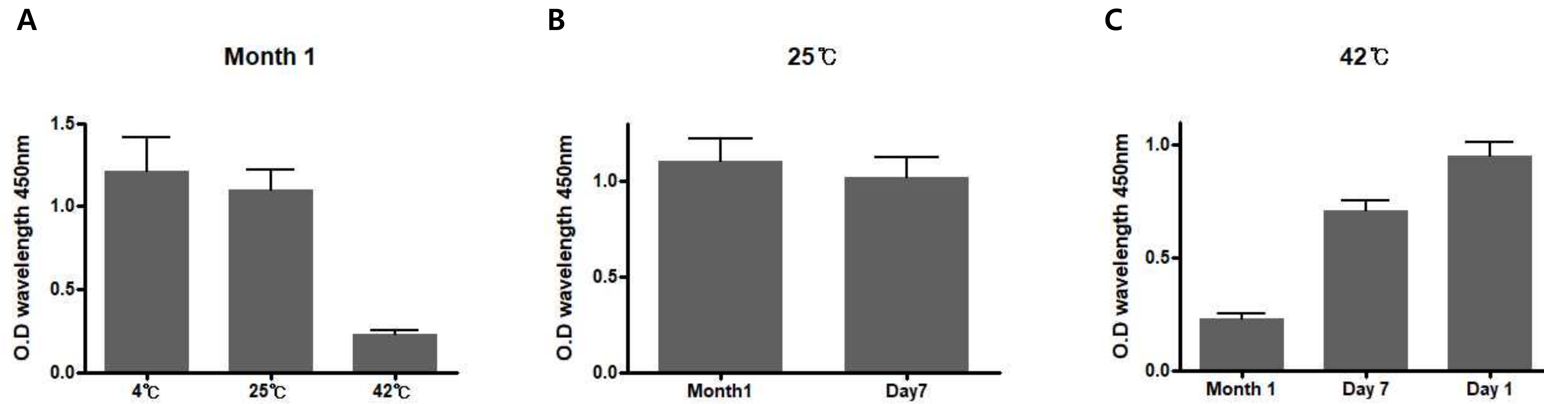


Figure. 13. IgG response in immunized mice serum after first immunization

After first immunization with 2.6 μg VLP exposed to temperature, IgG antibody response was detected by ELISA using immunized mice serum. 4℃ was used as positive control. (A) IgG response in month 1 was collected for compared with positive control more briefly. (B)-(C) IgG response in 25℃ and 42℃, respectively. IgG response level of 42℃ groups in month 1 were significantly different from 4℃ and 25℃.

Immune response after boost immunization

After second immunization for boost, antibody response of all groups was almost two fold higher or more than first immunization (Fig. 14). It means that second immunization makes enhance efficacy of vaccinization than vaccinization at once.

