Master's Thesis of Science in Agriculture

Efficiency Evaluation of 5BT, a Genetically Recombinant Multi-epitope-protein, for Its Application as a Subunit Vaccine against Foot and Mouth Disease

유전자 재조합형 다중 항원결정기 단백질, 5BT의 구제역 대응 아단위 백신으로써의 활용을 위한 효율성 평가

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Efficiency Evaluation of 5BT, a Genetically Recombinant Multi-epitope-protein, for Its Application as a Subunit Vaccine against Foot and Mouth Disease

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Abstract

Foot-and-Mouth Disease (FMD) is a highly infectious disease of cloven-hoofed animals like cattle, sheep, and pigs in the livestock industry causing serious loss of productivity and large-scale economic damage. Although vaccination is the reliable solution to prevent FMD, FMD is still hard to control and prevent because of a variety of subtypes and high mutation in the FMD virus. Recombinant protein vaccine is one of the good alternatives to the attenuated viral vaccine in many ways including safety issues, which deals with no virus for vaccine production. 5BT is composed by five B cell epitopes (amino acid residues 130 to 160) derived from GH loop in VP1 from the various O type FMDV and one T cell epitope (amino acid residues 21 to 350) of 3A, one of the non-structural proteins (NSPs), in FMDV.

The production of the 5BT protein from *E. coli* BL21 (DE3) was optimized by determination of proper IPTG induction and purification conditions. The optimal conditions for the IPTG induction and His-Tag affinity purification were achieved with 1.0mM IPTG treated at OD_{600} 0.5 for 4 hours and subsequent 3 time-washing by 20, 30, and 50mM imidazole after binding the sample to the resin, respectively. The 5BT could be obtained as soluble proteins with 52.89mg/L yield and 95% of purity by the condition optimization.

To evaluate the efficiency of 5BT protein as FMD vaccine, we compared the serum antibody induction levels of 5BT protein with commercial vaccine (type O + type A). 8-week-old SD rats were immunized by intramuscular administration with 5BT protein and commercial vaccine in different dose levels, with 50, 100, 150, 200, or 250μg, of 5BT protein, with 20, 40, 60, 80, or 100μl, of commercial vaccine, respectively. At the 6-week after initiation of immunization, the group treated with 200μg of 5BT protein showed the highest serum IgG level among the various dose group for 5BT protein. The commercial vaccine showed the equivalent level of serum IgG with 200μg of 5BT even in the group treated with 20μl.

Usually, the adjuvants help 5BT protein antigen to enhance the

immunogenicity. We also optimized the vaccine with the different adjuvants with 8 groups of SD-rats, the 200μg of 5BT but with different tendencies-treated groups in combination with various adjuvants such as CFA/IFA, Alum, MF59, MPLA, and AS04 (MPLA+Alum) were compared with a 20μl of commercial FMD (type O+type A)-treated group and a 50μg of 5BT-treated group, additionally. After vaccination, only the group of CFA/IFA with 200μg of 5BT protein showed the early antibody induction on the 2nd week. After 6 week-vaccination, the IgG level in the group of 20μl of commercial vaccine was similar with the group with MF59. To validate the immunization efficacy of 5BT protein antigen as FMD vaccine, the levels of FMDV serotype O specific antibodies in serum were analyzed. Our 5BT protein groups with CFA/IFA and MF59 showed higher immunization efficacy than the commercial vaccine.

All effective vaccines induce inflammatory responses. In this study, all of the vaccines showed a balanced induction between humoral and cellular immune response. The commercial vaccine showed the tendency of Th2 type immune response with dominant induction of IL-4 and IgG1. However, 5BT alone, 5BT with CFA/IFA and 5BT with MF59 groups showed the tendency of Th1 type with dominant induction of IFN-γ and IgG2a.

In conclusion, the recombinant 5BT protein were produced in *E. coli* host as a soluble form efficiently with high yield and purity and immunization results showed the balanced induction between humoral and cellular immune responses. Therefore, recombinant 5BT protein has considered as having a potential to be as an FMD vaccine to control and prevent FMD.

Key words: 5BT, Multi-epitope-recombinant protein, FMD vaccine, Adjuvant, Optimization of production, Immunization, His-tag affinity column chromatography, Neutralizing antibody

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List of Abbreviations

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

AEI: Acetylethyleneimine

BHK: Baby hamster kidney

BSA: Bovine serum albumin

CFA: Completely Freund's adjuvant

E. coli: Escherichia coli

ELISA: Enzyme linked immune- sorbent assay

FMD: Foot- and- mouth disease

FMDV: Foot- and- mouth disease virus

HPV: Human papillomavirus

IFA: Incompletely Freund's adjuvant

IPTG: Isopropyl β- D- 1- thiogalactopyranoside

IRES: Internal ribosome entry site

LB: Luria- Bertani

LPS: Lipopolysaccharide

MPLA: Monophosphoryl lipid A

NEVT: Neutralizing antibody

NSP: Non-structure protein

NTA: Ni-nitrilotriacetic acid

ORF: Open reading frame

PBS: Phosphate buffered saline

PK: Pseudoknot

RGD: Arginine- glycine- aspartic acid

RT-PCR: Reverse- transcription polymerase chain reaction

SAT: Southern African Terrieories

SD: Standard deviation

SD-rat: Sprague dawley rat

SDS- PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP: Structural protein

Th1: Helper T cell type 1

Th2: Helper T cell type 2

UTR: Untranslational region

Introduction

Foot-and-Mouth Disease (FMD) is considered one of the most important diseases of cloven-hoofed animals; it affects cattle, buffaloes, pigs, sheep, goats and about 70 wildlife species, e.g. African buffaloes (Jamal and Belsham, 2013). FMD, an acute, violent, highly contagious disease of cloven-hoofed animals, remains widespread in most parts of the world (Han et al., 2015), leading to enormous economic losses including reduced animal productivity and the restrictions on international trade in animal product when incursions occur into countries (Jamal and Belsham, 2013).

The disease is caused by infection with foot-and-mouth disease virus (FMDV), a picornavirus. There are seven serotypes, including type O, A, C, Asia-1, Southern African Territories (SAT-1), SAT-2, and SAT-3. And they have a very rapid replication rate and multiple transmission routes (Domingo et al., 2005). It is reported that Types O and A have the broadest distribution, occurring in many parts of Africa, southern Asia, the Far East and South America (Knowles and Samuel, 2003). According to the statistics, the seven serotypes of FMDV contains approximately 2,000 sequences. Especially, over 1,000 complete or partial VP1 sequences, one of the structural proteins of FMDV, were included in serotype O (Samuel and Knowles, 2001).

Facing with this disease, vaccination is one of the options to control and prevent FMD. With the emergence of various FMD virus serotypes and subtypes, inactivated vaccines usually were usually used in which the pathogenic virus are inactivated by removing their pathogenicity. And

inactivated vaccines need to have a high level of safety to prevent the risk of virus leakage. Therefore, biosafety may be a serious concern. Moreover, inactivated vaccine delayed onset of immune response and also had shorter duration of immunity. Besides, because of the high mutation rate of FMDV strains (Maes and Mesquita, 1970), it is difficult to develop a new vaccine with a novel type of FMDV in a short time (Park and research, 2013).

In recent years, many researchers developed to produce the newly designed recombinant subunit vaccines to solve the disadvantages of inactivated vaccine(Mason et al., 2003a; Li et al., 2008). The recombinant protein vaccine can be easily applicable for mass production in *Escherichia coli* (*E. coli*) and vector. Due to the high mutation rate of FMDV, a multi-epitope vaccine was developed (Ryan and Drew, 1994).

The FMD subunit multi-epitope vaccine composed of five representative B cell epitopes of VP1 on the surface of FMDV and one T cell epitope derived in non-structure proteins (NSP) of FMDV. The artificial 5BT multi-epitope protein was developed (Lee et al., 2017)

To evaluate the efficiency of a multi-epitope subunit vaccine, the 5BT was researched in this study. In chapter 1, to get a large amount and high yield of 5BT proteins, the culture condition and purification condition has been optimized.

Usually, antigen has the immunologic tolerance, the dosage of vaccine should be considered (Schwartz and Dameshek, 1963). Another, the adjuvant can help the vaccine to improve the protective effect and increase the

immunogenicity (Weinberg and Merigan, 1988).

