A Dissertation
for the Degree of Doctor of Philosophy

Researches on Development of Multi-epitope Vaccine and Validation of Its Immuno-protective Efficacy against Foot-and-Mouth Disease

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Summary

Livestock farms in Korea are constantly suffering from outbreaks of foot-and-mouth disease (FMD) since 2010. Especially, the FMD that occurred in November 2010 caused massive economic damage of 2.4 billion US Dollars and stamping out 3.5 million of domestic animals. Republic of Korea lost the status of FMD free country from Office International des Epizooties (OIE). Although Korean government supplied emergency FMD vaccine, FMD constantly reoccurred. All FMD vaccines in Korea are imported from abroad and commercial FMD vaccine have several drawbacks. First, the mass culture of FMDV takes a long time and the vaccine is expensive because it can be cultured only in a facility with high biosafety level. Second, vaccination with commercial FMD vaccine may be disadvantageous to international trade of livestock products because it is difficult to distinguish between infected and vaccinated animals (DIVA). Thus, it is necessary to develop new FMD vaccine.

In study I, the recombinant proteins as FMD subunit vaccine were produced in *Escherichia coli*. FMDV capsid composed of four structural proteins. VP1, one of those structural proteins, has GH loop containing RGD motif. GH loop is a representative B cell epitope recognized by neutralizing antibodies, and can bind integrin of host cells to cause an infection. GH loop has low cross-immunity among the FMDV variants since the region has highly mutable amino acid sequence. To widen the spectrum of protection against FMDV variants,
recombinant proteins composed of multiple B cell epitopes were designed. Seventy-one VP1 protein sequences of FMDV serotype O which has occurred across the globe were collected in the US National Center for Biotechnology Information (NCBI), the peptide sequences of GH loop (amino acid residue 136 to 162 of VP1) were clustered by similarity analysis of amino acid sequences and the five GH loops as B cell epitope were randomly selected in each clustering. A recombinant gene consisted of five selected B cell epitopes and one T cell epitope was synthesized and introduced into *Escherichia coli* BL21 (DE3) to produce the recombinant protein. Four subunit vaccine candidates for FMD were successfully produced and they were named as 5BT, M5BT, B5BT and MB5BT. M5BT has M cell targeting peptide at the N terminus of 5BT protein, which was developed to induce mucosal immunity subsequently. B5BT has BmpB at the N terminus of 5BT protein which is membrane protein B of *Brachyspira hyodysenteriae* and was introduced as fusion protein to enhance stability and solubility of 5BT protein.

The characteristics of 5BT and B5BT proteins were analyzed. The results show that 5BT was more easily degraded by endogenous proteolytic enzymes than B5BT, and the ratio of inclusion body formation of 5BT protein was higher than that of B5BT. Although 5BT was unstable, the production amount of soluble protein was higher than B5BT. Since conjugating BmpB to 5BT showed little effect in mouse immunization, B5BT was excluded in later studies. Mass production of 5BT protein was optimized by exploring culture conditions, such as induction time, concentration of inducer, culture temperature, and harvest time. In
addition, the purification conditions were also optimized for high purity of recombinant protein which eventually came to more than 90%.

In study II, the efficacy of M5BT protein was evaluated through various in vivo immunization using mice and pigs. Mice and pigs were intramuscularly vaccinated with M5BT mixed with commercial adjuvant, CFA and IMS1313, and the sera of the animals were analyzed. The results show that M5BT specific antibodies and neutralizing antibodies for FMDV were detected in the sera of animals vaccinated with M5BT, and it was similar level with the sera of animals vaccinated with commercial FMD vaccine. These results suggest that the M5BT protein can be used as the FMD vaccine.

Since the FMDV is mainly infected through aerosol route, the studies have been conducted to develop a vaccine to induce mucosal immunity through the intranasal immunization. Because the M5BT protein is unstable as according to the results in study I, it is degraded by proteolytic enzymes in mucosal environment. To overcome the problem, the delivery carrier consisting of mannan-decorated acetylated inulin microparticles (M-INAC MPs) was used to transfer the M5BT safely into the mucosa and induce the immune response. M5BT loaded in M-INAC MPs was immunized via intramuscular and intranasal route of mice. The data showed that M5BT loaded in M-INAC MPs induced more effective immune response for M5BT and the production of anti-FMDV antibodies was higher than animals vaccinated with naked M5BT protein. The mucosal IgA production was also increased in the nasal vaccination.
In this study, the strategy of multi-epitope vaccine showed efficacy of vaccine and the new concept could be applied to develop a vaccine against not only FMDV but also other mutated viruses. If this multi-epitope vaccine is used to prevent disease, it can suggest insight in research to develop designed peptide vaccine.

Key words: foot-and-mouth disease, FMD virus, FMD vaccine, recombinant protein, multi-epitope vaccine, artificially designed vaccine, GH loop, B cell epitope

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List of Abbreviations

5BT: Multi-epitope vaccine composed of five B cell epitopes and one T cell epitope

Ad5: Adeno virus type 5

APCs: Antigen presenting cells

BALT: Bronchus-associated lymphoid tissue

BAL: Bronchoalveolar lavage

BHK21: Baby hamster kidney cell line

BmpB: Membrane protein B of *Branchyspira hyodysenteriae*

BW: Bronchoalveolar lavages wash

B5BT: BmpB-5BT

CALT: Conjunctival-associated lymphoid tissue

CAP: Cellulose acetate phthalate

CFA: Complete Freund’s adjuvant

cre: cis-acting replication element

CTB: Cholera toxin subunit B

DCs: Dendritic cells
DCM: Dichloromethane

DMF: Dimethyl formamide

DIVA: Distinguish the infected and vaccinated animals

FAE: Follicle-associated epithelium

FDCs: Follicular dendritic cells

FMD: Foot-and-mouth disease

FMDV: Foot-and-mouth disease virus

GALT: Gut-associated lymphoid tissue

GST: Glutathione S-transferase

HPMCP: Hydroxypropylmethyl cellulose phthalate

IFA: Incomplete Freund’s adjuvant

iFMDV: Inactivated FMD vaccine

INAC: Acetyl inulin

IRES: Internal ribosomal entry site

IW: Intestine wash

LALT: Larynx-associated lymphoid tissue

L.pro: Leader protease
MALT: Mucosal-associated lymphoid tissue

MBP: Maltose binding protein

M cells: Microfold cells

ME-SA: Middle-east-south Asia

MHC: Major histocompatibility complex

MPs: Microparticles

MRs: Mannose receptors

MTP: M cell targeting peptide

M5BT: MTP-5BT

MB5BT: MTP-BmpB-5BT

NALT: Nasal- or nasopharynx-associated lymphoid tissue

NaOAc: Acetic anhydride, Sodium acetate

NSP: Non-structural protein

NW: Nasal wash

PAMP: Pathogen-associated molecular pattern

PCP-FMD: Progressive control pathway-FMD

PD_{50}: 50% protective dose
PI: Percentage of inhibition

PK: Pseudoknot

PLGA: Poly (lactic-co-glycolic acid)

PMSF: Phenylmethylsulfonyl fluid

PRRs: Pattern recognition receptors

PVA: Poly (vinly alcohol)

RGD motif: Arginine-Glycine-Aspartic acid motif

RT-PCR: Reverse-transcription polymerase chain reaction

SALT: Skin-associated lymphoid tissue

SAT: Southern African Territories

SP: Structural protein

SEA: South-east Asia

SUMO: Small ubiquitin modified protein

TCID50: 50% tissue culture infective dose

TMB: Tetramethylbenzidine

VALT: Vulvo- or vaginal-associated lymphoid tissue

VW: Vaginal wash

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Introduction

Foot-and-mouth disease (FMD) causes loss of productivity of animals, leads to large-scale economic damage in livestock industries and induces disadvantages in national trade of livestock products as it is an acute contagious disease to cloven-hoofed animals such as pigs, cattle, and sheep (Kitching et al., 2007; Knight-Jones and Rushton, 2013).

FMD virus (FMDV) has seven serotypes (namely, O, A, C, Asia-1, SAT-1, SAT-2, and SAT-3) and rapid mutation rate of serotypes derived numerous variants of serotypes (Kitching et al., 2007; Knight-Jones and Rushton, 2013). Serotype O is known as the main serotype of FMDV breaking out in East Asia, Middle Asia, Africa and Europe (Samuel and Knowles 2001a). The serotype O has three major topotypes, namely, Pan-Asia (ME-SA), Cathay, and SEA, in Asia. The VP1, one of the structural proteins of FMDV, has sequence difference of 15 to 20% between topotypes (Samuel and Knowles, 2001a; Samuel and Knowles, 2001b).

In Republic of Korea, after outbreaks of FMDV serotype O infection in October, 2010 the FMDV had re-occurred in 2011, 2014, 2015 and 2017. There are two reasons for these reoccurrences. First, there are differences between the topotypes of the vaccine strain and the virus strain in field. Inactivated FMD vaccines used in Korea were mainly made of the Manisa O1 strain, which is topotype Pan-Asia. However, topotype SEA is the main reoccurring strain in Korea (Park, 2013). Second, many antigenic variants have been recognized within the same serotype,
and some of these antigenic variants are important influential factors for cross-protection (Jamal and Belsham, 2013).

Vaccination is one of the options to control and prevent FMD. Inactivated virus vaccine for the prevention of FMD has been commercialized (Rodriguez and Grubman, 2009). However, it is expensive because the production of inactivated vaccine requires a high level of biological safety facility to prevent the risk of leakage of live virus, and a long time to adapt the virus to cells. The inactivated vaccine is produced by only using the SPs and removing the NSPs of FMDV, which can be obtained from killing the virus through chemical treatments. If the NSPs are not completely removed in the process, it would cause a serious biosafety concern, which would hinder efforts to employ serology to DIVA (Distinguish between infected and vaccinated animals) (Doel, 2003; Purcell et al., 2007). This fact derives to classifying all the countries as FMD free or not by the use of vaccination by OIE. For this reason, most of countries have introduced a policy of the stamping out of FMD infected animals to keep the FMD free statue rather than employing vaccination (Knight-Jones and Rushton, 2013).

Recombinant subunit proteins produced in bacteria have been suggested as an alternative vaccine to solve these problems (Rodriguez and Grubman, 2009). These vaccines are free from DIVA concern and easy for mass production. However, subunit vaccine with fixed amino acid sequence may have limited efficacy for certain FMD strains because of high mutation rate. For this reason, the antibodies produced by existing subunit vaccines have low specificity for
neutralizing the mutated FMDV (Domingo et al., 1990). To overcome this weakness, many researchers have tried to produce newly designed recombinant subunit vaccines which are more effective to FMD (Hansson and Nygren, 2000). However, there are two shortcomings of a recombinant protein vaccine. First, it has the low immunogenicity effect (Blanco et al., 2013). Second, it has a narrow spectrum for preventing a highly mutable virus such as FMDV. Thus, we have developed a multi-epitope vaccine that can overcome the drawbacks of a recombinant protein vaccine, while still maintain the advantages.

The FMD subunit vaccine candidates were developed with artificially designed multi-epitope proteins in study I (Figure 1). Multi-epitope vaccine composed of five representative B cell epitopes of FMDV VP1 and one T cell epitope derived in NSP of FMDV. The proteins were optimized for their characteristics and production amounts by exploring culture conditions and in vivo immunization to mice.

Since the FMDV infects into animals through aerosol and oral routes, the FMD vaccine need to induce mucosal immune responses. Polymer-microparticles and M cell targeting peptide were introduced for inducing mucosal immunity in multi-epitope vaccine system. MTP can bind M cells in mucosal associated lymphoid tissues (MALTs) and improve antigen transportation to immune cells through transcytosis in mucosal immunity (Yoo et al., 2010).

NALT is representative mucosal immune system in nasal route. There are
advantages compare to oral administration because of the absence of acidity and lack of abundant secreted enzymes. The points result in a low dose requirement of antigen because there are few factors that affect stability of the vaccine. In addition, nasal vaccination is easily accessible.

Another adjuvant concept, particulated adjuvants, was introduced. Particulated adjuvants may function as efficient adjuvants as TLR agonist by enhancing the antigen uptake by immune cells such as macrophages and dendritic cells. Also, the particles can protect protein vaccines loaded in particles from proteolytic enzymes of host and be released in a sustained manner known to induce long-term immune response of the antigen (Wu and Lee, 2000; Robert et al., 2012). The particles was consisted of acetyl inulin microparticles (INAC MPs) and mannan.

In study II, the multi-epitope vaccine was evaluated its protective efficacy by analyzing sera from *in vivo* immunization into mice and swine. To implement a mucosal vaccine through intranasal vaccination, delivery carrier with adjuvant ability was introduced to protect recombinant protein from enzymatic degradation and enhance immune response (Figure 1).

The study I and study II are summarized in Figure 1. The need for the production of more effective vaccines has increased due to the onset of persistent FMD and the failure to prevent the disease through the use of existing commercial FMD vaccines. In this study, multi-epitope vaccine and particulated adjuvant may suggest insights to develop subunit vaccine system against FMD.
Figure 1. Experimental flow chart of the studies

In study I, the vaccine candidates were developed by producing multi-epitope protein in *E. coli* and characterized by various analysis. In study II, M5BT protein was evaluated by *in vivo* immunization and M-INAC MPs was used as delivery and adjuvant of multi-epitope vaccine to intranasal vaccination.
Review of Literature

1. Foot-and-mouth disease

1) Foot-and-mouth disease virus

(F) FMDV genome

FMDV is the prototype member of the genus *Aphthovirus* of the family *Picornaviridae*. The genome of FMDV is composed of a single positive-strand RNA. Its size of the RNA genome is approximately 8.3 to 8.5 kb. The genomic structure consists of a 5′-untranslational region (5′-UTR), an open reading frame (ORF), and a 3′-untranslational region (3′-UTR) (Figure 2) (Grubman, 2005).

The 5′-UTR is about 1300 nucleotides (nt) in length and consists of an S fragment at its 5′ end, a poly C tract, a series of RNA pseudoknot (PK) structure, a *cis*-acting replication element (*cre*), and the internal ribosomal entry site (IRES). The S fragment is 360 nt in length and is predicted to fold to form a large hairpin structure. The poly C tract is of variable length from 150 to 250 nt, but is composed of over 90% C residues. The function of the PK is unknown at the moment. The *cre* is a stable stem-loop element of about 55 nt and contains a conserved AAACA motif which acts as a template for uridylylation of VPg by the viral RNA polymerase. The IRES is about 450 nt and is responsible for cap-independent initiation of viral protein synthesis by using host`s protein synthesis machineries (Jamal and Belsham, 2013).
The ORF is a coding region following the 5’-UTR. It is the major portion of the RNA genome and is about 7000 nt. It encodes one polyprotein which is consisted of structural proteins (SPs) and non-structural proteins (NSPs). The polyprotein is cleaved by viral proteases to form four different SPs and eleven different NSPs. After translation, four primary products are formed, namely, L\textsuperscript{pro}, P1-2A, P2 and P3. L\textsuperscript{pro} is called leader protease which is the N-terminal component of the polyprotein. The L\textsuperscript{pro} inhibits host cell protein synthesis by inducing the cleavage of the host protein, eIF4G, which is a translation of the capped cellular mRNAs for using the host cell’s protein synthesis machinery to synthesize own protein. The P1-2A is capsid precursor, which is cleaved by 3C protease (3C\textsuperscript{pro}) to produce SPs, VP0, VP3, and VP1. The VP0 is cleaved to make VP4 and VP2 during encapsidation of the genome. 2A is a very short peptide. The 2A is self-cleaved from the P2 to divide between P1 and P2 with unknown factor. The P2 and P3 regions of the polyprotein are the NSPs. The P2 region is composed of 2B and 2C. The proteins are responsible for genome replication. The P3 region is composed of 3A, 3B\textsubscript{1-3}, 3C\textsuperscript{pro}, and 3D\textsuperscript{pol}. The 3A, 3B\textsubscript{1-3}, and 3D\textsuperscript{pol} are responsible for genome replication. The 3C\textsuperscript{pro} cleaves SP into VP0, VP1, and VP3 as well as the formation of the different NSPs (Grubman, 2005; Jamal and Belsham, 2013).

The 3’-UTR is much shorter than others. It is about 90 nt in length and folds to form a specific stem-loop structure by a poly A tract, has variable length. The 3’-UTR plays a role in genome replication (Jamal and Belsham, 2013).
The size of the RNA genome is approximately 8.3 to 8.5 kb. The genomic structure consists of a 5’-UTR, ORF, and a 3’-UTR. The 5’-UTR is about 1300 nt in length and consists of an S fragment at its 5’ end, a poly C tract, a series of PK structure, a cre, and the IRES. The ORF is a coding region following the 5’-UTR. It is the major portion of the RNA genome and is about 7000 nt. It encodes one polyprotein which is consisted of SP and NSP. The 3’-UTR is much shorter than others. It is about 90 nt in length and folds to form a specific stem-loop structure by a poly A tract, variable length. The figure is modified from MJ. Grubman, 2005.
(2) FMDV variants

The nucleotide sequences of the VP1, one of the SPs, have been used for genetic district of FMDV strains due to their significance for protective immunity and serotype specificity. Analysis of VP1 sequence can trace evolutionary dynamics, epidemiological relationships among the genetic lineages, the origin and movement of outbreak strains by using phylogenetic analysis (Samuel and Knowles, 2001a).

FMDV has seven different serotypes which are O, A, C, Asia-1, SAT-1, SAT-2, and SAT-3. Serotypes O and A were initially discovered. They named these serotypes after their place of origin (O for the department of Oise in France and A for Allemagne, the France word for Germany) (Jamal and Belsham, 2013). Serotype A is antigenically the most diverse (Mohapatra et al., 2011). Waldmann and Trautwein discovered third serotype which was named serotype C. Serotype C is apparently disappeared (Jamal and Belsham, 2013). Three additional serotypes were identified in samples originating from South Africa and were named as Southern African Territories (SAT) 1, 2, and 3 (Vangrysperre and De Clercq, 1996). The last serotype, Asia-1, was discovered in a sample collected from Pakistan in 1954. Serotype Asia-1 is antigenically the less diverse (Figure 3) (Jamal et al., 2011). There are 30 to 50 % genetic difference of VP1 sequence among the serotypes (Jamal and Belsham, 2013). Thus, infection or vaccination with one serotype does not confer protection against the other serotypes. In addition, there are many subtypes in each serotype.
Serotypes have been classified into genotypes based on up to 15% difference in VP1 coding sequences called topotypes. There are 7.5 to 25% genetic difference of VP1 sequence among the topotypes (Knowles and Samuel, 2003). FMDV serotype O have been classified into eight genetically and geographically distinct genotypes on the basis of the VP1 coding sequence, Middle East-South Asia (ME-SA), South-East Asia (SEA), Cathay, Indonesia-1, Indonesia-2, East Africa, West Africa and Europe-South America (Eura-SA) (Knowles and Samuel, 2003). Among these, ME-SA, SEA, and Cathay are circulating nearby Republic of Korea (Park, 2013). FMDV serotype A has genetically and antigenically the most diverse. The serotype A have been classified into 26 genotypes based on 15% difference in VP1 nucleotide sequences and form three geographically distinct topotypes, Asia, Eura-SA and Africa. There are about 24% genetic difference of VP1 sequence among the topotypes (Mohapatra et al., 2011). Topotype Asia-1 is the most prevalent in the Middle East and South Asian region and exists in various lineages. FMDV serotype Asia-1 has genetically and antigenically the less diverse. It was reported that 44 subtypes in Asia-1 serotype. The serotype Asia-1 is classified into two geographical distinct, Pakistan and other nations (Jamal et al., 2011). FMDV serotype C is classified into five genetically and geographically distinct genotypes, C1 (German), C2 (Uruguayan), C3 (South America), C4 (Argentina), and C5 (Argentina). Serotype C is apparently disappeared and exists in lab (Jamal and Belsham, 2013).
Figure 3. FMDV variants.

(A) Analysis of VP1 sequence can trace evolutionary dynamics, epidemiological relationships among the genetic lineages, the origin and movement of outbreak strains by using phylogenetic analysis. (B) FMDV has seven serotypes. There are many topotypes in each serotype. The topotypes are classified by genetically and geographically distinct.
(3) The pathogenesis of FMDV

The FMDV particle is spherical in shape and about 25 to 30 nm in diameter (Jamal and Belsham, 2013). The virus transmission occurs through respiratory aerosols (Alexandersen et al., 2002). Aerosol transmission of FMDV over distances as great as 50 km is believed to occur under certain weather conditions. It also is orally contagious through direct or indirect contact with infected animals and contaminated fomites, including infected animal products and feed (Abubakar et al., 2012).

The RNA genome of FMDV is surrounded by a protein shell or capsid. The capsid is composed of 60 copies of the capsomers (Grubman, 2005). The capsomers composed of four SPs, VP1, VP2, VP3 and VP4. While the VP1, VP2 and VP3 are exposed on the surface of the virus, VP4 is located internal capsid. Exposed capsid proteins, VP1, VP2 and VP3, contribute to the antigenic properties of the virus. The VP1 contains an important immunogenic sites, the GH loop (amino acid residue 141 to 160) (Grubman, 2005). The GH loop is an important epitope for producing neutralizing antibodies and has integrin binding sequence, arginine-glycine-aspartic acid (RGD) motif. The RGD motif of FMDV is required for attachment of the virus to the host cell via an integrin receptor (Baranowski et al., 2000; Baranowski et al., 2001). Integrin is heterodimeric glycoprotein on the surface of the cells consisted of two subunits, α and β subunit. 15 α subunits and 8 β subunits combine to form 20 different integrins (Takada et al., 2007). FMDV majorly recognizes three heterodimeric integrins, αvβ3, αvβ6
and αvβ8 integrins (Figure 4) (Duque et al., 2004). The αvβ6 integrin is a receptor for the extracellular matrix proteins expressed in epithelial cells and it is a most important receptor to bind and infect into cells for FMDV. However, the virus can also infect cells in an RGD-independent route through alternative receptors, heparan sulphate proteoglycan (Baranowski et al., 2000).