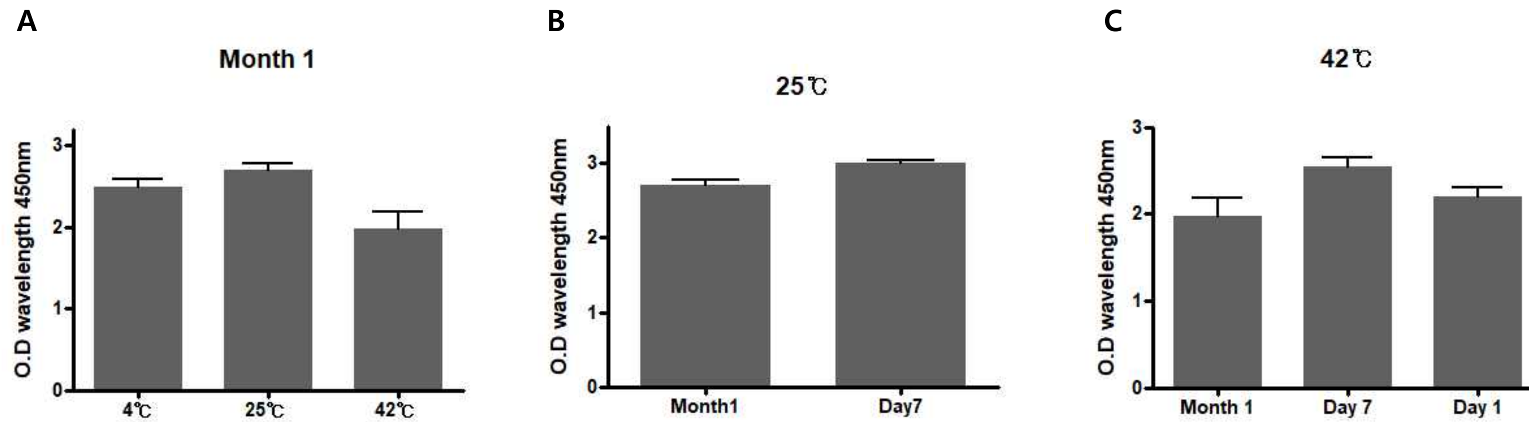


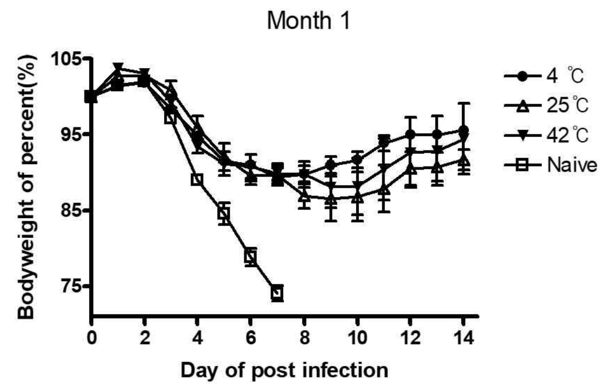
Figure. 14. IgG response in immunized mice serum after second immunization

After second immunization for boost, IgG antibody response was detected by ELISA using immunized mice serum. 4℃ was used as positive control. (A) IgG response in month 1 was collected for compared with positive control more briefly. (B)-(C) IgG response in 25℃ and 42℃, respectively. IgG response level of all groups was significantly increased.

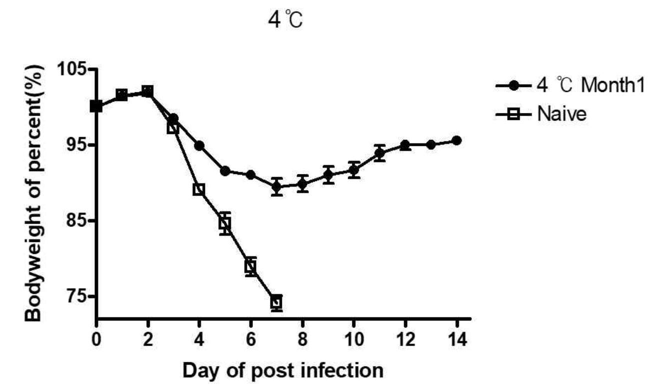
Vaccinization with PR8 VLPs exposed to temperature condition protects mice from challenge

The group of mice immunized with VLPs exposed at 25℃ for month 1 and day 7 lost approximately 15% and 10% bodyweight, respectively. The 42℃ group showed less than 9%, 8% and 8% loss in bodyweight. All mice survived after lethal virus challenge. Mice in the naive group died from lethal challenge (Fig. 15). These results suggest that the VLPs retain capacity of vaccine while expose to room or high temperature.

A



B



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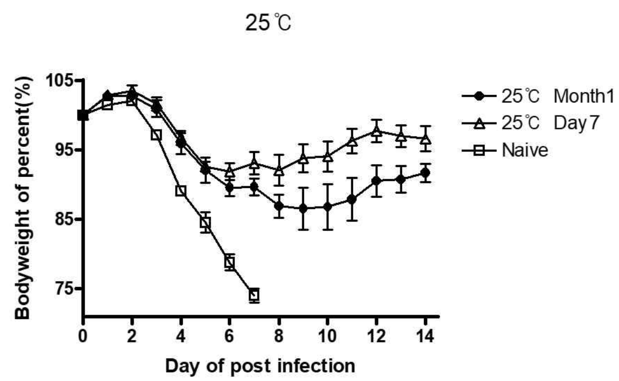