Thus, in chapter 2, the subunit multi-epitope vaccine was evaluated from *in vivo* immunization through intramuscular injection into the animals, Sprague Dawley Rat (SD-rat). Firstly, to determine equivalent dose of 5BT as an FMDV vaccine, antibody induction efficacy of 5BT has been compared with commercial vaccine with various doses after *in vivo* immunization. Secondly, optimal formula of 5BT has been also explored by combination with various commercial adjuvants.

Overall procedures of chapter 1 and chapter 2 are depicted in **Figure 1**.

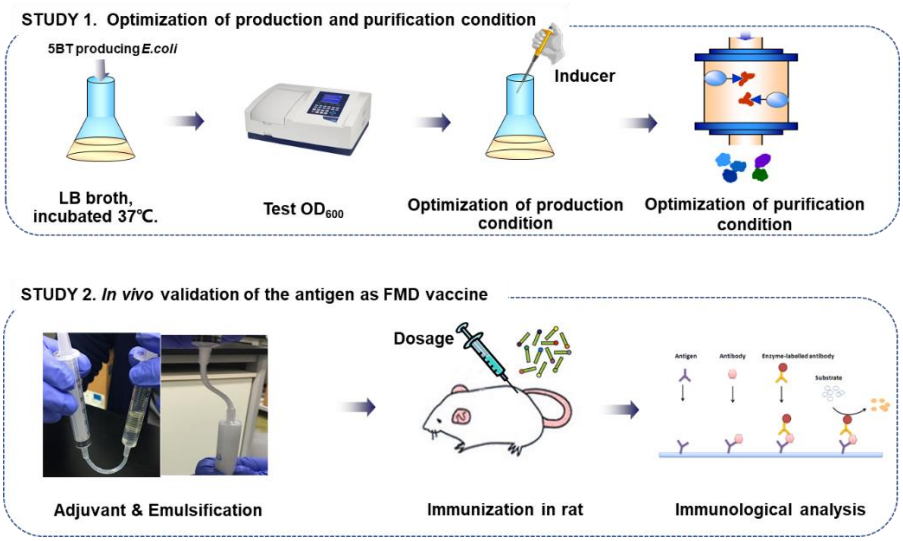


Figure 1. Experimental flow chart of the studies

Literature Review

1. Foot-and-Mouth Disease

1.1 Foot-and-Mouth Disease Virus

The etiological agent, foot-and-mouth disease virus (FMDV), is a nonenveloped ribonucleic acid (RNA) virus, which belongs to the Aphthovirus genus within the family Picornaviridae.

1.1.1 FMDV genome

The virus is composed of a positive-sense, single-stranded RNA genome of about 8.3 kb. The genomic structure consists of a 5'-untranslational region (5'-UTR) about 100nt long, an open reading frame (ORF) about 7,000nt long, and a 3'-untranslational region (3'-UTR) about 1,300nt long (**Figure 2**) (Mason et al., 2003b; Han et al., 2015).

In 5'-URT, it can be divided into five functional elements which play roles in virus translation and RNA replication. Firstly, S-fragment is about 360 bases and fold into a long stem-loop. Although its function is not known, it is reported that it may affect viral pathogenicity. Another is the poly (C) tract comprising over 90% C residues. The length of poly (C) can be extremely variable. The element, cis-Acting Replication Element (*cre*), was predicted to form a stable stem-loop structure that contains a conserved motif of AAACA acting as the template for the uridylylation of VPg (3B). It is essential for RNA genome replication. The function of pseudoknot (PK) is unknown. And the internal ribosome entry site (IRES) about 45nt is an initiation of protein synthesis on

the FMDV RNAs (Grubman and Baxt, 2004).

The 3'-UTR is composed of two stem-loops and a poly (A) tract. And it plays a key role in viral replication and infectivity.

The open reading frame (ORF) about 7000nt is the major portion of the RNA genome. It includes the structural proteins (SPs) which encodes one polyprotein and non-structural proteins (NSPs). The polyprotein is cleaved by viral proteases to form four different SPs and eleven different NSPs. ORF is responsible for genome translation. Finally, the SPs main divide into VP1, VP2, VP3, and VP4.

1.1.2 FMDV structure

The virus has an icosahedral capsid (Alam et al., 2013). This capsid is composed of 60 copies of four different structural polypeptides, including VP1, VP2, VP3, and VP4 (Jung et al., 2013). Vp1, VP2, and VP3 proteins are exposed on the surface, but VP4 is completely located inside the capsid (**Figure 3**), (Han et al., 2015). In Particularly, The VP1 was studied by many researchers (Carrillo et al., 1998; Sun et al., 2003; Yin et al., 2014).

VP1 contains an important immunogenic site, the GH loop (amino acid residue 141 to 160). The GH loop of VP1 possesses the consensus arginine-glycine-aspartic acid (RGD) motif which plays a key role in the interaction of viruses with host cells via an integrin receptor. There are four types of integrin receptors, αvβ1, β3, αvβ6, and αvβ8. The αvβ6 may be the primary receptor and can mediate the delivery of the virus into endosome. Therefore, it is

suggested that virus infection can be inhibited by preventing the binding of RGD motif to the host cells(Han et al., 2015).

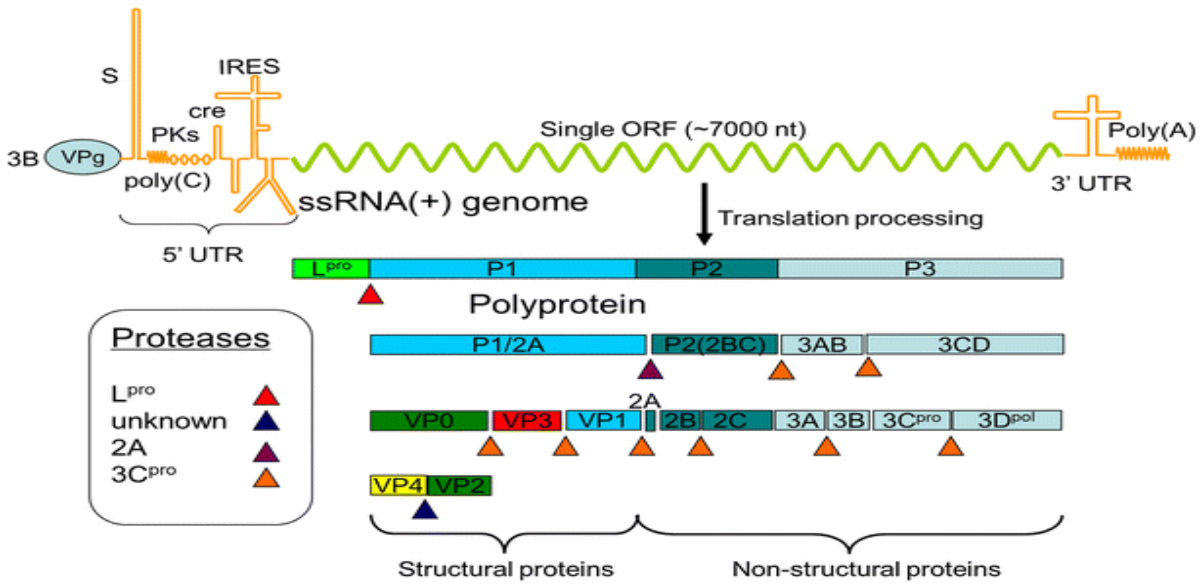


Figure 2. Schematic map of the FMDV genome,

about 8.3kb. The structure consists of the 5'-UTR, including the S- fragment, poly(C), PKs, *cre*, and IREs, and the 3'-URT, composed of two stem-loops and a poly (A) tail, and ORF, including the SP and NSP. The process of post-translational proteolytic cleavage is also illustrated. The figure is modified from Shi-Chong Han,2014.