FMDV replication occurs mostly in tongue and mouth cells. After the virus attaches to its host cell, it enters by endocytosis and its coating from around the genome is removed. After the viral RNA is in the cytoplasm of the cell, ribosomes are recruited to make negative-strand RNA to serve as viral templates. Much like other picornaviruses, the virus ‘hijacks’ the cell and redirect vesicles to form reproduction sites within the cell. Vesicles redirected from the endoplasmic reticulum are housing the process where new viral RNA is being made, while ribosomes are synthesizing P1-encoded proteins. When the capsids are ready, their proteins are quickly rearranged so that the positive-strand RNA can be inserted. From there, the virions burst out of the cell and travel all around the body, or if they were being produced in a blister, the fluid released by the blister exposes the virion to the air, feed, and other animals (Andino et al., 1999; Jamal and Belsham, 2013)
Figure 4. The infectious mechanism of FMDV

(A) The FMDV can be infected by aerosol route, oral route and injuries. (B) The VP1 has GH loop, contain RGD motif to attach integrin receptor of host cells. FMDV mainly recognizes three heterodimeric integrins, αvβ3, αvβ6 and αvβ8 integrins. (C) The virus attaches the integrin receptors of host cells and internalizes into the cell via endocytosis mechanism. Then, the viral capsid is degraded and the RNA genome get out from endosome via endosomal escape into cytoplasm.
2) FMDV ecology and pathology

(1) Clinical symptoms

Clinical signs of infected cattle include pyrexia of ~ 40°C, followed by vesicular development on the tongue, hard palate, dental pad, lips, gums, muzzle, coronary band, interdigital cleft, and teats in lactating cows. Acutely affected individuals may salivate profusely, stamp their feet, and prefer to lie down. Ruptured oral vesicles can form, but rapid recovery, roughly 11 days after vesicle formation. Feet vesicles take longer to heal and are susceptible to bacterial infection leading to chronic lameness (Pacheco et al., 2005; Aftosa, 2007).

Infected pigs show mild lameness and blanching around the coronary band and may develop a fever of up to 42°C. The pigs become lethargic and have little interest in feed. The vesicles develop on the coronary band, heel of the foot, and tongue. Infected younger pigs commonly show heart failure and may die without clinical signs of illness because of viral damage to the developing myocardium (Figure 5) (Kitching and Alexandersen, 2002; Aftosa, 2007).

Infected sheep and goats usually show lameness as the first clinical sign of FMDV infection. This is followed by fever and vesicular development. Vesicles may also form on the heel bulbs, coronary band, mouth, and teats of lactating animals. Secondary infections result in reduced milk production and chronic lameness. Immature sheep and goats results in death without clinical signs due to heart failure (Kitching and Hughes, 2002; Aftosa, 2007).
(2) Diagnostic technique

There are many methods to diagnose FMD (Table 1). The tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle on the tongue, buccal mucosa or feet. Where epithelial tissue is not available from animals, samples of OP fluid can be collected by means of a sputum cup for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). The serum sample can be collected to diagnose FMD in case viraemia by means of RT-PCR or virus isolation (Commission and Committee, 2008; Jamal and Belsham, 2013).

Liquid Phase Blocking (LPB) enzyme linked immune-sorbent assay (ELISA), NSP blocking ELISA, Virus neutralizing assay, Penside (NSPs), and etc. are used to diagnose FMDV specific antibodies. The FMDV specific antibodies is measured to determine whether the animals is infected with the virus or to distinguish infected or vaccinated animals (DIVA). Thus, when an antibodies to the NSPs of FMDV is detected, it is diagnosed that the animals was infected by FMDV, not inoculated with FMD vaccine (Commission and Committee, 2008).

Virus isolation test, RT-PCR, Real-time PCR, Typing PCR, Antigen ELISA, Pen-side (SPs), and etc. are used to diagnose FMDV antigen. In this case, it is done to check the exact serotypes or strain of the FMDV of the confirmed animals. The nucleotide sequence is confirmed by PCR and the topotypes is identified.
through phylogenetic classification and the infection route is deduced (Reid et al., 2001).

![Figure 5. Typical clinical signs of infected animals by FMDV](image)

Suppurational sialorrhea and rhinorrhea of cattle, vesicles on the feet of pigs and ruptured vesicles on the tongue of cattle. These signs induce lameness and decrease of feed intake due to pain. The Figures were obtained by DEFRA, UK. (Department for Environment, Food and Rural Affairs)

**Table 1. Diagnostic methods of FMD**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Assay</th>
<th>Commercial products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis of specific antibodies</td>
<td>LPB ELISA</td>
<td>WRL Pirbright UK</td>
</tr>
<tr>
<td></td>
<td>NSP antibodies ELISA</td>
<td>Median diagnostics, Bionote, Prionics</td>
</tr>
<tr>
<td></td>
<td>Virus neutralizing titer</td>
<td>IBRS-2</td>
</tr>
<tr>
<td></td>
<td>Pen-side (NSP)</td>
<td>BPM east</td>
</tr>
<tr>
<td>Diagnosis of FMDV antigen</td>
<td>Virus isolation test</td>
<td>ZZR, IBRS-2, BHK</td>
</tr>
<tr>
<td></td>
<td>RT-PCR, Real-time PVE, Typing PVR</td>
<td>Primers: IRES, 3D region</td>
</tr>
<tr>
<td></td>
<td>Antigen ELISA</td>
<td>WRL Pirbright UK</td>
</tr>
<tr>
<td></td>
<td>Pen-side (SP)</td>
<td>PBM east</td>
</tr>
</tbody>
</table>
(3) Outbreak in Republic of Korea

Korea has been affected, in 2000 and 2002, by FMD outbreaks involving of Pan-Asia serotype O. Since 1990, many countries in Asia have been affected by the topotype ME-SA, other namely, topotype Pan-Asia. Early 2010, serotype A was detected in Pocheon city, and the isolates were confirmed as similar variants detected in neighbouring countries by sequencing. Before beginning in April 2010, Korea, Japan and Monglia were sequentially affected by outbreaks of FMD, Korea was recognized as a FMD free country without vaccination from the OIE, world organization for animal health (Park, 2013).

Thirteen outbreaks of FMD were reported in pigs and cattle in Korea between 8 April and 4 June 2010. The virus isolates were of serotype O, indicating that they were related to the virus strains of topotype SEA. The topotype SEA was circulating in East Asian countries. The FMD that recurred in Andong, Gyeongsangbuk-do in November 2010, continued until April 2011 and caused massive economic damage, 2.4 billion US Dollars, to livestock industries (Park, 2013; Park et al., 2014a). The Korean government provided emergency supplies of vaccines and various controversies, but the FMD decreased as the rainy season began. After, the end of huge epidemic of FMD, the Korea was recognized as a FMD free country with vaccination at the general meeting of OIE held in May 2014 in Paris (Yoon et al., 2016).

However, on 23 July 2014, animals in suspicion of infection were notified from
a pig farm, and FMD was confirmed. The FMD lasted until April 2015, and the virus strain was detected as topotype SEA. Korea was recognized as a no endemic FMD in domestic livestock. Subsequently, FMDV serotype A occurred in cattle from November 2016 to February 2017. There was a concern about naturalization of virus in 2010 as occurred in Pocheon city. The government has provided emergency supplies of vaccine against FMDV serotype A to cattle farms. Since then, Korea has remained an endemic FMD country (Table 2).

### Table 2. Current outbreak of FMD in Republic of Korea

<table>
<thead>
<tr>
<th>Occurrence Yr.</th>
<th>Period term</th>
<th>Stamping out (head)</th>
<th>Cause</th>
<th>Total processing cost (million USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mar. 2000</td>
<td>23 days</td>
<td>2,216</td>
<td>Imported hay</td>
<td>240.1</td>
</tr>
<tr>
<td>May. 2002</td>
<td>53 days</td>
<td>160,155</td>
<td>Migrant worker</td>
<td>93.4</td>
</tr>
<tr>
<td>Jan. 2010</td>
<td>28 days</td>
<td>5,956</td>
<td>Migrant worker</td>
<td>23.5</td>
</tr>
<tr>
<td>Apr. 2010</td>
<td>29 days</td>
<td>49,874</td>
<td>Travel in endemic country</td>
<td>91.1</td>
</tr>
<tr>
<td>Oct. 2010</td>
<td>145 days</td>
<td>3,479,962</td>
<td>Travel in endemic country</td>
<td>2,412</td>
</tr>
<tr>
<td>Jul. 2014</td>
<td>15 days</td>
<td>2,009</td>
<td>Oversea inflow</td>
<td>1.5</td>
</tr>
<tr>
<td>Dec. 2014</td>
<td>147 days</td>
<td>172,798</td>
<td>Oversea inflow</td>
<td>56.2</td>
</tr>
<tr>
<td>Jan. 2016</td>
<td>44 days</td>
<td>33,073</td>
<td>Unknown</td>
<td>5.2</td>
</tr>
<tr>
<td>Feb. 2017</td>
<td>9 days</td>
<td>195</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
</tbody>
</table>

The information in table were supplied by Ministry of Agriculture, Food and Rural Affairs and modified by author.
(4) Progressive control pathway for FMD

There are three main criteria for classifying the status of the countries in relation to the outbreak of FMD (Figure 6). The highest status is FMD free countries without vaccination. The second status is FMD free countries with vaccination. The last status is endemic FMD countries. The reasons for classifying countries with vaccination or without vaccination are discussed in the DIVA section. FMD is classified as one of the most dangerous diseases in the livestock industries because of its high infectivity and rapidity. Since the outbreak of FMD can lose the pretext for protecting the domestic livestock industries from cheap imported livestock products of endemic FMD countries, the outbreak of FMD affects the trade of livestock products between countries (Jamal and Belsham, 2013). In order to maintain its status as FMD free countries without vaccination, the Korea government has suppressed the transmission by stamping out all infected animals and controlling movement of susceptible animals. However, since 2010, when the situation has become out of control, the emergency vaccine has been supplied and the status of FMD free country with vaccination had been maintained for a while. In present, Korea maintain endemic FMD country. In order to be certified as FMD free country with vaccination, there shouldn’t be outbreak FMD for 12 months after vaccination. It is necessary to provide specific plans and control processes for outbreak of FMD to OIE. Forecasting and prevention are also important. To be certified as FMD free country without vaccination, there shouldn’t be outbreak FMD for 12 months from last vaccination.
Figure 6. Progressive control pathway for FMD

The status of countries on the Progressive control pathway (PCP)-FMD is evaluated according to defined criteria. Countries with endemic disease are in status endemic countries and no endemic countries. FMD free countries is classified two criteria which are FMD free countries with vaccination and without vaccination. The outbreak of FMD affects status about the trade of livestock products between countries.
2. Development of vaccine for FMD

1) Inactivated vaccine

(1) Vaccine

There are many types of vaccine (Figure 7). Vaccines are divided into whole body vaccine and subunit vaccine. Whole body vaccine includes the attenuated vaccine and the inactivated vaccine (killed vaccine). The other types included in subunit vaccine. The attenuated vaccine is made by a method that attenuate their virulent of live microbe and the inactivated vaccine is produced using killed microbe by chemical treatment. These vaccines consist of all components of the microbe. The subunit vaccine consists of specific components of the killed microbe or are made of recombinant proteins expressed in bioreactor.

Vaccines are also divided into using live microbe or not. The vaccines using live microbe include whole body vaccine, subunit vaccine, and the vaccines using viral vector such as adeno virus. The vaccines not using live microbe include recombinant protein vaccine, DNA vaccine, designed vaccine and the vaccine using polymer vehicle.
The vaccines are classified whether they use live microbe or not. In case with using live microbe, there are live attenuated virus vaccine, inactivated vaccine, and DNA encoded subunits of target microbe (DNA vaccine) and subunit vaccine loaded in a viral vector such as adeno virus without virulent. In case without using live microbe, there are subunit vaccine, recombinant protein, multi-epitope vaccine, and DNA vaccine loaded in a vectors such as polymer microparticles.
(2) Commercial FMD vaccine

Vaccination is one of the options to control FMD. All commercialized FMD vaccines are inactivated FMD vaccine. Because the inactivated vaccine consists of SPs of FMDV, it is made by using live FMDV. Varying requirement relating to quality, safety and efficacy apply in particular countries or regions in order for manufacturers to obtain an authorization or license for a FMD vaccine.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as baby hamster kidney (BHK) cell line. They are expanded in nutrient medium to a volume and cell density appropriate to seeding the main culture. When the virus is expected to have reached its maximum yield, the culture is clarified, often by chloroform treatment or ethyleneimine followed by centrifugation or filtration. The inactivation procedure is not satisfactory unless the decrease in virus titer, plotted logarithmically, is linear and extrapolation indicates that there is less than 1 infectious virus unit per $10^4$ liters of liquid preparation at the end of inactivation. After inactivation any residual chemicals in the harvest can be removed, or neutralized. The inactivated virus may be concentrated and purified by procedures such as ultrafiltration and chromatography. The concentrated and purified antigens can be formulated into vaccines by dilution in a suitable buffer and addition of adjuvants (Commission and Committee, 2008).

To vaccinating FMD vaccine in pigs, when pigs reach 8 to 12 weeks old, they
are vaccinated with 6PD$_{50}$ (50% protective dose) through intramuscular nearby ears. Then, boosting shot is vaccinated two weeks later. However, the animals are vaccinated once and are expected to be protected for a reason of farm management in Korea. The calf is inoculated with the first vaccine at 2 months old and the second vaccine after 4 weeks. Sows and adult cows are vaccinated once a seven month (Kitching et al., 2007).

In 2010, since the outbreak of FMD in Korea, Korean government has controlled outbreak of FMD by supplying inactivated FMD vaccine. There is a controversy over the low efficacy of the FMD vaccine. In addition, abnormal meats have been occasionally found such as the pus and necrosis tissue (Figure 8).
Figure 8. The FMD vaccine and controversy.

(A) Site of vaccination. To vaccinating FMD vaccine in pigs, when pigs reach 8 to 12 weeks old, they are vaccinated with 6PD$_{50}$ through intramuscular nearby ears. (B) Abnormal meats have been found such as the pus and necrosis tissue in sites injected the vaccine. The figures were obtained from Korea pork producers association.
(3) Distinguish infected and vaccinated animals (DIVA)

It is important that detection of animals have been infected with FMDV for the prevention of FMD especially in FMD free countries with vaccination and without vaccination. Both previously infected animals and vaccinated animals can detect neutralizing antibodies for FMDV in their sera, but previously infected animals can become “carriers”. The animals are clinical normal and can maintain this state for a lone period. It is possible that such animals can transmit FMDV to other animals. It is important to exactly distinguish infected and vaccinated animals (DIVA) for trade livestock products and animals (Figure 9) (Blanco et al., 2013).

Inactivated vaccine consists of purified preparations of inactivated 146S virions induce antibodies almost exclusively against SPs of the virus. Animals inoculated with inactivated vaccine have SPs specific antibodies in their sera. In infected animals, viral replication during infection results in the production of both SPs and NSPs. They have both antibodies against SPs and NSPs in their sera. Thus, it can be possible to discriminate between infected and vaccinated animals based on the detection of NSPs specific antibodies by NSP blocking ELISA such as 2C and 3ABC (Grubman, 2005; Jamal and Belsham, 2013). However, it can be difficult to differentiate between infected animals and vaccinated animals if the vaccine used has been prepared from insufficiently purified supernatant and it contaminated by viral NSPs especially. For this reason, OIE establishes Progress Control Pathway for FMD, and classify FMD free countries with or without vaccination.
Figure 9. Distinguish infected and vaccinated animals (DIVA)

Previously infected animals can have FMD virus and become “carriers”. They have neutralizing antibodies, SPs specific antibodies and NSPs specific antibodies in their sera. DIVA could be distinguished by detecting NSPs specific antibodies. However, animals inoculated with unsanitary vaccine included with NSPs can also have NSPs specific antibodies in their sera. It is difficult to DIVA.
2) Other types of vaccine

(1) Recombinant protein vaccine

Many researchers have attempted to develop alternative FMD vaccine due to address the above concerns of using live FMDV by new approaches. Although these new approaches can successfully produce neutralizing antibodies in laboratory scale, more researches are needed to demonstrate the efficacy of these strategies.

Recombinant protein vaccine is one of the novel FMD vaccine strategies. FMDV capsid proteins and structure are well known, including the prominent surface exposure of epitopes recognized neutralizing antibodies. A number of strategies have employed VP1 subunit and partial VP1 subunit as recombinant protein vaccine. These include bioengineered VP1, VP1 peptides, or chemically synthesized VP1 peptides. VP1 has GH loop which can bind integrin receptor of host cells. GH loop is representative neutralizing epitopes. Thus, animals vaccinated with recombinant VP1 have neutralizing antibodies in their sera. VP1 is produced by many platforms such as bacteria, animal cells, insect cells, chemical synthesis, and plant cells (Mor et al., 1998; Santos et al., 2002; Wang et al., 2003; He et al., 2007; Jung et al., 2013). Research has studied to utilize VP1 recombinant protein producing plants as feed.

However, many researchers have attempted to develop recombinant whole body vaccine because subunit vaccine has low immunogenicity. SP (P12A) of
FMDV is processed by 3C protease, one of the NSPs, and processed SP can form empty capsid like live FMDV (Figure 10). There are no concerns about DIVA and biosafety of inactivated vaccine because empty capsids do not contain viral genome and NSPs. In addition, the vaccine can produce various neutralizing antibodies of whole body vaccine. P12A3C protein (SPs and 3C protease) can be expressed in bacteria, plant and animal cell line (Wigdorovitz et al., 1999; Li et al., 2008).

Generally, recombinant protein has low immunogenicity. Recombinant protein can fuse various functioned protein or peptide to enhance immune response. SUMO protein, MBP, Cholera toxin B, flagellar protein, and GST are representative fusion protein to enhance solubility and stability of target protein. Various studies have accepted fusion protein to produce VP1, as FMDV subunit vaccine. Functional peptide can also fuse to enhance immunogenicity. M cell targeting peptide can improve mucosal immune responses by binding M cells in peyer’s patch of GALT (Yoo et al., 2010).

The solubility of protein vaccine is important. VP1 was produced in inclusion body from in E.coli (Wang et al., 2003; García-Fruitós et al., 2005). Inclusion body was produced by aggregation of miss folded proteins. Aggregated proteins have low bioactivity because it has insoluble character. In this reasons, many researchers have tried to make soluble protein from inclusion bodies using high concentration of urea and acetone precipitation (Vallejo and Rinas, 2004; Cao et al., 2012; Chen et al., 2016). Since inclusion bodies also have different structure
from original soluble proteins, neutralizing antibodies could not be produced. Therefore, solubilization is very important step for the production of subunit vaccine, and the addition of re-solubilizing step in increases the production cost.
Figure 10. Procedure of producing FMDV empty capsid vaccine

(A) FMDV RNA genome, (B) P12A polypeptide (SP, VP1 – VP4 and 2A) and 3Cpro were cloned into DNA and the gene was inserted into expression vector. The constructed vector was introduced into bioreactors. (C) Recombinant SPs were expressed in the cells and processed by 2A and 3Cpro. (D) The SPs were self-assembled. (E) Self-assembled SPs can form empty capsid.

Table 3. FMDV epitopes

<table>
<thead>
<tr>
<th>Subunit of FMDV</th>
<th>B cell epitopes</th>
<th>T cell epitopes (MHC class II)</th>
<th>T cell epitope (MHC class I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP2 (218 a.a)</td>
<td>190 – 192</td>
<td>40 – 50</td>
<td></td>
</tr>
<tr>
<td>VP3 (220 a.a)</td>
<td>69 – 71, 193 – 197</td>
<td>26 – 39</td>
<td></td>
</tr>
</tbody>
</table>

These epitopes correspond to the FMDV serotype O
(2) Designed vaccine

Designed vaccine is one of the recombinant protein vaccines. There are many epitopes recognized B cells and T cells in FMDV (Table 3). B and T cell epitopes is important to induce immune response for producing neutralizing antibodies against FMDV. B cell epitopes of FMDV were almost located in SP and exposed on the surface of virus. Generally, the B cell epitopes of FMDV are hyper-variable region. Amino acid sequence of the regions is different among the FMDV variants. Amino acid residues between 144 and 159 of VP1 known as GH loop are representative B cell epitope (Tang et al., 2012). T cell epitope has more conserved sequence of NSPs. T cell epitopes can divide CD8 T cell epitope and CD4 T cell epitope. CD8 T cell epitopes are loaded on MHC class I and induce response of cytotoxic T cells. CD4 T cell epitopes are loaded on MHC class II and induce response of helper T cells. There are many B and T cell epitopes in FMDV. The Table 3 shows FMDV epitopes.

Designed vaccine is specific concepts such as tandem repeated epitope protein, multi-epitope protein, and synthesized peptide. Blanco et al. developed synthesized epitope FMD vaccine that consisted one B cell epitope and one T cell epitope (Blanco et al., 2013). Multimerization approach consisting of a dendrimeric peptide bearing four copies of the B cell epitope and one T cell epitope was explored. Shao et al. developed multiple epitope peptide vaccine expressed in E.coli (Shao et al., 2011). The vaccine is consisted of two B cell epitopes of VP1 and is repeated three times of two epitopes. The vaccine
effectively induced neutralizing antibodies and protected from challenging the virus.

There are advantages of artificially designed multi-epitope vaccine. First, the strategies can concentrate immune response to epitopes by increase epitope density. Animals vaccinated with multi-epitope can produce epitope specific antibodies for viral neutralization. Second, the vaccine strategies can improve protective spectrum for viral mutation through combination with epitopes from various FMDV variants (Cao et al., 2012; Fang et al., 2015). However, simple peptide vaccines have low immunogenicity. It need to improve their immunogenicity by using strong adjuvant and fusion protein. Fusion protein, such as maltose binding protein (MBP), small ubiquitin modified protein (SUMO) and glutathione S transferase (GST), can help to improve protein solubility and stability. Some fusion protein, such as cholera toxin (CT), cytokines and flagellar protein, can help to improve immune response against the protein vaccine (Sun et al., 2003; Guo et al., 2013).

(3) DNA vaccine

Many researchers have employed DNA vaccine strategy to develop novel FMDV vaccine. DNA vaccination is a technique for protecting against disease by injection with genetically engineered DNA so cells directly produce an antigen, producing a protective immunological response. DNA vaccine can be targeted to various cellular compartments to improve antibody or cytotoxic T cell response.
Cytotoxic T-cell responses can be improved by targeting antigens for cytoplasmic degradation and subsequent entry into the major histocompatibility complex (MHC) class I pathway. It is expected to be more effective because it is similar to the immune response mechanism in the host infected with FMDV (Li et al., 2006; Pan et al., 2014).

DNA vaccine for FMDV can express antigens, such as FMDV whole body (empty capsid) and subunit protein (SPs), in host cells. The DNA vaccines are considered as a subunit vaccine or a recombinant protein vaccine.

However, DNA vaccines are difficult to use because they are easily degraded by DNase in the animal body. Various DNA delivery vehicles have been studied to overcome this problem. Wang et al. and Li et al. reported cationic PLGA microparticles loaded FMDV DNA vaccine to intranasal delivery (Wang et al., 2015; Li et al., 2016). The DNA vaccine loaded in PLGA particles effectively induced protective immune response against FMDV challenge.

