A Dissertation
for the Degree of Master of Science

Development and characterization of trivalent subunit vaccine for defending various serotypes of Foot-and-Mouth disease viruses

다양한 혈청형의 구제역 바이러스 방어를 위한 3가형 아단위 백신의 개발 및 특성규명

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Summary

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals like pig, cow, horse, sheep and goat. It is classified number 1 communicable disease and International Enzootic des Office (OIE) is trying to control its contagion to abroad. Clinical symptoms of FMD are the development of lesions resembling blisters on the feet and around of the mouth, fever, depression and lameness. Adult animals can recover from FMD within two weeks after infection. But, because of high contagion and mutant, slaughter of infected and susceptible animals is came into action and it causes economic losses to livestock industry. The country which is not recognized as FMD-free by OIE is placed restrictions on international trade. DIVA (Differentiation Infected from Vaccinated Animals) problem is a main reason of restriction on international trade. Vaccine used in farm is live attenuated or inactivated FMD vaccine. Because live attenuated or inactivated FMD vaccine contains non structural proteins, it is impossible to distinguish live attenuated or inactivated vaccinated animals from infected animals. In addition, live attenuated or inactivated FMD vaccine can't defend various serotypes and subtypes of FMDV.

To overcome disadvantages of live attenuated or inactivated FMD vaccine, subunit vaccine strategy was introduced. Because producing subunit vaccine is simple, disease control is more faster than live attenuated or inactivated vaccine. By not using non structural protein of FMDV, it is possible to distinguish

subunit vaccinated animals from infected animals. But the main problem of subunit vaccine is low immunogenecity. To overcome low immunogenecity of subunit vaccine, we introduced multi-epitope vaccine strategy which is linked epitopes having high antigenecity. Also, to defend various serotypes and subtypes of FMDV, trivalent vaccine strategy was introduced. Serotype O, serotype A and Asia-1's three epitopes of three subtypes were combined, respectively. As a result three vectors were constructed; recombinant protein of serotype O was named O9BT, recombinant protein of serotype A was named A9BT and recombinant protein of Asia-1 was named I9BT.

We used *E. coli* expression system to produce trivalent multi-epitope subunit vaccine. Multi-epitope subunit vaccine is not a native structure of FMDV but a artificial form of vaccine. The problem of artificial vaccine is that it is expressed as a form of inclusion bodies. Because trivalent multi-epitope subunit vaccine we constructed also expressed as a form of inclusion bodies, we introduced solubilization methods; chaperone co-expression system and alkaline-pH acetone precipitation. Production efficiency of soluble O9BT was the highest among three recombinant proteins, we used O9BT to compare solubilized proteins with soluble proteins. First, chaperone co-expression system was used to induce production of soluble protein by delaying proteins folding or transforming misfolded and aggregated proteins. Because pTf16, which delays protein folding when it is extruded from exit site of ribosome, was the most effective among five types of chaperones plasmids, it was

selected for mass production and *in vivo* immunization. Second, inclusion bodies were solubilized by introducing alkaline-pH acetone precipitation. After solubilization by alkaline buffer (> pH 12.5), cold acetone was added to precipitate proteins. Precipitated proteins can solubilize in PBS or D.W because of protein refolding. Especially alkaline-pH acetone precipitation uses inclusion bodies, so it's production efficiency is higher than other production methods. We compared solubilized proteins produced by chaperone co-expression system and alkaline-pH acetone precipitation with soluble proteins through *in vivo* immunization. Regardless of solubilization methods, soluble proteins induced similar immune responses. Finally, alkaline-pH acetone precipitation was selected for trivalent multi-eptitope subunit vaccine production. Because it's production efficiency was 4 times higher than other methods and it didn't need purify step.

Trivalent multi-epitope subunit vaccine (O9BT, A9BT, I9BT) were produced by alkaline-pH acetone precipitation and 20 μg or 45 μg of cocktail trivalent multi-epitope subunit vaccine was injected to mice intramuscularly. Even though amount of each protein were reduced one third, the efficiency of trivalent multi-epitope subunit vaccine corresponded to monovalent multi-epitope subunit vaccine. Expecially, in neutralization assay, only 20 μg of trivalent multi-epitope subunit vaccine was effective. Interestingly, the vaccine efficiency was not necessarily proportional to amount of vaccine, because 20 μg and 45 μg of trivalent multi-epitope subunit vaccine showed similar immune responses.

We have shown through this study that trivalent multi-epitope subunit vaccine can overcome the antigenic variation of FMDV. In order to be used as a practical commercial vaccine, additional experiments such as a neutralizing antibody assay and challenge assay will be necessary. But it is expected that trivalent multi-epitope FMD subunit vaccine can increase immunogenecity of subunit vaccine and effectively defend various serotypes of FMDV in livestock industry.

Keywords : Foot-and-mouth disease, subunit vaccine, multi-epit ope, trivalent vaccine, chaperone co-expression system, alkaline-pH acetone precipitation, inclusion bodies, solublization

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List of Abbreviations

BCA : Bicinchoninic acid

bp : Base pair

BSA : Bovine serum albumin

CCV : Clathrin-coated vesicle

CFA : Complete freund's adjuvant

E. coli : *Escherichia coli*

EDTA : Ethylenediaminetetraacetic acid

ELISA : Enzyme-linked immunosorbent assay

FBS : Fetal bovine serum

FMD : Foot-and-mouth disease

FMDV : Foot-and-mouth disease virus

His : Histidine

HRP : Horseradish peroxidase

iFMDV : Inactivated FMD vaccine

IBs : Inclusion bodies

IFA : Incomplete freund's adjuvant

IPTG : Isopropyl β-D-1-thiogalactopyranoside

kb : Kilobase

kDa : Kilodalton

LB : Luria-Bertani

NSPs : Non structural proteins

OD : Optical density

OIE : International Enzootic des Office
(World Organization for Animal Health)

P/S : Penicillin-Streptomycin

PBS : Phosphate-buffered saline

PBST : Phosphate-buffered saline containing 0.1% Tween 20

PCR : Polymerase chain reaction

PI : Percentage inhibitor titer

RGD : Arginylglycylaspartic acid (Arginine-Glycine-Aspartate)

RPMI : Roswell Park Memorial Institute medium

SAT : South African Territories

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPs : Structural proteins

TBST : Tris-buffered saline containing 0.1% Tween 20

Th1 : Type 1 T helper

Th2 : Type 2 T helper

TMB : 3,3′,5,5′-Tetramethylbenzidine

Ⅰ. Introduction

Foot-and-mouth disease (FMD) is a highly transmissible vesicular disease of cloven-hoofed animals. It is economically devastating disease and classified number 1 communicable disease. Foot-and-mouth disease virus (FMDV) is etiological agent of FMD. FMDV is classified into seven serotypes; serotype O, serotype A, serotype C, Asia-1, SAT-1, SAT-2, SAT-3. In addition, there are numerous subtypes within one serotype and FMDV is continuously mutating and evolving. Antigenic variation of FMDV makes it difficult for commercial live attenuated or inactivated FMD vaccine to protect animals from disease. Also, it is difficult to differentiate infected animals from vaccinated animals. In this aspect, FMD subunit vaccine strategy is developed and commercially have been used in China (Li and Liu, 2011).

However, subunit vaccine also has some limitation for practical application. Immunogenecity is lower than inactivated vaccine and antigenic spectrum is narrow. So, enhancement of immunogenecity and antigenic spectrum is important for FMD subunit vaccine development.

To increase antigenic spectrum of FMD subunit vaccine, we used trivalent subunit vaccine strategy. We designed three types of subunit vaccine corresponding to serotype O, serotype A and Asia-1. To improve immunogenecity, we used multi-epitope strategy. In previous study, it was proved that FMDV serotype O multi-epitope vaccine has cross-protection effects against three topotypes of FMDV serotype O (Cao *et al.,* 2014). The subunit

vaccine applied two strategy is called trivalent multi-epitope subunit vaccine. Each multi-epitope subunit vaccine contains nine B-cell epitopes and one T-cell epitope from SPs region of FMDV. Selected epitopes are known as having high antigenecity. For example, VP1 G-H loop is representative epitope of FMDV, because FMDV infects to host mucosal epithelial cell through integrin receptor by binding with RGD motif of VP1 G-H loop in FMDV (Fowler *et al.,* 2010). Because T-cell epitope can present the processed antigen from dendritic cells to B-cells and induce differentiation, proving T-cell epitope is important for enhancing immunogenecity (Blanco *et al*., 2013).

In this study, we used *E. coli* expression system to produce trivalent multi-epitope subunit vaccine and the recombinant protein expressed as a form of inclusion bodies. In the first study, we tried to solubilize inclusion bodies and compare proteins produced by various methods; soluble protein, chaperone-assisted protein, alkaline-pH acetone precipitated protein and inclusion bodies. Production efficiency, protein purification and immune responses were evaluated. In the second study, the broaden antigenic spectrum of alkaline-pH acetone precipitated trivalent multi-epitope subunit vaccine was evaluated by vaccinating mice with monovalent or trivalent multi-epitope subunit vaccine.

Ⅱ. Review of Literature

1. Foot-and-mouth disease

1) Feature of foot-and-mouth disease

Foot-and-mouth disease (FMD) is a highly contagious and devastating disease of cloven-hoofed animals which lead to severe economic losses to affected countries (Sutmoller *et al.,* 2003). It is classified number 1 communicable disease and International Enzootic des Office (OIE) is trying to control its contagion to abroad (Grubman and Baxt, 2004). FMD-free countries are trying to control the disease by vaccination, international trade regulation, slaughter of infected and susceptible animals and movement controls. Also vaccination often plays a key role to control the disease in endemic countries where slaughter and movement are not always realistic (Kitching *et al.,* 2005). Outbreak of FMD in a FMD-free country can lead to significant economic losses because of morbidity and limitation of international trade with other FMD-free countries (Knowles and Samuel, 2003).

Clinical symptoms of FMD are the development of lesions resembling blisters on the feet and in and around of the mouth, fever and lameness. Lesions may also appear on the mammary gland of females, so it causes a sudden decrease of milk production. After 1~3days, lesions rupture and create painful maceration. Above 90% of mortality rates are recorded among young livestock, because FMDV has a penchant for heart tissue

of young livestock and its replication there could cause collapse of heart function which leads to heart failure and death. But the mortality rates of adult livestock is under 3%, because adult livestock can recovery from disease within 2 weeks (Belsham *et al.,* 2015). Ruminant animals like cattle, sheep and goats can become carriers among livestock species. Pigs can have detectable viral RNA and capsid proteins in lymph nodes for an extended period, but they are not considered to become carriers. Vaccination does not prevent the carrier establishment and its state is independent of host immune status. Also the duration of carriage depends on the host species; sheep can carry viruses for 9 months, cattle for 3 years and african buffalo for 5 years. But the fact that carriers paly a key role in initiating new outbreaks is still disputed (Dekker *et al.,* 2008)

2) Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is a member of the genus *Aphthovirus* of the family Picornaviridae. Picornaviruses only infect birds and mammals. FMDV has seven serotypes; serotype O, serotype A, serotype C, SAT-1, SAT-2, SAT-3 and Asia-1. Each serotype has multiple subtypes reflecting significant antigenic variation. FMD viral genome is approximately 8.5 kb long and one molecule of single-stranded RNA. Viral genome encodes four structural proteins named SPs (VP1, VP2, VP3 and VP4) and ten mature non-structural proteins named NSPs (L, 2A, 2A, 2C, 3A, 3B, 3C, 3D, 3AB and 3ABC) (Ding *et al.,* 2013). This viral genome is surrounded by single-layered protein shell

which is called capsid. The viral RNA genome play a role of messenger RNA, and polyprotein which is encoded by a single long open reading frame produce the viral protein's entire complement. After that, virus-encoded protease cleaved viral proteins. The genome has genome-linked viral protein at 5' end and poly (A) tail at 3'-terminus (Belsham *et al.,* 2015).

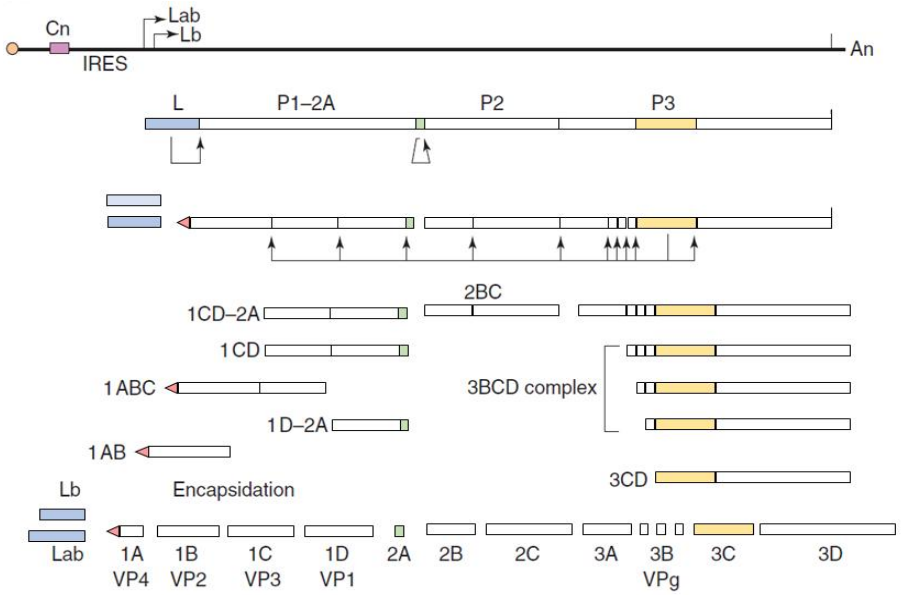

Figure 1. Scheme of foot-and-mouth disease virus proteins synthesis (Belsham *et al.*, 2015).

3) Infection of the host cell

By repeating cell infection and release cycles, FMDV can proliferate itself like most animal viruses. Because FMDV viral RNA has all the information needed to initiate productive viral infection, the aim of infecting virus particle is to release viral RNA and deliver it to the cytoplasm where protein production

and RNA replication occur. Virus particle uses a flexible RGD motif to bind host cell receptor on the surface of mucosal epithelial cells, identified as integrin αvβ1, αvβ3 αvβ6 and αvβ8. Especially αvβ6 showed high affinity with RGD motif (Jackson *et al.*, 2003). Then, the virus receptor complex captured by clathrin-coated vesicle (CCV) fuses with early endosome whose environment is relatively acidic. These conditions trigger capsid disassembly and viral ribonucleic acid is released and delivered to the cytoplasm. Before cell lysis and virus release, viral protein synthesis, RNA replication and particle assembly occur in advance (Belsham *et al.,* 2015).

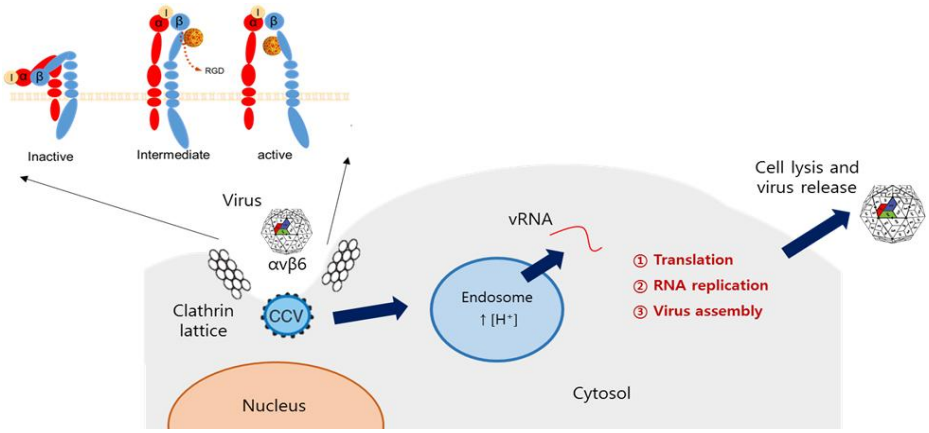

Figure 2. Infection route of FMDV (Belsham *et al.*, 2015).

4) Serotypes

(1) Serotype O

Among seven serotypes of FMDV, serotype O is internationally the most prevalent and widely distributed, especially throughout the Middle East-South Asia (ME-SA) region (Valarcher *et al.*,

2004). Three topotypes of serotype O were provoked in Korea and its neighbouring countries. O/Mya/98 strain, the Southeast Asia (SEA) topotype of serotype O, caused severe outbreaks of FMD during 2010 to 2011 in Korea, China, Japan, Mongolia and Russia (Valdazo-González *et al.*, 2013). During 2000 to 2006, Korea, Japan, Mongolia, Russia, France, United Kingdom and South Africa reported the outbreak of PanAsia topotype of serotype O (Knowles *et al.,* 2005). In 2001, the outbreak of PanAsia topotype of serotype O was observed in China. The cathay topotype of serotype O which also called the porcinophilic strain still remains in some regions of China and its neighbouring countries. Especially cathay topotype of serotype O can infect ruminant animals without any clinical symptoms. These three topotyepes (SEA, PanAsia and Cathay) is a major threats to animal health in Middle East-South Asia. Because major neutralizing antigenic sites of these three topotypes are different, inactivated FMD serotype O vaccine only confer a limited cross-protection against each topotype (Cao *et al.,* 2014). Even though many efforts like vaccination is enforced to control the movement of FMDV, outbreaks of serotype O reoccurred in Korea and neighbouring countries every year (Paton *et al.,* 2010).