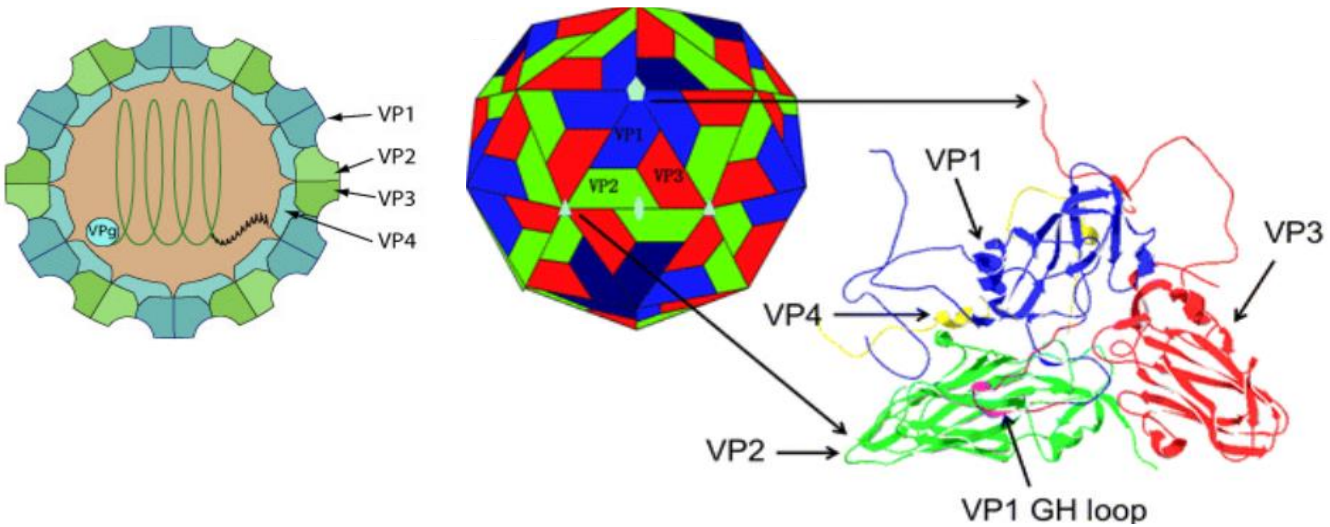


Figure 3. Schematic depiction of the viral icosahedral capsid of four structural proteins (VP1-4).

VP1: blue; VP2: green; VP3: red; VP4: yellow. This figure is modified from Shi-Chong Han,2014 and ViralZone at https://viralzone.expasy.org/98?outline=all_by_species.

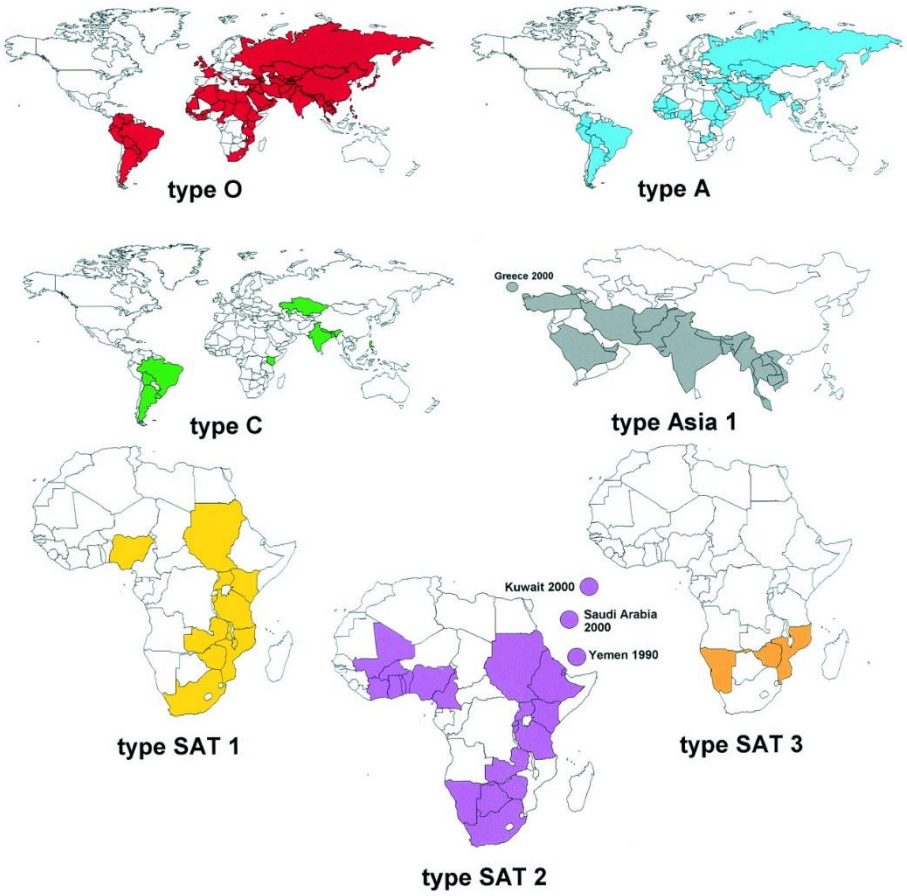


Figure 4. Countries in which FMD were reported between 1990 and 2002.

This figure is cited from the website at www.iah.bbsrc.ac.uk/virus/picornaviridae/aphthovirus.

1.2 FMDV variants and ecology

The virus exists in seven distinct serotypes: O, A, C, Asia-1, and Southern African Territories (SAT)1, SAT2 and SAT3 (**Figure 4**). Since the beginning of the 20th century, FMD has been a concern to many countries. In 1997, in Taiwan the FMD outbreak. Usually, Taiwan had been in free of the disease for 68 years. More than 4 million pigs slaughtered at the cost of U.S. $6 billion (Grubman and Baxt, 2004). In 2001, a large of FMD outbreak started in the United Kingdom and the losses were EUR 3.1 billion (Guo et al., 2013). In the Republic of Korea, the outbreak of FMD occurred in 2010, 2011, 2014, 2015 and 2017 (Lee et al., 2017). In 2019, the FMD also re-occurred. Facing these problems, vaccination is one of the options to control and prevent FMD.

1.3 FMDV clinical symptoms

The incubation period for FMD is highly variable and rapid. Usually, the spread period of infectious disease within-farm is generally 2–14 days but may be as short as 24 h, especially in pigs and under very high challenge conditions (Alexandersen et al., 2003). When FMD occurred, the most visible clinical signs are the lameness, vesicles and sialorrhea. And the body temperature can be raised in the region of 40 °C, lasting one or two days. Feeding and ruminant stop. The vesicles form on the mouth, tongue, hard palate, lips, gums, muzzle, coronary band and interdigital space (Kitching, 2002). The FMD can be affected by air, feed, and other animals. And the speed of spreading is so fast. Affected rams can’t walk and lactating animals suffer a temporary loss of milk yield (Kitching and Hughes, 2002).

1.4 FMDV diagnostic methods and control

The FMD can be diagnosed by reverse-transcription polymerase chain reaction (RT-PCR) to confirm the genome of virus and enzyme linked immune-sorbent assay (ELISA) to confirm the serotype (Reid et al., 1999). It also can be identified by the exact serotypes or strain of the FMDV. Virus isolation tests, Real-time PCR, Typing PCR, Pen-side (SPs), etc. are also used to diagnose FMDV antigen (Lee et al., 2017).

The procedures for the control of FMD will depend on the existing disease status of the affected zone before the outbreak. In countries free of the disease, all infected and in-contact susceptible animals are slaughtered, whereas vaccination would be used to control an outbreak in an endemic area and to prevent the virus spreading (Kitching, 2002).

2. FMDV Vaccine

There are many different types of vaccines. The main types of vaccine include live attenuated vaccine, inactivated vaccine, and recombinant subunit vaccine. The characters were compared in the **Table 1**.

2.1 Live attenuated vaccine

The intensity vaccine was the earliest applied. In ancient China, it was popular in folk that powdered smallpox scabs were inoculated to the health. The patients would develop a mild case of the disease and from then on were immune to it. But the risk using strong toxic to immunize was so high, so it was abandoned to use. Then the attenuated vaccine is used widely.