(4) Viral vector vaccine

Various FMD vaccine candidates introduced in the section, other types of vaccine, can delivered into host body by viral vector, such as replication-defective human adenovirus type 5 (Ad5). Surface of Ad5 vector can include VP1, one of the SPs, or P12A polypeptide, capsid of FMDV. The vaccines induced stronger immune response against FMDV antigens on the surface of Ad5 than recombinant protein vaccine (Sanz-Parra et al., 1999). Pacheco et al. reported that Ad5 vector
containing the FMDV capsid were protected when challenged 7 days later with homologous virus (Pacheco et al., 2005).

In addition, the DNA vaccine utilizing the viral vector can be used to induce an immune response similar to a live virus infection. It not only transfers DNA to the host cell, but also it replicates in the host cells and induces the immune response. Unlike polymer vectors, viral vectors are more efficient for transfection of DNA encoded antigens into the host genome. However, this vaccine has potential risk of virulent of viral replication.
3. Mucosal immunity and mucosal adjuvant

1) Mucosal immunity

(1) General description

The mucosal surfaces of the body are vulnerable to infection due to thin and permeable barriers to the interior of the body. Mucosal membranes are huge surface covering the aero-digestive and the urogenital tracts, which have been less explored as routes of vaccine administration. Mucosal membrane are the major sites of entry for most pathogens because mucosal organs need to permeability of the surface lining these sites. It is important route of infection which goes with systemic immune response. Therefore, these vulnerable surfaces are associated with a large and highly specialized innate and adaptive mucosal immune system that protects the surface and the body against potential destructive agents and harmless substances from the environment. A healthy human contributes almost 80% of all their immunocytes to mucosal immune response. These immune cells accumulate in a particular mucosa or circulate between various mucosa-associated lymphoid tissues (MALT) (Figure 11) (Holmgren and Czerkinsky, 2005b; Czerkinsky and Holmgren, 2010).
The antigens are initially blocked by mucus layer. Antigens can be introduced through M cells and epithelial cells. Introduced antigens in lamina propria and antigens in lumen are captured by DCs. DCs migrate into lymph nodes and affect T cells activation. The antigens directly displace to FDCs in germinal center. B and T cells recognize the antigen by DCs and FDCs. Activated B cells and plasma cells migrate into effector sites and mesenteric lymph nodes. Plasma cells produce antigen specific secretory IgA (sIgA). Dimeric sIgAs are exported into lumen and intercept antigens and pathogens. Mucosal immune response affects both other sites of mucosal immune system and systemic immune response.
(2) MALT

The MALT is a highly compartmentalized immunological system and it functions independently of systemic immune response. The MALT is sub divided with gut-associated lymphoid tissue (GALT) (Liang et al., 2012), bronchus-associated lymphoid tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), genital tract (VALT, vulvo-vaginal-associated lymphoid tissue), and the mammary gland. The organized lymphoid follicles are considered as the principal inductive sites of mucosal immune response and also serve as inductive sites of local immune response (Figure 12) (Brandtzaeg et al., 2008).

The structure of MALT resemble lymph node with B cell follicles, T cell zone, and a variety of antigen presenting cells (APCs). It is follicle-associated epithelium (FAE). FAE has M cells to deliver exogenous antigens into immune cells. The antigens are transported actively to reach APCs, including dendritic cells (DCs), macrophages, B cells and follicular dendritic cells (FDCs). In addition, sub-epithelial DCs may capture antigens at the effector sites and migrate into lymph node and follicles. The DCs become active APC in lymph node and stimulate T cells for productive or down regulatory immune responses. Naïve B cells recognize antigens on the FDCs and are activated. Activated B cells were differentiated into plasma cells by cytokine signals secreted from activated T cells. The B cell zone form nearby FDCs presenting antigens. The region develop into germinal center from boundary of T cell zone. After being primed to become memory or effector B and T cells, they migrate from MALT and lymph nodes to
Peripheral blood for subsequent extravasation at mucosal effector sites. The plasma cells produce antibody in the mucosal immunity. The major antibody isotype is secretory immunoglobulin A (sIgA). IgA daily secreted is approximately 40 mg per Kg of body weight (Bombardieri et al., 2007; Brandtzaeg, 2010).

Figure 12. The categorization of MALT

There are many MALTs by organ in the body. CALT; conjunctival-associated lymphoid tissue, NALT; nasal- or nasopharynx-associated lymphoid tissue, LALT; larynx-associated lymphoid tissue, SALT; skin-associated lymphoid tissue, BALT; bronchus-associated lymphoid tissue, GALT; gut-associated lymphoid tissue, and VALT; vulvo-vaginal-associated lymphoid tissue. The figure was modified from longstarboars (www.longstarboars.com)
(3) NALT

NALT represents immune system of nasal mucosa and is a well-organized structure consisting of B cell and T cell enriched zone, which are covered by an FAE containing epithelial M cells and erythrocytes. The function of M cells seems to be similar to those found peyer’s patch in GALT. M cells are typical for antigen intake from mucosa (Kiyono and Fukuyama, 2004).

NALT has strategic position for incoming pathogens and it’s the first site of recognition and elimination of inhaled pathogens. It is important to inducing mucosal and systemic immunity. Lymphocytes in NALT proliferate and differentiate after antigen recognition. The lymphocytes start to produce cytokines such as IFNγ, IL-2, IL-4 and etc. B cell receptor go through isotype switching and produce antigen specific IgA mainly. Activated B cells can migrate through others mucus organ to respiratory and genital tract. Intranasal immunization is effective way how to activate respiratory immune system (Csencsits et al., 1999; Zuercher et al., 2002).

There are advantages compare to oral administration because of the absence of acidity and lack of abundant secreted enzymes. The points result in a low dose requirement of antigen because there are few factors that affect stability of the vaccine. In addition, nasal vaccination is easily accessible. However, to be effective following intranasal vaccination, the stability of antigen should be ensured and retained long enough time to interact with the lymphatic system.
Furthermore, there is a risk of passing into the brain through olfactory nerves and could induce side effects (Nacer et al., 2014). The nasal route holds great promise from the perspective of vaccination.

2) Mucosal adjuvant

(1) Adjuvant

An adjuvant is vaccine component to enhance immune response through co-administrated. There are many types of adjuvant. Representative adjuvant is alum, the most common adjuvant used in current human vaccine. However, alum is difficult to use as an adjuvant to induce mucosal immunity. The most studied adjuvant to induce mucosal immune response is cholera toxin (CT) (Holmgren et al., 1993; Holmgren and Czerkinsky, 2005a). Cholera toxin is protein complex secreted by the Vibrio cholera and has strong virulence. These enterotoxins promotes induction of antigen specific IgA antibodies and long-lasting memory to co-administrated antigens (Di Tommaso et al., 1996). In addition, heat-labile enterotoxins from Escherichia coli (E.coli) or bacterial flagellum can be used as adjuvants (Lee et al., 2006). These adjuvants act as ligands of Toll-like receptors (TLRs) on the surface of the immune cells and enhance immune response. However, there is the limitation of actually use of these adjuvant candidates due to the bio-safety issue and they is used only at the laboratory (Lawson et al., 2011; Norton et al., 2011).

Despite these problems, studies to develop adjuvants have employed a strategy
using TLR agonist (Orr et al., 2014). Promising mucosal adjuvant is the TLR 9 agonist CpG. CpG is small oligodeoxynucleotide sequences induce strong Th1 responses and have been effective in animal vaccine studies (Stern et al., 2002). In recent years, inulin was also reported as a novel TLR-4 agonist. Inulin is a group of naturally occurring polysaccharides produced by many types of plant including chicory, dahlia, wheat, etc. It consists of chain-terminating glucosyl moieties and a repetitive fructosyl moiety (GFn), which are linked by β (2-1) bonds. Some researchers have adapted microparticles consisting of inulin as an adjuvant (Kumar and Tummala, 2013).

Other adjuvants for inducing mucosal immune response include recombinant cytokines. There are many examples of producing recombinant cytokines through bioreactors such as bacteria. Li et al. reported that the immune response was increased by oral co-administration of IL-6 producing lactic acid bacteria and antigen (Li et al., 2015). Kayamuro et al. reported that recombinant IL-1 family cytokines increased IgA and IgG antibodies after administrating influenza subunit vaccine by intranasal route (Kayamuro et al., 2010).

(2) Carrier for mucosal delivery

The development of vaccine delivery carrier strategies to induce mucosal immunity remains a major challenge, especially after vaccination via mucosal tract since bioavailability is limited by the mucus layer, epithelial barriers of the mucus tracts and enzymatic degradation. In recent years, it was observed that
approaches to engineering delivery carriers to overcome the hindrances so that vaccines and adjuvants can be delivered to the mucus tracts. The carriers can be made of a variety of materials such as lipids, polymer, inorganic materials (Woodrow et al., 2012).

Polymer nanoparticle (10 – 1000 nm) or microparticle (1 – 10 µm) allow encapsulation of the drugs inside a polymeric matrix, protecting them from enzymatic degradation and acidic environment (des Rieux et al., 2006; Singh and Lillard, 2009). There are many polymer candidate substances, such as polyesters, polyanhydrides, and natural polymers. It has properties such as biodegradable and bioeliminable. Hydroxypropylmethyl cellulose phthalate (HPMCP) and cellulose acetate phthalate (CAP) are widely used for oral delivery of drugs, the proteins delivery with the polymers are protected from low pH and released at pH 5.5 in the duodenum and neutral pH due to the solubility of the polymers. The carriers can be used as oral vaccine delivery vehicle due to controlled released property. Conjugation of cysteine in polymers can induce disulfide bond with thiol groups of mucus. It assigned mucoadhesive property of polymer vehicle. The mucoadhesive property can increase the retention time of the polymers in the mucosa and maintains immune response via constantly releasing the vaccine (Singh et al., 2015; Lee et al., 2017b).

Chitosan can be also used to vaccination via intranasal route. Chitosan have already been proposed as vehicles for nasal immunization. Chitosan is a linear polysaccharide polymer consisting of randomly distributed β-(1 -4)-linked D-
glucosamine and $N$-acetyl-$D$-glucosamine. Besides its low toxicity and susceptibility to biodegradation, chitosan has shown mucoadhesive properties as well as an important drug penetration enhancement capacity across mucosal barriers. A number of studies show the efficacy of chitosan as an adjuvant and delivery vehicle for mucosal vaccine (Van der Lubben et al., 2001; Vila et al., 2004).

PLGA, copolymer of poly (d, l-lactide-co-glycolide), is the most commonly used polymeric materials. PLGA has attracted considerable attention due to its attractive properties. First, it is biodegradability and biocompatibility. Second, it was approval in drug delivery system by FDA. Third, the polymer are well described formulation and methods of production adapted to drug. Fourth, it protects the drug from degradation and has property of sustained release. Fifth, it is possible to modify surface properties (Jaganathan and Vyas, 2006; Slütter et al., 2010).

In recently, many studies have been conducted to conjugate substance with adjuvant property to the vaccine delivery system to enhance mucosal immune response (Figure 13).
Figure 13. Development of carrier with adjuvant function

Protein vaccines have low immunogenicity due to enzymatic degradation in mucus. The protein vaccines are also poorly immunogenic if they do not have a trait that alert the immune system. Many researchers have studied to conjugate substance with adjuvant property to the polymers and to develop new mucosal delivery carrier with adjuvant ability.
Study I. Development and Production of Multi-epitope Vaccine Candidates against FMD

1. Introduction

Recombinant subunit proteins produced in bacteria have been suggested as an alternative vaccine to solve biosafety problems of inactivated FMD vaccine which are discussed at literature review (Rodriguez and Grubman, 2009). These vaccines are free from DIVA concern and easy for mass production. However, subunit vaccine with fixed amino acid sequence may have limited efficacy for certain FMDV strains because of high mutation rate. For this reason, antibody produced by existing subunit vaccines have low specificity for neutralizing the mutated FMDV (Domingo et al., 1990). To overcome this weakness, many researchers have tried to produce newly designed recombinant subunit vaccines which are more effective to FMD (Hansson and Nygren, 2000). Therefore, artificially designed multi-epitope vaccine candidates were developed to address this viral mutation problem in this study I (Figure 15). The Table 4 shows that the advantages and disadvantages of inactivated vaccine, subunit vaccine and multi-epitope vaccine are compared.

Some researchers have reported to multi-epitope vaccine discussed at review of literature. Their multi-epitope vaccine is consisted of repeated epitope box, composing one or two B cell epitopes of FMDV and one T cell epitope (Shao et
al., 2011). The multi-epitope vaccine was expressed as inclusion body in *E. coli* and their researches focused on developing efficient vaccines to prevent single FMDV strains.

However, multi-epitope vaccine candidates in this study are mainly composed of five B cell epitopes derived from GH loop (commonly known as amino acid residues 130 to 160) in VP1 of FMDV. Several studies already showed that B cell epitopes are important to produce neutralizing antibodies (Wang et al., 2007; Tang et al., 2012). The GH loop is a representative B cell epitope containing RGD motif, which is an essential sequence to bind integrin of host animal cells for infection (Bittle et al., 1982; Verdaguer et al., 1995; Tang et al., 2012). RGD motif region is conserved in most FMDV variants although the rest regions of GH loop are highly variable (Lee et al., 2015). The representative GH loop as B cell epitope (amino acid residue 136 to 162 of VP1) among the epidemic strains existing throughout the world wide for protection against FMDV variants considering its hyper-variability were selected. The multi-epitope vaccine composed of tandemly repeated selected B cell epitopes will produce specific neutralizing antibodies for various FMDV serotype O variants (Figure 14).

T cell epitopes are necessary to develop a more efficient vaccine by activating T cells, enhancing the immune response. T cell epitope (amino acid residue 21 to 35 of 3A) of FMDV in one of the FMDV NSPs can enhance the immune response of the recombinant vaccines (Blanco et al., 2013). The multi-epitope vaccine, namely 5BT, developed in this study was tandemly composed five B cell epitopes
and one T cell epitope.

There are several huddles to producing artificial recombinant proteins in a soluble form using *E.coli* system. Recombinant proteins expressed in *E.coli* often form inclusion bodies and, in some cases, are not accumulated (Sørensen and Mortensen, 2005). The use of fusion protein is one of the options to overcome this problem. In addition to problems with the production of soluble proteins, a variety of fusion proteins or peptides can be used to enhance immunogenicity as a protein vaccines (Cuadros et al., 2004; Ma et al., 2005). In this study, BmpB was introduced as fusion protein (namely B5BT). BmpB is a membrane protein B of *Brachyspira hyodysenteriae*, and is produced with soluble protein in *E.coli* (Kim et al., 2009). There are fusion proteins selected according to the routes and targets of vaccination. Since FMDV can be infected into host cell through aerosol and oral route, it is important to induce mucosal immune responses. M cell targeting peptide (MTP) is typically used to induce mucosal immune responses. Conjugating MTP (CKSTHPLSC) to mucosal vaccine can induce higher chances to encounter immune cells because M cells conduct antigen transportation to the immune cells in lamina propria (Yoo et al., 2010). The multi-epitope vaccine was conjugated with MTP (namely M5BT and MB5BT).

The aims of the study I are design of multi-epitope vaccines and analysis of its characteristic. Multi-epitope subunit vaccine candidates were artificially designed and produced. Their properties were analyzed via expression pattern and resistance from enzymatic degradation.
Figure 14. Graphical abstract of study I

The developing process (A) and concept (B) of multi-epitope FMDV vaccine. i) Pooling the FMDV serotype O variants. ii) Pooling the GH loop sequences of the variants. iii) Grouping through analyzing similarity of amino acid sequence. iv) Selection of representative epitopes. v) Production of artificially protein. vi) Production of antibodies for FMDV variants. vii) Wide protection for FMDV variants.
Figure 15. The reasons for developing multi-epitope vaccine

Although recombinant protein subunit vaccine is novel alternative to overcome the problems of classical vaccines, subunit vaccine with fixed amino acid sequence may have limited efficacy for certain FMD strains because of high mutation rate. Multi-epitope vaccine composed of representative B cell epitopes can compensate the FMDV mutation.

Table 4. Comparison of inactivated vaccine, subunit vaccine and multi-epitope vaccine

<table>
<thead>
<tr>
<th>Type</th>
<th>Inactivated vaccine</th>
<th>Subunit vaccine</th>
<th>Multi-epitope vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Biosafety</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>DIVA</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Strong antigenicity</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cross-reactivity</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
</tbody>
</table>

Y: Effective; N: Ineffective
2. Materials and methods

1) 1st multi-epitope FMD vaccine candidates

(1) Exploration of representative epitopes

Seventy-six peptide sequences of partial GH loop (residue 132 to 151) of VP1 were collected from NCBI database (Table 5), and analyzed to selection of representative epitopes by method of sequence array and epitope randomly grouping in silico (Figure 16). The analysis was carried out by Corp. Insilicotech (Insilicotech, Gyeonggi-do, Korea). The fifteen peptide sequences were selected.

(2) Vector construction for vaccine candidates

The fifteen selected B cell epitopes were divided into three epitope boxes of each five epitopes, composing multi-epitope vaccine candidates. To minimize interference between adjacent epitopes, each epitope was separated by three glycines. To induce mucosal immune response, MTP was introduced in N terminus and C terminus of epitope box with 3GS (three glycines and one serine) linker. MEP was multi-epitope recombinant protein with MTP introduced in N terminus. EPM was multi-epitope recombinant protein with MTP introduced in C terminus. 378 base pair (bp) synthetic genes, MEP1, MEP2 and MEP3, and 381 bp synthetic genes, EPM1, EPM2, and EPM3, were synthesized in pIDTSMART-AMP (IDT, CA, USA). These gene contains Nde I and Xho I restriction sites. The genes were cut out by Nde I and Xho I and ligated with pET21a precut with Nde
I and Xho I resulting in pET21-MEP1, MEP2, MEP3, EPM1, EPM2, and EPM3 (Figure 21B). EP1, EP2, and EP3 genes without MTP were amplified by PCR from MEP1, MEP2, and EPM3 genes using an upstream primer engineering to introduce an Nde I site and a downstream primer engineered to introduce a Xho I site (Table 6). PCR product was cloned into Nde I and Xho I of pET21a precut with the same enzymes, resulting in pET21a-EP1, EP2, and EP3 (Figure 21A). The recombinant plasmids were confirmed by DNA sequencing at the National Instrumental Center for Environmental Management (NICEM, Seoul, Korea). The PCR products analyzed by gel-electrophoresis at 100 V in 1% agarose gel.

(3) Protein expression in E.coli

The vectors were transformed into E.coli BL21 (DE3) (Novagen, CA, USA) using heat-shock transformation at 42 °C. And, 7 ml of overnight culture was inoculated in 150 ml of Luria-Bertani (LB) broth containing 100 ng/ml of ampicillin in 500 ml Fernbach flask. Cultures were agitated at 230 rpm until A600 reached 0.6 and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by centrifugation at 6500 rpm for 10 min at 4 °C. The cell pellets were resuspended in 1 ml of PBS and sonicated on ice (60 × 2 seconds). Lysates were centrifuged at 17000 rpm at 4 °C for 10 min and the supernatants were collected.
Table 5. Seventy-six amino acid sequences (132 to 151) of partial VP1 GH loop

<table>
<thead>
<tr>
<th>Partial VP1 GH loop</th>
<th>Partial VP1 GH loop</th>
<th>Partial VP1 GH loop</th>
<th>Partial VP1 GH loop</th>
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<tr>
<td>GECKYSSXAVPNVRGDLNVL</td>
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<td>GNCKYGESNPVTNLRGDLQVL</td>
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</table>
Figure 16. Selection of representative epitopes by method of sequence array and epitope randomly grouping

(A) Pooling the FMDV epitopes, (B) Grouping the epitopes by method of sequence array and epitope randomly grouping, and (C) Selection of representative epitopes. In study I, fifteen epitopes were selected for consisting of three vaccine candidates.
(4) Analysis of protein expression with SDS-PAGE and western blot

The 20 µl of supernatants were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue by 3 times of heating in a microwave oven for 70 seconds, cooled down on a rocker for 5 min and destained with 25% methanol and 7.5% acetic acid solution overnight. The target protein was confirmed by western blot assay using His-tag antibody (Abcam. MA. USA). The protein was separated in a 15% SDS-PAGE and then transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was blocked by 5% skim milk in tris buffered saline (TBS) contacting 0.05 % Tween 20 (TBST) for 1 h on a rocker and then washed three times with TBST. The membrane was incubated with a 1:1000 diluted his-tag antibody overnight at 4 °C, washed three times with TBST, and incubated with a 1:2000 dilution of rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (Abcam, MA, USA) for 1 h. After washing three times with PBST, the signal was developed tetramethylbenzidine (TMB).

2) 2nd multi-epitope FMD vaccine candidates

(1) Selection of representative epitope by analyzing similarity of amino acid sequences

Seventy-one peptide sequences of GH loop (residues 136 to 162) of VP1 were collected from NCBI database and characteristics of amino acids in sequences were analyzed (Figure 17), and hierarchical clustering for analyzing the amino
acid sequence through R software (Figure 25) was performed. The final selection was conducted to include one representative GH loop sequence from each major cluster in the phylogenetic tree.