(2) Serotype A

Globally, serotype A is second prevalent serotype and endemic in many developing countries of Asia and Africa (Chitray *et al.*, 2014). Serotype A has the highest antigenic variation among seven serotypes. Even though recombination of serotype A is

occurs frequently more than other serotypes, the origin of the recombinant virus is undisclosed (Jackson *et al.*, 2007). Provoke of serotype A in dairy cattle and deer in Korea since January 2010 has been revealed by Epidemiological investigations. This was confirmed by clinical symptoms, isolation and identification of samples, nucleotide sequencing of the complete capsid protein VP1 coding region and antigen detection through ELISA. China have reported no major outbreaks of serotype A since 1958. However, there was an emergence outbreak of serotype A in Hubei and Shanghai in early 2009. Phylogenetic analysis revealed that the strain isolated from these area belonged to the Southeast Asia topotype of serotype A (Bai *et al.*, 2011).

(3) Asia-1

Asia-1 was first found in a sample from Pakistan in 1954 and identified to be the last FMDV serotype. It was named FMDV Asia-1 because this new serotype was primarily isolated from Asia (Brown, 2003). Recently, incursions of Asia-1 are mainly restricted to Asia and neighbouring countries like Korea, Mongolia, Russia, Middle East region, Greece and Turkey (Lee *et al.,* 2009). Asia-1 has two topotypes; Southeast Asian and South Asia. After Asia-1 spread into Iran, Turkey and Saudi Arabia, the virulence of Asia-1 was enhanced and the disease spread rapidly (Knowles and Samuel, 2003). Especially Asia-1 has risk of co-infection. Animals infected with Asia-1 can be infected by other serotypes of FMDV at the same time. In Malaysia, it was reported that serotype O and Asia-1 circled and co-infected simultaneously (Guo *et al.,* 2006). Susceptible animals of Asia-1

are livestock animals such as pigs, cattle, goats, sheep, buffaloes and camels. Also, cloven-hoofed wildlife species like wild pig, deer and antelope can become infected (Thomson *et al.,* 2003). The outbreak of Asia-1 made prevention and controlling of Asia-1 an important issue in veterinary safety and provoked aggressive control action. As a result the outbreak of Asia-1 has been gradually controlled (Guo *et al.,* 2006).

5) Foot-and-mouth disease vaccine

In endemic countries, vaccination is key strategy to control FMD. Current FMD vaccines contain one or more strains of chemically inactivated virus adjuvanted with oil or aluminum hydroxide (Doel, 2003). Because duration of FMD vaccine is relatively short, two vaccination with an 4-week interval between two and three months of age is recommended for pigs and cattle. However, due to vaccine cost and supply, growing piglet were vaccinated only once at ages between 8 and 12 weeks (Lee *et al.,* 2013).

Even though inactivated vaccine induce strong humoral immune response, there are major disadvantages; necessity of propagating virulent virus in the facilities, risk of virus escape from manufacturing facilities (Bartelin and Vreeswijk, 1991), DIVA (Differentiation Infected from vaccinated animals) problem (Shen *et al.*, 1999), difficulty of protecting against all strains within a certain serotype (Doel, 2003). Especially, DIVA problems is a major reason for restriction of international trade with FMD-free countries, so it can be a cause of severe economic impact in

endemic countries (Thompson *et al.*, 2002). Because of all these reasons, effort is made to develop alternative vaccine which are safe and efficient based on plasmid DNA, replicating vectors, recombinant proteins (Grubman, 2005). Some promising results of alternative vaccines were obtained. Adenovirus vectors expressing capsid proteins and 3C protease (mason *et al.*, 2002) and expression of virus-like particles in insect cells (Porta *et al.,* 2013) showed the effects in pigs. Adenovirus vectors expressing type 1 and type 3 interferons were also effective and showed the possibility of rapid infection control (Perez-martin *et al.,* 2012).

2. Recombinant subunit vaccine

Recombinant subunit vaccine is consisted of essential epitopes of an antigen. Subunit vaccine can be the alternative vaccine for live attenuated vaccine or inactivated vaccine. Most of all subunit vaccine is more safer than live attenuated vaccine or inactivated vaccine. Because subunit vaccine doesn't use virus, virus replication is prevented. Therefore, there is no risk of virus leakage from facilities and it is suitable for the person who has immunosuppression (Lal *et al.*, 2015). Subunit vaccine has production efficiency. Large-scale production and engineering requirements are fast and simple. By producing highly purified target recombinant protein, subunit vaccine can induce antigen specific immune response (Agger and Andersen, 2001). Moreover, subunit vaccine could not contain non structural proteins, thereby it is possible to differentiated infected from vaccinated animals (Cao *et al.,* 2013).

Even though subunit vaccine has many advantages, it may have several limitations. Because subunit vaccine uses part of antigen, antigenic spectrum is narrow and cross protection level is low (Cao *et al.,* 2013). Because of low immunogenecity and shorten duration, multiple injection of subunit vaccine is needed. To overcome these disadvantages, several strategies have been explored such as addition of adjuvants, developing multivalent subunit vaccine (Govindarajan *et al.,* 2015) and using polymeric adjuvant for delivering to target organ (Adams and Mallapragada, 2014).

3. Solubilization

1) Inclusion bodies

For the production of recombinant proteins which do not need post-translational modification, *E. coli* expression system have been most widely used (Swarta, 2001). *E. coil* produces high level expression of recombinant proteins, but recombinant proteins are often produced as a form of inclusion bodies (Fahnert *et al.*, 2004). Even though inclusion bodies are considered undesirable because of their low bioactivity, their formation can be an advantage for the following reasons. First, expression level of inclusion bodies are very high. More than 30 % of the cellular protein are expressed as a form of inclusion bodies. Second, isolation of inclusion bodies is more convenient than soluble proteins. Third, target proteins expressed as a form of inclusion bodies have lower degradation level. Fourth, inclusion bodies have resistance to proteolytic attack by cellular proteases. Fifth, because target proteins expressed as a form of inclusion bodies have homogeneity, the number of purification step can be reduced. Because of above advantages, recombinant proteins expressed as a form of inclusion bodies in *E. coli* are widely used for the commercial production of proteins (Walsh, 2003). However, inclusion bodies have no bioactivity, so recovery of bioactive protein from inclusion bodies is needed. Especially, solubilization of the inclusion bodies and refolding of the solubilized protein into bioactive form are two main issues of the recovery (Singh and Panda, 2005).

2) Traditional method of protein recovery from inclusion bodies

Traditional method of protein recovery from inclusion bodies consists of the following four steps; isolation of inclusion bodies from *E. coli* cells, solubilization of inclusion bodies, refolding of the solubilized proteins and purification of solubilized proteins (Vallejo and Rinas, 2004). To obtain high level of recovered bioactive proteins, solubilization and refolding are the most important steps among four steps. Traditionally, inclusion bodies were solubilized by using high concentrations (6~8 M) of chaotropic reagents like Urea and guanidine hydrochloride (Rudolph *et al.*, 1997) and detergents like *N*-acetyl trimethyl ammonium chloride (Cardamone *et al.,* 1995), SDS (Stöckel *et al.,* 1997)) and sodium *N*-lauroyl sarcosine (Burgess, 1996). Also, reducing agents such as beta-mecaptoethanol, cysteine and dithiothreitol and chelating agents like EDTA (Clark, 2001) are used for solubilization buffer (Fischer *et al.,* 1993). However, high concentration of chaotropic reagents causes loss of secondary structure. It can be the main reason for the low recovery efficiency of bioactive protein from inclusion bodies. In case of refolding, refolding followed by purification is more preferable. Because some of the high molecular weight aggregates along with contaminants, co-purified in a single step is needed (Singh and Panda, 2005).

3) Chaperone co-expression system

Chaperone proteins bind to the polypeptides, prevent their

aggregation and help them to fold. By doing this, production of inclusion bodies decreases and production of soluble proteins increases. There are five types of commercial chaperone plasmids which express different chaperone proteins. pG-Tf2 expresses GroES, GroEL and trigger factor. pGKJE7 expresses DnaK, DnaJ and GrpE. pG-KJE8 expresses DnaK, DnaJ, GraE, GroEL and GroES. pTf16 expresses trigger factor and pGRO7 expresses GroES and Gro EL.

Trigger factor, DnaK, DnaJ and GrpE are well known to protect nascent poplypeptide from aggregation and misfolding (Deuerling *et al.*, 1999). Without chaperone proteins, folding of poplypeptides is rapid but inefficient and default co-translational pathway. Trigger factor delays folding of proteins by scanning a nascent poplypeptide when it is extruded from exit site of ribosome (Maier *et al* 2001). DnaK also binds to polypeptide extruded from ribosome and delays protein folding (Teter *et al.*, 1999). Polypeptide temporarily are bound to DnaJ which is J-domain co-chaperone of DnaK and transfers onto DnaK. And GrpE is nucleotide-exchange factor of DnaK existed in *E. coli* (Bukau and Horwich, 1998). As newly synthesized proteins are released from ribosomes, multimeric chaperonin GroEL-GroES help to keep them soluble (Ullers *et al.,* 2004). GroEL function is encapsulating polypeptide in a cavity and it is capped by GroES which is heptameric cofactor of GroEL. In a cavity, poplypeptides are allowed the completion of folding and non-productive interactions with other unfolded poplypeptide are prevented (Hartl and Hayer-Hartl, 2002).

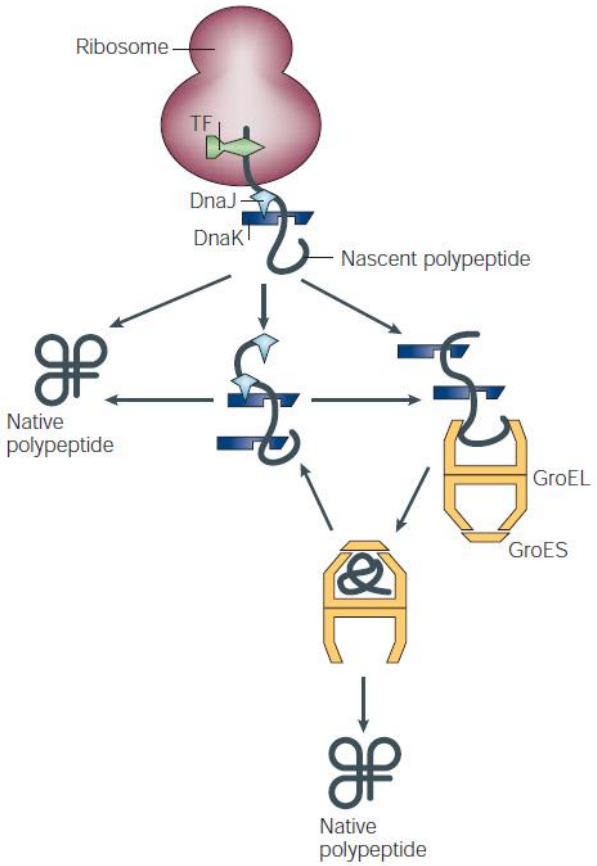

Figure 3. Chaperone network of the prokaryotic cytosol (Young *et al.,* 2004).

4) Alkaline shock solubilization

Alkaline shock solubilization is a novel solubilization method using alkaline buffer of pH 12.5 to solubilize inclusion bodies. Briefly, 1 g of inclusion bodies pellet was resuspended in buffer of pH 8. Then, the pH was increased to 12.5 by using NaOH

and pellet of inclusion bodies was completely solubilized within seconds. After centrifugation, supernatant was filtered and precipitated by cold acetone. After centrifugation, precipitated protein can dissolved in distilled water or PBS.

Because alkaline solubilization methods is mild solubilization method which doesn't require Urea or guanidine hydrochloride, it can preserve native-like secondary structure of protein which refold after precipitation by distilled water or PBS. Therefore, alkaline shock solubilization can recovery as many as inclusion bodies into bioactive form. Also, combination of alkaline solubilization and acetone precipitation is cost effective and time saving methods. Dilution-based refolding methods require expensive chemicals, large volumes of cost-prohibitive refolding buffers and additives which do not need for alkaline solubilization and acetone precipitation (Figure 4). Through combination of alkaline solubilization and acetone precipitation which called alkaline-pH acetone precipitation, maximum gram amounts of protein can be obtained (Heiker *et al.,* 2010).

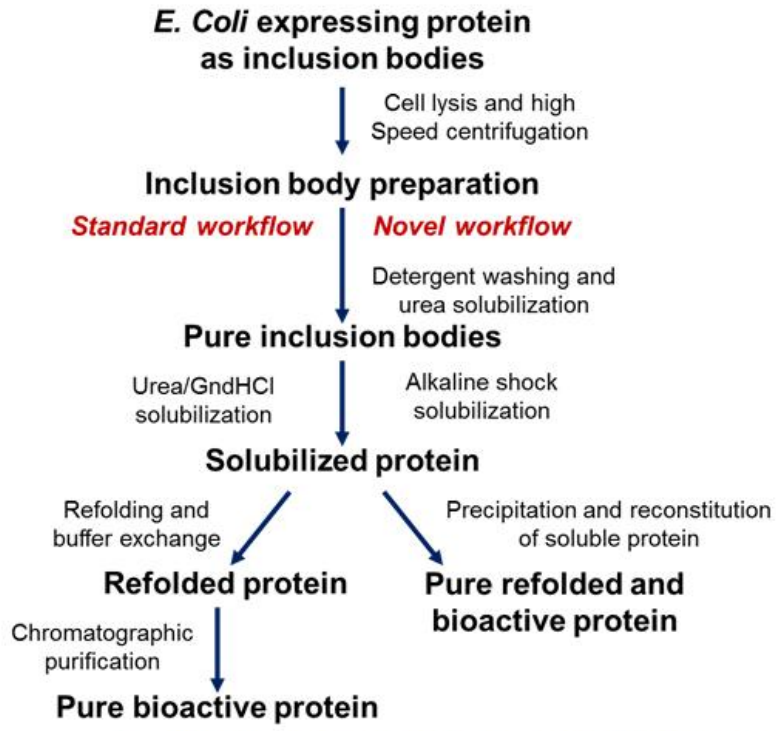

Figure 4. Workflow comparison of the alkaline shock protocol with standard refolding protocol (Heiker *et al.*, 2010).

Ⅲ. Materials and Methods

1. Cloning of the trivalent multi-epitope subunit vaccine

1) Design and synthesis of the multi-epitope genes

T-cell epitope of type O FMDV (O-UKG 11/01) was used in order to increase immunogenecity of the multi-epitope proteins (Blanco *et al.*, 2013). Each nine B-cell epitope of FMDV serotype O, serotype A, Asia-1 (Table 1) were linked together by two glycine (G) residues according to the order in Table 2. VP1 G-H loop is well known to have high antigenecity, but it can not be the essential part of protection against FMDV and there is possibility of presenting other important antigenic sites (Fowler *et al.*, 2010). In this reason, we combined three epitopes per strain. In previous study, combination of VP1 G-H loop and C-terminal showed high protection level against FMDV (Cao *et al.,* 2013). When screening B cell epitope of FMDV by using DNAStar Protean system, VP1 C-termianl showed high reactivity with anti FMDV-P1 (four structural proteins) serum (Zhang *et al.,* 2012). VP2 and VP3 also have epitopes showing high antigenecity. When using VP2 E-F loop for multi-epitope vaccine, it showed high protection level (Cao *et al,* 2012). By using 24 monoclonal antibodies of FMDV, four neutralizing sites in FMDV were identificated; VP1 G-H loop, VP3 B-B knob, VP2 B-C loop and VP3 C-terminal (Grazioli *et al.,* 2013). Serotype A vaccine viruses were analyzed by matching 20 amino acids of serotype A located on the viral surface. As a result, VP2 B-C

loop and VP3 B-B knob were related to high immunologic reactivity (Lee *et al.,* 2015).

Three different serotypes DNA sequence of the designed multi-epitope proteins were synthesized by GenScript Incorporation (www.genscript.com) according to the most commonly occurring codons in *E. coli.*

Table 1. Epitopes of FMDV used in this study.