In the early times 1700s, the live attenuated vaccines used successfully. Subsequently, many live attenuated vaccines were created against human and animal disease. In 1996, a serotype A 12 virus lacking the sequence encoding the leader (L), A12-LLV2, was evaluated as a live-attenuated FMDV as a vaccine. To test the clinical and immunological response, A12-LLV2 and A12-IC (its wild-type parent) were inoculated in cattle. The result showed that it induced a neutralizing and fast protective antibody response (Mason et al., 1997). In 1998, according to A12-LLV2, the leaderless vLLCRM48-KGE was constructed as an attenuated vaccine to immunize swine. RGD- KGE happened mutation in the G-H loop of VP1. As a result, vLLCRM48-KGE induced a poor immune response and didn't produce high neutralizing antibodies (NEUT) (Almeida et al., 1998). The live attenuated vaccine had many limits including unstable phenotype or species differences in pathogenesis. And some virus is so attenuated that they can't induce the protective immune system. Because of these problems, the live attenuated vaccine didn't be researched for many years (Rodriguez and Grubman, 2009)

2.2 Inactivated vaccine

Inactivated vaccine is made by live virus. The virus is inactivated with chemical methods, such as *acetylethyleneimine* (AEI) treatment and still maintains the immunogenicity. The AEI neutralized with sodium thiosulphate. Virus (antigen) is generally prepared in baby hamster kidney (BHK) monolayers. (Anderson et al., 1971). After the inactivated antigen is vaccinated, the adjuvants should be added to improve the immune effect because

inactivated vaccine could not be reproduced inside animal's body.

Usually, the commercial vaccine is an inactivated vaccine consisting of SPs of FMDV. Also, the commercial vaccine called the emergency vaccine. During or around the time of an outbreak in FMD, the emergency FMDV vaccine was administered to immune the affected animal or prevent the outbreak of FMD. It is reported that the emergency vaccination with an inactivated vaccine showed a high protective effect in preventing clinical disease during the short period. And the neutralization titer is lower than routine vaccine (Elnekave et al., 2013).

2.3 Recombinant subunit vaccine

Subunit vaccines are safer than other types of vaccines because the subunit vaccine does not use a virus and easy to produce. Recombinant subunit vaccine divided into many types including nucleic acid vaccine (Hallengärd et al., 2012; Wahren and Liu, 2014), protein vaccine, virus-like particle vaccine (Guo et al., 2013), transgenic vaccine (Carrillo et al., 1998; Wigdorovitz et al., 1999; Dus and Wigdorovitz, 2005) and multiple epitope vaccine (Blanco et al., 2013) (Cao et al., 2013). The recombinant subunit vaccine refers to only essential epitopes of the antigen used to inoculate the host. In recent years, the recombinant multiple epitopes vaccine is popular subject among the researchers. Esther Blanco (2013) was reported that B4T ([VP1(136-154)]4-3A (21-35)), B-cell epitope (VP1 136 to 154) linked to a T-cell epitope (3A 21 40 35) of type O FMDV was constructed. Also, bivalent B2T ([VP1(136-154)]2-3A (21-35)) was created. To identify that the multi-epitope vaccine whether can enhance the

effectiveness of presentation of the immune system for B-and T-cell epitopes, these two kinds of vaccine tested in CD1 mice by inducing the neutralizing antibodies and the IFN-γ level (Blanco et al., 2013). There are some novel vaccines such as a live vector vaccine that uses a chemically weakened virus to transport pieces of the virus to stimulate an immune response, an nucleic acid vaccine that are genetically DNA to produce an immunological response, and novel attenuated vaccine that are engineered to knock out some regions or oligonucleotides of virus by biotechnology but not continuously cultured in a non-native susceptible host just as traditional attenuated vaccines. (Zhang et al., 2011).

In this study, the artificially recombinant multi-epitope-vaccine, 5BT protein, which had been originally developed by Lee et al (Lee et al., 2017) was evaluated as FMD vaccine compared with commercially available inactivated FMD vaccine.

Table 1. The characters between inactivated vaccine, subunit vaccine and multi-epitope vaccine.

Type	Attenuated live & Inactivated vaccine	Subunit vaccine	Multi-epitope vaccine
Cost	High	Low	Low
Biosafety	Low	High	High
Strong antigenicity	Strong	Medium	Medium
Cross-reactivity	Yes	Case by	Yes

3.1 Inclusion bodies

Recombinant subunit vaccines, the essential epitopes of the antigen, are used to inoculate the host. Usually, the multiple-epitope proteins are expressed by the *Escherichia coli* (*E. coli*) system. What result we want to get is the soluble recombinant protein. However, for some gene sequences, the inclusion bodies are easy to form in *E. coli*. Inclusion bodies are dense aggregates of A misfolded polypeptide which have little biological activity. The formation of inclusion bodies is a dynamic equilibrium between the addition and removal of folded proteins (Middelberg, 2002), like **Figure 5**. To re-establish the lost biological activity, inclusion bodies should be refolded back to the bioactive native structure with isolation and purification and the ratio of refolding is more than 40% (Singh and Panda, 2005). But it needs much of the time and labors. It is best to achieve a high ratio of soluble protein to insoluble proteins to avoid refolding steps.

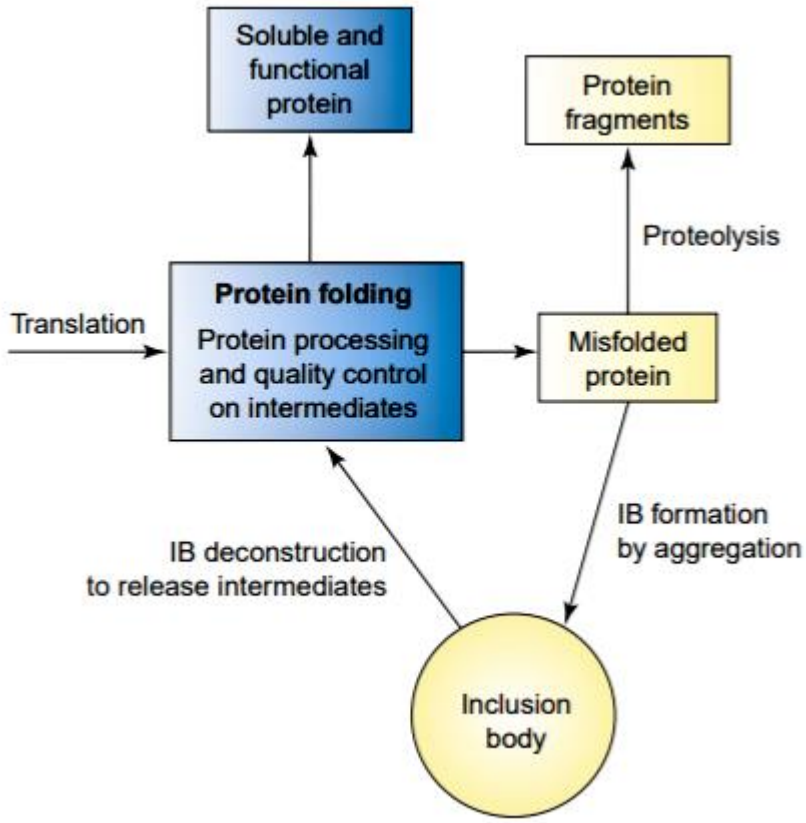


Figure 5. Inclusion body formation as a dynamic process involving the addition and removal of folded proteins.

This figure is cited from this article (Anton P.J. Middelberg 2002)

3.2 The environment of medium factors

If more bioactive soluble proteins were obtained in *E. coli*, it needs to balance these three processes: DNA transcription, protein translation and protein folding (**Figure 6**). Correct folding of many proteins requires the correct formation of disulfide bonds and glycosylation, which cannot be satisfied in the intracellular expression environment of *E. coli*. Therefore, a large number of recombinant proteins showed incorrect folding to form inclusion bodies. In conclusion, it is important to express genes in culture conditions. Usually, it is a good way to avoid the formation of inclusion bodies by controlling the velocity of protein synthesis.