(2) Vector construction for expressing vaccine candidates

A 504 base pair (bp) synthetic gene, 5BT, which consists of five B-cell epitopes and one T-cell epitope in tandem array, was synthesized in pIDTSMART-AMP (IDT, CA, USA). A T-cell epitope (amino acid residues 21 to 35) was selected from 3A of type O FMDV (O-UKG 11/01) (Blanco et al., 2013). This gene contains two Xho I restriction sites. To minimize interference between adjacent epitopes, each epitope was separated by two glycines, and T-cell epitope was separated from five B-cell epitopes by two glycines and one glutamate. 5BT gene was cut out by Xho I and ligated with pET21a-BmpB precut with Xho I (Kim et al., 2009) resulting in pET21a-BmpB-5BT and pET21a-MBmpB-5BT (Figure 26A). 5BT gene was amplified by PCR from the pIDTSMART-AMP using an upstream primer engineered to introduce an Nde I site and a downstream one with a Xho I site (Table 6). PCR product was cloned into Nde I and Xho I of pET21a precut with the same enzymes, resulting in pET21a-5BT and pET21a-M5BT (Figure 26A). The recombinant plasmids were confirmed by DNA sequencing at the National Instrumental Center for Environmental Management (NICEM, Seoul, Korea). The PCR products analyzed by gel-electrophoresis at 100 V in 1% agarose gel.
Figure 17. Analysis of amino acid characterization in seventy-one GH loop sequences of FMDV serotype O

The amino acids in sequences was analyzed by Mega X software. Amino acids of blue color have positive charged side chain, amino acids of red color have negative charged side chain, amino acids of green color have polar uncharged side chain, amino acids of yellow color have hydrophobic side chain, and amino acid of purple color has glycine, special case.
Table 6. Primers to synthesize the genes of vaccine candidates

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<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Tm</th>
<th>Length</th>
<th>GC%</th>
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<td>catatggGGGAACTGTAATACTTGAGACC</td>
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<td>EP1-R</td>
<td>CTCGAGCGAGACCTGGGAGAT</td>
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<td>EP2</td>
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<td>EP3</td>
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<td>M5BT</td>
<td>M5BT-F</td>
<td>CATATGGCGTGAATAATCAACCCAC</td>
<td>63.6</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>M5BT-I</td>
<td>GGGGATTTGCCATACTCCCAACCA CAGCTCA</td>
<td>63.8</td>
<td>23</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>M5BT-R</td>
<td>GGTCACTCGTCCATCAAACCTCGAGCAATCTC</td>
<td>64.0</td>
<td>25</td>
<td>52</td>
</tr>
</tbody>
</table>

Tm: melting temperature; GC%: The ratio of guanine and cytosine
(3) Protein expression and purification

The vectors were transformed into *E. coli* BL21 (DE3) (Novagen, CA, USA) using heat-shock transformation at 42 °C. And, 7 ml of overnight culture was inoculated in 1 L of Luria-Bertani (LB) broth containing 100 ng/ml of ampicillin in 2.8 L Fernbach flask. Cultures were agitated at 230 rpm until $A_{600}$ reached 0.6 and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by centrifugation at 6500 rpm for 10 min at 4 °C. Cell pellets were resuspended in 100 ml of binding buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9) and sonicated on ice (48 × 10 seconds). Lysates were centrifuged at 17000 rpm at 4 °C for 20 min and supernatants (soluble fraction) were filtered through a 0.45 µm filter (Corning, NY, USA). 100 ml of binding buffer was added to soluble fraction to purify two target proteins, 5BT and B5BT. The Ni-nitrilotriacetic acid (NTA) agarose resin (Novagen, CA, USA) was packed into a column and equilibrated with binding buffer. The sample was loaded into a column and the column was washed with specific volumes of wash buffers. Target protein was eluted with 20 ml of elution buffers. The eluted protein was dialyzed using a membrane tube (molecular cut-off: 6-8000 kDa, Spectrum, CA, USA) against the distilled water at 4 °C overnight. Desalted solution was lyophilized and stored at -20 °C until used. Lipopolysaccharide (LPS) was removed using ToxinEraser™ Endotoxin removal kit (Genscript, NJ, USA) and detected by using ToxinSensor™ Chromogenic LAL endotoxin assay kit (Genscript, NJ, USA). $OD_{280}$ was detected by a
spectrophotometer (Implen, Munchen, Germany) and protein concentration was calculated using extinction coefficient (Gill and Von Hippel, 1989). To analyze the inclusion body formation, sonicated cell debris was dissolved in 100 ml of solubilization buffer (10 mM tris-base, pH 12.5) and centrifuged at 17000 rpm at 4 °C for 20 min. Supernatant containing dissolved inclusion body (inclusion body fraction) was transferred to other tubes.

3) Characterization and optimization to produce vaccine candidates

(1) Analysis of solubility and stability of recombinant proteins

The 20 µl of soluble and inclusion body fractions were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue by 3 times of heating in a microwave oven for 70 seconds, cooled down on a rocker for 5 min and destained with 25% methanol and 7.5% acetic acid solution overnight. Bands were analyzed by image J software (NIH) to compare target protein quantity (Zhang et al., 2011). The target protein was confirmed by western blot assay using His-tag antibody (Abcam. MA. USA). The protein was separated in a 15% SDS-PAGE and then transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was blocked by 5% skim milk in tris buffered saline (TBS) contacting 0.05 % Tween 20 (TBST) for 1 h on a rocker and then washed three times with TBST. The membrane was incubated with a 1:1000 diluted his-tag antibody overnight at 4 °C, washed three times with TBST, and incubated with a 1:2000 dilution of
rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (Abcam, MA, USA) for 1 h. After washing three times with PBST, the signal was developed tetramethylbenzidine (TMB). To test stability of the proteins, the cell pellets from 50 ml culture were resuspended in 10 ml of PBS and distributed in 1 ml aliquot into the micro tube. The tubes were centrifuged at 13000 rpm for 1 min at ambient temperature. The supernatant were removed and cell pellets were stored at -70 °C until use. Every day one frozen tube was resuspended in 1 ml of PBS, sonicated and supernatants after centrifugation were stored at 4 °C. This was repeated for 7 days to investigate the protein degradation by endogenous proteases of E. coli. After 7 days proteins were analyzed by 15% SDS-PAGE, bands of target proteins in gel images were analyzed by Image J software.

(2) Optimization of culture conditions to improve quantity of acquired 5BT protein

The transformed E.coli was cultured under various conditions to improve quantity of acquired target protein. The conditions were temperature, induction time, culturing time and IPTG concentration. The E.coli was cultured in 25 ml of LB broth containing 100 ng/ml of ampicillin in 150 ml Fernbach flask. Extracted proteins were analyzed by SDS-PAGE and image J software.

(3) Optimization of purification conditions to improve purity of acquired 5BT protein

The extracted proteins were carried out by purification methods under various
conditions to enhance their purity. The Ni-nitrilotriacetic acid (NTA) agarose resin (Novagen, CA, USA) was packed into a column and equilibrated with binding buffer. The sample was loaded into a column and the column was washed with specific volumes of wash buffers. Target protein was eluted with 20 ml of elution buffers. The conditions show in Table 11.

4) *In vivo* evaluation as FMD vaccine

(1) Mouse immunization

Six-week old BALB/C mice were used for the immunization following the policy and regulations for the care and use of laboratory animal (Laboratory Animal Center, Seoul National University, Korea). All of the protocols were reviewed and approved by the Animal Care and Use Committee at Seoul National University (SNU-141201-1). The mouse was immunized intramuscularly at days 0, 14 and 28 with 20 µg (0.5 µg/µl) of each peptide emulsified in Complete Freund’s Adjuvant (CFA, priming) or Incomplete Freund’s Adjuvant (IFA, boosting) and sacrificed on day 42. Five mice in the negative control group were immunized with PBS and positive control group of 5 mice were immunized with 40 µl of inactivated FMDV vaccine (iFMDV, Daesung, Gyeonggi-do, Korea). Blood samples were collected before priming (day 0) and on days 13, 27, and 42 (Sacrifice) from intra-petosal veins with a disposable syringe and delivered into sterilized tube (Figure 18 and Table 7). Serum was separated by centrifugation at 12000 rpm for 3 min using serum separate tube (BD microtainer, NJ, USA).
Figure 18. The immunization schedule of mouse *in vivo*

The mouse was immunized intramuscularly at days 0, 14 and 28. Blood samples were collected before priming (day 0) and on days 13, 27, and 42 (Sacrifice) from intra-petrosal veins with a disposable syringe and delivered into sterilized tube.

Table 7. Mouse *in vivo* immunization group

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen (Annotation)</th>
<th>Dose</th>
<th>Adjuvant</th>
<th>Animals (Age)</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>40μl</td>
<td>CFA/IFA (equal volume)</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5BT</td>
<td>20μg (0.5 μg/μl)</td>
<td>CFA/IFA (equal volume)</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>B5BT</td>
<td>20μg (0.5 μg/μl)</td>
<td>CFA/IFA (equal volume)</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>IFMDV</td>
<td>40μl</td>
<td>Included</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
</tr>
</tbody>
</table>
(2) ELISA assay

Antibody production was examined by ELISA in serum samples collected at days 0, 13, 27, and 42. Briefly, 96 well immuno-plate was coated with purified 5BT in carbonate-bicarbonate buffer (CBB) for 1 h at 37 °C (0.1 µg/well) or to evaluate peptide specific antibody production about five B cell epitopes in the 5BT were separately synthesized (Peptron, Daejeon, Korea) and dissolved in DMSO. Plates were coated with 50 pmole/well of each peptide in the CBB. Then, wells were washed with PBS and blocked with 0.5 % skim milk in PBS for 1 h at room temperature (RT). Series of five-fold dilution of each serum sample were prepared, starting at 1/50 and volume adjusted to 100 µl with PBST (0.5 % tween 20 in PBS) containing 0.5 % skim milk. Plates were incubated for 2 h at RT and HRP conjugated goat anti-mouse antibody diluted 1:5000 in PBST containing 0.5 % skim milk was added. The color was developed with 100 µl/well of the TMB (Sigma, MO, USA) and stopped by an equal volume of 0.16 M H₂SO₄. Plates were read at 450 nm in a Microspectrophotometer (Tecan, Austria). Titer of specific antibody was calculated by Softmax Pro version 5.4.1. Antibody titers were reported as log₁₀ of the reciprocal of the highest dilution. Serum of days 0, 13, and 27 were analyzed with above methods according to time by detecting 5BT specific IgG titers.

In addition, anti-FMDV O type antibodies were detected by competition ELISA using VDPro FMDV type O ELISA kit (Median diagnostics, Gangwon-do, Korea), following the manufacturer`s protocol. Briefly, each plate of the kit was pre-
coated with FMDV type O P13C protein. Serum sample, negative control, and positive control were diluted by 1:5 in dilution buffer and prepared samples were incubated in wells for 1 h at RT. Then, wells were washed with washing buffer, 100 µl of HRP conjugated anti-FMDV antibody was added and samples were incubated for 1 h at RT. Color was developed with 100 µl/well of the TMB substrate and stopped by 50 µl of stop solution. All reagents were provided in the kit. Plates were read in a Microspectrophotometer at 450 nm. PI (%) means the percent inhibition (Jung et al., 2013).
3. Results and Discussion

1) 1st multi-epitope FMD vaccine candidates

(1) Exploration and selection of epitope candidate

Functions and structure of the proteins consisted of FMDV have been thoroughly studied. VP1 is major capsid protein of FMDV and has revealed into details. There are many neutralizing epitopes in VP1. Representative epitopes is GH loop, C terminus and BC loop (Tang et al., 2012). Comparing the VP1 protein sequences of various FMDV serotype O variants, it can be seen that the sequence mutation of these epitopes is the most severe regions (Figure 19). Even if it is the same strain, there are mutation of one or two amino acids in these regions. The regions are exposed on the surface of VP1 and can bind neutralizing antibodies (Parry et al., 1989). The FMDV can avoid cross-reactivity because of the high variability of these epitopes (Leippert et al., 1997). GH loop is an important and representative region as B cell epitope of VP1 and is exposed on the surface of VP1 (Figure 20). The GH loop has RGD motif which can bind to integrin of host epithelial cells and cause infection of animals (Burman et al., 2006).

The sequence information of VP1 were gathered at NCBI GenBank and the peptide sequences (amino acid residue 132 to 151 of VP1) were analyzed by the method of sequence array and random epitope grouping (Figure 16). The fifteen peptides with the most overlapping or similar sequences were selected (Table 8).
Figure 19. Mutation regions in the FMDV VP1

(A) BC loop (amino acid residue 21 to 44 of VP1), (B) GH loop (amino acid residue (130 to 160) and (C) C terminus (amino acid residue 200 to 213). The data was produced by protein Blast at NCBI. The sequence of Query is VP1 of FMDV serotype O at Nov. 2010 and the sequence of Subject is VP1 combination. X mean variable amino acid sequences.
Figure 20. Structure of FMDV VP1

(A) Secondary structure of VP1 analyzed by free online tool (Psipred). The sequence is VP1 of FMDV type O in Korea at Nov. 2010. GH loop has helix structure containing eight amino acids (148 to 155) following RGD motif. (B) Tertiary structure of FMDV Asia 1 VP1 (Alam et al., 2013) and (C) Tertiary structure of FMDV A VP1 (Fowler et al., 2010). The open quadrangles show the GH loop.
Table 8. Information of fifteen selected B cell epitopes

<table>
<thead>
<tr>
<th>No</th>
<th>Year</th>
<th>Accession number</th>
<th>Country</th>
<th>Peptide sequence</th>
<th>*No. of Similar strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2001</td>
<td>CAC51271</td>
<td>Korea</td>
<td>GNCKYGESPVTNVNRGDLQVL</td>
<td>13 (6)</td>
</tr>
<tr>
<td>2</td>
<td>2013</td>
<td>AGI96743</td>
<td>United Kingdom</td>
<td>GECRYSRNAV/PNLAVRGLQVL</td>
<td>9 (5)</td>
</tr>
<tr>
<td>3</td>
<td>2010</td>
<td>ADA62474</td>
<td>Taiwan</td>
<td>GSQKYGDSSTSNVVRGDLQVL</td>
<td>7 (4)</td>
</tr>
<tr>
<td>4</td>
<td>2010</td>
<td>ADO43949</td>
<td>Iran</td>
<td>GDCKYGESRTTNRGDLQVL</td>
<td>6 (2)</td>
</tr>
<tr>
<td>5</td>
<td>2012</td>
<td>AFM36798</td>
<td>Egypt</td>
<td>GNCKYGESRTTNVRGDLQVL</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>2011</td>
<td>AEA48888</td>
<td>Korea</td>
<td>GNCKYAGGLPVRGDLQVL</td>
<td>5 (4)</td>
</tr>
<tr>
<td>7</td>
<td>2011</td>
<td>ADZ276042</td>
<td>Vietnam</td>
<td>GNCKYAGGLTNVRGDLQVL</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>2006</td>
<td>AAR85359</td>
<td>Kenya</td>
<td>GNCKYGEVSRTTNVRGDLQVL</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>2013</td>
<td>AGO58328</td>
<td>Kenya</td>
<td>GNCKYGEASRTTNVRGDLQVL</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>2006</td>
<td>ABA46739</td>
<td>Pakistan</td>
<td>GNCKYGQGVPNTVRGDLQVL</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>2004</td>
<td>AAQ92301</td>
<td>Kenya</td>
<td>GNCKYGRPSRTTNVRGDLQVL</td>
<td>3 (2)</td>
</tr>
<tr>
<td>12</td>
<td>2004</td>
<td>AAP81678</td>
<td>Cameroon</td>
<td>GSCKYSGAVPNNVRGDLHVL</td>
<td>3 (2)</td>
</tr>
<tr>
<td>13</td>
<td>2006</td>
<td>ABA46746</td>
<td>Uganda</td>
<td>GNCKYSDSRTTNVRGDLQVL</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>2008</td>
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<td>Afghanistan</td>
<td>GNCKYGSSPTTVNRGDLQVL</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>2007</td>
<td>CAJ51080</td>
<td>Afghanistan</td>
<td>GECKYTNGANPVRGDLQVL</td>
<td>2</td>
</tr>
</tbody>
</table>

*The number of similar sequences (the number of perfectly matched sequences)
(2) Design of recombinant proteins and vector construction

The selected B cell epitopes were classified into three groups and each groups were synthesized to genes. The genes were introduced into pET21a expression vector (Figure 21A). Introduced genes in pET21a vector were analyzed by PCR and agarose gel electrophoresis (Figure 21B).

(3) Analysis of protein expression

The vector were transformed into *E.coli* BL21 (DE3) using heat-shock method. MEP1 and EPM2 proteins were expressed and analyzed by SDS-PAGE and western blot assay (Figure 22). The proteins were confirmed to be expressed in western blot, compared with proteins without induction by IPTG. However, the expression amounts of MEP1 and EPM2 proteins seemed to be extremely low in the SDS-PAGE analysis. High amount of expression is one of the advantages of using bacteria as expression host, but MEP1 and EPM2 proteins did not correspond to this merit. Thus, new strategy to produce recombinant protein was needed to be devised.
Figure 21. Vector construction of 1st vaccine candidates

(A) Scheme of vector construction. (B) Amplified target genes in pET21a vector by PCR were analyzed by agarose gel. bp: base pair; M: marker; 1: MEP1 gene; 2: MEP2; 3: MEP3 (530 bp); 4: EPM1; 5: EPM2; 6: EPM3 (533 bp); 7: EP1 (488bp); 8: empty vector (140 bp)
Figure 22. Analysis of expressing protein

(A) SDS-PAGE and (B) western blot. M: protein marker; 1: MEP1 in *E.coli* extract with induction by IPTG; 2: EPM2 in *E.coli* extract with induction by IPTG; 3: MEP1 in *E.coli* extract without induction by IPTG; 4: naïve *E.coli* extract.
2) 2\textsuperscript{nd} multi-epitope FMD vaccine candidates

(1) Re-exploration and re-selection of epitope candidate

Recombinant proteins containing five B cell epitopes covering amino acid residue 132 to 151 were designed and tried to express, but this construction was not expressed in \textit{E.coli}. There is a classical hypothesis for the mechanism of folding in which secondary structure, such as helices, turns, and sheets, are formed first and then dock to form the tertiary structure (Fersht and Daggett, 2002). It was reported that in VP1 3D structure, GH loop region (amino acid residues 136 to 162) is mostly un-structured on the surface of VP1 (Fowler et al., 2010), but 8 amino acids (amino acid residues 148 to 155) following RGD motif which forms alpha-helical structure (Tang et al., 2012) (Figure 23A). New peptide as B cell epitope (amino acid residues 136 to 162) containing the secondary structure region might help the structural formation of artificially designed protein (Figure 23B).

The amino acid sequence similarity of various GH loop peptides was analyzed through hierarchical clustering (Figure 25). The peptide sequences were classified into five clusters and one representative peptide was selected from each cluster to cover all five clusters of FMDV variants (Table 9). One epitopes (KOR, 2010) of five selected B cell epitopes is identical with FMDV strain from Andong city. It is a topotype SEA. The B cell epitope (KOR, 2000) is similar with MANISA O1 strain used in inactivated vaccine. It is a topotype Pan-Asia. And the epitope (CHN, 2000) is a topotype Cathay. The multi-epitope composed of selected peptides is
expected to cross-protection against various FMDV serotype O strains.

However, it needs to analyze seventy-one sequences (amino acid residues 136 to 162 of VP1) of VP1 from NCBI GenBank, since the GH loop sequences have both conserved region and variable region (Figure 24).
Figure 23. Selection of B cell epitope region

(A) α-helix (pink column) in GH loop of predicted VP1 secondary structure. (B) Selection of new epitope. 8 amino acids (amino acid residues 148 to 155) following RGD motif forms alpha-helical structure. New peptide as B cell epitope (amino acid residues 136 to 162) containing the secondary structure region might help the structural formation of artificially designed protein.

Table 9. Information of five selected B cell epitopes

<table>
<thead>
<tr>
<th>No.</th>
<th>Year</th>
<th>Accession number</th>
<th>Country</th>
<th>Peptide sequence</th>
<th>note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2000</td>
<td>ADV5920</td>
<td>China</td>
<td>YGKSPVTNLRLQVLQVTQKAARTLPTS</td>
<td>Group A</td>
</tr>
<tr>
<td>2</td>
<td>1963</td>
<td>ACC53126</td>
<td>Belgium</td>
<td>YSRNAVPLNLRLQVLQVAQRTLPTS</td>
<td>Group B</td>
</tr>
<tr>
<td>3</td>
<td>2000</td>
<td>CAC51271</td>
<td>Korea</td>
<td>YGESPVTNLRLQVLQVAQARTLPTS</td>
<td>Group C</td>
</tr>
<tr>
<td>4</td>
<td>2010</td>
<td>AFD50726</td>
<td>Korea</td>
<td>YAGGSPLNVRLRLQVLQVAQARPLPTS</td>
<td>Group D</td>
</tr>
<tr>
<td>5</td>
<td>2010</td>
<td>AAP92301</td>
<td>Kenya</td>
<td>YGARPVNLRGLQVLQVAARPLPTS</td>
<td>Group E</td>
</tr>
</tbody>
</table>

Outbreak nation, year, NCBI accession number of FMDV and amino acid sequences included in 5BT design.
Figure 24. Amino acid mutational frequencies of GH loop (136 to 162)

GH loop has both variable region and conserved region. RGD motif and its following sequences have conserved region or predictable amino acid mutation. Variable region has many number of cases
Figure 25. Phylogenetic tree via the correlation analysis of seventy-one GH loop sequence (reside 136 to 162) from FMDV type O VP1 protein

Height, y axis means the number of different amino acids among GH loops. Open square boxes mean variant cluster and arrows indicate five sequences incorporated in 5BT design.
(2) Design of the recombinant proteins and vector construction

It cannot be predicted whether the artificial protein, 5BT, will be expressed in soluble or inclusion body form, or not accumulated in the recombinant host cells because this peptide is an artificially designed protein that does not exist in nature. Recombinant proteins expressed in E.coli often form inclusion bodies and, in some cases, are not accumulated (Sørensen and Mortensen, 2005). The use of fusion protein is a common method to overcome this problem. Solubility and stability of recombinant proteins have been improved by conjugating fusion protein (Kapust and Waugh, 1999; Sørensen and Mortensen, 2005). Membrane protein B of Brachyspira hyodysenteriae (BmpB) which caused swine mucosal-hemorrhagic dysentery was introduced as a fusion protein of multi-epitope vaccine at N-terminus of the recombinant protein (Kim et al., 2009). Total four constructions was made up (Figure 26A) and introduced in pET21a expression vector. The genes were amplified by PCR using T7 primer and analyzed by agarose gel (Figure 26B). Since 5BT cannot prevent all strains of FMDV, mixed vaccine composed various combinations of multi-epitope vaccine is worth consideration. It is important to induce cytotoxic T cells responses for viral diseases. FMDV has many T cell epitopes discussed at review of literature. It needs to be explored and introduced effective T cell epitopes for inducing both helper T cells and cytotoxic T cells responses.
Figure 26. Vector construction

(A) Schematic diagram for construction of recombinant proteins expression vector system. Four constructions: pET21a-5BT, M5BT, BmpB-5BT (B5BT), MBmpB-5BT (MB5BT). (B) Amplified PCR analysis using 1% agarose gel. M: marker; 1: 5BT (659 bp); 2: M5BT (689 bp); 3: B5BT (1418 bp); 4: MB5BT (1452 bp); 5: BmpB (920 bp); 6: MBmpB (971 bp); 7: empty vector (140bp).
(3) Protein expression

The expressed proteins were purified by his-tag purification (Figure 27). The solubility, stability, thermos-stability, and half-life of the vaccine candidates were predicted by online tool (Table 10). Because the 5BT and M5BT are artificially designed recombinant protein, these proteins were predicted to have low solubility and stability. In addition, the half-life of the proteins was predicted to be short. Conjugating BmpB on the N terminus of artificial protein was predicted to improve the solubility, stability, and half-life. 5BT and M5BT have high pI point. This property will affect purification step of mass production through ionic chromatography.