Epitopes	Amino acid sequence	Origin of epitopes
$VP2_{129\sim140}$	LCSIERRELFQL	E-F loop of O/Mya/98
$VP1_{133\sim160}$	NCKYAGGSLPNVRGDLQVLAQKAAWPLP	G-H loop of O/Mya/98
$VP1_{193\sim211}$	AVHPSAARHKQKIVAPVKQ	C terminal of O/Mya/98
$VP2_{129\sim140}$	LCSIDKRELYQL	E-F loop of O/Tibet/1/99
$VP1_{133\sim160}$	NCKYDESPVTNVRGDLQVLAQKAARTLP	G-H loop of O/Tibet/1/99
$VP1_{193\sim211}$	AIHPSEARHKQKIVAPVKQ	C terminal of O/Tibet/1/99
$VP2_{129\sim140}$	LCSIQKRELYQL	E-F loop of Manisa O1
$VP1_{133\sim160}$	NSKYGDGTVANVRGDLQVLAQKAARALP	G-H loop of Manisa O1
$VP1_{193\sim211}$	AIHPDQARHKQKIVAPVKQ	C terminal of Manisa O1
$VP2_{68\sim80}$	FDWTKDKPFGHME	B-C loop of A/Malaysia/97
$VP3_{55\sim79}$	LCFDGGKPYVETRTDDQRLLAKFDV	B-B knob of A/Malaysia/97
$VP1_{132\sim160}$	YNGTSKYSTPGARRGDLGSLAARDAAQLP	G-H loop of A/Malaysia/97
$VP2_{68\sim80}$	FDWTPDKAFGHLE	B-C loop of A22 Iraq
$VP3_{55\sim79}$	LCFDEGKPYVVTRTDEQRLLAKFDV	B-B knob of A22 Iraq
$VP1_{132\sim161}$	YNGTSKYSAGGTGRRGDLGPLAARVAAQLP	G-H loop of A22 Iraq
$VP1_{30\sim50}$	HHTDVSFIMDRFVQIKPVSPT	B-C loop of A/HuBWH/CHA/09
$VP1_{132\sim160}$	YNGTSKYSAPATRRGDLGSLAARLAAQLP	G-H loop of A/HuBWH/CHA/09
$VP1_{193\sim211}$	AVEVTSQDRHKQKIIAPAKQ	C terminal of A/HuBWH/CHA/09
$VP2_{68\sim80}$	FDWTPNLAFGHCY	B-C loop of Asia-1/YNBS/58
$VP1_{132\sim160}$	YNGKTTYGEESTSRGDPSALAQRLSGRLP	G-H loop of Asia-1/YNBS/58
$VP1_{193\sim211}$	ALDTTQDRRKQEIIAPEKQ	C terminal of Asia-1/YNBS/58
$VP2_{68\sim80}$	FDWTPGLSFGHCH	B-C loop of Asia-1/WHN/CHA/06
$VP1_{132\sim160}$	YNGKTTYGEESSRRGDLAALARRVDNRLP	G-H loop of Asia-1/WHN/CHA/06
$VP1_{193\sim211}$	ALDTTQDRRKQEIIAPEKQ	C terminal of Asia-1/WHN/CHA/06
$VP2_{68\sim80}$	FDWTPNLAFGHCY	B-C loop of Asia-1/Shamir/89
$VP1_{132\sim160}$	YNGKTAYGETTSRRGDMAALAQRLSARLP	G-H loop of Asia-1/Shamir/89
$VP1_{193\sim211}$	ALDTTQDRRKQEIIAPEKQ	C terminal of Asia-1/Shamir/89
$3A_{21\sim35}$	AAIEFFEGMVHDSIKLEHHHHHH	3A protein of O-UKG 11/01

Table 2. The composition of trivalent multi-epitope subunit vaccine.

Proteins	Sequence
O9BT	(O/Mya/98)VP2$_{129-140}$-GG-(O/Mya/98)VP1$_{133-160}$-GG-(O/Mya/98)VP1$_{193-211}$-GG-(O/Tibet/1/99)VP2$_{129-140}$-GG-(O/Tibet/1/99)VP1$_{133-160}$-GG-(O/Tibet/1/99)VP1$_{193-211}$-GG-(Manisa O1)VP2$_{129-140}$-GG-(Manisa O1) VP1$_{133-160}$-GG-(Manisa O1)VP1$_{193-211}$-GG-(O - UKG 11/01)3A$_{21-35}$-6x His
A9BT	(A/Malaysia/97)VP2$_{68-80}$-GG-(A/Malaysia/97)VP3$_{55-79}$-GG-(A/Malaysia/97)VP1$_{132-160}$-GG-(A22 Iraq)VP2$_{68-80}$-GG-(A22 Iraq)VP3$_{55-79}$-GG-(A22 Iraq)VP1$_{132-161}$-GG-(A/ HuBWH/CHA/09)VP1$_{30-50}$-GG-(A/HuBWH/CHA/09)VP1$_{132-160}$-GG-(A/HuBWH/CHA/09)VP1$_{193-211}$-GG-(O - UKG 11/01)3A$_{21-35}$-6x His
I9BT	(Asia-1/YNBS/58)VP2$_{68-80}$-GG-(Asia-1/YNBS/58)VP1$_{132-160}$-GG-(Asia-1/YNBS/58)VP1$_{193-211}$-GG-(Asia-1/WHN/CHA/06)VP2$_{68-80}$-GG-(Asia-1/WHN/CHA/06)VP1$_{132-160}$-GG-(Asia-1/WHN/CHA/06)VP1$_{193-211}$-GG-(Asia-1/Shamir/89)VP2$_{68-80}$-GG-(Asia-1/Shamir/89)VP1$_{132-160}$-GG-(Asia-1/Shamir/89)VP1$_{193-211}$-GG-(O - UKG 11/01) 3A$_{21-35}$-6x His

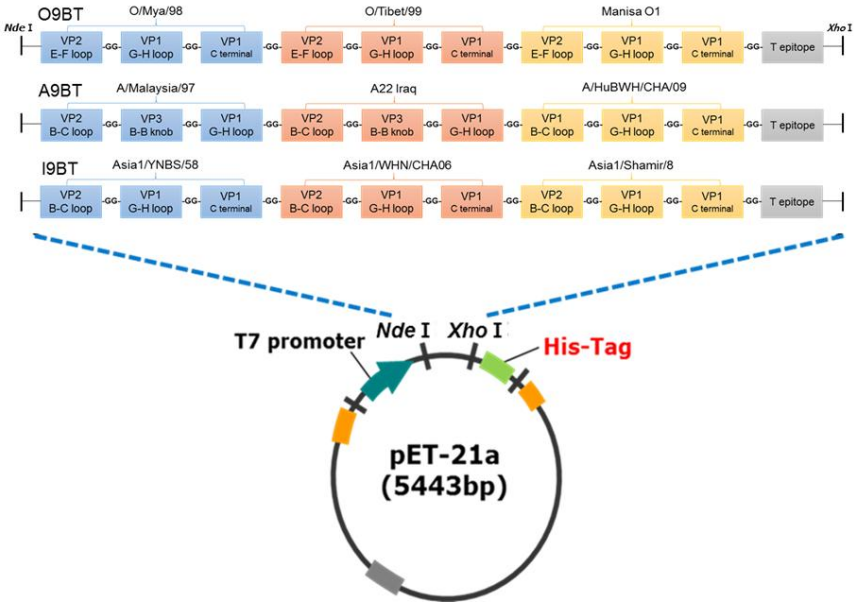

Figure 5. Scheme of the vector construction.

2) Plasmids and strains

O9BT, A9BT and I9BT were cloned into pET21a (+) (Novagen, USA) vector by using *Nde* I and *Xho* I restriction sites, respectively. Briefly, synthesized genes were amplified by PCR. 20 ng of gDNA was added to 20 μl of a PCR reaction mixture containing 10 μl of i-Taq 2x PCR master mix solution (Intron Biotechnology, Korea), 500 nM of forward primer pUC57F (5'-CTTAACTATGCGGCATC-3') and reverse primer pUC57R (5'-GTCATAGCTGTTTCCTG-3'). PCR was performed as follows: 94 °C for 2 min; 25 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min; 72 °C for 5 min. The amplified genes and pET21a (+) vectors were digested with *Nde* I and *Xho* I for overnight at 37 °C. After purification, genes were cloned into pET21a (+) vectors using T4 DNA ligase (NEB, USA). The

target genes transferred into pET21a (+) vectors were confirmed by sequencing and no mutations were induced. The primers for confirmation of the target gene were; T7promoter, 5'-TAATACGACTCACTATAGGG-3' and T7terminator, 5-'GCTAGTTATTGCTCAGCGG-3'. Expression vectors were transformed into *E. coli* BL21 (DE3) competent cell (Novagen, USA) by using heat shock transformation method. The transformation was confirmed by colony PCR using T7promoter and T7terminator.

3) Protein expression

A seed culture was prepared by inoculating with a single colony of recombinant *E. coli* strains and grown overnight in LB broth (BD, USA) containing ampicillin (100 μg/ml). 1 L of LB broth containing 100 μg/ml of ampicillin was routinely inoculated with 1% seed culture. Cells were grown with shaking at 230 rpm and 37 °C and the growth was monitored by measuring the absorbance of OD at 600 nm with a spectrophotometer. When the OD600 of the culture reaches set point, target proteins were induced by adding 1 mM IPTG at 37 °C for 4 hr.

Cells were harvested and washed by PBS. Then cells from 1 L culture were lysed in 40 ml PBS containing 40 mg lysozyme. After incubation with shaking at 230 rpm and 37 °C for 15 min, cells disrupted on ice by sonication (VCX 750, SONICS, USA) using a program (32 cycles of 10 sec On/5 sec Off, amp 40%) and centrifuged at 14,000 rpm and 4 °C for 10 min. The IBs pellet and supernatant were separated and stored at - 20 °C until

use.

4) SDS-PAGE and western blot assay

Protein expression were analyzed using band intensity of recombinant proteins on SDS-PAGE from independent and/or parallel induction samples. SDS-PAGE was made of 5% stacking gels and 15% resolving gels under reducing condition and run in a Mini-PROTEAN electrophoresis system (BioRad, USA). Gel were stained with Coomassie Blue R250 (AMRESCO, USA) and images were analyzed by image analysis software of a ChemiDoc^{TM} MP System (BioRad, USA).

In the case of western blot assay, proteins after SDS-PAGE run were transferred to a nitrocellulose membrane (Whatman, USA) in a XCell II^{TM} Blot Module (Invitrogen, USA). Then the membrane was blocked with TBST and 5% skim milk (BD/Difco, USA) for 1 hr. After 3 times washing with TBST, membrane was probed with mouse anti-His monoclonal antibody (abcam, UK) in TBST at 1:2,000 dilutions for overnight at 4 °C. After 3 times washing with TBST, HRP-conjugated goat anti-mouse IgG (abcam, UK) diluted in TBST at 1:5,000 were used as secondary antibody. After 3 times washing with TBST, detection was carried out using an ECL detection kit (GE Healthcare, Sweden).

2. Introduction of solubilization methods

1) Chaperone co-expression system

Five types of chaperone plasmids were extracted by Plus

Plasmid Purification kit (NucleoGen, Korea) from BL21/pG-KJE8, BL21/pGro7, BL21/pGKJE7, BL21/pG-Tf2 and BL21/pTf16 competent cell (TaKaRa, Japan). Extracted plasmids were transformed into the *E. coli* BL21 (DE3) competent cell respectively. The expression vector of O9BT was transformed into the *E. coli* BL21 (DE3)/pG-KJE8, BL21 (DE3)/pGro7, BL21 (DE3)/pGKJE7, BL21 (DE3)/pG-Tf2 and BL21(DE3)/pTf16 competent cell respectively. *E. coli* BL21 (DE3)/pG-KJE8 strain was designed to produce DnaK-DnaJ-GrpE/GroEL-GroES chaperone complex under the control of L-arabinose and tetracyclin inducible promoter. *E. coli* BL21 (DE3)/pGro7 and BL21 (DE3)/pGKJE7 strains were designed to produce GroEL-GroES and DnaK-DnaJ-GrpE chaperone complex, respectively, under the control of L-arabinose inducible promoter. *E. coli* BL21 (DE3)/pG-Tf2 strain was designed to produce GroEL-GroES-trigger factor chaperone complex under the control of tetracyclin inducible promoter. *E. coli* BL21 (DE3)/pTf16 strain was designed to produce the trigger factor under the control of L-arabinose inducible promoter.

A seed culture was prepared by inoculating with a single colony of recombinant *E. coli* strain and grown overnight in LB broth (BD, USA) containing ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml). For chaperone co-expression, 1% seed culture was inoculated to 25 ml or 1 L of LB broth containing 100 μg/ml of ampicillin and 20 μg/ml of chloramphenicol, followed by adding 0.5 mg/ml of L-arabinose and/or 10 ng/ml of tetracyclin for the induction of chaperone

proteins. Cells were grown with shaking at 230 rpm and 37 °C and the growth was monitored by measuring the absorbance of OD at 600 nm with a spectrophotometer. When the OD600 of the culture reaches set point, cultures were cooled at 4 ECL for 30 min and target proteins were induced by adding 0.5 mM IPTG at 15 °C for 24 hr.

Cells were harvested and washed by PBS. Then cells from 1 L culture were lysed in 40 ml PBS containing 40 mg lysozyme. After incubation for 15 min, cells disrupted on ice by sonication (VCX 750, SONICS, USA) using a program (32 cycles of 10 sec On/5 sec Off, amp 40%) and centrifuged at 14,000 rpm and 4 °C for 10 min. The IBs pellet and supernatant were separated and stored at - 20 °C until use.

Production efficiency of soluble protein was measured by western blot assay according to method mentioned above.

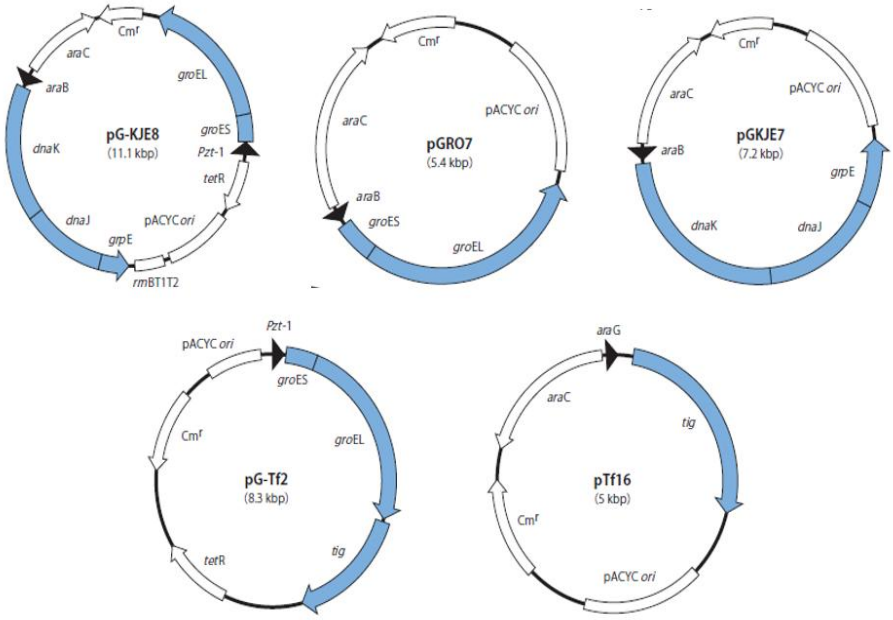

Figure 6. Type of chaperone plasmids.

2) Alkaline-pH acetone precipitation

IBs pellet was washed by PBS and purified IBs was collected at 14,000 rpm and 4 °C for 10 min. The IBs pellet from 500 ml cell culture was solubilized in 20 ml of alkaline-pH buffer (10 mM Tris-NaOH, pH 12.5). Then supernatant was collected at 14,000 rpm and 4 °C for 10 min and incubated on ice for 5 min. 4 volume of ice-cold acetone was added to precipitate the solubilizated proteins and incubated at -20 °C for 1 hr. After centrifugation at 17,000 rpm and 4 °C for 15 min, the supernatant was removed and the resulting pellet was washed once by adding 20 ml of ice-cold acetone. After cenrfiguation at 17,000 rpm and 4 °C for 15 min, the supernatant was removed and remained acetone was removed by air-drying for 30 min. Then

the pellet was dissolved immediately. The supernatant was collected by centrifugation at 14,000 rpm and 4 °C for 10 min (Heiker *et al*., 2010).

3. Protein purification

1) His-tag affinity chromatography

The crude protein of 1 L culture was purified by using His-tag affinity chromatography. Before purification, the crude protein was filtered by 0.45 μm filter to remove cell debris. 3 ml of His-bind resin (Novagen, USA) was used for soluble protein, 4 ml of His-bind resin was used for soluble protein produced by chaperone co-expression system and 8 ml of His-bind resin was used for protein resolubilized by alkaline-pH acetone precipitation. His-bind resin was equilibrated with 3 volume of binding buffer and charged with 5 volume of charging buffer. After washing with 5 volume of binding buffer to remove uncharged nickel ions, column was loaded with crude protein. Then column was washed with washing buffer containing different imidazole concentration to remove non-specific protein. 6x His-tag bearing O9BT was eluted with elution buffer. Buffer composition are listed in Table 3. Each fraction was analyzed by SDS-PAGE to check the purification quality and purity of protein. The purified protein was dialyzed against 5 L of distilled water at 4 °C for 24 hr with water changes for three times to remove the salts in elution buffer followed by lyophilization.

Table 3. His-tag affinity chromatography buffer composition.