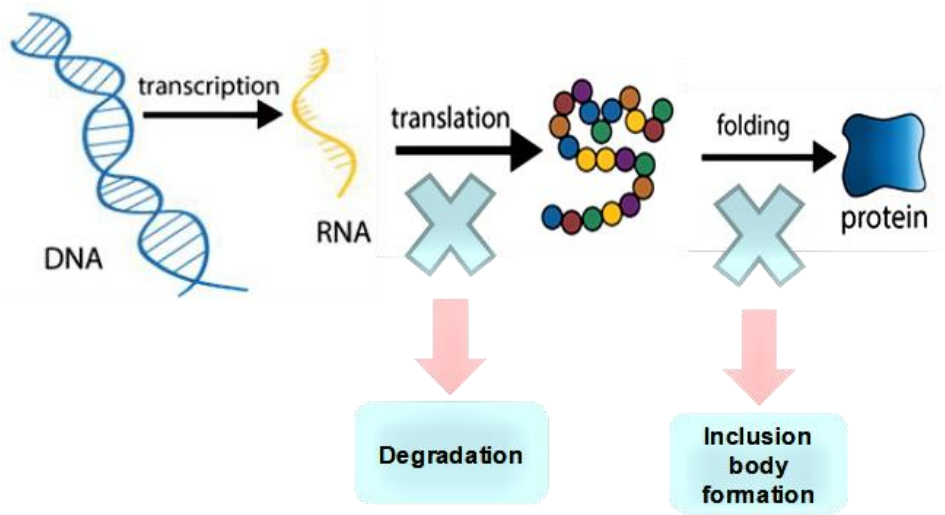


Figure 6. The process of protein synthesis

3.2.1 Induction time affects the expression of recombinant protein

Typical growth curve of *E. coli* in flask culture system has shown in **Figure 7**. There are three phases: lag phase, log phase, and stationary phase. The cell density increases slowly in lag phase and grow rapidly in the log phase in which the doubling time is approximately 20 minutes and the cells are healthiest and are actively producing proteins. The nutrients in the medium are depleted and metabolic products are accumulated. Usually, induction is performed early in the logarithmic phase of cell growth, but some research groups have also been reported that it can be induced at the end of the logarithmic phase or even stationary phase (Studier, 2005).

Therefore, the concentration of bacteria before induction is a very important factor for the expression of recombinant proteins. That needs to find the optimal inducing time.

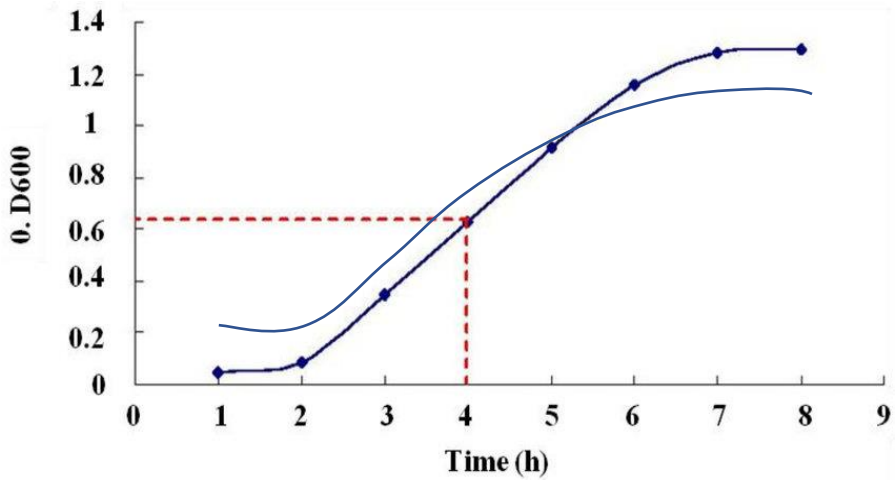


Figure 7. The growth curve of E. coli

3.2.2 Inducer concentration affects the expression of recombinant protein

Generally speaking, the frequently-used inducer included lactose and Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG used more as inducer to induce into protein. The inducer has a significant role for expression of recombinant proteins. Low inducer concentration may reduce the efficiency of induction and reduce the production of recombinant protein (**Figure 7**). Besides, inducer added in excess will not only increase the cost because inducer is expensive, but also bring about toxicity to inhibit the growth of bacteria, affecting the expression of recombinant proteins. Therefore, the concentration of inducer should be strictly controlled to the cell growth rate and cell concentration.

3.2.3 Incubation temperature after induction and harvest time affect the expression of recombinant protein

Two important factors that affect the soluble expression of proteins is incubation temperature after induction and harvest time after induction. The rate of protein synthesis can be reduced, and the formation of inclusion bodies can be reduced by reducing the incubation temperature after induction. A variety of difficult-to-express proteins have been successfully expressed by this method (Vasina and Baneyx, 1997). High temperature can promote the growth of bacteria, but it is easy to cause plasmid loss, which is not conducive to the expression of plasmids containing foreign genes. This effect is even more obvious during continuous culture. In general, the high temperature and temperature-dependent hydrophobic interactions are easily associated with

aggregate precipitation (Kiefhaber et al., 1991). In the fungus, there are some proteins suitable for slow, long-term induction, so it will inevitably have to choose under low temperature conditions (Schein and Noteborn, 1988).

4. Adjuvant system

4.1 Commercial adjuvants

The adjuvants play important roles in the immune response (**Table 2**) such as long-lasting adaptive immune response, high specific antibody production, and making vaccine more cost effective with fewer doses. To improve the immune effect, the adjuvants were studied. In currently licensed vaccine adjuvants, aluminum salt had a long history of being used as an adjuvant (Mbow et al., 2010). Oil in water emulsions include completely Freund's adjuvant (CFA), incompletely Freund's adjuvant (IFA), MF59, AS03, AS04 and Monophosphoryl Lipid A (MPLA).

Freund's adjuvant is a solution of antigen emulsified in mineral oil and used as an immunopotentiator. CFA is composed of inactive and dried mycobacteria whereas the IFA lacks the mycobacterial components. Also, CFA and IFA are common laboratory adjuvants. The squalene-based oil in water emulsion MF59 has been licensed in Europe for an adjuvanted flu vaccine. The immune response in elderly subjects was improved by the addition of MF59 to subunit influenza vaccines without causes clinically important changes in the safety profile of the influenza vaccine (Podda, 2001). MF59 elicits both cellular (Th1) and humoral (Th2) immune response (Calabro et al., 2013). A similar squalene-based oil in water emulsion called AS03 has been licensed in Europe. AS03 is an adjuvant system containing α- tocopherol and squalene in an oil in water emulsion. It achieved the highest antibody response and modulated the expression of some cytokines, including CCL2, CCL3, IL-6 and CSF3 (Morel

et al., 2011). MPLA is extracted from lipopolysaccharide (LPS) produced by the reluctant of a rough strain Salmonella Minnesota R595. Lipid A is a disaccharide with fatty acid side chains, which is the component responsible for the endotoxic activity of LPS (Martin et al., 2003). Removal of one phosphate group from Lipid A produces MPLA has reduced toxicity while retaining the ability to stimulate the immune system. It is reported that MPLA can act as carriers to induce a long-lasting IgG response against the antigen (Friede et al., 1993). Adjuvant system 04 (AS04) combines the TLR4 agonist MPL and aluminum salt. It is a new generation TLR-based adjuvant licensed for use in a human vaccine. When the human papillomavirus (HPV) vaccine Cervarix was used to elucidate the mechanism of action of AS04 in human cells and mice, AS04 induced local NF-kB activity and cytokine production. Aluminum salt with or inhibit MPL enhanced the vaccine response by rapidly triggering a local cytokine response leading to an optimal activation of APCs (Didierlaurent et al., 2009).

Table 2. The basic information of various adjuvants

Name	Major component	Company	Type
Alum	Aluminum salt	InvivoGen	Ab, Th2
MF59	Squalene oil-in-water	InvivoGen	Ab, Th1+Th2
MPLA	Squalene oil-in-water	InvivoGen	Ab, TLR4
CFA	Water-in-oil emulsion	Sigma-Aldrich	Ab, Th1
IFA	Water-in-oil emulsion	Sigma-Aldrich	Ab, Th2

4.2 Artificial synthetic adjuvants

CpG DNA is a novel adjuvant that promotes Th1 type immune responses which secret the cytokine IFN-γ, IFN- α and IL-12, strong CTL induction and opsonizing antibodies. In the immune system, the lymphocyte activation is caused by unmethylated CpG dinucleotides, at the expected frequency in bacterial DNA. B cell activation can be triggered by synthetic oligodeoxynucleotides (ODNs), containing an unmethylated CpG dinucleotide in a particular sequence context. CpG DNA activates all subsets of B cells and the mechanism is followed as **Figure 8** (Krieg et al., 1998). By Rose S. Chu was reported the effects of coadministered CpG ODN on the differentiation of Th responses to hen egg lysozyme (HEL). Only immunization with IFA-HEL plus CpG ODN induced a Th1-dominated cytokine pattern with high levels of IFN-γ secretion and decreased IL-5 production and induced IgG2a (a Th1-associated isotype), which was not induced IgG2a by IFA-HEL alone (Chu et al., 1997). However, there is also another report that CpG DNA gave mixed Th1/Th2 responses (IgG1 and IgG2a). Also, the Th1 response was predominant (Weeratna et al., 2000).