The recombinant proteins were produced mostly in soluble form. 5BT and B5BT were purified and analyzed by SDS-PAGE (Figure 28A). All the peptides except BSA were detected by western blotting assay using anti His-tag antibody (Figure 28B). The concentration of the acquired proteins were measured with the previously described method, protein extraction and purification (Grimsley and Pace, 2004). It was determined that purified 5BT was produced by 42 mg per litter culture and purified B5BT was produced by 11.6 mg per litter culture.
**Figure 27. Purified vaccine candidates**

M: marker; 1: 5BT protein (18.1 kDa); 2: M5BT (19.1 kDa); 3: B5BT (45.8 kDa); 4: MB5BT (47.4 kDa); 5: BmpB (28.7 kDa)

**Figure 28. Confirmation of target proteins**

(A) SDS-PAGE gel stained with Coomassie Brilliant Blue. Each lane was loaded with 2 µg of purified recombinant proteins. M: marker; 1: 5BT (18.1 kDa); 2: B5BT (45.8 kDa); 3: BmpB (28.7 kDa); 4: BSA (66.5 kDa). (B) Western blot analysis. Recombinant proteins were detected with His-tag using anti-His-tag antibody. BmpB is a positive control and commercial BSA is a negative control
Table 10. Information of predicted properties of proteins through amino acid sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Residues</th>
<th>Molecular weight</th>
<th>pI</th>
<th>Ext. coefficient</th>
<th>Half-life</th>
<th>Instability index</th>
<th>Aliphatic index</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB5BT</td>
<td>443</td>
<td>47.36</td>
<td>5.56</td>
<td>40800</td>
<td>30 Hours</td>
<td>29.28</td>
<td>68.98</td>
<td>In this study</td>
</tr>
<tr>
<td>B5BT</td>
<td>426</td>
<td>45.79</td>
<td>5.47</td>
<td>40800</td>
<td>30 Hours</td>
<td>27.16</td>
<td>88.38</td>
<td>In this study</td>
</tr>
<tr>
<td>M5BT</td>
<td>183</td>
<td>19.13</td>
<td>10.07</td>
<td>7450</td>
<td>&gt;10 Hours</td>
<td>40.19</td>
<td>79.45</td>
<td>In this study</td>
</tr>
<tr>
<td>5BT</td>
<td>173</td>
<td>18.10</td>
<td>10.27</td>
<td>7450</td>
<td>&gt;10 Hours</td>
<td>36.97</td>
<td>81.21</td>
<td>In this study</td>
</tr>
<tr>
<td>MBmpB</td>
<td>277</td>
<td>30.24</td>
<td>4.71</td>
<td>33350</td>
<td>&gt;30 Hours</td>
<td>23.69</td>
<td>88.38</td>
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</tr>
<tr>
<td>BmpB</td>
<td>260</td>
<td>28.66</td>
<td>4.64</td>
<td>33350</td>
<td>&gt;30 Hours</td>
<td>19.85</td>
<td>90.77</td>
<td>In previous study</td>
</tr>
</tbody>
</table>

Aliphatic index means thermos-stability index through alanine, isoleucine, valine and leucine.
The table analyzed by online tool (web.expasy.org/protparam)
3) Characterization and optimization to produce vaccine candidates

(1) Characterization of proteins

To examine the effect of BmpB as a fusion protein, the soluble and insoluble fractions of 5BT, B5BT and BmpB were prepared and analyzed by SDS-PAGE (Figure 29). The previous report showed that BmpB was expressed fully as a soluble protein (Kim et al., 2009). 5BT protein was produced mostly in the soluble form. However, approximately 36% of 5BT were produced as inclusion bodies. BmpB was introduced as fusion protein into 5BT to improve the solubility of protein. B5BT in which BmpB was conjugated at N-terminus of 5BT was expressed as 98% of soluble protein. 5BT was expressed into soluble protein by introducing B-cell epitope containing secondary structure sequence. But most of other 5BT region was unstructured in native VP1, thus a potentially unstable structure was predicted by the online tool. As predicted, the 5BT proteins were degraded when incubated the crude protein extracts at 4°C (Figure 30A). 5BT was rapidly degraded with 45% loss in 24 h whereas BmpB and B5BT were stable for at least 6 days (Figure 30B). Thus BmpB as a fusion protein increased the solubility of 5BT and protected the artificial proteins against endogenous host proteases. 5BT was unstable in crude protein extract and degraded during the production process. In most organism, mis-folded and unstable proteins are degraded by endogenous proteases (Dougan et al., 2002; Goldberg, 2003). This makes it complicated to produce and store the recombinant subunit vaccine candidate, e.g. cell harvest and purification steps. To overcome this problem,
BmpB was introduced as a fusion protein for improving the solubility and stability of 5BT. Conjugation of the fusion protein is one of frequently used methods to express soluble protein by improving the stable-structure. The BmpB is known to express in soluble form in large amount in *E. coli*, and the result indicated that BmpB could resist endogenous proteases. The stability and solubility of 5BT protein were greatly improved by BmpB conjugation although the expression level was lower compared to 5BT alone. This can be overcome by controlling culture condition such as culture temperature, induction time, inducer concentration, and culture time. Furthermore, it may be possible that re-cloning with a codon optimized fusion protein, BmpB, will improve the expression level. The following studies were focused mainly on 5BT and M5BT proteins because producing yield is very important in the industrial part.
Figure 29. SDS-PAGE analysis for the expression pattern of recombinant proteins.

5BT protein expressed in soluble fraction (64%) and inclusion body fraction (36%). B5BT protein expressed in soluble fraction (98%) and inclusion body fraction (2%). BmpB expressed in almost soluble fraction. M: marker; S:soluble fraction; IB: inclusion body fraction.

Figure 30. BmpB effect on the stability of recombinant proteins

(A) SDS-PAGE gel stained with Coomassie Brilliant Blue. Crude protein extracts were incubated at 4 °C for 6 days and daily sample was analyzed by SDS-PAGE. Lanes: M, protein marker. (B) Graph showing intact proteins in (A) analyzed by image J software.
(2) Optimization of culture conditions

Yield of recombinant protein can be improved by controlling culture conditions such as culture temperature, induction time, inducer concentration, and harvest time (Jana and Deb, 2005). Thus, the transformed *E.coli* was cultured at a variety of conditions to improve yield of 5BT vaccine candidate (Figure 31). 5BT protein is unstable. Unstable proteins can be degraded by endogenous enzymatic reaction. It is an option to express recombinant proteins under lower temperature which were expressed as inclusion bodies at normal condition, since growth and metabolic rate of *E.coli* decrease in low temperature (Ratkowsky et al., 1982). The culture temperature was controlled to reduce enzymatic activity, but protein expression was not observed at low temperature. In addition, it was confirmed that when the culture time was prolonged, the target proteins were degraded. Because 5BT is unstable, it is easily degraded by endogenous protease. The culture time of the highest protein expression was confirmed to be 4 hour at 37 ℃. The induction time and the IPTG concentration for the highest protein expression were also confirmed. Induction time was also analyzed to select the highly productive *E.coli* in log phase of growth curve. IPTG is a powerful inducer that inhibits the growth and metabolism of *E.coli* and focuses only on the production of the target protein. Induction time is 0.5 to 0.6 OD<sub>600</sub> and IPTG concentration is 0.5 mM (Figure 31).
Figure 31. Exploration of highest 5BT protein expression in flask level.

(A) Culture temperature (37 °C, 30 °C, and 23 °C) and culture time (3h, 6h, 12h, and 24h) (B) The culture time to the highest protein expression (C) Graph showing intact proteins in (B) analyzed by image J software. (D and E) Induction time (0.3, 0.5, 0.6, and 0.8 of OD₆₀₀) and IPTG concentration (1mM, 0.5mM, 0.1mM and 0.05mM) (F) Graph showing intact proteins in (D and E) analyzed by image J software.
(3) Optimization of purification conditions

Purification is as important as yield of protein production for industrialization. The vaccine candidates have his-tag due to being produced in pET21a vector. It can be purified by using Ni-NTA resin. His-tag is polyhistidine tag, an amino acid motif in proteins that consists of at least six histidine residues. Ni-NTA resin containing nickel ion and nickel ion can bind to his-tag of the recombinant protein through coordinate covalent bond (Gaberc-Porekar and Menart, 2001). Purification conditions were explored with many trials to improve purity of target protein (Table 11). The purity of 5BT in optimized condition was approximately 90% according to the analysis using image tool (Figure 32). Inferring the amount of target protein contained in E.coli extracts, it need to set up proper volume of resin to improve purity of target protein.
Table 11. Conditions to purity of 5BT protein

<table>
<thead>
<tr>
<th>Trials</th>
<th>Washing protocol</th>
<th>Elution protocol</th>
<th>Note</th>
<th>Bed volume (per 1L LB culture)</th>
<th>The purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>① 10 vol. of wash 1 buffer</td>
<td>20ml of elution A buffer</td>
<td>Low purity</td>
<td>7ml bed volume</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>② 6vol. of wash 6 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>① 10 vol. of wash 1 buffer</td>
<td>20ml of elution B buffer</td>
<td>Low purity</td>
<td>7ml bed volume</td>
<td>61.7</td>
</tr>
<tr>
<td></td>
<td>② 6 vol. of wash 6 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>③ 2 vol. of wash 2 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>④ 6 vol. of wash 7 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>① 8 vol. of wash 1 buffer</td>
<td>20ml of elution B buffer</td>
<td>Loss of target protein in wash 7 buffer</td>
<td>5ml bed volume</td>
<td>70.6</td>
</tr>
<tr>
<td></td>
<td>② 5 vol. of wash 2 buffer</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>③ 10 vol. of wash 6 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>④ 4 vol. of wash 7 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>① 20 vol. of wash 1 buffer</td>
<td>20ml of elution B buffer</td>
<td>Loss of target protein in wash 7 buffer</td>
<td>4ml bed volume</td>
<td>64.6 - 80.0</td>
</tr>
<tr>
<td></td>
<td>② 10 vol. of wash 2 buffer</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>③ 20 vol. of wash 6 buffer</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>④ 5 vol. of wash 7 buffer</td>
<td></td>
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<tr>
<td>5</td>
<td>① 20 vol. of wash 1 buffer</td>
<td>20ml of elution C buffer</td>
<td>Loss of target protein in wash 7 buffer</td>
<td>2.5ml bed volume</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td>② 10 vol. of wash 3 buffer</td>
<td></td>
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<tr>
<td></td>
<td>③ 10 vol. of wash 5 buffer</td>
<td></td>
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<tr>
<td></td>
<td>④ 10 vol. of wash 6 buffer</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>⑤ 4 vol. of wash 7 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>① 10 vol. of wash 1 buffer</td>
<td>20ml of elution C buffer</td>
<td>Optimized condition</td>
<td>3ml bed volume</td>
<td>88.8</td>
</tr>
<tr>
<td></td>
<td>② 10 vol. of wash 4 buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>③ 10 vol. of wash 6 buffer</td>
<td></td>
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</tr>
</tbody>
</table>

**Wash 1 buffer:** 5mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Wash 2 buffer:** 10mM imidazole, 1M NaCl, 10% glycerol, 10mM Tris·Cl, pH 7.9;  **Wash 3 buffer:** 20mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Wash 4 buffer:** 30mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Wash 5 buffer:** 40mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Wash 6 buffer:** 60mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Wash 7 buffer:** 70mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Elution A buffer:** 1M imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Elution B buffer:** 200mM imidazole, 0.5M NaCl, 10mM Tris·Cl, pH 7.9;  **Elution C buffer:** 1M imidazole, 10mM Tris·Cl, pH 7.9
Figure 32. SDS-PAGE analysis of optimized purification condition

The purity of 5BT in optimized condition was approximately 90% by analyzing using image tool. 3 ml of Ni-NTA resin was used to purify 1L culture volume. Total yield was 64 mg of 5BT protein. M: marker; S: start material; SFT: sample flow-through; W1: wash 1 buffer; W2: wash 4 buffer; W3: wash 6 buffer.
4) *In vivo* evaluation as FMD vaccine

(1) Evaluation of 5BT and B5BT as antigens

The immunogenic effect of 5BT and B5BT as subunit vaccines was tested in mice via the intramuscular injection. PBS and inactivated FMD vaccine (iFMDV) were used as a negative and a positive control. 5BT specific antibodies in the serum collected at day 0, 13, 27 and 42 after priming immunization were determined by ELISA (Figure 3). 5BT specific antibodies increased in 5BT, B5BT, and iFMDV injection group. 5BT specific antibody titer was the highest in 5BT group, and the lowest in iFMDV group.

Figure 3 showed that antibodies produced in the group injected iFMDV, vaccine produced with MANISA O1 strain, recognized 5BT although it does not contain GH loop peptide of MANISA O1. Furthermore, BmpB fusion protein did not affect the production of 5BT specific antibodies in B5BT group. To evaluate the route of immune response, total IgG and IgG subtype titers were determined until day 42 (Figure 3). All treatment groups induced the balanced immune response of IgG1 and IgG2a, which indicates the balanced activation of Th1 and Th2 route.

From the mouse immunization experiment, there were four noteworthy results regarding the effects of the artificial proteins. Firstly, 5BT specific antibodies were detected in the sera of 5BT and B5BT groups. B cell recognizes an epitope of antigens with B cell receptors and is activated by cytokines from T cells. Activated B cell differentiates into plasma cells which secrete antibodies. The
antibodies bind to the epitope recognized by B-cell receptors (Packard and Cambier, 2013). B-cell epitopes should be exposed on the surface of antigen for recognition by B-cell receptors (Abacioglu et al., 1994). It is indicated that 5BT region of B5BT is exposed and BmpB does not hinder the access of B cell to the epitope. Secondly, although 5BT does not have the GH loop sequence of Manisa O1 strain, which is the source of iFMDV vaccine, the sera of iFMDV group showed specific binding to 5BT and B5BT probably through the antibodies recognizing the conserved region containing RGD motif. It also suggests that 5BT and B5BT vaccine can protect other strains not included in 5BT, through the reaction to this conserved region. Moreover, multi-epitope vaccine has higher density of epitopes, which enhances the chances of being recognized by B cell receptors (Taherkhani and Farshadpour, 2015). It is important to prevent a wide spectrum of FMD variants.
Figure 33. Validation of multi-epitope proteins as FMDV subunit vaccine candidates in vivo

(A) Antibody titer analysis by ELISA. Specific antibody titers against 5BT was measured by ELISA in serum samples collected at days 0, 13, 27 and 42 post-immunization. Antibody titers were expressed as the reciprocal log10 of the last dilution calculated by interpolation to give an absorbance of 1 above background. Each point corresponds to the geometric mean of each groups. Error bars represent standard error of the mean. Validation of immune response routes of recombinant proteins. (B) 5BT specific total IgG titers detected by ELISA at day 42 post-immunization, (C) IgG1, and (D) IgG2a. Endpoint titers were expressed as the reciprocal log10 of serum dilutions. Each symbol represents the value of individual mouse. Horizontal lines indicate the mean of each group of animals.
(2) Evaluation of 5BT and B5BT as FMD vaccine

Anti-FMDV type O antibodies were evaluated using a SP competition ELISA (Figure 34A). Structure proteins originated from MANISA O1 strain were coated on the wells of the immuno-plate for competition ELISA. Antibodies in the serum of the three groups were significantly higher compared to the PBS group. FMD vaccine contains more epitopes other than GH loop unlike 5BT proteins, but there was no significant difference between the iFMDV and 5BT or B5BT group in this competition assay. Sera of 5BT and B5BT group successfully competed with antibodies from the commercial antigens that bound to VP1 protein of FMDV in competition ELISA.

The accessibility of B-cell receptors to each GH loop peptide of 5BT was estimated using an ELISA to access the production of epitope specific antibodies (Figure 34B). If epitopes are exposed on the surface of the protein, it may bring out higher B-cell specific antibodies than those buried inside the protein. All the epitopes of 5BT induced production of their own specific antibodies. Sera from 5BT and B5BT groups showed the similar peptide specific antibody titers with anti-BEL 63 antibodies being the lowest among the groups. Sera of iFMDV group reacted very similarly to all GH loop peptides of 5BT.

It was confirmed that immunization with 5BT and B5BT elicited the production of FMDV specific antibodies in mice. Sera of 5BT and B5BT groups successfully competed with FMDV specific antibodies from commercial antigens that bound to VP1 protein of FMDV in competition ELISA kit (Jung et al., 2013). It was
confirmed that immunization with 5BT and B5BT elicited production of meaningful antibodies such as neutralizing antibodies against FMDV, in animals.

In addition, antibodies from all three antigenic groups showed more or less similar specific binding affinity to each synthetic peptide composing 5BT. Although exposure of epitope is influenced by protein folding pattern (ABACIOGLU et al., 1994), B5BT and 5BT groups showed similar results. It means that BmpB increases the solubility and stability of 5BT without the conformational binding inhibition. In both 5BT and B5BT groups, BEL63 specific antibody had the lowest titers compared to other peptides. 5BT protein is expected to have a secondary structure in which linear structure and alpha helix structure are repeated five times, followed by terminal T cell peptide. Kloss et al. suggested insight of protein structures consisting of repeating peptides. Adjacent repeats packed together in a more-or-less linear array, facilitating a simple linear representation of energetics, similar to that of DNA double helix formation. 5BT proteins or 5BT region in B5BT might form single super secondary structure loop (Kloss et al., 2008). Therefore, it can be suggested that BEL63 epitope in the second position from N-terminus of 5BT is probably least exposed to the B-cell receptors for the antibody reaction.

This study did not confirm that NSP specific antibodies were not produced to confirm that multi-epitope vaccine could overcome biological safety issue, DIVA. This assay should be supplemented.
Figure 3.4. Evaluation of 5BT and B5BT as FMD vaccine

(A) Detection of FMDV specific antibody response in serum analyzed by competition ELISA assay at day 42 post-immunization. The PI (%) means the percent inhibition. \( \text{PI} = 100 - 100 \times \left( \frac{\text{OD}_{450} \text{ of sample serum}}{\text{OD}_{450} \text{ of negative control}} \right) \). Negative and positive controls were satisfied with standard recommending of manufacturer manual. Each symbol represents the value for individual animals. Horizontal lines describe the mean value for each group of animals. **: \( P < 0.01 \); ***: \( P < 0.001 \), one way ANOVA. (B) Antibody response to each peptide in 5BT in serum analyzed by ELISA assay at day 42 post-immunization. Antibody titers were expressed as the reciprocal log10 of the last dilution calculated by interpolation to give an absorbance. Error bars represent standard deviation.
4. Conclusion

The shortcomings of inactivated vaccine such as high production cost, safety issue, and low protection rate due to high mutation rate of FMDV have been discussed to prevent FMD for a long time. Various strategies have been brought up to overcome these problems. Subunit vaccine is the safest and the cheapest strategy among them. Especially, production of recombinant proteins in *E. coli* as bioreactor is most popular way and the protocols have been standardized enough. The popularity of *E. coli* system comes from relatively inexpensive development costs, simple cultivation procedures, and easy extraction of recombinant proteins.

Many researchers have developed subunit vaccines to prevent FMD and showed that multi-epitope vaccine was the good strategy for the livestock industry. Functions and structure of the proteins composing FMDV have been thoroughly studied. VP1, a structural protein, has a GH loop region which binds to integrin of host cells and causes infection of animals. The surface region of VP1 containing GH loop has been related to neutralizing antibodies. VP1 containing the linear and conformational epitope region is a good subunit vaccine candidate, which has comparable effect with the inactivated vaccine. However, it was reported that whole VP1 was produced in inclusion body form in *E. coli*. Inclusion body is known to be caused by aggregation of misfolded proteins. Aggregated proteins have low bioactivity because it has insoluble character. Because of this reason, many researchers have tried to make soluble protein from inclusion bodies using
high concentration of urea and acetone precipitation. Therefore, solubilization is very important step for the production of subunit vaccine, and the addition of re-solubilizing step increases the production cost.

There are two detailed aims in this study. First, it was a concrete strategy to develop vaccines against FMDV. Second, the vaccine should be produced as a soluble protein.

The strategy of multi-epitope vaccine was employed. GH loop was selected as representative B cell epitope. GH loop has both variable region and conserved region. Five representative B cell epitopes were selected through analyzing similarity of amino acid sequence. T cell epitope was also introduced in multi-epitope to enhance immune responses. Multi-epitope vaccine, 5BT, was developed. Though 5BT are mostly in soluble form, about 36% of 5BT was produced as the inclusion body. Moreover, 5BT was so unstable in crude protein extract and degraded during the production process. To overcome this problems, BmpB was introduced as a fusion protein for the solubility and stability of 5BT. The stability and solubility of 5BT protein were greatly improved by BmpB conjugation although the expression level was lower compared to 5BT alone. However, subsequent studies are mainly focused on 5BT and M5BT proteins considering the producing yield for industrialization. 5BT protein improved its purity and yield through optimizing culture condition and purification condition (Figure 35).
However, there are the limitation in this study. The length of epitope recognized by antibody is known with amino acid residue 5 to 20 (Gupta et al., 2013). The fifteen representative epitopes need to be re-selected through computationally analyzing mutation-pattern of variable region and length of epitopes recognized by B cell receptor. Mixing vaccine composing three multi-epitope vaccines will be good alternative vaccine. This study also lacks research on T cell epitope. There should be results about immune response by T cell epitope, and the search for better candidates was needed to develop virus vaccine such as FMD. It is also necessary to analyze antibodies about various GH loop peptides to evaluate as universal FMD vaccine.

In conclusion, the artificial peptide containing five different epitopes from worldwide FMDV epidemic strains were designed in a logical way and successfully expressed in soluble and stable form in E.coli. Through immunization of purified protein in mice, the peptide`s potential as a FMD subunit vaccine candidate was verified. This study provides insight about the design and selection of multipotent artificial recombinant protein as a vaccine for a highly mutable viral disease such as FMD.
Figure 35. Summary of Study I
Study II. *In vivo* evaluation of M5BT multi-epitope vaccine for FMD and researches on nasal vaccine system

1. Introduction

The concept of multi-epitope design was applied to developing a recombinant subunit vaccine to overcome these problems discussed at study I. The recombinant protein can overcome representative weak points of an inactivated vaccine, which include serious biosafety concerns such as, the risk of viral release during virus mass culturing, and the difficulty of serological distinction between infected and vaccinated animals (DIVA) (Rodriguez and Gay, 2011; Li et al., 2014). However, there are two shortcomings of a recombinant protein vaccine. First, it has low immunogenicity effect (Blanco et al., 2013). Second, it has a narrow spectrum for preventing a highly mutable virus such as FMDV. Thus, we have developed a multi-epitope vaccine that can overcome the drawbacks of a recombinant protein vaccine, while still maintaining the advantages.