Buffer		Imidazole	Tris-Cl	NaCl	$NiSO_4$	EDTA	pH	Volume	Flow rate (ml/min)
Charging buffer		-	-	-	50 mM		-	5	10
Binding buffer		5 mM						5	10
Washing buffer	1	5 mM						5	3-4
	2	10 mM						5	3-4
	3	20 mM						5	3-4
	4	30 mM	20 mM	0.5 M			7.9	5	3-4
	5	40 mM						5	3-4
	6	60 mM						3	3-4
Elution buffer		1 M						3	3-4
Strip buffer		-				100 mM		5	10

* 1 volume = resin volume

2) Endotoxin removal

Endotoxin was removed by 0.5 ml of Pierce^{TM} High Capacity Endotoxin Removal Spin column (Thermo, USA) according to manufacturer's instructions. Briefly, equilibrated the spin column at room temperature and placed the spin column into a collection tube. After centrifugation at 500 g for 1 min, 0.2 N NaOH in 95% ethanol was added to regenerate and incubated 2 hr at room temperature. After centrifugation at 500 g for 1 min, 2 M NaCl was added and the column was inverted until the resin was suspended in solution. After centrifugation at 500 g for 1 min, endotoxin free water was added and the column was inverted until the resin was suspended in solution. After centrifugation at 500 g for 1 min, endotoxin free buffer (30 mM Tris-Cl containing 0.2 M NaCl, pH 6~8) was added and the column was

inverted until the resin was suspended in solution (repeated this step for 3 times). After centrifugation at 500 g for 1 min, sample was added and incubated with gently end-over-end mixing at room temperature for 1 hr. Column was placed in a collection tube and centrifuged at 500 g for 1 min. Then the collected sample was stored at 4 °C until use.

Protein concentration was measured by BCA assay (Thermo, USA) using BSA as a standard before mouse immunization. BSA standards were prepared according to manufacturer's instructions. Protein sample was diluted 1:5, 1:10, 1:20, 1:50 and 1:100. 25 μl of each standard and diluted protein samples were added into a 96 well microplate (SPL life science, Korea). 200 μl of working reagent (50:1, Reagent A:B) was added to each well and mixed thoroughly for 30 sec. Plate was covered with plate sealing film (BioRad, USA) and incubated at 37 °C for 30 min. The absorbance was measured at 562 nm using Infinite 200 PRO (TECAN, Switzerland). The amount of protein was calculated from the standard curve.

4. *In vivo* immunization

1) Mouse immunization

Two independent experiments were performed. Mice were purchased from Samtako (Korea) and the experiment were performed in accordance with the guideline for the care and use of laboratory animals under the approval of animal ethics committee at Seoul National University (SNU-160202-1).

In the first experiment, 5 female BALB/c mice of seven-week-old were female BALB/c mice were used per group. Under standard pathogen-free conditions, mice were provided with free access to food and water during the experiments. After 1 week of acclimatization, mice were immunized intramuscularly with 20 μg of O9BT protein suspended in 40 μl PBS two times at two weeks intervals. 40 μl CFA was mixed with antigens for the priming of animals and IFA was mixed with antigens for the boosting of animals. Inactivated vaccine produced in Green Cross (Korea) was used as the positive control. General information of first mouse immunization showed in Table 4.

In the second experiment, 5 female BALB/c mice of seven-week-old were female BALB/c mice were used per group. Under standard pathogen-free conditions, mice were provided with free access to food and water during the experiments. After 1 week of acclimatization, mice were immunized intramuscularly with 20 μg of protein suspended in 40 μl PBS three times at one week interval. 40 μl CFA was mixed with antigens for the priming of animals and IFA was mixed with antigens for the boosting of animals. Inactivated vaccine produced in Komipharm (Korea) was used as the positive control. General information of second mouse immunization showed in Table 5.

Table 4. General information of first mouse immunization.

No.	Group	Vaccine	Mice	Dose (μg)	Note
1	Untreated	-	5	-	Negative control
2	SP	O9BT	5	20	Soluble protein
3	CHA		5	20	Chaperone co-expression method
4	APP		5	20	Acetone precipitation method
5	IB		5	20	Inclusion bodies
6	M5BT	M5BT	5	20	Positive control
7	iFMDV (Green Cross)	Inactivated FMDV type O vaccine	5	80 μl	Positive control

Table 5. General information of second mouse immunization.

No.	Group	Vaccine	Mice	Dose (μg)	Note
1	Untreated	-	5	-	Negative control
2	O9BT	O9BT	5	20	
3	A9BT	A9BT	5	20	Acetone precipitation method
4	I9BT	I9BT	5	20	
5	TSV (20 μg)	O9BT, A9BT, I9BT	5	6.6 μg per protein	Acetone precipitation method
6	TSV (45 μg)	O9BT, A9BT, I9BT	5	15 μg per protein	
7	iFMDV (Komipharm)	Inactivated FMDV type O vaccine	5	80 μl	Positive control

2) Blood and spleen sampling

In the first experiment, blood samples of immunized mice were collected at 0, 2 and 4 weeks after immunization. Each blood was collected before each immunization. The blood sample collection was conducted from tail vein using microtainer (BD, USA) followed by isolation of serum from blood by centrifugation at 14,000 rpm and 4 ℃ for 3 min and stored in －20 ℃ until use.

In the second experiment, blood samples of immunized mice were collected at 0 and 4 weeks after immunization. Each blood was collected before each immunization. The blood sample collection was conducted from tail vein using microtainer (BD,

USA) followed by isolation of serum from blood by centrifugation at 14,000 rpm and 4 ℃ for 3 min and stored in －20 ℃ until use.

For detection of cytokine production, the spleen was isolated from 3 mice per group at 4 weeks after immunization. The spleen was stored in 5 ml of pre-warmed RPMI 1640 medium (Gibco, USA) containing 10% FBS (GenDEPOT, USA) and 5% P/S until use. The spleen was placed on slide glass and place with plunger end of a syringe. Cells were filtered through the cell stainer (Falcon, USA) with the extra 5 ml medium. After centrifugation at 1,000 rpm for 5 min, supernatant was removed and added 1 ml of ACK lysis buffer (Gibco, USA). After ice incubation for 10 min, 5 ml of medium was added followed by centrifugation at 1,000 rpm for 5 min. After the supernatant was removed, cells were resuspended in PBS at $1x10^7$ per ml. After centrifugation at 1,000 rpm for 5 min, 1 ml of medium with antigens or without antigens (Table 6) were added to cell pellet. Then resuspended cells were added into 24 well cell culture plate (SPL life science, Korea) and incubated at 37 ℃ for 3 days. After centrifugation at 1,000 rpm for 5 min, the supernatant was collected and stored in －20 ℃ until use.

Table 6. Dose of antigen used in splenocyte proliferation assay.

	No.	Group	Antigen	Mice	Dose (μg)
1st experiment	1	Untreated	O9BT	3	20
	2	SP			
	3	CHA			
	4	APP			
	5	IB			
	6	M5BT			
	7	iFMDV (Green Cross)			
2nd experiment	1	Untreated	O9BT, A9BT, I9BT	3	10 μg per protein
	2	O9BT			
	3	A9BT			
	4	I9BT			
	5	TSV (20 μg)			
	6	TSV (45 μg)			
	7	iFMDV (Komipharm)			

3) Detection of serum antibody level

The induction of antigen specific serum IgG, IgG_1 and IgG_{2a} level was measured by indirect ELISA. The 96 well immunoplates (SPL life science, Korea) were coated with 1 μg/ml of proteins in 0.05 M carbonate-bicarbonate buffer (Sigma, USA) (100 μl/well) at 37 ℃ for 2 hr. In first experiment, 96 well immunoplates were coated with soluble protein of O9BT. In second experiment, 96 well immunoplates were coated with O9BT, A9BT and I9BT produced by alkaline-pH acetone precipitation method, respectively. After 3 times washing with PBS (200 μl/well), plates were blocked with 1% BSA in PBS (150 μl/well) at room temperature for 1 hr. After 3 times

washing with PBS (200 μl/well), serum with a 1:100 dilution in PBS containing 1% BSA were added to wells (100 μl/well) and incubated at 37 °C for 1 hr. After 3 times washing with PBST (200 μl/well), HRP conjugated goat anti-mouse IgG or goat anti-mouse IgG_1 or goat anti-mouse IgG_{2a} (Santa Cruz, USA) diluted at 1:5,000, 1:5,000 and 1:2,500, respectively, were used as secondary antibody (100 μl/well). After incubation at room temperature for 1 hr, 96 well immunoplates were washed with PBST for 3 times (200 μl/well). Then plates were incubated with the TMB substrate solution (Sigma, USA) (100 μl/well) at room temperature for 6 min in the dark and reaction was stopped by adding stop solution (0.16 M H_2SO_4; 100 μl/well). Absorbance at 450 nm was measured in an Infinite 200 PRO microplate reader (TECAN, Switzerland).

4) FMDV serotype O specific antibody production

FMDV type O specific antibody level was measured by using PrioCHECK® FMDV Type O ELISA Kit (Thermo, USA) according to the manufacturer's instructions. Briefly, 90 μl of ELISA buffer was added to all wells of the test plate. Then 10 μl of reference serum or test serum was added to all wells of the test plate and incubated at 25 °C for 1 hr. After 6 times washing with washing solution (300 μl/well), conjugated dilution was added to all wells (100 μl/well) and incubated 25 °C for 1 hr. After 6 times washing with washing solution (300 μl/well), plate was incubated with the TMB substrate solution (100 μl/well) at 25 °C for 15 min in the dark and reaction was stopped by

adding stop solution (100 µl/well). Absorbance at 450 nm was measured in an Infinite 200 PRO microplate reader (TECAN, Switzerland). The vaccination efficacy was presented as percentage inhibition titer (PI). PI value was calculated by following equation: PI = 100 - (corrected OD450 test sample/corrected OD450 max)*100.

5) Detection of cytokine production

The cytokine production level was measured by sandwich ELISA. To detect IL-4 and IFN-gamma production level, Mouse IL-4 ELISA Kit and Mouse IFN-gamma ELISA Kit (KOMA, Korea) were used according to manufacturer's instructions. Briefly, pre-coated plate was washed with PBST for 4 times (300 µl/well). Standard and splenocyte samples were added (100 µl/well) and incubated at room temperature for 2 hr. After 4 times washing with PBST (300 µl/well), detection antibody were added (100 µl/well) and incubated at room temperature for 2 hr. After 4 times washing with PBST (300 µl/well), streptavidin-HRP was added (100 µl/well) and incubated at room temperature for 30 min. After 4 times washing with PBST (300 µl/well), plate was incubated with the TMB substrate solution (100 µl/well) at room temperature for 8 min in the dark and reaction was stopped by adding stop solution (2 M H_2SO_4; 100 µl/well). Absorbance at 450 nm was measured in an Infinite 200 PRO microplate reader (TECAN, Switzerland).

5. Statistical analysis

All results are expressed as mean ± standard deviation (SD). Statistical significance was assessed using t-test or a one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison test. All statistical analysis was performed using GraphPad PRISM software (GraphPad Software, Inc.) All statistical significance is denoted by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Ⅳ. Results and Discussion

1. Cloning of the trivalent multi-epitope subunit vaccine

1) Vector construction

The synthesized gene was inserted into pUC57 vector when delivered from GenScript. After genes were amplified by PCR with specific primer, amplified genes and pET21a (+) vectors were digested with *Nde* I and *Xho* I and ligated. To confirm whether synthesized genes were ligated into pET21a (+) vector or not, precipitated plasmids were sequenced from NICEM (National instrumentation center for environmental management) and the sequence were aligned with reference sequence we constructed.

2) Transformation of expression vector

The expression vectors of pET21a/O9BT, pET21a/A9BT and pET21a/I9BT were transformed into *E. coli* BL21 (DE3), respectively, by using heat-shock transformation method. Three types of transformed cells were spread on LB agar plate containing ampicillin, respectively. After incubation for overnight at 37 ℃, one colony of O9BT, four colonies of A9BT and six colonies of I9BT were obtained. The transformation was confirmed by colony PCR using T7promoter and T7terminator primer. The PCR product was analyzed on 2% agarose gel, and 816 bp band corresponding to O9BT, 900 bp band corresponding to A9BT and 834 bp band corresponding to I9BT were observed

(Figure 7). Unfortunately, pET21a/A9BT was transformed only one colony among four colonies. Among six transformed colonies of I9BT, colony of lane 10 was selected.

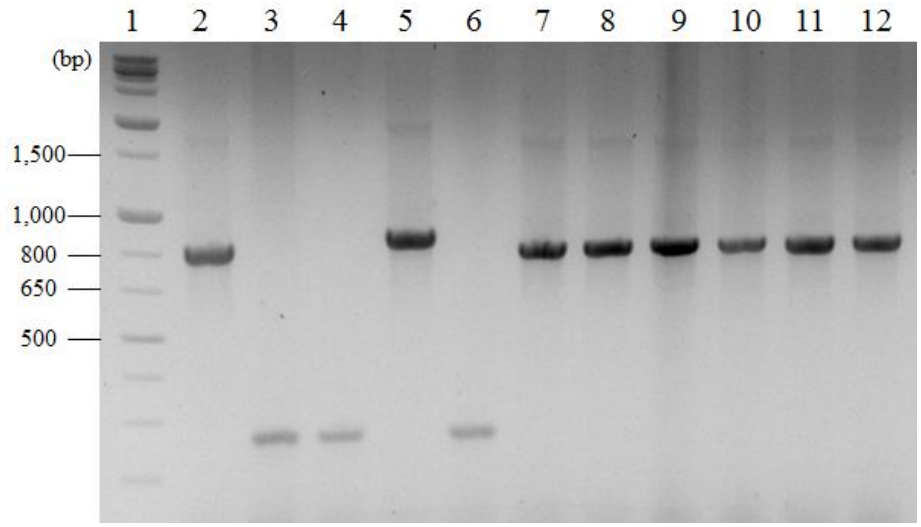

Figure 7. Transformation of pET21a/O9BT, pET21a/A9BT and pET21a/I9BT into *E. coli* BL21 (DE3). Lanes: 1, 1 kb DNA ladder; 2, *E. coli* BL21 (DE3) transformed O9BT; 3-6, *E. coli* BL21 (DE3) transformed A9BT; 7-12, *E. coli* BL21 (DE3) transformed I9BT.

3) Expression of O9BT, A9BT and I9BT in *E. coli*

Expression of O9BT, A9BT and I9BT were analyzed by SDS-PAGE (Figure 8A). 23.58 kDa band corresponding to O9BT, 26.5 kDa band corresponding to A9BT and 24.66 kDa band corresponding to I9BT were observed, whereas no band was found in no-induced *E. coli* BL21 (DE3). Previously, we ligated 5BT (constructed in our laboratory) having five B cell epitopes and one T cell epitope and constructed 5BT-2x, 5BT-3x and 5BT-4x. Through protein expression, we demonstrated 10 epitope

can be express as a form of soluble protein. Even though we followed 10 epitopes strategy, almost recombinant proteins were expressed as a form of inclusion bodies.

The expression of recombinant proteins were analyzed thoroughly by anti-His antibodies as shown in western blot assay (Figure 8B). O9BT and I9BT were expressed as a form of soluble protein to some degree. However, all proteins of A9BT was expressed as a form of inclusion bodies.

Even though inclusion bodies have advantage of isolation from cell homogenate, inclusion bodies are known as having lower bioactivity than soluble proteins. To refold inclusion bodies, we introduced two solubilization methods; Chaperone co-expression system and alkaline-pH acetone precipitation. Because O9BT had the most production yield of soluble protein, it was selected to compare solubilized proteins with soluble protein among three recombinant proteins.

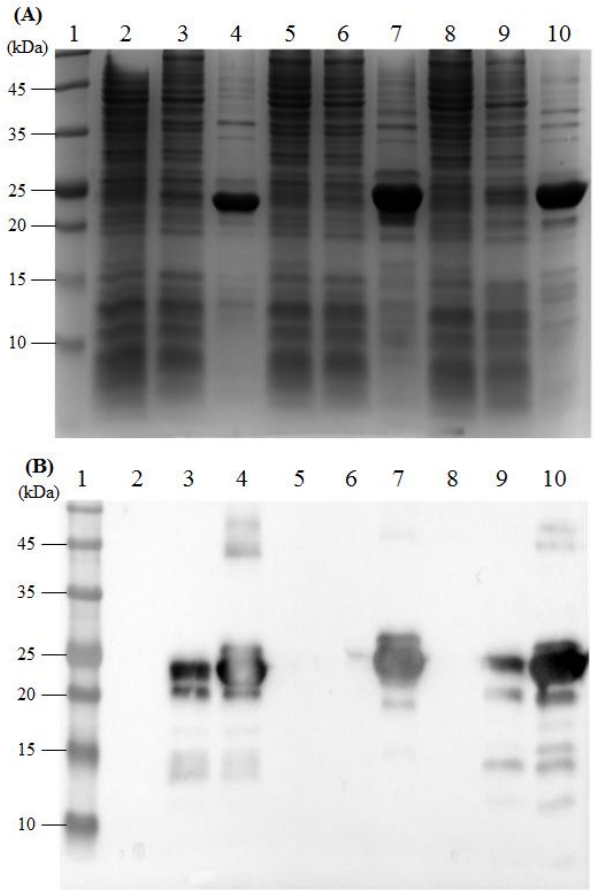

Figure 8. Confirmation of recombinant protein expression in *E. coli* BL21 (DE3) by SDS-PAGE (A) and western blot assay (B). Lanes: 1, protein ladder; 2, soluble fraction of no-induced *E. coli* BL21 (DE3) expressing O9BT; 3, soluble fraction of induced *E. coli* BL21 (DE3) expressing O9BT; 4, insoluble fraction of induced *E. coli* BL21 (DE3) expressing O9BT; 5, soluble fraction of no-induced *E. coli* BL21 (DE3) expressing A9BT; 6, soluble fraction of induced *E. coli* BL21 (DE3) expressing A9BT; 7, insoluble fraction of induced *E. coli* BL21 (DE3) expressing A9BT; 8, soluble fraction of no-induced *E. coli* BL21 (DE3) expressing I9BT; 9, soluble fraction of induced *E. coli* BL21 (DE3) expressing I9BT; 10, insoluble fraction of induced *E. coli* BL21 (DE3) expressing I9BT.