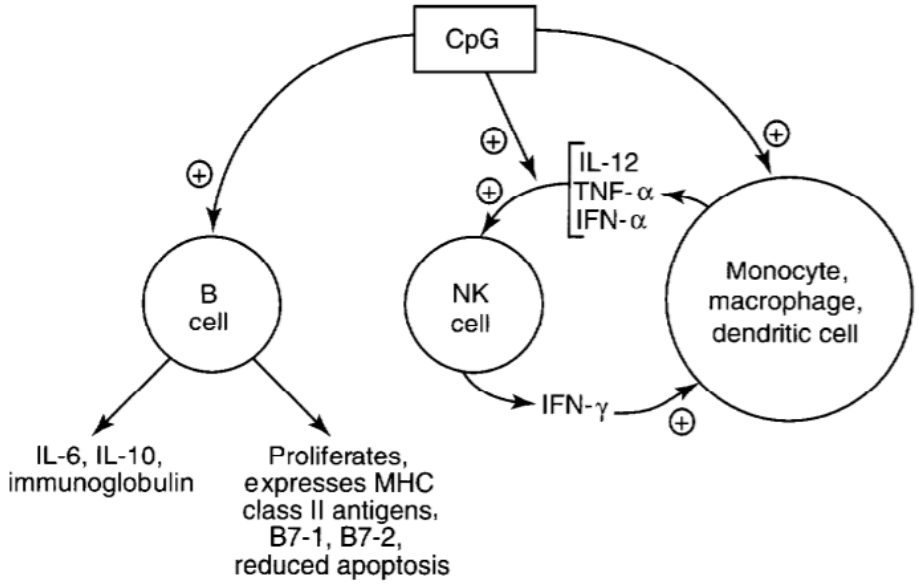


Figure 8. Overview of the adjuvant immune effects of CpG DNA.

The figure is cited from (Krieg et al., 1998)

Inulin was also reported as a novel TLR-4 agonist. Inulin is a polysaccharide consisting of a family of linear β-D-(2-1) polyfructofuranosyl α-D-glucose, in which an unbranched chain of up to 100 fructose moieties is linked to single terminal glucose. Inulin is the storage carbohydrate of *Compositae* and is obtained in high molecular weight from dahlia tubers. The form obtained by precipitation from water was referred to as alpha inulin, and the form obtained by precipitation from ethanol as beta inulin. Beta inulin is insoluble in water at 37°C but is soluble in concentrated solution at 70-80°C. Beta inulin is an effective vaccine adjuvant. It can boost both humoral and cell-mediated immunity, showing higher IgG, IgG2a, IgG2b and IgG3 responses than antigen alone. In a word, Inulin-based adjuvants are the potential to use in a wide variety of pathogen and cancer vaccines.

Chapter 1. Optimization for high yield of target protein, 5BT

1. Introduction

Nowadays, in order to solve the biosafety problems of inactivated FMD vaccine, recombinant subunit proteins produced in bacteria is a good alternative vaccine. FMD is still hard to control because of the high mutation rate with variety of subtypes in FMD virus. To overcome these weaknesses, a recombinant multi-epitope subunit vaccine against FMDV, designed as 5BT, has been newly designed in our research group. Previously.

5BT consists of five different sequences as B cell epitopes extracted from the GH loop in VP1 of various O type FMDV and another one sequence as T cell epitope derived from 3A in one of FMDV type O (**Figure 9**). 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 like **Figure 10**. Then, 5BT protein was expressed using *E. coli* system as a soluble form. The information of 5BT protein showed on the **Table 3**, (Lee et al., 2017).

In chapter 1, for effective control of foot-and-mouth disease (FMD), large yield of the 5BT protein, with high solubility, needs to be optimized. On the protein expression, to get a high amount of 5BT protein, protein production conditions need optimization, including A concentration of inducer (IPTG) and induction time point. After the 5BT protein expression, the protein includes two forms, soluble and insoluble form, the inclusion body. To get a high yield of

purified 5BT soluble protein, the condition of purification need optimization to remove other non-target proteins and get high purity 5BT target proteins.

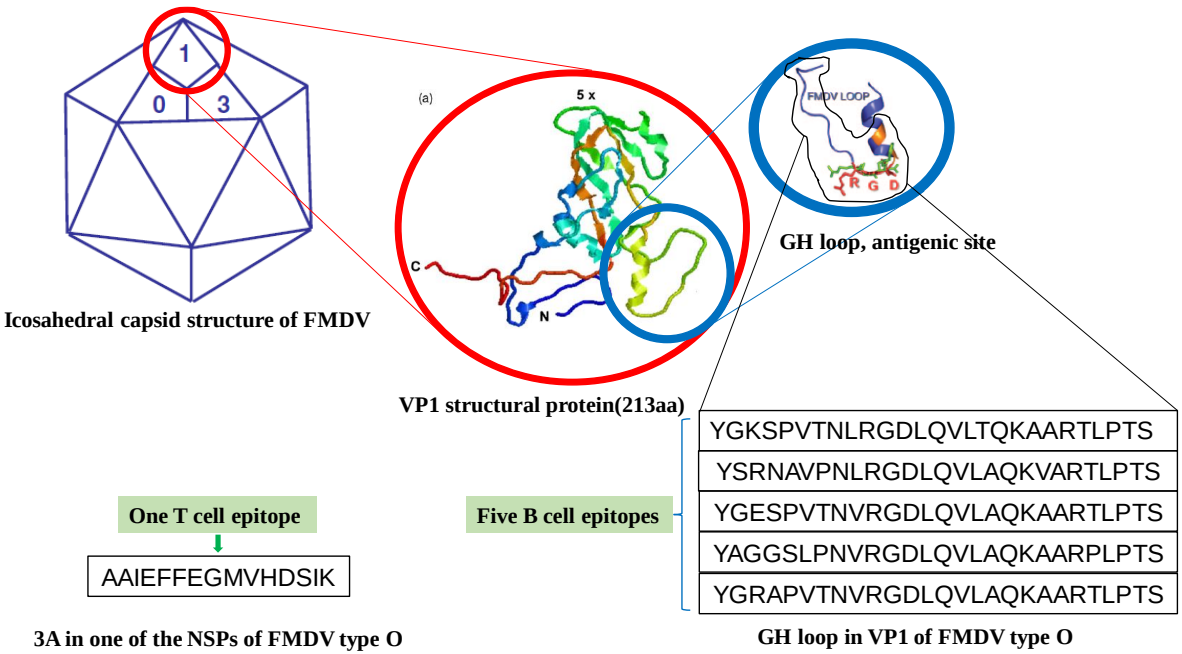


Figure 9. The three-dimensional structure of FMDV.

FMDV is an icosahedral capsid structure. VP0, VP1 and VP3 are exposed in the surface of the FMDV. The GH loop in VP1 has helix structure. containing RGD motif that binds to cell attachment proteins.

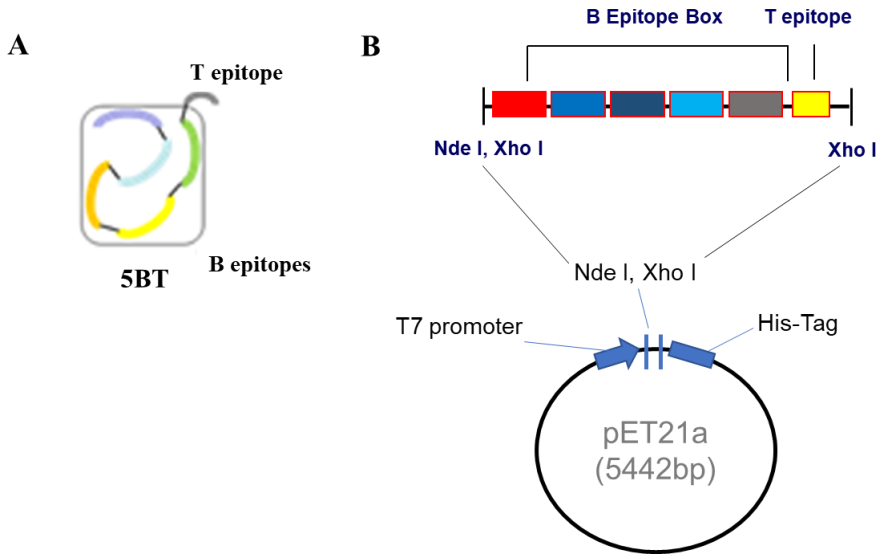


Figure 10. The schematic illustration of expression vector system for 5BT production.