In study I, recombinant protein, 5BT, consisted of five B cell epitopes, residue 136 to 162 of VP1 of each representative strains of serotype O and one T cell epitope, residue 27 to 42 of 3A protein was developed (Lee et al., 2017a). The multi-epitope vaccine can improve the immunogenicity through high-
epitope density and expand the protection spectrum against virus mutation rate by using epitope selection.

The aim of the study II is evaluation of multi-epitope vaccine against FMD via *in vivo* immunization. *In vivo* immunization to mice and porcine was conducted to evaluate production of protective immunity. According to the results, the M5BT protein elicited immune responses in animals containing anti-FMDV specific antibodies and neutralizing antibodies as well as in animals intramuscularly vaccinated with inactivated FMD vaccine.

M cell targeting peptide (MTP, CKSTHPLSC) in N terminus of the 5BT protein was also introduced. MTP can bind M cells in mucosal associated lymphoid tissues (MALTs) and improve antigen transportation to immune cells through transcytosis in mucosal immunity (Yoo et al., 2010). While the M5BT protein has improved the resistance from proteolytic agents and been expressed with comparable quantity to 5BT in *E. coli*, M5BT did not induce anti-FMDV immunity through oral vaccination due to the unstable characteristic of the protein in gastro-intestine. Another route, nasal vaccination, was evaluated with particulate system having adjuvant ability. NALT is representative mucosal immune system in nasal route. There are advantages compared to oral administration due to the absence of acidity and lack of secreted enzymes. These characteristics lead to low dose requirement of antigen because there are few factors that affect stability of the vaccine. In addition, nasal vaccination is more accessible than other vaccination route.
An adjuvant concept, particulated adjuvant, was introduced to enhance the efficiency of nasal vaccination. Particulated adjuvants may function as efficient adjuvants by enhancing the antigen uptake by immune cells such as macrophages and dendritic cells. Also, the particles can protect the loaded protein vaccines from proteolytic enzymes of host and be released in a sustained manner known to induce long-term immune response of the antigen (Wu and Lee, 2000; Robert et al., 2012). The particles are consisted of acetyl inulin microparticles (INAC MPs) and mannan.

Inulin is a group of naturally occurring polysaccharides produced by many types of plants including Jerusalem artichoke, chicory, dahlia, wheat, etc (Roberfroid, 2007). It consists of chain-terminating glucosyl moieties and a repetitive fructosyl moiety (GFn), which are linked by β (2-1) bonds. Because of the linkages, inulin is not digested by digestive enzyme in the animals (Barclay et al., 2010). Since some bacteria can use inulin as energy source, researchers have studied inulin as prebiotics (Gibson, 1999). Recently, it was also reported that inulin is a novel TLR-4 agonist. Depending on its solubility, particulated inulin can act as an efficient adjuvant for vaccine (Kumar and Tummala, 2013).

Acetyl inulin (INAC) is an acetylated form of inulin. INAC can be particulated in water due to self-assembled mechanism and it is soluble in various organic solvents including dichloromethane, in which INAC MP can be made by double emulsion method. As it is known that INAC MPs can function as a vaccine adjuvant when antigen is encapsulated into INAC particles, antigen-loaded INAC
MPs can enhance immune response of the subunit vaccine by overcoming its low immunogenicity (Kumar and Tummala, 2013; Tummala and Kumar, 2013).

Mannans are chains of up to several hundreds of mannoses that are added to fungal proteins via N- or O-linkages (Levitz, 2010). So, mannan from cell wall of *Saccharomyces cerevisiae* is a carbohydrate that can be recognized as pathogen-associated molecular patterns (PAMPs) by immune cells (van de Veerdonk et al., 2009). In this aspect, INAC MPs decorated with mannan as a TLR-4 specific ligand can have a synergistic effect to enhance immune response.

The need for the production of more effective vaccines has increased due to the onset of persistent FMD and the failure of preventing the disease through the use of existing inactivated vaccines. In study II, The potential of effective and safe M5BT protein was evaluated by *in vivo* immunization. M5BT protein was loaded in M-INAC MPs by double emulsion solvent evaporation method and evaluated through intramuscular and intranasal vaccination (Figure 36).
Figure 36. Graphical abstract of particulate system in study II
2. Materials and methods

1) *In vivo* evaluation of M5BT as vaccine candidate

(1) Purification of M5BT

A transformant was selected and 5 ml of overnight culture was inoculated in 500 ml of Luria-Bertani (LB) broth containing 100 ng/ml of ampicillin in a 2.8L Fernbach flask. Cultures were agitated at 230 rpm until $A_{600}$ reached 0.5 and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by centrifugation at 6500 rpm for 10 min at 4 °C. Cell pellets were resuspended in 20 ml of binding buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9) and sonicated on ice (48 × 10 seconds). Lysates were centrifuged at 17000 rpm at 4 °C for 20 min and supernatants were filtered through a 0.45 μm filter (Corning, NY, USA). 20 ml of binding buffer was added to the supernatants to purify the target protein, M5BT. The 3 ml bed volume of Ni-nitrilotriacetic acid (NTA) agarose resin (Novagen, CA, USA) was packed into a column and equilibrated with binding buffer. The sample was loaded into a column and the column was washed with 10 resin volume of binding buffer followed by 10 resin volume of wash buffer (30 mM and 60mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). Target protein was eluted with 20 ml of elution buffer (1M imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9) in Table 11. The eluted protein was dialyzed using a membrane tube (molecular cut-off: 6-8000 Da, Spectrum, CA, USA) against distilled water at 4 °C overnight. Desalted solution
was lyophilized and stored at -20 °C until used. Lipopolysaccharide (LPS) was removed using Triton-X 114 (Magalhães et al., 2007) and detected by using ToxinSensor™ Chromogenic LAL endotoxin assay kit (Genscript, NJ, USA). OD$_{280}$ was detected by a spectrophotometer (Implen, Munchen, Germany) and protein concentration was calculated using the extinction coefficient (Gill and Von Hippel, 1989).

(2) Analysis of solubility and stability of M5BT protein

The 20 µl of soluble and inclusion body fractions were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue by 3 times of heating in a microwave oven for 70 seconds, cooled down on a rocker for 5 min and destained with 25% methanol and 7.5% acetic acid solution overnight. Bands were analyzed by image J software (NIH) to compare target protein quantity (Zhang et al., 2011).

To test stability of the proteins, the cell pellets from 50 ml culture were resuspended in 10 ml of PBS and distributed in 1 ml aliquot into the micro tube. The tubes were centrifuged at 13000 rpm for 1 min at ambient temperature. The supernatant were removed and cell pellets were stored at -70 °C until use. Every day one frozen tube was resuspended in 1 ml of PBS, sonicated and supernatants after centrifugation were stored at 4 °C. This was repeated for 7 days to investigate the protein degradation by endogenous proteases of E. coli. After 7 days proteins were analyzed by 15% SDS-PAGE, bands of target proteins in gel images were
analyzed by Image J software.

The target protein was confirmed by western blot assay using His-tag antibody (Abcam, MA, USA) and serum of swine immunized with M5BT and inactivated FMD vaccine (iFMDV, Komipharm, Gyeonggi-do, Korea). The protein was separated in a 15% SDS-PAGE and then transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was blocked by 5% skim milk in tris buffered saline (TBS) contacting 0.05 % Tween 20 (TBST) for 1 h on a rocker and then washed three times with TBST. The membrane was incubated with a 1:1000 diluted his-tag antibody overnight at 4 °C, washed three times with TBST, and incubated with a 1:2000 dilution of rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (Abcam, MA, USA) for 1 h. After washing three times with TBST, the signal was developed tetramethylbenzidine (TMB).

2) M5BT-loaded INAC MPs as vaccine delivery system and adjuvant

(1) Materials for preparing mannan-decorated INAC MPs

Inulin from chicory (inulin), acetic anhydride, sodium acetate (NaOAc), dimethyl formamide (DMF), dimethyl sulfoxide-d6 (DMSO-d6), poly(vinyl alcohol) (PVA), mannan from Saccharomyces cerevisiae (mannan), Pluronic® F-127, dichloromethane (DCM).
(2) Synthesis of INAC

The synthesis of acetyl inulin was carried out according the previously reported method with a little modification (Wu and Lee, 2000). Briefly, 1 g of inulin was added to 5 ml of dimethyl formamide (DMF) with the addition of 0.2 ml of 5% acetic anhydride. 5 % (w/v) sodium acetate was used as a catalyst for the reaction. The acetylation reaction was carried at 40 °C for 24 h under nitrogen. After 24 h, INAC was dialyzed against DMF for 24 h to remove free acetic acid and against distilled water to remove DMF and unreacted inulin. After dialysis, INAC was lyophilized and stored at –20 °C until use. The conjugation of acetyl group was confirmed by 600 MHz ¹H NMR spectroscopy (AVANCE 600, Bruker, Germany) and acetyl group content in INAC was quantified.

(3) Preparation of M-5BT loaded M-INAC MPs

M5BT-loaded INAC and M5BT-loaded M-INAC MPs were prepared by a double emulsion solvent evaporation method (Figure 37) (Sander and Studart, 2014; Li et al., 2016). Briefly, 100 µl of 50 mg/ml M5BT solution was mixed with 100 µl of 10% (w/v) Pluronic F-127 solution as an aqueous phase (W₁). This aqueous phase was emulsified with 5 ml of DCM as an oil phase (O) containing 100 mg of INAC by using an ultrasonic processor (Sonics, Vibra cells™) (4 output watts) on ice for 1.5 min to form 1st W₁/O emulsion. This primary emulsion was then added dropwise into another aqueous (W₂) phase (50 ml water) containing 0.75% (w/v) PVA solution containing 0.25% mannan, with continuous
homogenizing at 13,000 rpm using Turrax resulting in the formation of double emulsion (W₁/O/W₂). In case of M5BT-loaded INAC MPs, 1% PVA solution was used. The stirring was continued overnight for complete evaporation of the organic solvent. Resulting MPs were collected via centrifugation at 6,000 rpm for 10 min at 4 ℃. The pelleted M5BT-loaded INAC or M5BT-loaded M-INAC MPs were washed with distilled water and centrifuged. The final MPs were resuspended in 10 ml of distilled water and frozen by liquid nitrogen followed by lyophilization under vacuum.

4) Determination of loading content and encapsulation efficiency

Loading content was determined as follows. The MPs (10 mg/ml) were dispersed in dimethyl sulfoxide (DMSO) and the clear solution was used for measurement of protein concentration using spectrophotometer (NanoPhotometer™). The encapsulation efficiency of the M5BT into MPs was determined by measuring the unloaded protein concentration in the supernatant during the double emulsion method steps. The loading content and encapsulation efficiency was calculated using the following equations:

\[
\text{Loading content (wt. −%) = } \frac{\text{amount of vaccine in MPs}}{\text{amount of MPs}} \times 100
\]

\[
\text{Encapsulation efficiency (wt. −%) = } \frac{\text{amount of vaccine in MPs}}{\text{amount of vaccine initially used}} \times 100
\]
Figure 37. Preparation steps for M5BT-loaded INAC MPs by double emulsion

(A) Addition of M5BT solution (water phase, W1) into INAC solution in DCM (organic phase, O). (B) 1st emulsification (W1/O emulsion). (C) Addition of W1/O solution into PVA solution (water phase, W2). (D) 2nd emulsification (W1/O/W2 emulsion). (E) Hardened microsphere by evaporating DCM and centrifugation. The illustration is modified from the figure from Sander et al., 2014 (Sander and Studart, 2014)
(5) **Analysis of morphology and size of MPs**

The surface topography was observed by field-emission scanning electron microscope (FE-SEM) using SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). MPs were mounted on metal stubs with thin adhesive copper tape and coated with platinum under vacuum using coating chamber (CT 1500 HF, Oxford Instruments Oxfordshire, UK). And, the sizes of MPs were measured with a dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Japan).

(6) **In vitro release behavior**

The *in vitro* release of M5BT from M5BT-loaded INAC or M5BT-loaded M-INAC MPs was determined as follows. The M5BT-loaded MPs (10 mg/ml) suspended in PBS (pH 7.4) were incubated at 37 ℃ with 100 rpm shaking. A 0.5 ml aliquot was withdrawn and same volume of PBS was supplemented at a predeterumined time. The protein amount released from MPs was measured using spectrophotometer.

3) **In vivo immunization**

(1) **Peroral immunization in mouse**

Peroral immunization study was conducted with six-week old BALB/C mice (female, n=5) to validate the effect of the M5BT protein as oral vaccine. Mice was fasted for 6 h before orally administration and immunized on days 0, and 14 with
200 µg (1 µg/µl) of M5BT protein in PBS. Before 30 min of every peroral immunization, each mouse was gavaged with 500 µl of neutralizing solution (7.5% NaHCO₃: PBS = 1:4) to reduce stomach acidity. Blood and fecal samples were collected before priming (day 0) and on day 28. The fecal samples were homogenized in resuspension buffer (1µg/ml, 1% BSA and 1mM phenylmethylsulfonyl fluoride) and incubated at 4 °C for overnight. The fecal pellet removed by centrifugation (14000 rpm, 10 min and 4 °C). The supernatant were stored at -70 °C until use to analyze quantity of IgA.

(2) Mouse immunization for M5BT protein vaccine

Six-week old BALB/C mice (female) were used for the immunization following the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University, Korea). The mouse was intramuscularly immunized at days 0 and 14 with 20 µg (0.5 µg/µl) of M5BT protein emulsified in Complete Freund’s Adjuvant (CFA, priming) or Incomplete Freund’s Adjuvant (IFA, boosting) and sacrificed on day 28. Five mice in the negative control group were not immunized (NT) and a positive control group of 5 mice were immunized with 40 µl of iFMDV. Blood samples were collected before priming (day 0) and on day 28 (Sacrifice) from intra-petrosal veins with a disposable syringe and delivered into a sterilized tube (Figure 38 and Table 12). Serum was separated by centrifugation at 7000 rpm for 3 min using serum separate tubes (BD microtainer, NJ, USA).
The mouse was immunized intramuscularly at days 0, 14, and 28. Blood samples were collected before priming (day 0) and on days 13, 27, and 42 (sacrifice) from intra-petrosal veins with a disposable syringe and delivered into sterilized tube.

Table 12. Mouse in vivo immunization group

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen (Annotation)</th>
<th>Dose</th>
<th>Adjuvant</th>
<th>Animals (Age)</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>Balb/c mouse female (6 week)</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>MSBT</td>
<td>20μg (0.5 μg/μl)</td>
<td>CFA/IFA (equal volume)</td>
<td>Balb/c mouse female (6 week)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>IFMDV</td>
<td>40μl</td>
<td>Included</td>
<td>Balb/c mouse female (6 week)</td>
<td>6</td>
</tr>
</tbody>
</table>
(3) Porcine immunization

Nine swine weighing 20 to 30 kg and free of antibodies against the SP and 3ABC in NSP of FMDV were chosen. The immunization assay was carried out in Farm Yang-Sung with the Animal and Plant Quarantine Agency of Korea. The swine were immunized intramuscularly on days 0, 14 and 28 with 10 mg (5 µg/µl) of peptide emulsified in IMS1313 (SEPPIC, Paris, France). The negative control group was not immunized and the positive control group was immunized with 2 ml of iFMDV (Komipharm, Gyeonggi-do, Korea). Blood samples were collected before priming (day 0) and on days 14, 28, 42 from the jugular veins with a disposable syringe and delivered into sterilized tubes (Table 13 and Figure 39). Serum was separated by centrifugation at 6000 rpm for 5 min using serum separate tubes (BD microtainer, NJ, USA). The serum was analyzed to evaluate M5BT specific antibodies and FMDV specific antibodies using the previously described methods.
Figure 39. The immunization schedule of porcine in vivo

The pigs were immunized intramuscularly nearby ears at days 0, 14, and 28. Blood samples were collected before priming (day 0) and on days 14, 28, and 42 (sacrifice) from intra-petrosal veins with a disposable syringe and delivered into sterilized tube.

Table 13. Swine in vivo immunization group

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen (Annotation)</th>
<th>Dose</th>
<th>Adjuvant</th>
<th>Animals (Age)</th>
<th>Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>30 kg growing pigs (8 – 12 weeks)</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>MSBT</td>
<td>10 mg (5 μg/μl)</td>
<td>IMS1313 (equal volume)</td>
<td>30 kg growing pigs (8 – 12 weeks)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>iFMDV</td>
<td>2 ml</td>
<td>Included</td>
<td>30 kg growing pigs (8 – 12 weeks)</td>
<td>3</td>
</tr>
</tbody>
</table>
(4) Mouse immunization for mannan-decorated INAC

Five female BALB/c mice (6 weeks) were used for each group. Mice were raised in cages providing ad libitum access to feed and water in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). All of the protocols were reviewed and approved by the Animal Care and Use Committee at Seoul National University (SNU-141201-1). The mice were immunized intramuscularly at days 0, 7 and 14 with 10 µg (0.125 µg/µl) of peptide emulsified in Complete Freund’s Adjuvant (CFA, priming) or Incomplete Freund’s Adjuvant (IFA, boosting), MPs resuspended in 80 µl of PBS, and inactivated FMD vaccine (iFMDV group) as positive control. The mice were immunized intranasally at days 0, 7 and 14 with 10 µg (0.5 µg/µl). 1 mg/ml of cholera toxin subunit B (CTB) was used as adjuvant in positive group. MPs resuspended in 20 µl of PBS, and the mice vaccinated via intranasal route with 10 µl per nostril (Figure 40 and Table 14). The mice were sacrificed on day 28. Blood samples were collected at 28 day (Sacrifice) from intra-petrosal veins with a disposable syringe and delivered into a sterilized tube. Serum was separated by centrifugation at 7000 rpm for 3 min using serum separate tubes (BD microtainer, NJ, USA).
**Figure 40. The immunization schedule of mice to evaluate MPs effects**

The mice were immunized intramuscularly and intranasally at days 0, 7, and 14. Blood samples were collected before priming 28 (sacrifice) from intra-petrosal veins with a disposable syringe and delivered into sterilized tube.

**Table 14. Mouse in vivo immunization to evaluate MPs effects**

<table>
<thead>
<tr>
<th>Group</th>
<th>Annotation</th>
<th>Dose</th>
<th>Adjuvant</th>
<th>Animals (Age)</th>
<th>Individuals</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>untreated</td>
<td>-</td>
<td>-</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>M5BT</td>
<td>10 µg (0.125 µg/µl)</td>
<td>-</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/M</td>
</tr>
<tr>
<td>3</td>
<td>iFMDV</td>
<td>80μl</td>
<td>Included</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/M</td>
</tr>
<tr>
<td>4</td>
<td>CFA</td>
<td>10 µg (0.125 µg/µl)</td>
<td>CFA/IFA</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/M</td>
</tr>
<tr>
<td>5</td>
<td>INAC</td>
<td>10 µg (0.125 µg/µl)</td>
<td>INAC MPs</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/M</td>
</tr>
<tr>
<td>6</td>
<td>M-INAC</td>
<td>10 µg (0.125 µg/µl)</td>
<td>M-INAC MPs</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/M</td>
</tr>
<tr>
<td>7</td>
<td>M5BT/n</td>
<td>10 µg (0.5 µg/µl)</td>
<td>-</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/N</td>
</tr>
<tr>
<td>8</td>
<td>CTB/n</td>
<td>10 µg (0.5 µg/µl)</td>
<td>CTB</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/N</td>
</tr>
<tr>
<td>9</td>
<td>INAC/n</td>
<td>10 µg (0.5 µg/µl)</td>
<td>INAC MPs</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/N</td>
</tr>
<tr>
<td>10</td>
<td>M-INAC/n</td>
<td>10 µg (0.5 µg/µl)</td>
<td>M-INAC MPs</td>
<td>Balb/c mouse female (6week)</td>
<td>5</td>
<td>I/N</td>
</tr>
</tbody>
</table>
(5) Collection of mucosal fluid

Bronchoalveolar lavage (BAL) samples were harvested from the lower respiratory tract of the mice. After the bronchi were exposed from the neck, a truncated needle with a syringe containing 500 µl of PBS was inserted through a small pore on each bronchus. Mucus exudation was harvested with a back-and-forth motion of the injector, and the supernatant were collected by centrifugation. The nasal washes were collected by 500 µl of PBS through the trachea toward the nose. For vaginal wash preparation, the vagina was washed 10 times with 200 µl of PBS and the obtained fluids were collected by centrifugation. Intestine wash was harvested from small intestine. The outside of the collected small intestine was washed with PBS and the inside of the intestine was wrapped into tube. The tube was centrifuged and supernatant was collected. The fecal were collected into tube and suspended in 10mM of PMSF (phenylmethylsulfonyl fluid) solution for overnight at 4 °C. And the tube was centrifuged and supernatant was collected. All of the samples were stored at -70 °C until analysis.

4) Serological analysis

(1) FMDV serotype O SP ELISA

Anti-FMDV O type antibodies were evaluated using a commercial FMDV ELISA kit (VDPro FMDV type O ELISA kit, Median diagnostics, Gangwon-do, Korea), a competition structure protein (SP) ELISA. ELISA was performed according to manufacturer’s instructions. Briefly, each plate of the kit was pre-
coated with FMDV type O structure proteins, P13C. Test serum samples, negative control, and positive control were diluted to 1:5 in dilution buffer and 100 µl of diluted sera were incubated in wells for 1 h at RT. Then, wells were washed with washing buffer. 100 µl of HRP conjugated anti-FMDV antibody was added, and plates were incubated for 1 h at RT. The color was developed with 100 µl/well of the TMB substrate and stopped by 50 µl of stop solution. All reagents were provided in the kit. Plates were read in a Microspectrophotometer at 450 nm. PI (%), the percent inhibition, was calculated using the formula:

$$\text{PI} (%) = 100 - \left( \frac{\text{OD}_{450} \text{of test sample}}{\text{OD}_{450} \text{of negative control}} \right) \times 100$$

Sera with PI ≥ 50 were considered as positive interpreted as the presence of anti-FMDV serotype O Abs in test serum.