2. Introduction of solubilization methods

1) chaperone co-expression system

(1) Transformation of expression vector

Because pET21a (+) vector has T7 promoter, expression of cloned target gene is extremely low in strains lacking a source of T7 RNA polymerase. Chaperone competent cell was *E. coli* BL21 strains lacking a source of T7 RNA polymerase. Therefore, precipitation of chaperone plasmids and transformation into *E. coli* BL21 (DE3) was conducted in advance.

After transformation of chaperone plasmid, we made five types of chaperone competent cell of *E. coli* BL21 (DE3). Then, pET21a/O9BT was transformed into five types of chaperone competent cell of *E. coli* BL21 (DE3) by using heat-shock transformation method. Five types of transformed cells were spread on LB agar plate containing ampicillin and chloramphenicol, respectively. After incubation for overnight at 37 °C, numerous colonies were obtained. The transformation was confirmed by colony PCR using T7promoter and T7terminator. The PCR product was analyzed on 2% agarose gel, and 816 bp band corresponding to O9BT was observed in all types of chaperone competent cell of *E. coli* BL21 (DE3) (Figure 9). Based on band intensity, colony of lane 14 was selected for protein expression except *E. coli* BL 21 (DE3)/pG-KJE8. Among fifteen colonies of *E. coli* BL21 (DE3)/pG-KJE8, colony of lane 13 was selected.

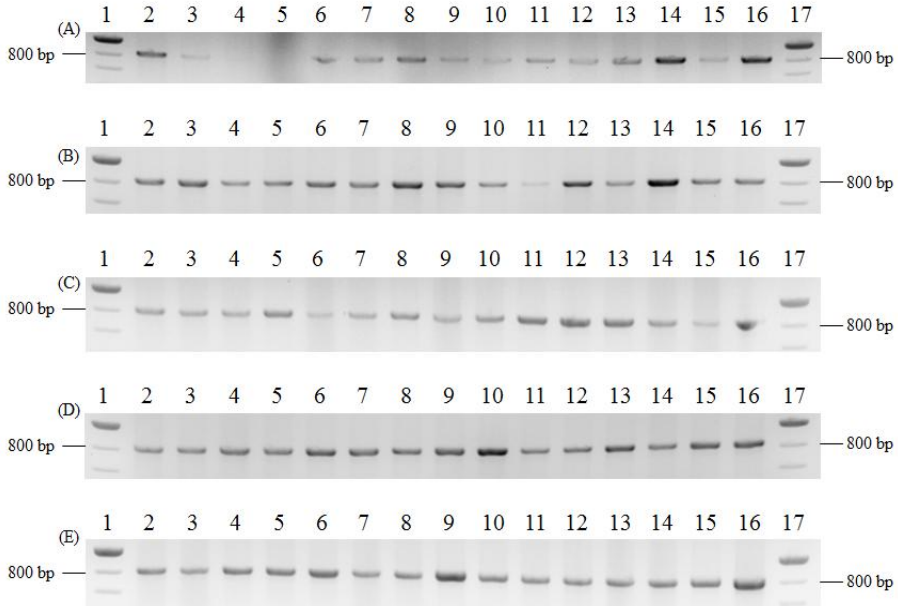

Figure 9. Transformation of pET21a/O9BT into chaperone competent cell of *E. coli* BL21 (DE3). (A) Lanes: 1, 1 kb DNA ladder; 2-16, *E. coli* BL21 (DE3)/pG-Tf2 transformed pET21a/O9BT; 17, 1 kb DNA ladder. (B) Lanes: 1, 1 kb DNA ladder; 2-16, *E. coli* BL21 (DE3)/pGKJE7 transformed pET21a/O9BT; 17, 1 kb DNA ladder. (C) Lanes: 1, 1 kb DNA ladder; 2-16, *E. coli* BL21 (DE3)/pG-KJE8 transformed pET21a/O9BT; 17, 1 kb DNA ladder. (D) Lanes: 1, 1 kb DNA ladder; 2-16, *E. coli* BL21 (DE3)/pTf16 transformed pET21a/O9BT; 17, 1 kb DNA ladder. (E) Lanes: 1, 1 kb DNA ladder; 2-16, *E. coli* BL21 (DE3)/pGRO7 transformed pET21a/O9BT; 17, 1 kb DNA ladder.

(2) Protein expression

Chaperone is known to promote and maintain the native conformation of cellular proteins. Briefly, trigger factor which has micromolar affinity for unfolded polypeptide substrates is considered that it has a function to scan a nascent chain

extruded from ribosome's exit site and shield hydrophobic stretches to keep protein soluble. Polypeptide substrates are temporarily bound to the cofactor protein DnaJ and transferred onto DnaK. DnaK is known as preventing protein misfolding and aggregation during translation by leading to delay the protein folding process relative to translation. Multimeric chaperonin GroEL-GroES helps to retain newly synthesized proteins's soluble form after they have been released from ribosomes. GroEL-GroES reaction is started after GroEL encapsulates polypeptide substrates in a cavity which is capped by the GroES (Young *et al.*, 2004).

According to combination of chaperone proteins, production efficiency of soluble protein is different. To identify which chaperone is most effective to produce soluble O9BT, expression of O9BT with chaperone co-expression of system was confirmed by SDS-PAGE (Figure 10). Interestingly, there were no production of inclusion bodies regardless of chaperone's existence. As reducing growth temperature is the easiest way to lower inclusion bodies formation (Schein, 1989), it can not be the effect of chaperone system but low growth temperature. Even though the production efficiency of soluble protein was increased by introducing chaperone co-expression system, total amount of O9BT expressed was decreased to 50~60% value of no chaperone introduction at 37 °C. Because band intensity of soluble proteins were too low, we couldn't confirm which chaperone system is the most effective to produce soluble protein among five types of chaperone plasmids.

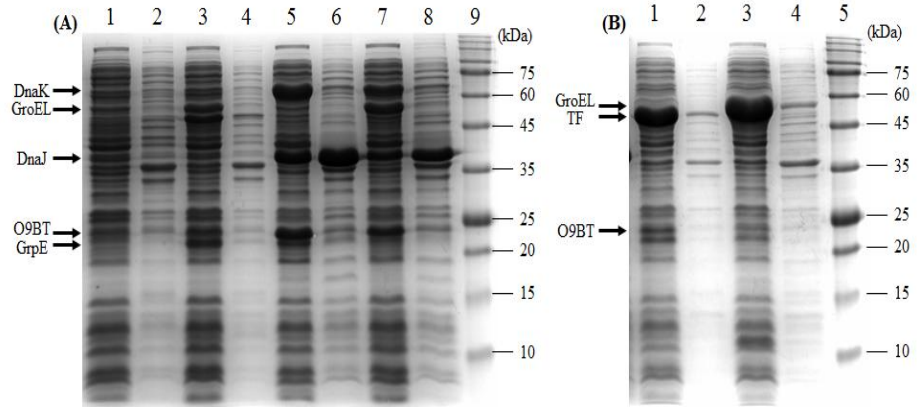

Figure 10. Confirmation of chaperone-assisted soluble O9BT expression. (A) Lanes: 1, soluble fraction of negative control, no chaperone; 2, insoluble fraction of negative control, no chaperone; 3, soluble fraction of co-expression with GroEL-GroES-trigger factor; 4, insoluble fraction of co-expression with GroEL-GroES-trigger factor; 5, soluble fraction of co-expression with DnaK-DnaJ-GrpE; 6, insoluble fraction of co-expression with DnaK-DnaJ-GrpE; 7, soluble fraction of DnaK-DnaJ-GrpE/GroEL-GroES; 8, insoluble fraction of DnaK-DnaJ-GrpE/GroEL-GroES; 9, protein ladder. (B) Lanes: 1, soluble fraction of co-expression with trigger factor; 2, insoluble fraction of co-expression with trigger factor; 3, soluble fraction of co-exrpression with GroEL-GroES; 4, insoluble fraction of co-exrpression with GroEL-GroES; 5; protein marker.

(3) Screening of chaperone co-expression system

To identify which type of chaperone induced the largest amount of soluble protein, western blot assay using anti-His tag antibodies was conducted (Figure 11). As shown in Figure 11B, we demonstrated that co-expression of trigger factor with O9BT at 15 °C was the most effective in production of soluble O9BT

compared to other types of chaperone proteins. Trigger factor system known as cold shock chaperone (Kandror and Goldberg, 1997), is simple and the most effective among the tested chaperone system for the production of soluble O9BT.

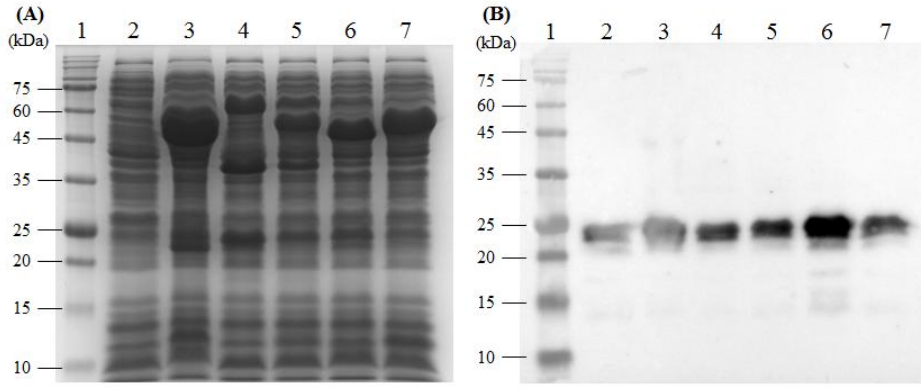

Figure 11. Expression of chaperone-assisted soluble O9BT. (A) SDS-PAGE and (B) western blot assay. Lanes: 1, protein ladder; 2, negative control, no chaperone; 3, co-expression with GroEL-GroES-trigger factor; 4, co-expression with DnaK-DnaJ-GrpE; 5, DnaK-DnaJ-GrpE/GroEL-GroES; 6, co-expression with trigger factor; 7, co-expression with GroEL-GroES.

2) Alkaline-pH acetone precipitation

Because protein yield of chaperone-assisted soluble O9BT was very low, introduction of another solubilization method was needed.

Alkaline-pH acetone precipitation is known as a method to obtain high concentration of protein (Jiang *et al.*, 2004). Previous study has demonstrated that gram scale production of soluble protein from inclusion bodies is possible through alkaline-shock solubilization (Heiker et al., 2010). Only a few amount of soluble

protein can be recovered by general solubilization procedure using 8 M Urea or 6 M guanidine hydrochloride, because the procedure of dialysis or dilution is needed. Therefore, alkaline-shock solubilization without dialysis or dilution is adopted as an alternative solubilization method for large scale production of soluble protein.

Solubilization of inclusion bodies using alkaline-pH buffer (pH 12.5, 10 mM Tris-NaOH) and the recovery of inclusion bodies was confirmed by SDS-PAGE (Figure 12). As shown in Figure 12, about 50% of inclusion bodies were recovered as soluble proteins. Because protein yield of O9BT inclusion bodies up to 550 mg from 1 L bacterial culture, to recover up to 275 mg of soluble proteins from per 1 L culture of inclusion bodies is possible.

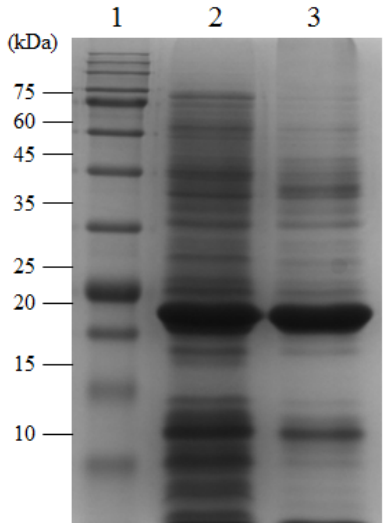

Figure 12. SDS-PAGE analysis of O9BT inclusion bodies recoverd as soluble proteins by alkaline-pH acetone precipitation. Lanes: 1, protein ladder; 2, O9BT inclusion bodies solubilized by alkaline-pH buffer (pH 12.5 10 mM Tris-NaOH) (sample was 2-fold diluted in alkaline-pH buffer and loaded); 3, alkaline-pH acetone precipitated O9BT dissolved in PBS.

3. Protein purification

1) His-tag affinity chromatography

To obtain sufficient O9BT soluble protein for mouse immunization, soluble O9BT was produced by 2 L culture at 37 °C for 4 hr. After isolation of crude protein, His-tag affinity chromatography was conducted for protein purification. Purification quality of purified O9BT was confirmed by SDS-PAGE (Figure 13) and approximately 85% pure proteins were obtained. His-tag affinity chromatography has a lot of

advantages. First, His-tag is easily added into the protein of interest. Second, His-tag affinity chromatography can provide up to 95% purity with 90% recovery of the tagged protein through a single purification step. Third, His-tag affinity chromatography is a rapid and inexpensive method compared to other affinity chromatography. However, His-tag chromatography also has disadvantages. If tagged proteins are not expressed at high levels, nonspecific binding can be occurred (Bornhorst and Falke, 2000). Because O9BT soluble protein were not produced sufficiently and other proteins having histidine were bound to His-hind resin, the purity of was lower than expected.

Preciously, we selected *E. coli* BL21 (DE3)/pTf16 strains expressing trigger factor for producing soluble O9BT. After 2 L culture at 15 °C for 24 hr and isolation of crude protein, His-tag affinity chromatography was conducted for protein purification. Purification quality of purified O9BT was confirmed by SDS-PAGE (Figure 14) and approximately 90% pure proteins were obtained. Interestingly, O9BT was released by 5 mM and 10 mM washing solution. According to His-bind resin manual (Novagen, USA), His-bind resin can be regenerated and reused many times. However there are no quantitative information about the number of reusing. Therefore we considered elution of O9BT by 5 mM and 10 mM washing solution is the problem of resin capacity. Other hypothesis is the portion of protein was too much for resin. We used 4 ml of His-bind resin and the capacity of His-bind resin is 8 mg/ml. As shown in Figure 13 and 14, production of proteins expressed by chaperone co-expression

system was much higher than soluble O9BT.

After alkaline-pH acetone precipitated O9BT was dissolved in binding buffer, His-tag affinity chromatography was conducted for protein purification. Production yield of inclusion bodies were higher than other soluble protein, so inclusion bodies produced through 500 ml culture was used. Purification quality of purified O9BT was confirmed by SDS-PAGE (Figure 15) and approximately 90% pure proteins were obtained. We considered alkaline-pH acetone precipitation do not have to purification step.

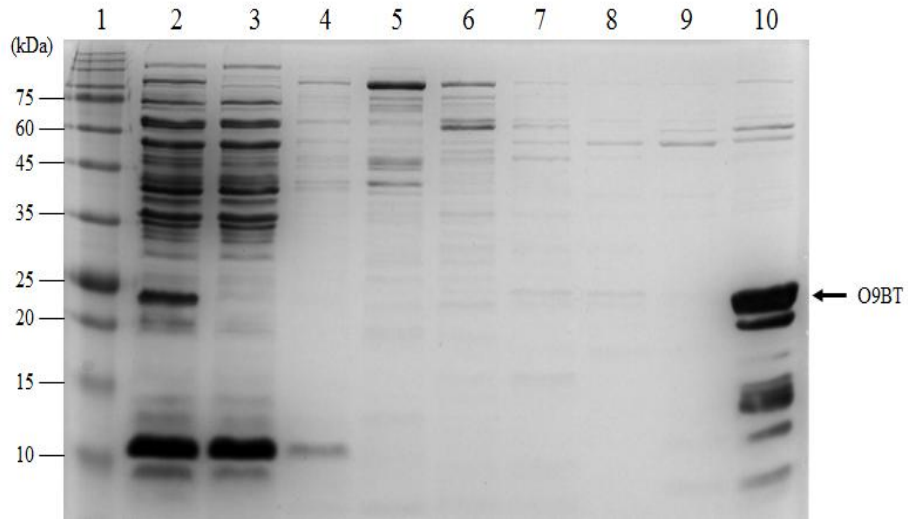

Figure 13. His-tag affinity chromatography of soluble O9BT. Lanes; 1, protein ladder; 2, crude protein sample; 3, sample flow through. 4-9, washing fraction; 10, elution fraction.