(A) Graphical illustration of 5BT (five B cell epitopes and one T cell epitopes).

(B) Recombinant multi-epitope- protein, 5BT, expression vector system.

Table 3. Information of properties of 5BT protein

Name	Residues	MW	pI	Ext.co	Half-life	Instability index	Aliphatic index	Note
5BT	173	18.10	10.27	7450	>10h	36.97	81.21	previous

MW: Molecular weight; p I: isoelectric point; Ext. co: extinction coefficient; Half-life: 10 hours.

The table was selected from Lee, H-B, 2017.

2. Materials and Methods

2.1 Production of proteins

2.1.1 Expression of 5BT protein

A stock culture was prepared by inoculation with a single colony of transformed recombinant *E. coli* BL21a (DE3). And, 5ml of overnight culture was inoculated into 500ml of Luria-Bertani (LB) broth which contained 100ng/ml of ampicillin in 2L Fernbach flask. Cultures were grown in a shaking incubator at 37℃ and 180rpm. Their growths were monitored by spectrophotometer. When A_{600} of the culture reached 0.5, the target proteins expressions were induced by adding 0.5mM concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) and then culture 4 hours in shaking incubator at 37℃ and 180 rpm.

Cells were harvested by centrifugation at 6,500rpm for 15min at 4℃. The cell pellets were resuspended in 20ml of phosphate buffered saline (PBS). Lysates were centrifuged at 6,500rpm for 10min. Then, the pellets were collected and stored at -80℃ until the use them.

2.1.2 Characterization of recombinant target protein

2.1.2.1 Optimization of culture conditions to improve 5BT protein production

5ml of the transformed recombinant *E. coli* was cultured in 500ml LB broth with 100ng/ml ampicillin in 2L Fernbach flask under different conditions to cultivate the proteins. The conditions included the induction time point

(OD_{600} 0.3, 0.5, 0.8, 1.0) and concentration of IPTG (0.1, 0.2, 0.5, 0.8, 1.0mM). The plan showed on **Table 4**.

2.1.2.2 Analysis of protein expression and protein solubility

After protein production, the cell pellets were collected. Then, the cells pellets were resuspended in 30ml of binding buffer (500mM imidazole, 500mM NaCl, 20mM Tris-HCl, pH 7.9) and sonicated on ice (10min; pluse: 5s 5s; Ampl: 30%). After centrifugation, the supernatants were collected. The 20µl of cellular extracts (supernatants) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE consisted of 12% resolving gels and 5% stacking gels and samples adding with 5×sample binding buffer were heating for 5 min at 95℃. Then, the samples were run in a Mini-Protean electrophoresis system (BioRad, USA) (Socking at 80V for 30min, Resolving at 120V for 50min). After finished running, the gels were stained with Coomassie Brilliant Blue by 3 times of heating in a microwave oven for 1 min and cooled down in 3min. Then, the gels destained with 25% methanol and 7.5% acetic acid solution with shaking overnight. Bands were analyzed by Image J software (NIH) to compare target protein quantity.

Table 4. The expression of recombinant protein under different culture conditions

Culture condition	**Various**	**Temp.**	**Inducer**	**Conc. of inducer**	**Induction Point (OD_{600})**	**Medium**	**Harvest time**
	Plan	37℃	IPTG	**0.1/0.2/0.5/ 0.8/1.0**	**0.3/0.5/0.8/ 1.0** (LB)	LB	4 hours (fix)

2.2 Optimization of purification condition to improve the purity of 5BT protein

2.2.1 Ion exchange chromatography

After the protein expression, the cell pellets that stored at -80℃ were resuspended in 30 ml binding buffer, and then 1ml of resuspension took out for protein quantity test, and another was sonicated on ice (10min; pluse: 5s 5s; Ampl: 30%). Lysates were centrifuged at 17000rpm for 20min at 4℃. The supernatants, soluble fractions, were filtered with 0.45μm filter as samples for purification. The anion exchange (QFF exchanger) column or cation exchange (CM-FF exchanger) was packed, and then the column equilibrated with binding buffer. The samples were completely loaded into a column and then washing column with 5ml of 10% eluted buffer. The target protein was eluted with 10 ml of 10%-50% elution buffer. Then, the column was cleaned with 5ml of eluted buffers. The buffers followed on **Table 5**. Finally, the quantity of target proteins in each fraction was checked with SDS-PAGE.

2.2.2 Optimization of His-Tag affinity purification

After the protein expression, the cell pellets that stored at -80℃ were resuspended in 20ml of binding buffer, and then 1ml of resuspension took out for protein quantity test, and another was sonicated on ice (10min; pluse: 5s 5s; Ampl: 30%). Lysates were centrifuged at 17,000rpm for 20min at 4℃. The supernatants, soluble fractions, were filtered with 0.45μm filter as samples for purification. The Ni-nitrilotriacetic acid (NTA) was packed into a column, and then the column equilibrated with binding buffer. The samples were completely

loaded into a column and then washing column with washing buffers. The target protein was eluted with elution buffers. Then, the column was cleaned with a strip buffer. The resin was infiltrated into 20% ethanol. The eluted fractions were collected and stored at 4℃. The detail conditions for ion exchange chromatography and His-Tag affinity purification were described in **Table 6**, **Table 7**, respectively. Finally, the quantity of target proteins in each fraction was checked with SDS-PAGE.

2.2.3 Protein dialysis and freeze drying

After the purification, the eluted fractions were dialyzed using a membrane tube (molecular cut-off: 6-8 KDa, Spectrum, CA, USA) against the distilled water (DW). Stirer bar was put in the DW and stirred at 4℃. The DW was changed every 3 hours. And the last stage of dialysis has been performed overnight. After dialysis, the dialyzed proteins were centrifuged at 13,000rpm at 4℃ for 20min. Dialyzed samples were freeze-dried and then stored at -20 ℃

2.2.4 Quantification of recombinant target proteins

The concentrated proteins were quantified by BCA protein kit based on the concentration of the BSA as a standard (Pierce TM BCA Protein Assay Kit, Thermo Scientific TM). The analysis procedure was followed by manufacturer's instruction.

Table 5. Buffers compositions for ion exchange chromatography

Ion exchange	Resin	Binding	Elution buffer
Anion exchange (QFF charge)	1	10mM Tris pH= 8.0	10mM Tris, 1M NaCl pH= 8.0
Cation exchange (CM-FF) charge)	1	10mM Tesh buffer. pH= 8.0	10mM Tes^{h} buffer, 1M NaCl pH= 8.0

Table 6. Conditions of Ni-NTA affinity chromatography

Buffer	Imidazole (mM)	Tris-base (mM)	NaCl (M)	$NiSO_4$ (mM)	pH
Charging buffer	-	-	-	10	7.9
Binding buffer	5	20	0.5	-	7.9
Elution buffer	500	10	-	-	7.9
Strip buffer	200	40	1	-	7.9

Table 7. Washing protocol with different conditions on Ni-NTA affinity chromatography

Trials	Washing protocol					
	Binding	Elution	Contained(mM)	pH	Resin(ml)	Wash(ml)
1	97%	3%	20	7.9	6	60
2	95%	5%	30	7.9	6	60
3	97%	3%	20	7.9	6	36
	95%	5%	30			30
	87%	13%	70			18
4	97%	3%	20	7.9	4	20
	95%	5%	30			20
	91%	9%	50			20
	87%	13%	70			20
5	97%	3%	20	7.9	4	32
	95%	5%	30			20
	91%	9%	50			16

3. Results and discussion

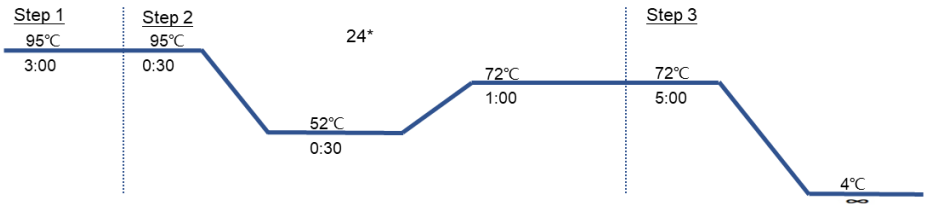
3.1 Confirmation of the target gene and protein expression

In order to identify whether stock culture has a 5BT gene or not, the transformant colonies harbouring 5BT expression vector system were randomly chosen and the 5BT genes were amplified through PCR with a specific primer set, then amplified genes were analyzed by agarose gel electrophoresis. technology with primer agarose gel electrophoresis (**Figure 11**). The generic protocol for protein production was followed. Briefly, *E. coli* cells were cultivated until they reached the set induction OD_{600} and add a set concentration of IPTG to induce protein expression. After incubation at 37℃ for 4 hours, cell pellets were collected by centrifugation. Then, PBS was added to suspend the cell pellets. The supernatants as soluble protein would be expressed on SDS-PAGE (**Figure 12** A) and analyzed the expressed bands with Image J software (**Figure 12** B). They showed that different amount of proteins was expressed in different induction point OD_{600} and concentration of IPTG. It means that the optimization of culture condition was significantly important for protein production.