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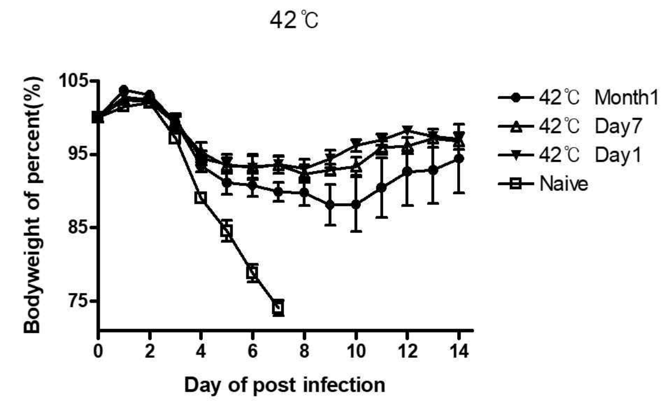

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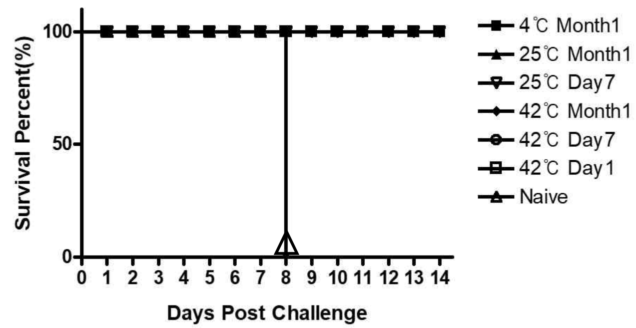

Figure. 15. Protective effect of immunization against lethal dose challenge

Protection of mice from PR8 live virus challenge. Post 2 weeks second immunization, each group of mice (n=5) was intranasally infected with 5 MLD_{50} of PR8 live virus. Daily bodyweight change of the immunized with VLPs exposed to 4℃ (B), 25℃ (C), and 42℃ (D) were shown. (A) Bodyweight change of month 1 was collected for compared with positive control more briefly. (E) Survival rate after challenge.

DISCUSSION

In 2009, considerable amounts of H1N1 vaccine were needed because influenza A virus, H1N1, spread widely to many other countries. But yield of vaccines produced by egg-based system was low and insufficient. In previously studies, to overcome these limitations, VLP vaccine platform was developed as alternative vaccines because VLP can be produced from such as yeast, insect or mammalian cell and also induce high neutralizing antibody response and strong protective immunity (3)(21). Because most influenza virus has Hemagglutinin (HA) and Neuraminidase (NA) as an antigen, most of VLP have been made using HA and/or NA or other viral protein. VLPs are made up of protein including components of constructure that are affected by thermal condition. In this study, H1N1 VLPs were prepared using baculovirus-insect cell expression system and thermal stability of H1N1 VLPs were identified. VLPs were confirmed by western blot analysis and HA assay. After confirmation, VLPs were preserved in temperature and humidity chamber for exposing with temperature condition. In vitro, immunogenicity was confirmed by HA assay. 4℃ group was used as positive control and its HA titer was the highest compared with other group. HA titer of 25℃ group was decreased

gradually by the day. 42℃ had no HA activity on day 7 and month 1. These results mean that high temperature affects HA activity of VLPs, in vitro. For confirmation in vivo, animal experiments was performed. BALB/c mice were immunized twice using VLPs exposed to temperature. Serum of mice was collected for identifying antibody response. At first bleeding, 25℃ induced significant level of IgG response but 42℃ showed low IgG titer, especially in month 1. After second immunization, both 25℃ and 42℃ were boosted ~2-3 fold. These data indicate that VLP is affected with 42℃ temperature compared with 25℃ but second immunization results in potential immune response. Moreover, all group of immunized mice lost weight but subsequently recovered health conditions. And all challenged mice showed complete protection against PR8 live virus challenge. Naive group had no protection as expected. These data indicate that VLPs provides sufficient protection against viral infection. Collectively, results of in vitro are identical with in vivo and it means that VLPs conserved immunogenicity against thermal condition. In conclusion, this study indicate that VLPs could maintain considerable stability at various temperature condition.