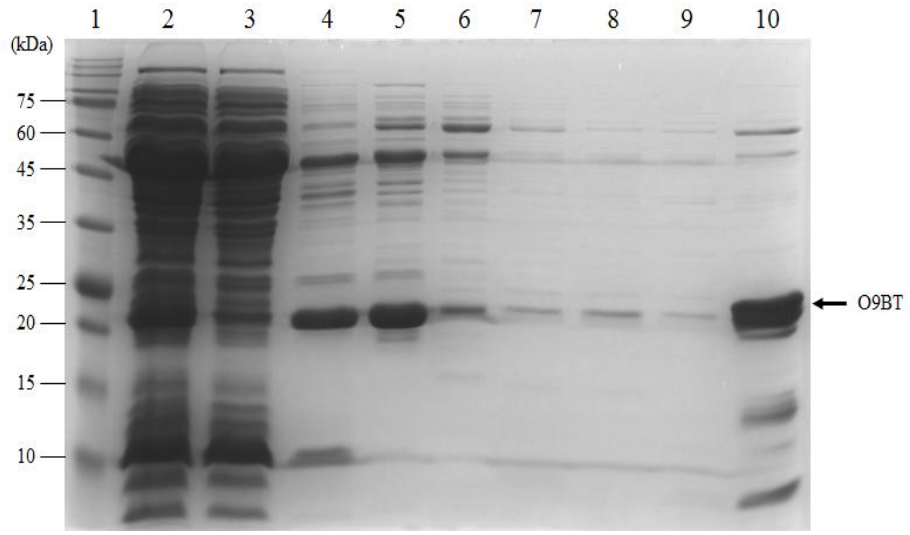

Figure 14. His-tag affinity chromatography of chaperone-assisted soluble O9BT. Lanes; 1, protein ladder; 2, crude protein sample; 3, sample flow through. 4-9, washing fraction; 10, elution fraction.

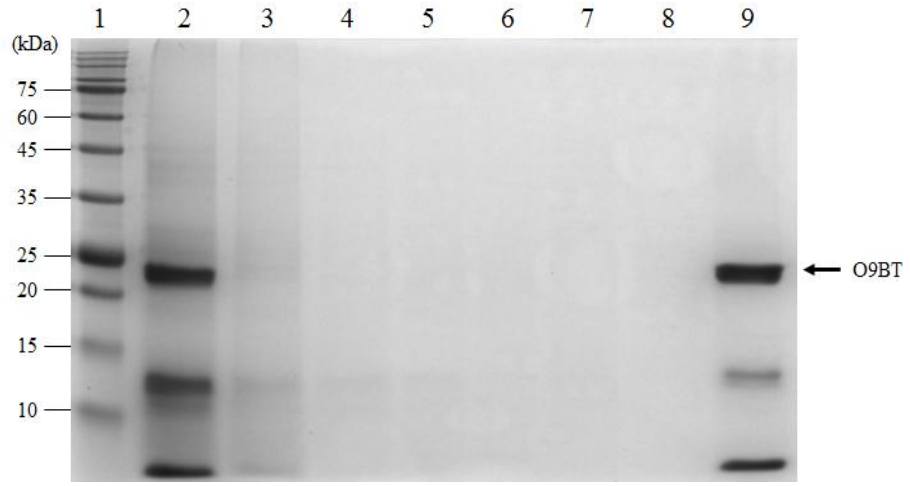

Figure 15. His-tag affinity chromatography of alkaline-pH acetone precipitated soluble O9BT. Lanes; 1, protein ladder; 2, crude protein sample; 3, sample flow through. 4-8, washing fraction; 9, elution fraction.

2) Protein quantification

After endotoxin removal with 0.5 ml of Pierce™ High Capacity Endotoxin Removal Spin column, the yield of purified protein was determined by BCA assay. Yield of purified soluble O9BT in 1 L flask culture was 3.12 mg/L and yield of chaperone-assisted soluble O9BT in 1 L flask culture was 1.99 mg/L. Yield of alkaline-pH acetone precipitated O9BT in 1 L flask culture was 8.34 mg/L and yield of inclusion bodies in 1 L flask culture was 78.5 mg/L (Table 7). Because inclusion bodies were not dissolved in PBS, endotoxin was removed with Triton X-114.

Production rate of alkaline-pH acetone precipitated O9BT was higher than other proteins. All proteins were obtained lower than expected because endotoxin was removed by column. Protein yield of chaperone-assisted soluble O9BT was lower than soluble protein. However, protein yield of chaperone-assisted soluble O9BT can be enhanced by establishing purification condition. We also anticipate production efficiency of alkaline-pH acetone precipitation will be enhanced because it doesn't need to purification step.

Table 7. Yields of purified proteins after endotoxin removal.

Type of protein	mg per culture liter
soluble O9BT	3.12
O9BT produced by chaperone co-expression system	1.99
Acetone precipitated O9BT	8.34
Inclusion bodies	78.5

4. *In vivo* immunization for comparison of solubilization methods

To compare protein efficiency of soluble proteins produced by various methods, mice were immunized intramuscularly with 20 μg of O9BT protein, 20 μg of M5BT protein and inactivated FMDV type O vaccine. M5BT protein, positive control, is FMDV serotype O subunit vaccine constructed in our laboratory and showed protection effect against FMD. Inactivated FMD vaccine produced in Green Cross was used as positive control.

Antigen-specific serum immunoglobulin from immunized mice was analyzed by indirect ELISA. Soluble O9BT-specific ELISA was conducted by coating immunoplate with soluble O9BT. M5BT-specific ELISA was conducted by coating immunoplate with M5BT.

1) Production of O9BT-specific serum IgG after immunization

Mice immunized with soluble O9BT, O9BT produced by chaperon co-expression system and alkaline-pH acetone precipitated O9BT showed high level of O9BT specific serum IgG titer after second injection of each antigen (Figure 16). Even though soluble O9BT was used as coating antigen in indirect ELISA, serum from mice immunized with chaperone-assisted soluble O9BT and alkaline-pH acetone precipitated O9BT showed high reactivity with coating antigen. Expecially serum from mice immunized with chaperone-assisted soluble O9BT showed higher reactivity than soluble O9BT. Therefore, protein recovered by solubilization methods could induce immune response like general soluble protein.

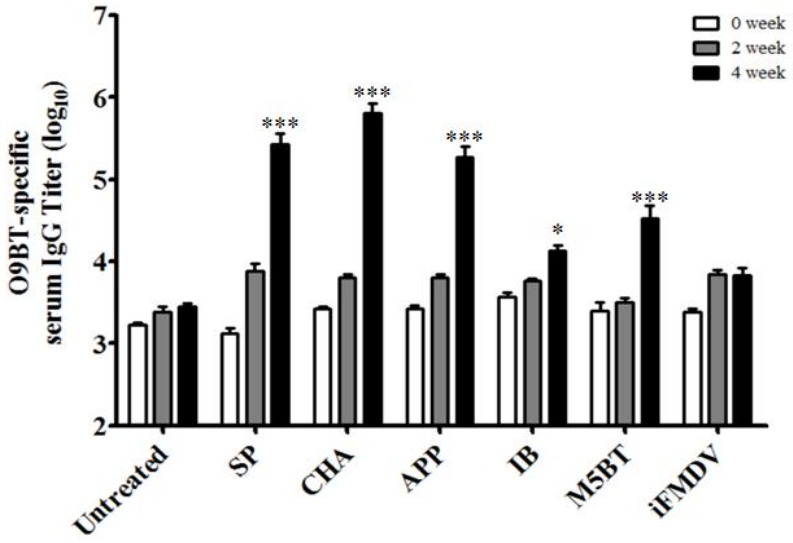

Figure 16. Soluble O9BT specific immune response after immunization with soluble O9BT, chaperone-assisted soluble O9BT, alkaline-pH acetone precipitated O9BT, inclusion bodies M5BT and, iFMDV (Green Cross). Anti-O9BT serum IgG levels at 0, 2, 4 weeks after immunization were measured by indirect ELISA. All values represent the means ± SD (n=5).

2) Production of O9BT-specific serum IgG subtype titer after immunization

Generally production of IgG_1 is used as an indicator of Th2 immune response and production of IgG_{2a} is used as an indicator of Th1 immune response (Ormstad *et al.*, 2003). If the ratio of IgG_1/IgG_{2a} is lower than 1, it means Th1 immune response was induced by immunization. If the ratio of IgG_1/IgG_{2a} is higher than 1, it means Th2 immune response was induced by immunization.

As shown in Figure 17A, production of IgG_1 is higher than

IgG_{2a} except inclusion bodies and iFMDV (Green Cross). Ratio of IgG_1/IgG_{2a} was higher than 1 (Figure 17B), it means O9BT induced Th2 immune response. Whereas, inclusion bodies and iFMDV (Green Cross) seemed to induce Th1 immune response.

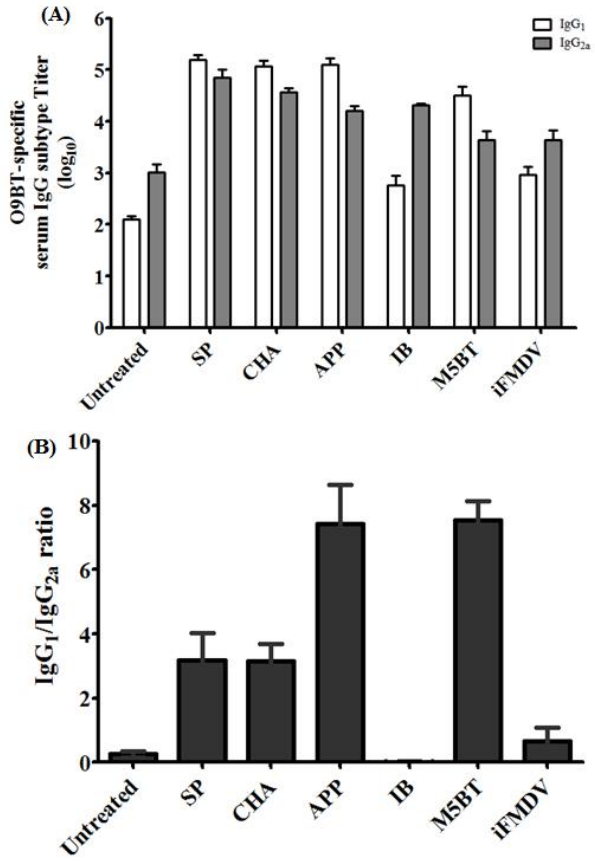

Figure 17. (A) Anti-O9BT serum IgG_1 and IgG_{2a} levels at 4 weeks after immunization were measured by indirect ELISA. (B) O9BT-specific IgG_1 and IgG_{2a} ratio. All values represent the means ± SD (n=5).

3) Production of M5BT-specific serum IgG titer after immunization

To evaluate cross-protection of O9BT, M5BT was used as a coating antigen for indirect ELISA. Even though O9BT and M5BT has no common strains or epitopes (Table 8), serum from mice immunized with soluble O9BT, chaperone-assisted soluble O9BT and alkaline-pH acetone precipitated O9BT showed reactivity with coating antigen (Figure 18). Expecially serum from mice immunized with soluble O9BT and chaperone-assisted soluble O9BT showed higher reactivity than mice immunized with M5BT. Considering serum IgG titer of 0 week after immunization, we interpreted that alkaline-pH acetone precipitated O9BT also has cross-protection like soluble O9BT or chaperone-assisted soluble O9BT.

Table 8. Comparison between O9BT and M5BT.

O9BT		M5BT	
Strain	Epitope	Strain	Epitope
O/Mya/98	VP2 E-F loop	O/CHA/00	VP1 G-H loop
		O/BEL/63	
O/Tibet/99	VP1 G-H loop	O/KOR/00	
		O/KOR/10	
Manisa O1	VP1 C terminal	O/KEN/10	

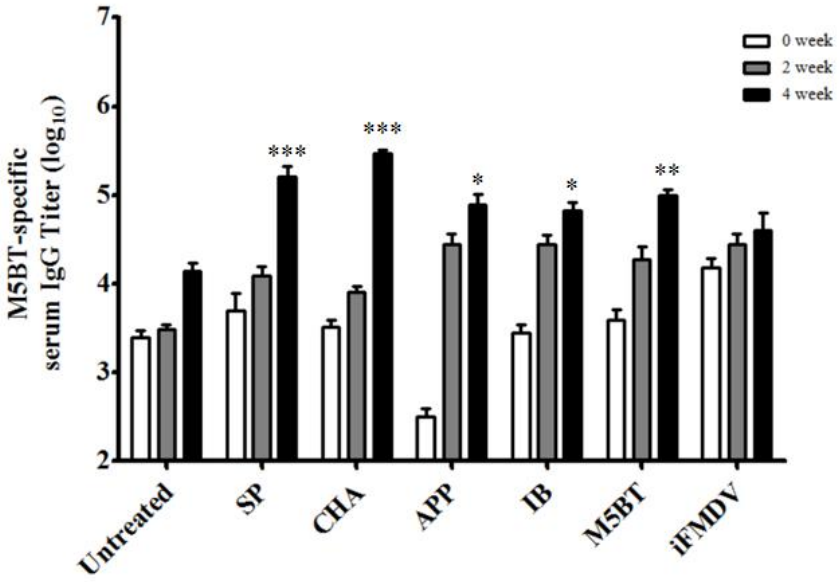

Figure 18. M5BT specific immune response after immunization with soluble O9BT, chaperone-assisted soluble O9BT, alkaline-pH acetone precipitated O9BT, inclusion bodies, M5BT and iFMDV (Green Cross). Anti-M9BT serum IgG levels at 0, 2, 4 weeks after immunization were measured by indirect ELISA. All values represent the means ± SD (n=5).

4) FMDV serotype O specific antibody production

Instead of neutralization assay, production of FMDV type O specific antibody was determined by blocking ELISA using FMDV serotype O antigen-coated plate (PrioCHECK). Percentage inhibitor titer (PI value) used as an indicator for vaccination efficacy.

As shown in Figure 19, only one mouse immunized soluble O9BT showed positive response (PI value > 50). However, serum

from mice immunized chaperone-assisted soluble O9BT and alkaline-pH acetone precipitated O9BT showed similar PI value with serum from mice immunized with iFMDV (Green Cross). Even inclusion bodies induced soluble O9BT-specific serum IgG, it didn't induce neutralization antibodies against FMDV serotype O.

It suggests that recovered proteins from inclusion bodies can enhance antigen-specific immune response and induce neutralization antibodies against antigen similar with soluble protein.

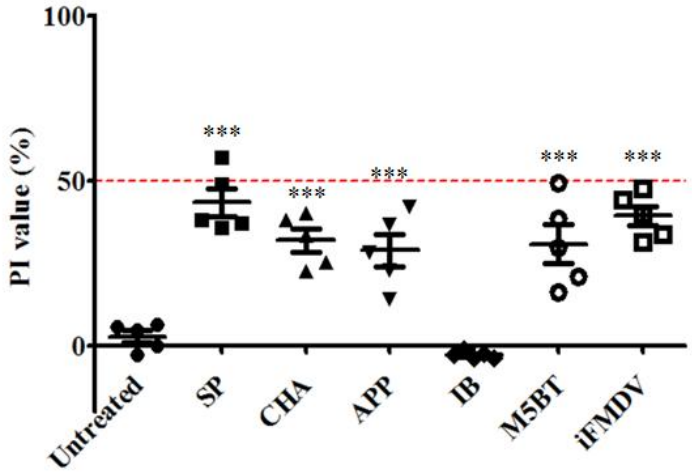

Figure 19. Detection of FMDV serotype O specific antibodies in serum from mice immunized with soluble O9BT, chaperone-assisted soluble O9BT, alkaline-pH acetone precipitated O9BT, inclusion bodies, M5BT and iFMDV (Green Cross). All values represent the means ± SD (n=5).

5) Detection of cytokine production

To detect cytokine production, the spleen was isolated from 3 mice per group at 4 weeks after immunization and incubated for 3 days. The cytokine production level was measured by sandwich ELISA by using Mouse IL-4 ELISA Kit and Mouse IFN-gamma ELISA Kit (KOMA, Korea). Th2 cells produce IL-4 and mediate humoral immune response, while Th1 cells produce IFN-gamma and mediate cellular immune response (Park *et al.*, 2005). Therefore, production level of IL-4 is used as an indicator of Th2 immune response and production level of IFN-gamma is used as an indicator of Th1 immune response.

As shown in Figure 20A, secretion of IL-4 was increased by soluble O9BT. Splenocyte from mice immunized with soluble O9BT produced higher level of IL-4 than splenocyte from mice immunized with other protein. Because soluble O9BT used as a antigen for inducing cytokine production, it was possible that splenocyte from mice immunized with O9BT showed higher reactivity than other protein. Interestingly, IL-4 level of iFMDV (Green Cross) group was decreased by introducing soluble O9BT.

As shown in Figure 20B, secretion of IFN-gamma was increased by soluble O9BT. Even though soluble O9BT used as a antigen for inducing cytokine production, splenocyte from alkaline-pH acetone precipitated O9BT immunized mice showed similar reactivity with splenocyte from soluble O9BT immunized mice.