(2) ELISA assay

Antibody production was examined by ELISA in serum and fecal samples collected at days 0 and 28. Briefly, 96 well immuno-plate was coated with purified M5BT in carbonate-bicarbonate buffer (CBB) for 1 h at 37 °C (0.1 µg/well). Then, wells were washed with PBS and blocked with 1 % BSA in PBS for 1 h at room temperature (RT). Series of five-fold dilution of each serum and fecal samples were prepared, starting at 1/100 and volume adjusted to 100 µl with PBST (0.5 % tween 20 in PBS) containing 1 % BSA. Plates were incubated for 2 h at RT and HRP conjugated goat anti-mouse IgG, IgG subtypes and IgA diluted 1:2000 in PBST containing 1% BSA was added. The color was developed with 100 µl/well
of the TMB (Sigma, MO, USA) and stopped by an equal volume of 0.16 M H$_2$SO$_4$. Plates were read at 450 nm in a Microspectrophotometer (Tecan, Austria). Titer of specific antibody was calculated by Softmax Pro version 5.4.1. Antibody titers were reported as log10 of the reciprocal of the highest dilution.

(3) Spleen isolation, cytokine ELISA and T cell proliferation assay

Spleen of the immunized mice were isolated and prepared as a single-cell suspension by gentle mechanical disruption using a 40 µm cell strainer (SPL, NJ, USA). Then, cells were treated with ACK lysis buffer (Gibco, USA) to remove red blood cells. Splenocytes were washed and resuspended at a density of 1 x 10$^7$ cells/ml in PBS. An equal volume of 5 µM carboxy-fluorescein diacetate succinimidyl ester (CFSE, Thermos Fisher Scientific, MA, USA) in PBS was added, and the cells were gently mixed for 20 min at 37°C. 5 x 10$^6$ cells/well of labeled splenocytes were re-stimulated with M5BT (33 µg/ml) in 24-well plates containing RPMI 1640 supplemented with 10% FBS and 1% of penicillin and streptomycin for 3 days. On the third day, the culture supernatant was collected and cytokines in the supernatant were measured. The level of IFN$\gamma$ and IL-4 concentration of mice was analyzed using a mouse ELISA Complete Kit (IFN-$\gamma$ Cat. No. K0331138; IL-4 Cat. No. K0331144) (KOMA BIOTECH, Korea). In addition, splenocytes were harvested for the analysis of T cell proliferation by centrifugation at 2000 rpm for 5min at RT. T cells in splenocytes were labeled with anti-mouse CD3e PE-efluor® 601 (Thermos Fisher Scientific, MA, USA) for 1h in a darkroom. The cells were washed with FACS buffer (3% BSA in PBS)
two times. T cell proliferation assay was analyzed by using fluorescence activated cell sorter (FACS, BD Biosciences, USA).

(4) Virus neutralizing test

Virus neutralization activity was determined in sera using a standard micro-neutralization test performed in 96-well plates by incubating serial twofold dilutions of each serum sample with 100 50% tissue culture infective doses (TCID50) of FMDV Manisa O1, for 1h at 37 °C. The remaining viral activity was determined in 96-well plates containing fresh monolayers of BHK-21 cells. Endpoint titers were calculated as the reciprocal of the final serum dilution that neutralized 100 TCID50 of FMDV in 50% of the wells (Golding et al., 1976).
3. Results and Discussion

1) \textit{In vivo} evaluation of M5BT protein as vaccine candidate

(1) Characterization of M5BT protein

To compare the expression type and expressed quantity of the M5BT and 5BT, the soluble and insoluble fractions of M5BT (19.1 kDa) and 5BT (18.1 kDa) were prepared and analyzed by SDS-PAGE (Figure 41). The expressed quantity of proteins were shown as relative ratio compared to band marked as unknown protein (17 kDa) expressed in \textit{E.coli}. The expressed quantity of soluble M5BT (3.71) was higher than soluble 5BT (3.10), analyzed by image J software. The total quantity (soluble protein and IB) of M5BT (7.43) was higher than that of 5BT (4.95). However, the IB ratio of M5BT (50.1%) was higher than that of 5BT (37.4%).

Un-stability of 5BT as an artificial recombinant protein was discussed in study I (Lee et al., 2017a). To analyze stability of M5BT, M5BT and 5BT were incubated in crude protein extract at 4 °C. All samples were analyzed by SDS-PAGE and image J software (Figure 42). M5BT showed improved resistance from proteolytic agents in some degree compared to 5BT. While M5BT was degraded by 35 % and 55 % in 24 and 48 h respectively, 5BT degraded by 39 % and 68 % in 24 and 48 h respectively.

M5BT also showed higher expression quantity and better resistance to
proteolytic degradation compared to 5BT, which is expected to be due to the stabilization of N-terminus of the protein.

M cell targeting peptide was selected by phage display technique which uses a set of genetically recombinant bacteriophages expressing randomized foreign peptide sequences on their surface (Yoo et al., 2010). Because the peptide sequences (CN7C) have two cysteines at both ends, the peptide can form disulfide bond and ring structure (McLafferty et al., 1993). The ring structure of MTP might contribute stability of artificial recombinant protein. However, the increased expression of unstable M5BT in E.coli led to the higher production of inclusion bodies. This can be overcome by controlling culture conditions (Jana and Deb, 2005).
Figure 41. SDS-PAGE analysis for the expression pattern of M5BT protein

(A) SDS-PAGE analysis to confirm the expression pattern of recombinant proteins. S, soluble protein; IB, inclusion body. (B) The expressed quantity of proteins were showed as relative ratio compared to band marked as unknown protein (17 kDa) commonly expressed in E.coli. (C) The ratio of expression pattern analyzed by image J software and inclusion body fractions were: M5BT, 50.9 %; 5BT, 37.4 %. 
Figure 42. MTP effect on the stability of recombinant proteins

Crude protein extracts were incubated at 4 °C for 6 days and daily sample was analyzed by SDS-PAGE. (A) M5BT and (B) 5BT. Lanes: M, protein marker. 0 to 6: days after sonication (C) Graph showing intact proteins in (A) and (B) analyzed by image J software.
(2) Purification and confirmation of M5BT protein

The purity of M5BT was above 95% according to SDS-PAGE result (Figure 43A). Target protein was confirmed by western blotting assay using anti-histag antibody. To confirm production of specific antibodies in sera of swine vaccinated with M5BT and iFMDV, the western blot assay was carried out (Figure 43B). The sera of animals vaccinated with M5BT and iFMDV recognized M5BT protein. The animals produced specific antibodies for M5BT protein. The quantity of purified M5BT (106.3 mg per litter culture) were higher than that of purified 5BT (65.2 mg per litter culture). The protein production yields can be increased using a high density culture system using a fermenter and effective culture media (Jana and Deb, 2005). In addition, the protein was easily purified with > 95% purity by using one-step affinity column chromatography. These are important advantages to industrialize the protein as a vaccine.
Figure 43. Purification and confirmation of M5BT protein

(A) Analysis of purified M5BT protein by SDS-PAGE. 1, un-induced cell extracts; 2, induced cell extracts; 3, purified M5BT protein; M, protein marker. (B) Western blot analysis with M5BT specific antibody. N.C., negative control (control sera from untreated animals); M5BT, sera of animals injected with M5BT protein; iFMDV, sera of animals vaccinated with inactivated FMD vaccine; P.C., positive control (commercial anti His-tag antibody).
(3) Antigenicity of M5BT in mice oral immunization

The FMDV transmission occurs through respiratory aerosols. It also is orally contagious through direct or indirect contact with infected animals and contaminated fomites including infected animal products and feed (Kitching et al., 2007). Mucosal vaccines can induce mucosal immunity, including antigen specific-secretory IgA production, to protect and neutralize the virus (Mishra et al., 2010). Mucosal immunity induced in one location can be spread to other mucosal immune systems (Li et al., 2016). Thus, it is important to induce mucosal immunity for preventing FMD. However, the M5BT specific antibodies were not detected in serum and fecal samples of oral group (data not shown). Although M5BT improved the expression level and resistance to endogenous proteolytic degradation compare to 5BT, it is not stable enough to induce mucosal immune response in gastro-intestine. In a follow-up study, we published the study about an oral vaccine using M5BT loaded mucoadhesive cellulose acetate phthalate (CAP) microparticles to safely deliver the proteins into the intestines (Lee et al., 2017b)

(4) Antigenicity of M5BT in mice intramuscular immunization

Anti-FMDV antibodies were analyzed using an SP ELISA kit (Figure 44A). The kit recognizes the neutralizing antibody production against FMDV when the PI value is greater than 50%. After 2 weeks of boosting inoculation, sera of the groups vaccinated with M5BT and iFMDV showed > 50% of PI value. M5BT specific IgG and IgG subtypes were also analyzed by ELISA using serum samples
collected 4 weeks after priming (Figure 44). Antibody production was significantly higher in groups injected with M5BT and iFMDV compared to NT group. IgG1 was produced about 10 times more than IgG2a in the group inoculated with M5BT. Conversely, IgG1 was only a tenth of IgG2a in the group vaccinated with iFMDV. The in vivo immunization assay showed that IgG1 titers were higher than IgG2a in the sera of animals inoculated with M5BT protein. M5BT protein was designed to maximize production of antibodies against epitopes by focusing the appropriate immune response via high-density epitopes. The IgG1 production is affected by IL-4 expressed in type 2 helper T cells (Th2 cells). B cells differentiate into plasma cells by IL-4 stimulation secreted by Th2 cells and secrete IgG1 after class switching. Th2 cells are the differentiated type 0 helper T cells (Th0 cells) in the IL-4 dominant immunological environment (Seder and Paul, 1994; Abbas et al., 1996). Thus, the higher IgG1 level indicates that Th2 cell mediated humoral immune response is prevalent in M5BT immunization. On the other hand, IgG2a titers were much higher than IgG1 in the sera of animals vaccinated with iFMDV. The IgG2a production is effected by IFNγ expressed in type 1 helper T cells (Th1 cells). B cells differentiate into plasma cells by stimulation of IFNγ and secrete their B cell receptor (BCR) through class switching to IgG2a. Th1 cells are the differentiated Th0 cells in the IFNγ dominant immunological environment (Trinchieri, 1995). This indicates that Th1 cells mediated cell-mediated immune response activation was higher. Thus, M5BT induced an efficient, but different immune response compared to iFMDV.
Figure 44. Serological analysis of M5BT immunization for mouse

(A) FMDV-specific antibody assay using SP competition ELISA. Each symbol represents the value for individual animal. M5BT specific antibody titers were measured by ELISA in serum samples collected at 4 weeks after immunization and expressed as the reciprocal log 10 of the last dilution calculated by interpolation to give an absorbance of 1 above background. The bars correspond to the geometric mean of each group. Error bars represent standard deviation. (B) Total IgG titer, (C) IgG1 titer, and (D) IgG2a titers. ***: P < 0.001, one way ANOVA.
(5) Cytokine ELISA and T cell proliferation assay

Spleens from sacrificed mice were extracted and splenocytes were cultured to analyze the proliferation of M5BT specific T cells. T cell proliferation was higher in iFMDV and M5BT groups compared to the NT group (Figure 45A). IL-4 and IFNγ in the supernatant of splenocyte culture media was also analyzed. Splenocytes of M5BT and iFMDV group was re-activated by the M5BT and produced high level of IL-4 and IFNγ compared to NT group (Figure 45B). The expressed cytokines were no significant difference between M5BT and iFMDV groups. The cytokines known to be induced in antigen stimulation, IL-4 and IFNγ, were re-induced with M5BT addition in the culture supernatants of splenocytes from mouse immunized with M5BT and iFMDV. The splenocytes of the iFMDV group were highly induced after reactivation of the M5BT than them of M5BT group. This may be the strong effect of the adjuvant contained in the commercial vaccine. There have been reports that suppuration had occurred around the region inoculated with inactivated FMD vaccine (Park et al., 2014b). This is considered to be a side effect of the over-induced immune response by the adjuvant in the FMD vaccine. Since it was reported that GH loop of VP1 acts as a T cell epitope (Collen et al., 1991), T cell epitopes should be synthesized and a more accurate study plan is needed. There is much evidence for cross-presentation through strong adjuvants (Jelinek et al., 2011). Since the subunit vaccine can induce the cytotoxic T cell responses, the introduction of the CD8 T cell epitopes are also worth consideration.
Figure 45. Analysis of memory immune response

(A) T cell proliferation in immunized splenocytes by M5BT re-stimulation. Splenocytes were treated with M5BT protein in a 3 day cell culture and T cells were analyzed by using Fluorescence-activated cell sorter using anti-CD3 antibody label. Cytokine assay: (B) IL-4 and IFNγ. Cytokine expression was assayed after 3d splenocyte culture stimulated with M5BT protein (33 µg/ml). The bars correspond to the geometric mean of each groups. Error bars represent standard deviation. *P <0.05; **: P < 0.01, one way ANOVA.
(6) Evaluation of M5BT as a vaccine in swine immunization

A porcine immunization assay was carried out to evaluate the efficacy of the M5BT vaccine. M5BT and iFMDV groups significantly produced more M5BT specific IgG compared to NT group (Figure 46A). Anti-FMDV antibody production level was similar to both the M5BT and the iFMDV groups which have 74.7 % and 77.5 % PI at 6 week, respectively (Figure 46B).

Neutralizing antibody production in sera collected every two weeks was also analyzed (Table 15). Both immunized groups showed similar level of neutralizing antibody production. The neutralizing antibody level was about 81.3 fold and 181 fold dilution in M5BT and iFMDV immunized groups, respectively. Only priming vaccination of M5BT elicited the production of neutralizing antibodies above the cut-off value (16 fold) in 2 out of 3 individuals.

When M5BT was inoculated, neutralizing antibody and anti-FMDV antibody were effectively produced in the similar level to iFMDV immunized animals, but without the excessive immune response from the splenocyte culture in a ground state and appropriately re-activated by the addition of M5BT protein antigen. However, the protein content of one dose is very high compared to the inactivated virus vaccine due to the low-immunogenicity of the subunit vaccine (Li et al., 2014). Thus, to reduce the dose level, further study may be needed to develop the effective adjuvants and delivery vehicle, such as polymer coating technologies.
Although multi-epitope vaccine has protective effects against FMD through these assays, the advantage, universal vaccine which was development intention of multi-epitope vaccine, was not confirmed. It should be verified that antibodies against other GH loops that do not constitute a multi-epitope vaccine are produced and neutralizing assays using other FMDV variants should be performed. In addition, it is necessary to confirm by challenge assay for industrialization.
Figure 46. Evaluation of M5BT in swine immunization

(A) Antibody titer analysis by ELISA. Specific antibody titers against M5BT was measured by ELISA in serum samples collected at days 0, 14, 28 and 42 post-immunization. Antibody titers were expressed as the reciprocal log10 of the last dilution calculated by interpolation to give an absorbance of 1 above background. Each point corresponds to the geometric mean of each group. (B) Detection of FMDV specific antibody response in swine serum analyzed by SP competition ELISA at weeks 0, 2, 4 and 6 post-immunization. The dotted line at 50% PI is standard significant level of anti-FMDV antibody. The bars correspond to the geometric mean of each group. Error bars represent standard error of the mean. **: P < 0.01, one way ANOVA.
Table 15. Analysis of neutralizing antibody titers using M5BT-immunized serum against FMDV MANISA O1 strain

<table>
<thead>
<tr>
<th>Group</th>
<th>Week</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
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<tbody>
<tr>
<td>NT</td>
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<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
</tr>
<tr>
<td>M5BT</td>
<td></td>
<td>&lt;16</td>
<td>22</td>
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<td>64</td>
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<tr>
<td></td>
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<td>&lt;16</td>
<td>&lt;16</td>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>iFMDV</td>
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<td>&lt;16</td>
<td>32</td>
<td>90</td>
<td>181</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>&lt;16</td>
<td>32</td>
<td>64</td>
<td>181</td>
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</tbody>
</table>

Effective value over 16 fold.
2) M5BT-loaded INAC MPs as vaccine delivery system and adjuvant

(1) Preparation and characterization of INAC

Acetyl inulin was synthesized by conjugation of acetyl anhydride with inulin at 40°C for 24 h using sodium acetate as a catalyst (Figure 47). The conjugation of acetyl groups with inulin was confirmed and calculated by 600 MHz $^1$H-NMR spectroscopy (Figure 48). The peak of three hydrogens from acetyl groups (-COCH$_3$) was identified at 2 ppm (87.390) in the NMR spectra of INAC whereas there is no peak at 2 ppm in that of inulin. And the peak of hydrogen of hydroxyl group of sixth carbon in hexoses is identified at 3.8 ppm (6.911). Therefore, the degree of acetylation was 80.8 mole%. Because inulin is soluble in water, however, MPs are not formed and proteins are not loaded. Acetylated inulin is produced by substituting acetyl group for the hydroxyl group of hexose in inulin (Wu and Lee, 2000). The acetylated inulin is water insoluble due to weak hydrogen bonding between hydroxyl groups of inulin and the water molecules compare to its precursor polymer inulin. Thus, it can form MPs by double emulsion evaporation method.
Figure 47. Chemical reaction scheme for synthesis of acetyl inulin

Figure 48. Confirmation of acetyl inulin synthesis by H-NMR

$^1$H 600MHz NMR spectra of (A) Inulin and (B) Acetyl inulin. The asterisk means three hydrogens in acetyl group with 2 ppm.
(2) The morphology and size of MPs

M5BT-loaded M-INAC MPs were prepared by double emulsion evaporation method. The characteristics of M5BT-loaded M-INAC MPs are summarized in Table 16. M5BT-loaded INAC MPs and M-INAC MPs showed a loading content of 2.47 ± 0.24 % and 3.25 ± 0.25% with an encapsulation efficiency of 49.45 ± 4.73% and 64.91 ± 5.01 %, respectively. Microparticle formation of MPs was observed by FE-SEM showing regular spherical-shaped microparticles as shown in Figure 49. The average particle size of M5BT-loaded INAC MPs and M-INAC MPs are 2.82 µm and 2.50 µm, respectively (Figure 50). The data showed that the size of MPs increased by loading M5BT. Self-aggregated MPs larger than 10 µm in diameter were observed. The actual size of MPs excepting self-aggregated MPs were 1.37 ± 0.21 µm and 1.20 ± 0.21 µm, respectively. Mannan was introduced as PAMP in INAC to synergistic effect as adjuvants. Mannan could be targeted to the pathogen recognition receptors (PRRs) and mannose receptors (MRs) of APCs (van de Veerdonk et al., 2009). In previous study, it was confirmed that the MPs coated with mannan were more untaken by the Raw264.7 cells, macrophage cell line derived from mouse than MPs only (Li et al., 2016). In addition, M-INAC MPs was formed spherical-shaped microparticles ranging from 1-2 µm. It was reported that MPs less than 5µm in diameter can be uptaken through phagocytosis by antigen presenting cells (APCs) such as macrophages and dendritic cells playing a crucial role in initiating innate immune response (Eldridge et al., 1990; Katare et al., 2005). The MPs of this size could be internalize into APCs.
Table 16. Determination of loading content and encapsulation efficiency

<table>
<thead>
<tr>
<th></th>
<th>Loading content (wt.-%)</th>
<th>Encapsulation efficiency (wt.-%)</th>
</tr>
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<tbody>
<tr>
<td>INAC</td>
<td>2.47 ± 0.24</td>
<td>49.45 ± 4.73</td>
</tr>
<tr>
<td>M-INAC</td>
<td>3.25 ± 0.25</td>
<td>64.91 ± 5.01</td>
</tr>
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Figure 49. Observation of morphology of MPs by FE-SEM

(A) M5BT loaded INAC MPs and (B) M5BT loaded M-INAC MPs (Magnification 2,000 X and scale bar (white bar): 10 µm).
Figure 50. Measurement of sizes of MPs by dynamic light scattering for MPs

(A) M5BT loaded INAC MPs and (B) M5BT loaded M-INAC MPs. INAC MPs and M-INAC MPs of average diameter is 2819.7 nm and 2501.8 nm, respectively.
(3) *In vitro* release behavior of vaccine from M5BT-loaded M-INAC MPs

To investigate the antigen release behavior in physiological conditions, *in vitro* release study was conducted at PBS (pH 7.4). As a result, the release of M5BT from M5BT-loaded M-INAC MPs was faster than that of M5BT-loaded INAC MPs (Figure 51). And 45.3 ± 8.03 % of M5BT was released from M5BT-loaded M-INAC MPs within 24 h, whereas 27.3 ± 2.11 % of M5BT was released from M5BT-loaded INAC MPs. More than 90 % of M5BT was released from M-INAC MPs within 6 days, while less than 60 % of M5BT was released from INAC MPs. Recombinant proteins in the MPs were also protected from enzymatic degradation. The sustained release properties of the MPs protect protein from enzymatic degradation and maintain immune responses for a long time. The recombinant protein to prevent FMD was easily degraded by endogenous protease in cell extract at 4°C (Lee et al., 2017a). The M-INAC MPs can protect the M5BT protein from enzymatic degradation due to sustained release properties of MPs. More than 90 % of M5BT was released from M-INAC MPs within 6 days, while less than 60 % of M5BT was released from INAC MPs. The results might be regarded that mannan-decoration affected fast release of M5BT from M5BT-loaded M-INAC MPs due to the hydrophilic property of the mannan.
Figure 51. *In vitro* release profile of M5BT protein from M5BT-loaded INAC MPs at neutral pH

MPs (10mg/ml) were suspended in PBS (pH 7.4). Protein concentration was measured by micro BCA assay. All values represent the means ± SD (n=3).
(4) FMDV serotype O specific antibody production

Mannan-decorated INAC MPs would enhance antigen-specific immune response by synergistic effect on TLR pathway in comparison to non-modified INAC MPs. *In vivo* immunization assay to mouse was conducted to validate efficacy of the M5BT loaded mannan-INAC MPs against FMD. The vaccine combination was vaccinated via intramuscular and intranasal route. To assess the immunization efficacy of MPs, levels of FMDV serotype O specific antibodies in sera from immunized mice was determined by FMDV SP ELISA with FMDV serotype O structure protein coated plate (Figure 52A). The vaccination efficacy was presented as percentage inhibition (P.I), which are considered as producing neutralizing antibody when P.I is more than 50.