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ABSTRACT

Influence of temperature on the antigenic stability of influenza virus-like particles

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Influenza A virus causes recurrent outbreaks and results in high risk to human. To prevent the infection of influenza, vaccination is the most effective way to control infection of influenza and vaccines have been developed. However, traditional vaccine platforms have several limitations because of egg-based production. To overcome limitations, virus-like particle (VLP) which is cell-based has been promised as an alternative vaccine platform. VLP of influenza virus elicits immune response by viral protein including hemagglutinin, neuraminidase and matrix protein that can be affected by temperature. Therefore, it is needed to identify thermal stability of VLPs for using VLPs as vaccine. In this study, using strain

A/Puerto Rico/8/1934 (PR8), influenza H1N1 PR8 VLPs were produced by baculovirus-insect cell expression system. Expression of VLPs was confirmed by western blot analysis. VLPs were exposed to temperature & humidity chamber at 4℃, 25℃, and 42℃. Immunogenicity of VLPs exposed to temperature was confirmed by hemagglutinin (HA) assay. HA titer of 42℃ was significantly decreased as exposed for a long time. BALB/c mice (n=5) were immunized twice with VLPs exposed to temperature and serum was collected by retro-orbital bleeding. IgG response was detected using immunized mice serum by enzyme-linked immunospecific assay (ELISA). After first immunization, IgG response of 42℃ group in month 1 was significantly decreased than 4℃ and 25℃. After second immunization, IgG response of all groups was almost two fold higher or more than first immunization. After lethal virus challenge, all mice survived. These results indicate that when VLPs were exposed to 4℃, 25℃, and 42℃ for a month, significant immunogenicity can be elicited by twice immunization.

감사의 글

내가 대학원을 정말 졸업 할 수 있을까, 내가 정말 논문을 쓸 수 있을까 하던 날이 엊그제 같은데, 이렇게 논문을 다 끝마치고 이렇게 감사의 글을 적고 있습니다. 참 많은 어려움도 있었고 즐거움도 있었습니다. 대학원 생활을 하면 할수록 부족함밖에 없는 나의 모습에 자신감을 잃고 낙담하던 적도 있었고, 중간에 포기할까도 수없이 고민하던 적이 있었습니다. 하지만 주변의 많은 도움들 덕에 무사히 여기까지 올 수 있었고, 도움주신 그 모든 분들에게 참으로 감사합니다.

먼저, 부족함 투성인 저를 이끌어주시고, 저의 고민을 다 들어주시며 대학원 생활을 잘 마무리 할 수 있게끔 격려해주신 지도교수님이신 송재민 교수님께 너무나 감사드립니다. 교수님께서 보내주신 격려와 응원이 무색해지지 않도록 졸업 후 겪어가게 될 과정 또한 열심히 나아가도록 하겠습니다. 그리고 항상 뒤에서 응원해주시고 챙겨주시는 전민영 교수님, 갑작스런 심사 부탁에도 친절히 받아주시고 많은 조언을 해주신 강창수 교수님께도 정말 감사드립니다.

2년간 즐거운 일, 힘든 일 등 모든 것을 함께 한 연구실 식구들에게도 고마움을 전합니다. 실험적으로 정말 많은 도움주시고, 항상 챙겨주시며 함께 고민해주신 이선화 박사님 정말 감사합니다. 그리고 바쁘신 와중에 항상 모든 것을 자신의 일처럼 챙겨주시고, 함께 고민해주시고 위로해주신 다빈선배 정말 감사드려요. 그리고 귀찮을 정도인 나의 질문에도 항상 웃으며 자세히 설명해주고 답해준 영찬오빠. 오빠 일도 정말 바쁜데 하는 부탁마다 거절 없이 모두 도와줘서 정말 고맙고, 오빠의 남은 과정 응원할게! 무엇보다 나의 너무나 큰 버팀목이었던 소화. 너의 도움과 응원, 그리고 함께 웃고 떠들던 시간이 없었다면 내가 이렇게 무사히 이 과정을 마치지 못했을 거야. 너무나 고맙고, 너의 남은 과정 너무나 응원한다! 이 외에도 대학원 생활 중 함께 했던 모든 분들에게 감사인사를 드립니다.

마지막으로 그 누구보다 진심으로 응원해주시고 기도해주신 부모님과 그 누구보다 저를 아껴주시고 안부를 물어주신 할머니, 그리고 뒤에서 항상 멋지다, 힘내라 응원해준 오빠 모두에게 감사의 인사를 드립니다.