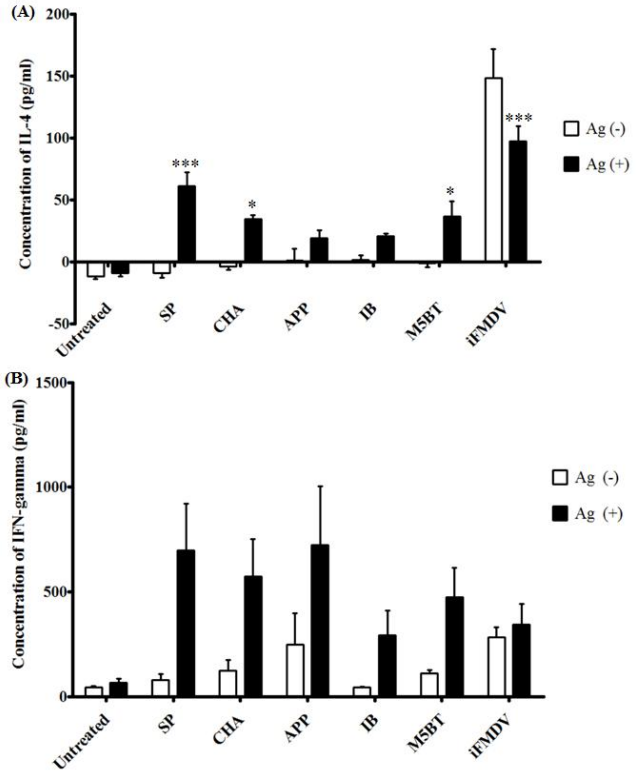

Figure 20. IL-4 (A) and IFN-gamma (B) production from O9BT stimulated murine splenocytes. Splenocytes ($1x10^7$ cells/ml) from immunized mice were incubated with 20 μg O9BT for 3 days. All values represent the means ± SD (n=5).

5. Production of trivalent multi-epitope FMD subunit vaccine

According to the reason mentioned below, alkaline-pH acetone precipitation was selected for production of trivalent multi-epitope FMD subunit vaccine. First, alkaline-pH acetone precipitated protein can enhance antigen specific immune response and induce neutralization antibodies. Second, production yield of alkaline-pH

acetone precipitated protein is highest because alkaline-pH acetone precipitation uses inclusion bodies. Third, alkaline-pH acetone precipitation doesn't need to purification step.

1) Alkaline-pH acetone precipitation

After solubilization of inclusion bodies using alkaline-pH buffer (pH 12.5, 10 mM Tris-NaOH), O9BT, A9BT and I9BT were recoverd by alkaline-pH acetone precipitation, respectively. Approximately 50% of inclusion bodies were recovered as soluble proteins. Protein yield of A9BT inclusion bodies up to 700 mg from 1 L bacterial culture, to recover up to 350 mg of soluble proteins from per 1 L culture of inclusion bodies is possible. Protein yield of I9BT inclusion bodies up to 520 mg form 1 L bacterial culture, to recover up to 260 mg of soluble proteins from per 1 L culture of inclusion bodies is possible.

2) Protein quantification

After endotoxin removal with 0.5 ml of Pierce^{TM} High Capacity Endotoxin Removal Spin column, the yields of purified protein were determined by BCA assay. Yield of purified alkaline-pH acetone precipitated O9BT in 1 L flask culture was 11.36 mg/L, yield of alkaline-pH acetone precipitated A9BT in 1 L flask culture was 23.08 mg/L and yield of alkaline-pH acetone precipitated I9BT in 1 L flask culture was 9.95 mg/L (Table 9). All alkaline-pH acetone precipitated proteins were recovered lower than amount of inclusion bodies because endotoxin was removed by column. Existence of alkalien-pH acetone precipitated

O9BT, A9BT and I9BT was confirmed by SDS-PAGE (Figure 21).

Table 9. Yields of alkaline-pH acetone precipitated proteins after endotoxin removal.

Type of protein	mg per culture liter
Alkaline-pH acetone precipitated O9BT	11.36
Alkaline-pH acetone precipitated A9BT	23.08
Alkaline-pH acetone precipitated I9BT	9.95

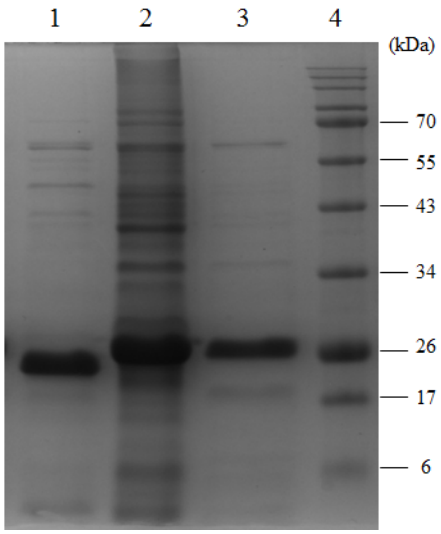

Figure 21. SDS-PAGE analysis of alkaline-pH acetone precipitated proteins after endotoxin removal. Lanes: 1, alkaline-pH acetone precipitated O9BT; 2, alkaline-pH acetone precipitated A9BT; 3, alkaline-pH acetone precipitated I9BT; 4, protein ladder.

6. *In vivo* immunization for development of trivalent multi-epitope FMD subunit vaccine

To identify efficiency of trivalent multi-epitope subunit vaccine, mice were immunized intramuscularly with 20 μg of O9BT protein, 20 μg of A9BT protein, 20 μg of I9BT protein, 20 μg of trivalent multip-epitope subunit vaccine, 45 μg of trivalent multi-epitope subunit vaccine, and inactivated FMDV type O vaccine produced in Komipharm (Korea).

Antigen-specific serum immunoglobulin from immunized mice was analyzed by indirect ELISA. O9BT-specific ELISA, A9BT-specific ELISA and I9BT-specific ELISA, was conducted by coating immunoplate with alkaline-pH acetone precipitated O9BT, alkaline-pH acetone precipitated A9BT and alkaline-pH acetone precipitated I9BT, respectively.

1) Production of O9BT-specific serum immunoglobulin titer after immunization

Mice immunized with alkaline-pH acetone precipitated O9BT and 20 μg of trivalent multi-epitope subunit vaccine showed high level of O9BT specific serum IgG titer after third injection of each antigen (Figure 22). Even though 20 μg of trivalent multi-epitope subunit vaccine used 6.6 μg of O9BT, mice immunized 20 μg of trivalent multi-epitope subunit vaccine produced high level of O9BT specific antibodies.

As shown in Figure 23A, production of IgG_1 is higher than IgG_{2a} except 45 μg of trivalent multi-epitope subunit vaccine and

iFMDV (Komipharm). IgG_1/IgG_{2a} ratio of O9BT was higher than 1 (Figure 23B), it means monovalent O9BT induced Th2 immune response. Whereas, 20 μg trivalent multi-epitope subunit vaccine's IgG_1/IgG_{2a} ratio was lower than O9BT, it means trivalent multi-epitope subunit vaccine induce more Th1 immune response than O9BT monovalent multi-epitope subunit vaccine. Th1 immune response which also referred as cell mediated immune response is important for protection against most intracellular pathogens like virus (Ashkar *et al.,* 2000). Therefore, trivalent multi-epitope subunit vaccine is more effective for protection against foot-and-mouth disease serotype O virus.

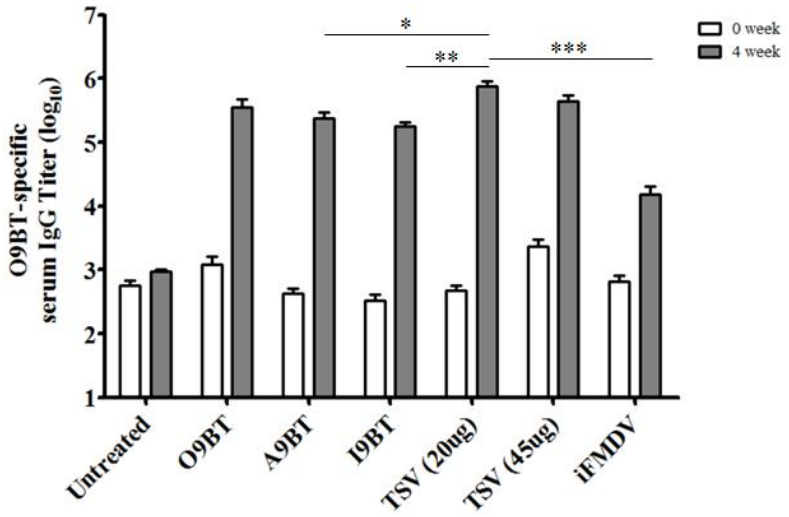

Figure 22. Alkaline-pH acetone precipitated O9BT specific immune response after immunization with O9BT, A9BT, I9BT, trivalent multi-epitope subunit vaccine and iFMDV (Komipharm). Anti-O9BT serum IgG levels at 0, 4 weeks after immunization were measured by indirect ELISA. All values represent the means ± SD (n=5).

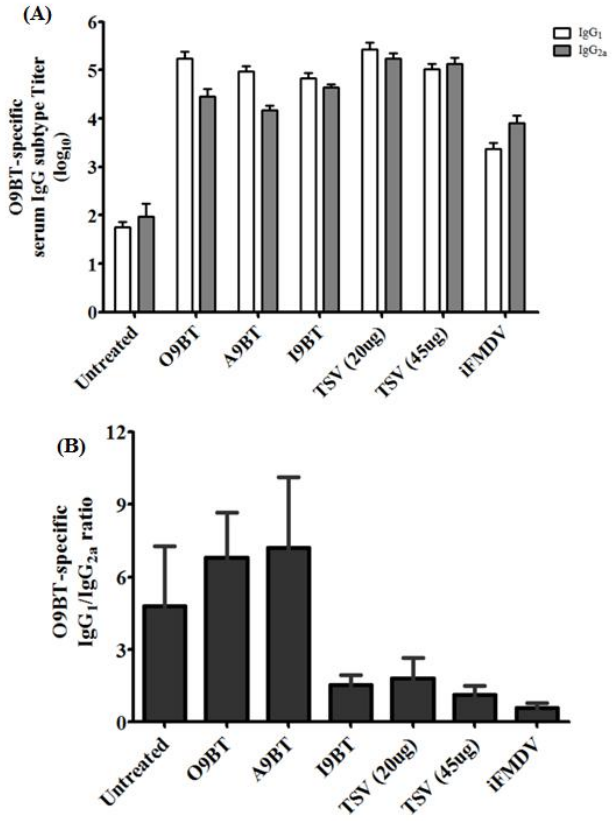

Figure 23. (A) Anti-O9BT serum IgG_1 and IgG_{2a} levels at 4 weeks after immunization were measured by indirect ELISA. (B) O9BT-specific IgG_1 and IgG_{2a} ratio. All values represent the means ± SD (n=5).

2) Production of A9BT-specific serum immunoglobulin titer after immunization

Mice immunized with alkaline-pH acetone precipitated A9BT, 20 μg and 45 μg of trivalent multi-epitope subunit vaccine showed high level of A9BT specific serum IgG titer after third injection of each antigen (Figure 24). Even though 20 μg of trivalent multi-epitope subunit vaccine used 6.6 μg of A9BT,

mice immunized 20 μg of trivalent multi-epitope subunit vaccine produced high level of A9BT specific antibodies more than mice immunized O9BT or I9BT.

As shown in Figure 25A, production of IgG_1 is higher than IgG_{2a} in O9BT, A9BT and I9BT group. However in 20 μg of trivalent multi-epitope subunit vaccine, 45 μg of trivalent multi-epitope subunit vaccine and iFMDV (Komipharm) group, IgG_{2a} is higher than IgG_1 and iFMDV (Komipharm). A9BT's ratio of IgG_1/IgG_{2a} was higher than 1 (Figure 25B), it means A9BT induced Th2 immune response. Whereas, trivalent multi-epitope subunit vaccine's IgG_1/IgG_{2a} ratio was lower than 1, it means trivalent multi-epitope subunit vaccine induce Th1 immune response and it is more suitable for protection against foot-and-mouth disease serotype A virus.

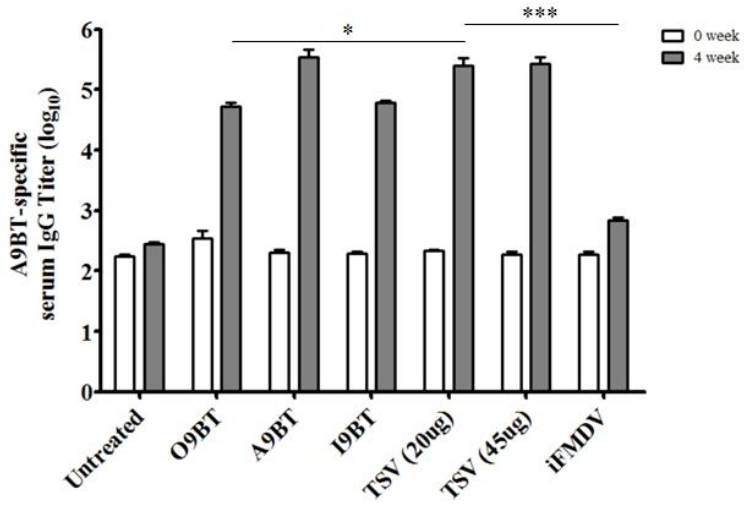

Figure 24. Alkaline-pH acetone precipitated A9BT specific immune response after immunization with O9BT, A9BT, I9BT, trivalent multi-epitpe subunit vaccine and iFMDV (Komipharm). Anti-A9BT serum IgG levels at 0, 4 weeks after immunization were measured by indirect ELISA. All values represent the means ± SD (n=5).

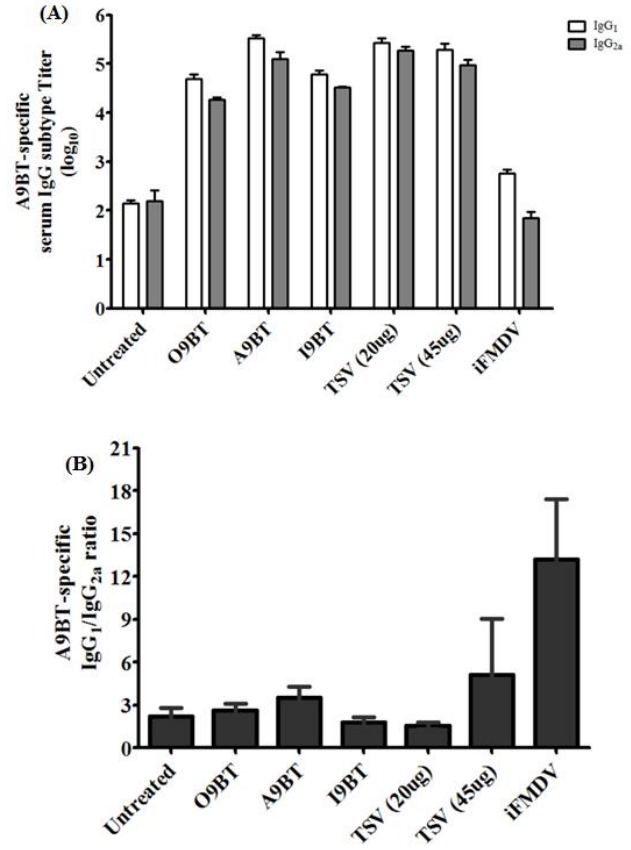

Figure 25. (A) Anti-A9BT serum IgG_1 and IgG_{2a} levels at 4 weeks after immunization were measured by indirect ELISA. (B) A9BT-specific IgG_1 and IgG_{2a} ratio. All values represent the means ± SD (n=5).

3) Production of I9BT-specific serum immunoglobulin titer after immunization

Mice immunized with alkaline-pH acetone precipitated I9BT, 20 μg and 45 μg of trivalent multi-epitope subunit vaccine showed high level of I9BT specific serum IgG titer after third injection of each antigen (Figure 26). Even though 20 μg of trivalent

multi-epitope subunit vaccine used 6.6 μg of I9BT, mice immunized 20 μg of trivalent multi-epitope subunit vaccine produced high level of I9BT specific antibodies more than mice immunized O9BT or A9BT.

As shown in Figure 27A, production of IgG_1 is higher than IgG_{2a} except iFMDV (Komipharm). A9BT's ratio of IgG_1/IgG_{2a} was higher than 1 (Figure 27B), it means I9BT induced Th2 immune response. Whereas, 20 μg trivalent multi-epitope subunit vaccine's IgG_1/IgG_{2a} ratio was almost 1, it means trivalent multi-epitope subunit vaccine induce Th1 immune response and it is more suitable for protection against foot-and-mouth disease Asia-1 virus.