The results indicate that group intramuscularly immunized with M5BT delivered by M-INAC MPs showed P.I value over 50 in all individuals. Anti-FMDV antibody production level was similar to both the M-INAC MPs and iFMDV, commercial vaccine. M-INAC MPs elicited higher level of FMDV antibodies than CFA group, commercial vaccine. In intranasal assay, the group vaccinated with M-INAC MPs elicited the highest level of P.I than others of intranasal vaccination (Figure 52B). But, all group elicited P.I value under 50. It means that neutralizing antibodies wasn’t produced in sera.
Figure 52. FMDV serotype O specific antibody assay using SP competition ELISA

(A) The groups vaccinated via intramuscular injection and (B) The groups vaccinated via intranasal injection. Test sera was collected at week 4 post-immunization. The PI (%) means the percent inhibition. Each symbol represents the value for individual animal. The dotted line describes 50 PI (%). Horizontal lines describe the mean value for each group of animals.
(5) M5BT specific immunoglobulin detection

To assess the systemic humoral immune response after immunization with M5BT-loaded M-INAC MPs, anti-M5BT serum IgG level in sera from immunized mice were analyzed by ELISA (Figure 53). Among the intramuscularly immunized groups, anti-M5BT IgG titer was improved in adjuvant group (iFMDV, CFA, INAC MPs, and M-INAC MPs) as compared to that of M5BT only group. And M5BT-loaded M-INAC MPs groups elicited significantly higher IgG titers suggesting the immune cell activating effect of mannan-decoration of INAC MPs as compared to the CFA group. Furthermore, to investigation the induction of type 1 helper cell (Th1) and type 2 helper T cell (Th2) response, anti-M5BT IgG2a and IgG1 titer were analyzed, respectively (Abbas et al., 1996). The anti-M5BT IgG1 titer was highest in M5BT-loaded INAC MPs. There is no significance between INAC MPs and M-INAC MPs group. The anti-M5BT IgG2a titers of mice immunized with M5BT loaded M-INAC MPs and iFMDV were significantly higher than that of untreated, M5BT only, and CFA group. In immunization assay, M-INAC MPs and iFMDV group produced anti-FMDV antibody in their serum. PI (%) above 50 means production of neutralizing antibody for FMDV. All individuals in group M-INAC MPs and iFMDV recorded PI (%) over 50. Although production of IgG and IgG subtype between INAC MPs and M-INAC MPs groups was similar as shown in Figure 53, M-INAC MPs worked more efficiently than INAC MPs. Furthermore, to understand the characteristics of the immune responses induced by M-INAC MPs, IgG1 and IgG2a was analyzed as
an indicator of Th2 or Th1 bias in the immune response (Abbas et al., 1996). While the Th1 response was induced higher than the Th2 response in the iFMDV group, the Th2 response was induced higher than the Th1 response in the CFA, INAC MPs and M-INAC MPs group. The immune response was increased higher in the particulated vaccine groups compared with the native antigen and CFA group.

The immunization assay via intranasal injection was also conducted to evaluate efficacy of inducing systemic immune response (Figure 53). The group vaccinated with M-INAC MPs elicited the highest IgG, IgG1 and IgG2a level than other groups of intranasal injection. Serum antibody production titer was much lower than intramuscular injection. This is consistent with the anti-FMDV serotype O specific antibodies ELISA. However, introduction of mannan resulted in increased immune response.
Figure 53. M5BT specific IgG and IgG subtypes immune response at 4 weeks post immunization.

Anti-M5BT serum immunoglobulin titers were measured using blocking ELISA. Note that the endpoint titers of the result were represented in log scale on the y-axis. (A) anti-M5BT total IgG titers, (B) IgG1 titers and (C) IgG2a titers in sera of groups vaccinated via intramuscular injection. (D) Total IgG titers, (E) IgG1 titers and (F) IgG2a titers in sera of groups vaccinated via intranasal injection. All values represent the means ± SD (n=5).
(6) Mucosal IgA analysis

To evaluate efficacy of intranasal vaccination, M5BT specific antibody titer was detected in nasal wash (NW), bronchoalveolar lavages wash (BW), small intestine wash (IW), vaginal wash (VW), and fecal extract (fecal) (Figure 54). The higher M5BT specific IgA titers in mucosal fluid of groups vaccinated with M-INAC MPs was detected than that of other groups. The results showed that the effect of introducing mannan improved mucosal immune response compared to INAC group. In addition, particulated vaccine is important to enhance mucosal immune response by comparing to CTB group. CTB was simple mix with M5BT protein. Particulated vaccine can uptake into dendritic cells and activates the NALP3 inflamasome, and this contributes to their enhancing effects on innate and antigen-specific cellular immunity (Sharp et al., 2009). FMD is an infectious disease that mainly affects aerosol pathway. Thus, the vaccine system that activates mucosal immunity is needed. Although mucosal immunization vaccine has been developed for the prevention of FMD, it is mainly carried out as a neutralizing antibody or SP ELISA test using serum in order to evaluate the efficiency of the vaccine. However, in order to accurately evaluate mucosal immunization vaccines, the methods for evaluating mucosal IgA titers should also be developed.
Figure 54. Analysis of IgA titers in mucosal fluid

M5BT specific IgA titers in mucosal fluid of various sites. (A) Nasal wash, (B) BAL wash, (C) small intestine wash, (D) vaginal wash, and (E) fecal extract. IgA levels in mice immunized with each indicated formulations were analyzed by ELISA and then calculated as optical density (450 nm). The error bar represents standard deviation.
(7) Secretory IL-4 and IFN-γ in supernatant of splenocyte cultures

To assess the immunization efficacy with mannan-decorated carrier, splenocytes were isolated from immunized mice and cultured in vitro for 3 days with antigen re-stimulation. IFNγ and IL-4 levels of splenocyte culture medium were analyzed by ELISA (Figure 55). The results were represented as relative cytokine levels of antigen-stimulated splenocytes compared to that of untreated controls. M5BT-loaded M-INAC MPs induced higher IFNγ and IL-4 level than others. In the groups vaccinated via intranasal injection, while IFNγ was activated in all groups compared to untreated group, IL-4 wasn’t activated in all groups. The confirmation of immune memory via reactivated splenocytes was carried out (Myeong—Heon and Duk—Young, 1998). The cytokines were highly expressed in the splenocytes of iFMDV, CFA and particulate vaccines groups through reactivation of M5BT protein compare to native protein group. This results suggest that M5BT loaded M-INAC MPs enhance immune response such as adjuvant and protect M5BT protein from enzymatic degradation. However, the intranasal vaccine may not be sufficient to induce a systemic immune response.
Figure 55. *In vitro* released cytokines in splenocyte culture supernatant

(A) The cytokines in splenocyte culture supernatant of animals vaccinated via intramuscular injection, and (B) the cytokines in splenocyte culture supernatant of animals vaccinated via intranasal injection. All values represent the means ± SD (n=5). Significant differences were compared to the untreated group. **: P < 0.01; ***: P < 0.001, one way ANOVA.
4. Conclusion

Vaccination is one of the selected policies for the control and prevention of FMD. However, the risks of large-scale cultivation to produce FMD vaccine are well known. This raises concerns with biosafety issues and United States prohibited vaccine production on its mainland. In addition, DIVA is a potential issue that can arise from inoculating a whole-body vaccine creating a disadvantage for imports and exports of livestock products. In study II, a multi-epitope vaccine, M5BT, was tested as an alternative subunit vaccine which is free from the problems that can occur with conventional vaccines. The results demonstrated that the multi-epitope vaccine was capable of generating neutralizing antibodies similar to commercial FMD vaccines. Although the multi-epitope vaccine does not contain the MANISA O1 strain used in vaccine, FMDV kit and neutralizing assay, it indirectly confirmed neutralizing antibodies production against various FMDV variants as originally intended. However, the limitation of this study are that it didn’t test neutralizing assay using various FMDV variants. It is also difficult to demonstrate the practical effects of this multi-epitope vaccine because it has not been studied to challenge assay. To confirm efficacy of the multi-epitope vaccine, the neutralizing assays and challenge assays will be conducted using various FMDV variants.

The FMDV transmission occurs through respiratory aerosols. It also is orally contagious through direct or indirect contact with infected animals and contaminated fomites including infected animal products and feed. Mucosal
vaccines can induce mucosal immunity, including antigen specific-secretory IgA production, to protect and neutralize the virus. Mucosal immunity induced in one location can be spread to other mucosal immune systems. Thus, it is important to induce mucosal immunity for preventing FMD.

M5BT consists of MTP and 5BT protein. M cells located on the follicle-associated epithelium (FAE) of the Peyer’s patch (PP) have an ability to uptake and deliver antigens into immune cells in PP via transcytosis. MTP (CKS9) was selected by the phage display technique. In previous study, MTP conjugated chitosan nonaparticles were transported more effectively across the M cell model in vitro and accumulated more specifically into PP region in gut-associated lymphoid tissue (GALT) in vivo. It was reported that the animals vaccinated with MTP conjugated BmpB produced more BmpB specific antibodies than those vaccinated with BmpB only. Oral immunization of M5BT for mice to induce mucosal immunity against FMDV was carried out with reference to previous studies using BmpB. While BmpB induced mucosal immunity compared to PBS control in the previous study, M5BT failed to induce mucosal immunity for FMDV in this study (data not shown).

Although M5BT improved the expression level and resistance to endogenous proteolytic degradation compare to 5BT, it is not stable enough to induce mucosal immune response in gastro-intestine. Because the Immunogenicity and antigenicity of M5BT for FMDV through intra-muscular injection into swine and murine model in vivo was evaluated in study II, novel delivery system using INAC
and mannan was developed that the microparticles have adjuvant ability and protection ability from proteolytic degradation. Although the recombinant protein vaccine will be next generation vaccine, the vaccine requires appropriate carrier to protect clearance by enzymatic degradation in host and adjuvant to overcome its low-immunogenicity. In this study, mannan-decorated acetylated inulin were successfully prepared and their delivery to the mannose receptors on APCs was evaluated in vitro and in vivo. It was found that vaccination with M5BT-loaded M-INAC MPs in mice induced enhanced serum IgG and anti-FMDV antibody responses as compared to commercial adjuvant. This indicate that vaccination of subunit vaccine via T-CAP MPs effectively delivered the vaccine to APCs and elicited humoral immune response. It will make a step forward into promising subunit vaccine development in livestock industries.

Nasal immunization with vaccine delivery system was conducted to effectively induce mucosal immune responses. MTP is a peptide targeting human M cells that have been shown to be effective in mice but not in porcine. Thus, finding a targeting peptide for a porcine M cell has become a problem in the future. Currently, there are many reports that porcine M cells have a large amount of TLR-2, and a porcine TLR-2 targeting peptide was found in our laboratory. By replacing this peptide with the current MTP, the vaccine will be developed to induce mucosal immune responses in pigs. In addition, the effective methods should be developed since nasal vaccination is difficult to animals.

In conclusion, the multi-epitope vaccine was developed that could induce a
strong neutralizing antibody reaction against FMDV serotype O. This antigen is easy to produce and purify and effectively demonstrated neutralizing antibody production comparable to conventional inactivated viral vaccines. In the future, the vaccine efficacy should be demonstrated through a challenge test in the target animal to register as an alternative to the inactivated vaccine (Table 17).
## Table 17. Summary of study II

<table>
<thead>
<tr>
<th>Category</th>
<th>Traits</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M5BT</strong> (study 2-1)</td>
<td>Recombinant designed protein vaccine</td>
<td>① M5BT can produce neutralizing antibodies</td>
</tr>
</tbody>
</table>
| Novel FMD vaccine system (study 2-2) | Dual-functioned            | ① Protection of M5BT protein from proteolytic degradation  
  ② Enhancement of immune responses  |
| Nasal vaccine                |                            | ① Enhancement of mucosal immune responses  
  ② Production of IgA in mucosal fluid |
Overall conclusion

The Korean government has been trying to prevent FMD through supplying inactivated FMD vaccine rather than stamping out infected animals. As a result, Republic of Korea lost the status of FMD free country without vaccination, the best status acknowledged by OIE. However, FMD continues to occur since 2010. The main reason why the government failed to early prevent the disease was poor responses, but it seems that they lacked understanding of the FMDV. The whole area of Asia is currently suffering from FMD and should have taken care of specific livestock quarantine due to increased travel abroad. In addition, the FMDV has a various variants due to their highly mutation rate. The vaccine for various FMDV variants had to be secured in consideration of various possibilities. Until 2010, Korea had a FMD vaccine composed of topotype Pan-Asia in virus serotype O, but the FMD that occurred after 2010 was topotype South-East Asia in virus serotype O. It is difficult to expect cross-immunity because the sequence difference between these two topotypes is about 15 to 20 %. The current commercialized FMD vaccine is not cross immunity, so new vaccines are needed.

Many researchers have studied to develop alternative vaccines through introducing various strategies. Although the subunit vaccines via recombinant protein system have demonstrated their efficacy through many studies, the strategy using the part of virus whole body may be efficient to production but cross-immunity is more difficult to expect. In this study, the multi-epitope vaccine
strategy was devised to maintain the advantages of recombinant subunit vaccine and to have cross-immunity against various FMDV variants.

In study I, several recombinant multi-epitope vaccine candidates were developed and their characteristics were analyzed. The GH loop, one of the most important regions in VP1 protein was selected as B cell epitope (amino acid residue 132 to 151) to develop effective designed protein vaccine for FMDV. Five representative B cell epitopes were selected by similarity analysis of amino acid sequences of seventy-one GH loops collected from NCBI. There are several huddles to producing artificial recombinant proteins in a soluble form using bacterial bioreactors. Recombinant proteins often form inclusion bodies and, in some cases, are not accumulated. In this study, originally designed recombinant proteins, MEP, EPM, and EP, were not accumulated. The peptide (amino acid residues 136 to 162) containing the secondary structure, \( \alpha \)-helix, region might help the structural formation of artificially designed protein. Thus, the new five epitopes were selected by hierarchical clustering. Because multi-epitope vaccine is the minimum unit to concentrate the immune response on the epitope, the T cell epitope was conjugated in C terminus of multi-epitope recombinant protein (5BT) to enhance immune response and BmpB as fusion protein was conjugated in N terminus of 5BT to enhance stability of the protein (B5BT, MB5BT). In addition, M cell targeting peptide was conjugated in N terminus of 5BT to develop mucosal vaccine (M5BT). These protein vaccine candidates were produced as soluble form and can purify through affinity chromatography using his-tag.
Analysis of the protein characteristics of 5BT and B5BT revealed that the 5BT was more easily degraded by endogenous proteolytic enzymes and formed as high degree of inclusion body formation than B5BT. From this analysis, it was concluded that BmpB as fusion protein is effective for the stabilization of artificial protein. However, 5BT is thought to be more industrially valuable because soluble 5BT was produced and obtained more than soluble B5BT. In addition, there was no effect of BmpB via in vivo immunization assay. 5BT was selected for further study.

Mass production of 5BT was optimized by controlling culture conditions, such as culture temperature, induction time, concentration of inducer, and harvest time. The optimal condition in flask level was inoculation of 0.5mM IPTG induction at 0.5 (OD_{600}) and 37°C and incubating for 4 hours. Based on this condition, fermentor condition, which is commercialization scale, will also be able to search for optimal conditions. In addition, this protein can easily be purified at over 90% purify through affinity chromatographic purification. Because this protein does not easily change its characteristics depending on the various buffer, purification can be done at high purity through various purification methods.

Specific antibodies against B cell epitopes constituting 5BT were identified using serum obtained from mouse in vivo immunization in study I. If antibodies to other B cell epitopes that do not constitute 5BT were identified, it would have been possible to assess whether the strategy met the original intent of the multi-epitope. It will require confirmatory evidence as universal vaccine in further
In study II, the efficacy of selected M5BT was confirmed in swine and mouse models. When M5BT protein was injected intramuscularly into mouse, it was confirmed that anti-FMDV antibody was produced in serum as accordance with inactivated FMD vaccine. The anti-FMDV antibodies and neutralizing antibody were produced in serum of swine vaccinated with M5BT mixed in IMS1313, swine adjuvant, as accordance with inactivated FMD vaccine. These studies have proven that M5BT can be used as FMD vaccine, but it is necessary to conduct challenge assay or virus neutralizing assay using various FMDV variants corresponding to the concept of this vaccine. It is also important to establish an appropriate dose of the multi-epitope vaccine and develop or use an adjuvant appropriate for that.

The M5BT was used as a mucosal immunization vaccine by introducing M cell targeting peptide. However, there was no effect in oral administration because M5BT is instability. To overcome the problem, M5BT was loaded in microparticles consisted of manna decorated acetyl inulin (M-INAC MPs) with adjuvant ability and vaccinated via intramuscular and intranasal routes. Because intranasal route has milder environment than oral route, it can compensate for the weakness of the unstable M5BT. In addition, vaccine delivery strategies that complement the low immunogenicity of recombinant proteins and protect them from proteolytic enzymes. The results confirmed that M-INAC MPs induced an immune response more effectively than other groups. It was also confirmed that
the anti-FMDV antibody increased significantly in both of intranasal and intramuscular routes. Although the multi-epitope vaccine showed promise as a mucosal vaccine, the stability of the protein was low and did not show a great effect. It is also necessary to introduce a fusion protein to increase protein stability. In addition, when inoculating the domestic animals, it is important to use it as a vaccine be substituting the MTP that targets the porcine M cells.

In this study, we developed a multi-epitope vaccine by randomly selecting five representative B cell epitope of seventy-one GH loops. However, since variants of FMDV are diverse, it is worth considering the production of a universal vaccine composing mixture of multi-epitope vaccines consisted of other B cell epitopes.

Multi-epitope vaccine and particulated adjuvant may suggest insights to develop subunit vaccine system against FMDV. This multi-epitope strategy can be used to develop a vaccine against a highly-mutable viral disease such as AI for humans or animals. Further studies should examine the effect of T cell epitopes on immune responses and search for better T cell epitopes.

In conclusion, the results confirmed that the multi-epitope vaccine has potential as a vaccine to prevent FMD and efficacy of the multi-epitope vaccine was indirectly confirmed to be able to prevent various FMDV variants due to conserved region of B cell epitope. However, further analysis is required for the challenge assay and cross-immunity against various variants. There are many limitations to the challenge assay due to the occurrence of persistent FMD.
Nevertheless, this protein vaccine is worthy of analysis. Many topotypes of serotype O that are prevalent in East Asia have the potential to spread to Korea and generate FMD at any time. By using more epitopes and securing pools of multi-epitope vaccines, we can control FMD more effectively. And FMDV serotype A occurred in 2017 could be controlled in the same way.
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Study I에서는 구제역 불활화 백신을 대체하기 위해 제조합단백질 생산기술을 이용한 아단위 백신 후보들을 개발하였다. 구제역바이러스는 4개의 구조단백질을 가지고 있고 그 중 VP1 단백질은 GH loop라는 구조에 속주세포의 integrin에 결합할 수 있는 RGD 서열을 가지고 있는 대표적인 중화항체 결합부분이다. GH loop은 아미노산 서열이 변화가 심하게 발생하기 때문에 바이러스 변종에 대한 교차면역이 생
기지 않는다는. 구제역 바이러스의 변종에 대한 폭넓은 예방을 위해 다중 B 세포 항원결정부 (epitope)로 구성된 제조합 단백질을 디자인하였다. 미국 국립생물정보센터의 데이터베이스에서 전세계에서 발생한 구제역 바이러스 혈청형 O의 VP1 단백질 서열 71개를 수집하였고 136 - 162 아미노산 서열에 대한 유사성 분석을 통해서 71개에 대한 교차면역을 가지도록 대표적인 서열 5개를 선택하였다. 선택된 5개의 서열과 T 세포 활성화를 위한 T 세포 항원결정부 1개를 연결하여 제조합 유전자를 합성하여 대장균 BL21 (DE3)에 도입하여 제조합 단백질을 생산하였다. 최종 구제역 백신 후보는 4종으로 5BT, M5BT, B5BT, 그리고 MB5BT이다. M5BT는 5BT의 N 말단에 M 세포 표적 펩티드를 연결한 것으로 주후에 점막면역 유도를 위해 개발되었고, B5BT는 5BT의 N 말단에 BmpB를 연결한 것이다. BmpB는 돼지적 리균의 막단백질로 5BT의 안정성을 높이기 위해 도입되었다.

5BT 단백질과 B5BT 단백질의 특성을 분석하였다. 5BT는 B5BT에 비하여 단백질분해효소에 의해 쉽게 분해되는 것을 확인하였고 봉입체 (inclusion body) 생성 비율이 높은 불안정성을 가지고 있었다. 하지만, 5BT 단백질은 B5BT 단백질에 비하여 L 배양당 더 많은 수용성 단백질을 생산할 수 있었다. 마우스 in vivo 분석에서는 BmpB가 연결되어 있는 것이 이렇다 할 장점이 없었기 때문에 추후의 연구에
서는 B5BT는 제외하기로 하였다. 5BT 단백질을 이용한 대장균 생산 최적화 조건을 분석하기 위해 대장균의 배양시간, 단백질 생산 유도 시점, 배양온도 그리고 유도체 농도를 조절하여 90% 이상의 정제도로 정제할 수 있는 조건을 최적화 하였다.

Study II 에서는 최종 결정된 단백질, M5BT를 이용한 마우스 및 돼지를 통한 다양한 in vivo 면역실험을 통해서 백신으로써의 효용성을 평가하였다. M5BT를 마우스와 돼지에게 상용 면역보강제 (adjuvant) 를 혼합하여 근육주사를 통해 주입하여 혈청분석을 실시한 결과, M5BT 단백질 특이적인 항체가 생산된 것을 확인하였고 구제역 바이러스에 대한 중화항체가 상용백신에 준하게 생산되는 것을 확인하였다. 이 결과를 통해서 M5BT 단백질이 구제역 백신으로서 사용이 가능하다는 것을 입증하였다.

구제역은 공기 전파를 통해 호흡기로 감염이 되기 때문에 비강을 통한 점막면역을 유도하는 백신의 개발을 수행했다. 이는 study I의 결과에서 알 수 있듯이 단백질의 안정성이 떨어지기 때문에 생기는 현상으로 생각되었다. 그래서 단백질을 안전하게 점막으로 전달하여 면역반응을 유도할 수 있도록 만난 (mannnan)이 장식된 이눌린 고분자미립자 (M-INAC MPs) 전달체를 이용하였다. M-INAC MPs에 담지된 M5BT 단백질은 근육주사와 비강접종을 통해서 마우스에서 그
효과가 평가되었다. 다른 그룹에 비해서 M-INAC MPs에 담지된 M5BT 단백질이 더 효과적으로 면역반응을 유도하였고 항구체역 항체 생산자가 높았음을 확인하였고 비강접종의 경우에는 점막 IgA 생산이 다른 그룹에 비해 증가한 것을 확인하였다.

이 새로운 개념의 재조합 단백질은 구제역뿐만 아니라 다른 변이가 심한 바이러스에 대한 백신 개발에 있어서 활용이 가능할 것이다. 이 연구에서 보여준 결과들은 단백질 백신의 디자인 연구에 대한 통찰력을 제공한다.

주요어: 구제역, 구제역바이러스, 구제역 백신, 재조합 단백질, 다중항원결정기 백신, 다자인 백신, GH loop, B 세포 항원결정기

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