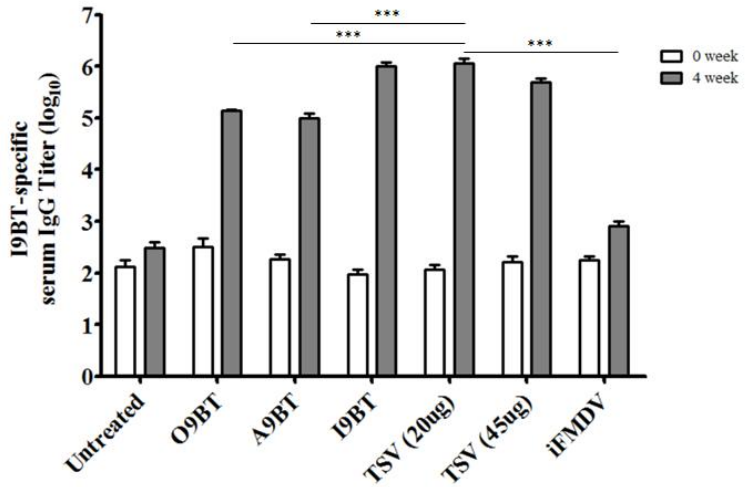

Figure 26. Alkaline-pH acetone precipitated I9BT specific immune response after immunization with O9BT, A9BT, I9BT, trivalent multi-epitope subunit vaccine and iFMDV (Komipharm). Anti-I9BT serum IgG levels at 0, 4 weeks after immunization were measured by indirect ELISA. All values represent the means ± SD (n=5).

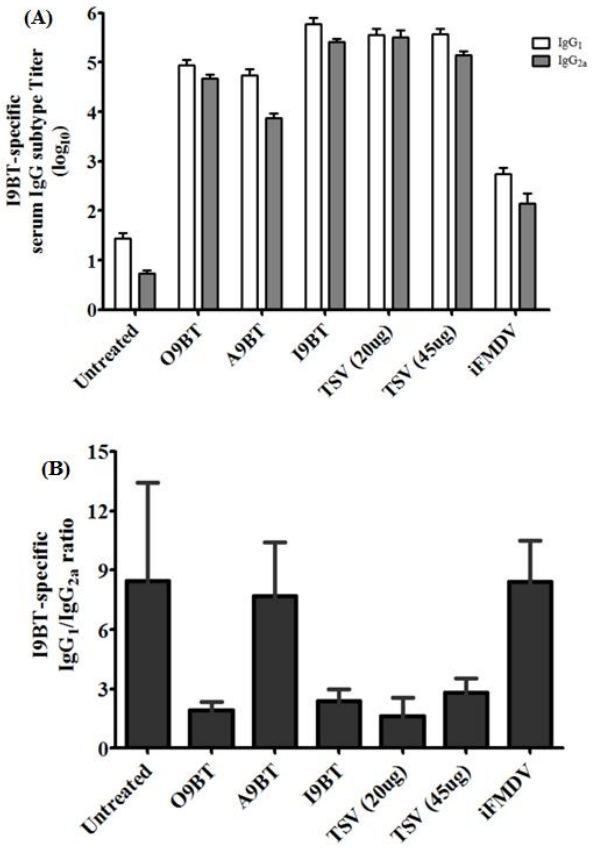

Figure 27. (A) Anti-I9BT serum IgG_1 and IgG_{2a} levels at 4 weeks after immunization were measured by indirect ELISA. (B) I9BT-specific IgG_1 and IgG_{2a} ratio. All values represent the means ± SD (n=5).

4) FMDV serotype O specific antibody production

Instead of neutralization assay, production of FMDV type O specific antibody was determined by blocking ELISA using FMDV serotype O antigen-coated plate (PrioCHECK). Percentage inhibitor titer (PI value) used as an indicator for vaccination efficacy.

As shown in Figure 28, three mice immunized 20 μg of

trivalent multi-epitope subunit vaccine, one mouse immunized O9BT and one mouse immunized 45 μg of trivalent multi-epitope subunit vaccine showed positive response (PI value > 50). Mice immunized 45 μg of trivalent multi-epitope subunit vaccine showed lower antigen-specific immune response, it means the vaccine efficiency was not necessarily proportional to amount of vaccine. There are many possible reasons for this phenomenon; 45 μg of trivalent multi-epitope subunit vaccine can be aggregated after intramuscular injection due to the large amount of proteins (Kijanka *et al.,* 2014) or vaccine efficiency can be saturated by 20 μg of trivalent multi-epitope subunit vaccine.

It suggests that trivalent multi-epitope subunit vaccine can enhance antigen-specific immune response with a small amount of vaccine.

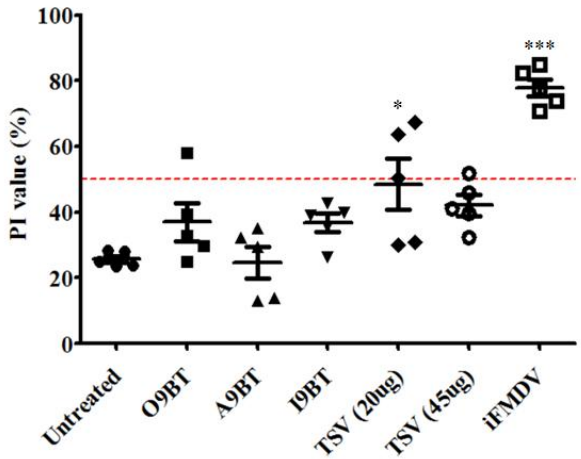

Figure 28. Detection of FMDV serotype O specific antibodies in serum from mice immunized with O9BT, A9BT, I9BT, trivalent multi-epitope subunit vaccine and iFMDV (Komipharm). All values represent the means ± SD (n=5).

5) Detection of cytokine production

As shown in Figure 29A, secretion of IL-4 was increased by 10 μg of O9BT, 10 μg of A9BT, and 10 μg of I9BT. There was no significant difference in IL-4 production between the groups.

As shown in Figure 29B, secretion of IFN-gamma was increased by 10 μg of O9BT, 10 μg of A9BT, and 10 μg of I9BT. Splenocyte from mice immunized with 20 μg of trivalent multi-epitope subunit vaccine produced higher level of IFN-gamma than splenocyte from mice immunized with other protein.

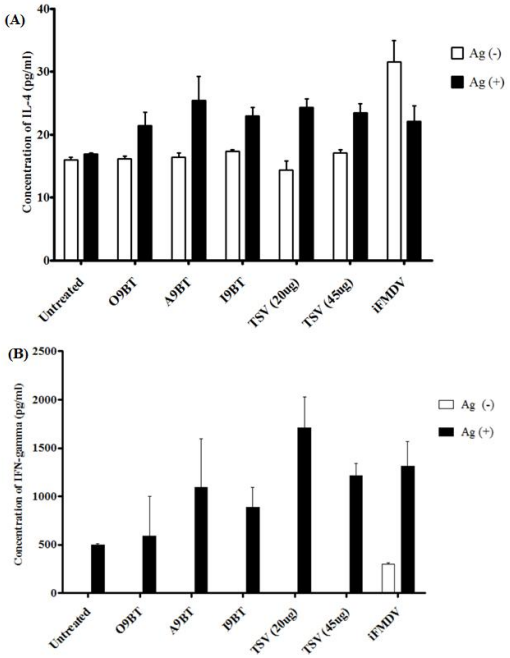

Figure 29. IL-4 (A) and IFN-gamma (B) production from trivalent recombinant protein stimulated murine splenocytes. Splenocytes ($1x10^7$ cells/ml) from immunized mice were incubated with 10 μg O9BT, 10 μg A9BT and 10 μg I9BT for 3 days. All values represent the means ± SD (n=5).

Ⅴ. Conclusion

In spite of many efforts to prevent FMD, outbreaks of the disease occur almost every year and cause economic losses in livestock industry. Vaccination is considered good way to prevent FMD and advancement in vaccine development was remarkable. However, preventing FMD through vaccination is difficult due to the antigenic variation of FMDV. In addition, difficulty of differentiating infected from vaccinated animals is the main reason of restriction on trade, followed economic losses. To overcome this problems, development of efficient FMD subunit vaccine is important. However, low immunogenecity of subunit vaccine is huge bottleneck of vaccine development. Therefore, aim of this study is to enhance efficiency of FMD subunit vaccine by using trivalent and multi-epitope strategy. We used trivalent multi-epitope subunit vaccine strategy for increasing antigenic spectrum of FMD subunit vaccine and multi-epitope strategy for improving immunogenecity of FMD subunit vaccine.

Recombinant proteins expressed mainly as a form of inclusion bodies. To increase production yield of soluble protein, we introduced chaperone co-expression system. There were many reports of chaperone's efficiency, but yield of recombinant protein we constructed was lower than expected. In this reason, we introduced combination of alkaline shock solubilization and acetone precipitation which is called alkaline-pH acetone precipitation. Because alkaline-pH acetone precipitation was using inclusion bodies, its productivity rate was higher than chaperone

co-expression system. In addition, recovery rate of bioactive proteins from inclusion bodies is higher than traditional solubilization method using chaotropic reagents like Urea and guanidine hydrochloride, because alkaline shock solubilization is mild solubilization without chaotropic reagents. To compare efficiency of soluble proteins produced by various methods, *in vivo* immunization was conducted. All soluble proteins induced antigen specific immune response. Among these solubilization methods, alkaline-pH acetone precipitation was selected for producing trivalent multi-epitope subunit vaccine by followed reasons. First, there no big difference between efficiency of solubilized proteins. Second, production efficiency of alkaline-pH acetone precipitation is 4 time higher than other methods. Third, acetone precipitated proteins don't need purification steps.

As a result, trivalent multi-epitope subunit vaccine were produced by alkaline-pH acetone precipitation. Through *in vivo* immunization, vaccine efficiency was confirmed. Even though trivalent multi-epitope subunit vaccine has one third of each protein, the efficiency of trivalent multi-eitope subunit vaccine corresponded to monovalent multi-epitope subunit vaccine. In addition, trivalent multi-epitope subunit vaccine showed cross-protection against FDMV serotype O.

Through this study, trivalent multi-epitope subunit vaccine showed the possibility of overcoming FMDV antigenic variation and enhanced immunogenecity. And it is possible to substitute inactivated FMD vaccine and be applied to livestock industry in Korea and neighbouring countries.

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Ⅶ. Summary in Korean

구제역은 돼지, 소, 말, 양, 염소 등의 우제류 동물에게서 발생하는 가축 전염병으로 제 1종 가축전염병으로 분류되어 있다. 구제역 바이러스에 감염되면 발과 입 주변에 수포가 발생하며 발열, 무기력함 및 절뚝거림의 증상을 수반한다. 성장을 마친 가축의 경우 구제역 바이러스에 감염되더라도 2주 이내에 자연치유가 된다. 하지만 구제역 바이러스의 강한 전염성과 변종 생성의 이유로 인해 살처분의 조치가 취해지며 이로 인해 축산업에 미치는 경제적 손실이 크다. 또한 구제역 청정국가로 분류 되지 않은 국가의 경우 수출 제약으로 인해 발생하는 경제적 손실도 큰데, 이는 구제역 바이러스에 감염된 후 자연 치유된 가축과 구제역 백신을 접종한 가축과의 구별이 어려워 수출에 많은 제약을 받고 있기 때문이다. 현재 농가에서 사용되고 있는 구제역 백신은 약독화/불활화 백신이 주를 이루고 있다. 상용 중인 구제역 백신은 구제역 바이러스가 가지고 있는 다양한 혈청형과 아형을 모두 방어하기 어렵다는 문제점을 지니고 있다.

약독화/불활화 백신이 가지고 있는 단점을 극복하기 위한 방안으로 서브유닛 백신이 각광을 받고 있다. 서브유닛 백신은 제조 방법이 약독화/불활화 백신에 비해 간단하기 때문에 질병발생 직후 빠른 백신 제조가 가능해 전염을 통제할 수 있다는 장점이 있다. 또한 구제역 바이러스가 가지고 있는 비구조 단백질을 사용하지 않아서 백신을 접종한 가축을 구별할 수 있다. 그러나 서브유닛 백신은 약독화/불활화 백신에 비해 면역원성이 낮다는 단점이 있다. 우리는 이를 극복하기 위한 방안으로 항원성이 높다고 알려진 항원결정기만을 연결한 다항원결정기 백신을 구축하였다. 또한, 구제역 바이러스의 항원 다양성을 극복하기 위한 방안으로 3가지 혈청형에 대한 백신을 구축하였으며, 각 혈청형마다 3가지 아형과 3가지 항원결정기

를 각각 조합하여 백신을 구축하였다. 최종적으로 총 3가지 종류의 단백질을 생산하기 위한 벡터를 구축하였으며, 구제역 바이러스 혈청형 O형 단백질은 O9BT, A형 단백질은 A9BT, Asia-1 단백질은 I9BT로 명명하였다.

우리는 구축한 다항원결정기 서브유닛 백신을 생산하기 위한 방안으로 대장균 발현 시스템을 이용하였다. 다항원결정기 서브유닛 백신은 본래 구제역 바이러스가 가지고 있는 구조가 아닌 항원성을 띄고 있는 항원결정기 일부분만을 사용해서 구축한 인위적인 구조의 백신이다. 이러한 백신을 미생물에서 발현할 때 가장 문제가 되는 점은 단백질이 soluble한 형태가 아닌 inclusion bodies의 형태로 생산이 된다는 점이다. 우리가 개발한 3가형 구제역 백신 또한 주로 inclusion bodies로 생산 되어 solublization 방법을 도입하였다. 3가형 백신 중 soluble한 형태의 단백질이 가장 많이 생산되는 O9BT만을 사용하여 soluble하게 재생산 된 단백질과 soluble한 단백질과의 효능을 비교하는 연구를 진행하였다. 먼저, 샤페론 co-expression system을 도입하여 단백질이 발현될 때 inclusion bodies가 아닌 soluble하게 발현되도록 유도하였다. 샤페론 단백질은 세포질에서 단백질이 생산될 때 발현 속도를 늦추거나 엉킨 단백질을 풀어서 다시 접히게 해줌으로써 soluble한 단백질로 생산되도록 유도하는 역할을 한다. 다섯 종류의 샤페론 벡터 중 리보솜에서 단백질 생산 속도를 늦춰주는 샤페론 단백질을 발현하고 있는 pTf16의 solubilization 효율이 가장 좋아 이를 사용해 대량생산하였다. 두 번째로는 염기성-pH 아세톤 침전법을 사용하여 inclusion bodies의 solubilization을 유도하였다. Inclusion bodies의 경우 pH 12.5 이상의 버퍼에 녹는 성질이 있으며, 이에 아세톤을 가해주면 침전이 일어나게 된다. 이 침전물을 PBS나 D.W 등의 버퍼에 다시 녹이면 단백질의 재구조화가 일어나면서 soluble한 단백질로 바뀌게 된다. 특

히 alkaline-pH 아세톤 침전법의 경우 inclusion bodies 자체를 사용함으로 생산량을 극대화 시킬 수 있고 염기성 버퍼를 사용함에 따라 단백질 재구조화 효율이 좋다는 장점이 있다. 위 두 가지 방법으로 생산된 단백질을 soluble한 단백질과 비교한 결과, 생산 방법에 상관없이 항원 특이적인 면역반응을 유도한다는 것을 검증할 수 있었다. 같이 비교된 inclusion bodies의 경우, 이전에 알려진 대로 생리활성도가 떨어지는 것으로 검증되었다. 위의 방법들 중에서 염기성-pH 아세톤 침전법은 다른 방법들에 비해 단백질 생산량이 4배 높고, 염기성-pH 아세톤 침전법은 정제할 필요가 없기 때문에 3가형 다항원결정기 서브유닛 백신을 개발하는 방법으로 선택되었다.

3가형 다항원결정기 서브유닛 백신 모두 염기성-pH 아세톤 침전법으로 생산한 뒤, 쥐에 근육주사하였다. 3가형 다항원결정기 서브유닛 백신의 경우 3가지 단백질을 칵테일 형식으로 섞어 두 가지 용량 (total 20 μg, 45 μg) 으로 나누어 접종해 주었다. 20 μg 3가형 다항원결정기 서브유닛 백신에 사용된 각 혈청형 단백질 양이 3분의 1로 줄었음에도 불구하고, 각 혈청형에 대한 방어효과가 단가형 다항원결정기 서브유닛 백신 효능에 상응하였다. 특히, 중화항체 반응의 경우 단가형 다항원결정기 서브유닛 백신보다 3분의 1로 단백질량을 줄인 20 μg 3가형 다항원결정기 서브유닛 백신에서 더 뛰어난 효과를 나타내는 것을 확인하였다. 특이한 점은 3가형 다항원결정기 서브유닛 백신의 양을 늘려주더라도 그에 비례하여 효능이 증가하지는 않는다는 점이었다. 이미 20 μg에서 백신 효능이 포화되었거나 45 μg의 경우 주사된 곳에 머물면서 단백질이 다시 응집되었기 때문이다.

우리는 이 연구를 통해서 3가형 다항원결정기 구제역 서브유닛 백신이 구제역 바이러스가 가지고 있는 항원 다양성을 극복할 수 있다는 가능성을 확인하였다. 실제 상용 백신으로 활용되기 위해서

는 중화 항체 및 돼지 공격 접종 실험 등의 추가적인 실험이 필요하겠지만, 이 연구에서 보여준 가능성으로 보아 우리가 개발한 3가형 다항원결정기 구제역 서브유닛 백신이 다양한 혈청형의 구제역 바이러스 효과적으로 방어할 수 있을 것이라고 기대된다.