(A)

Target	Primer name	Primer sequence	Tm	Length	GC%
5BT	T7-F	5'-DNA- TAATACGACTCACTATAGGGGA-3'	53.7	22	41
	T7-R	5'-DNA- GCTAGTTATTGCTCAGCGG-3'	55.3	19	53

(B)



(C)

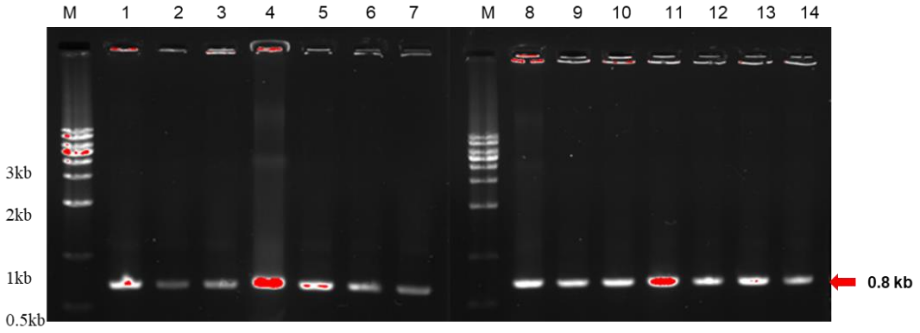


Figure 11. Identification of 5BT gene in stock culture

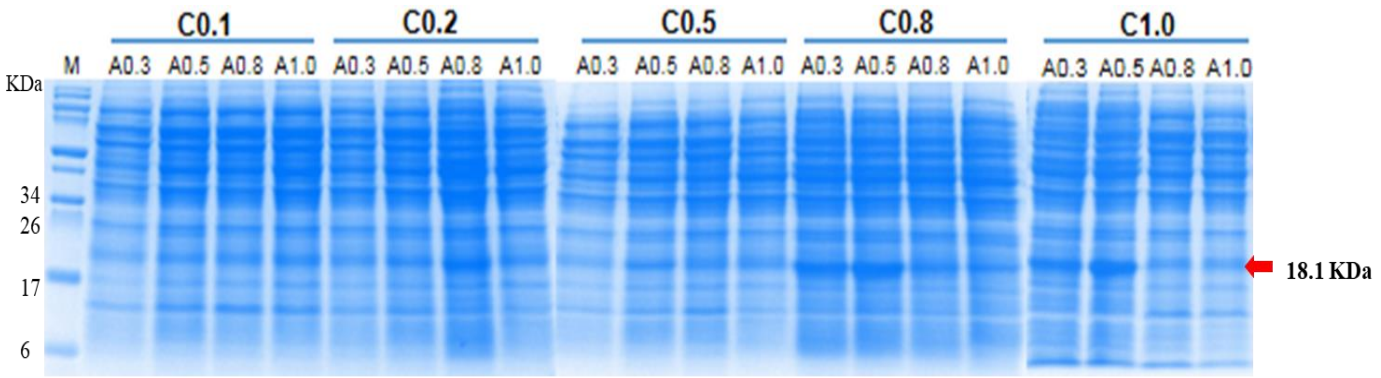
(A) Information of 5BT gene primers. (B) The conditions of PCR reaction.

(C) Amplified target 5BT genes analyzed by agarose gel. kb: kilo base pairs;

M: molecular marker; The numbers mean colonies; eg: 1: 1st colony, 2: 2nd colony,

3: 3th colony…Size of target gene (799bp).

(A)



C: Concentration of IPTG; A: Absorbance at wave length 600 nm; M: Marker;

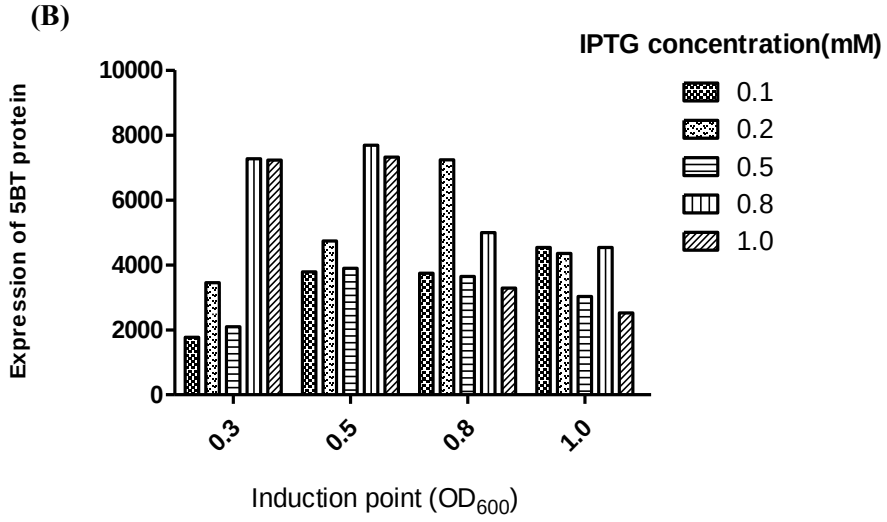


Figure 12. Expression profile of 5BT protein in *E. coli* with various induction time point and IPTG concentration

（A）SDS-PAGE; (B) Qualification analysis by Image J software.

3.2 Optimization of the 5BT protein expression condition

3.2.1 The solubility of recombinant protein

During the over expression of the recombinant protein in *E. coli*, insoluble form of protein in fraction, the inclusion body, could be formed. The inclusion body is considered as nonfunctional protein, thus optimal induction time point and concentration of the inducer (IPTG) was investigated to avoid the inclusion body formation and analyzed by SDS-PAGE (**Figure 13**) and analyzed by Image J software (**Figure 14**). The large amounts of soluble proteins were induced when 1.0mM of IPTG was added during the mid-log phase of growth rather than the lag phase. The **Figure 14** B showed that the protein was induced at OD_{600} 0.5 by adding 1.0mM IPTG, the protein was achieved to 98% solubility. When protein induced during lag phage, cells density increased slowly. As shown in **Figure 14** A, when the induction point OD_{600} was 0.3 and the concentration of IPTG was 0.1mM, the solubility of 5BT protein was only 57%. The differences in solubility represented as the percentage of soluble protein to the total amount of the target protein expressed. The solubility of 5BT protein with various induction time point and IPTG concentration was shown in **Table 8**.

Generally, it has been known that the stationary phase is not a good time point for induction, in which the nutrients in the medium were depleted, and this may prevent their growth. However, it is interesting in my experiment that when induction point OD_{600} 1.0 was 1.0 like **Figure 14** D, the solubility of 5BT

was not so low and the fluctuation of solubility was not obvious. I think maybe one of the reasons is that the OD_{600} is not a stationary phase for growth of *E. coli*.

3.2.2 Quantification of recombinant proteins

The amount of proteins was quantified by comparison with the standard curve using Bovine Serum Albumin (BSA) according to the protocol of BCA kit (Pierce ^{TM} BCA Protein Assay Kit, Thermo Fisher Scientific). **Figure 15** A showed the total 5BT protein in both soluble from and inclusion bodies. The result showed the concentration of IPTG 1.0mM and induction point OD_{600} 0.5 could produce high amount of target proteins. In **Figure 15** B, the amount of soluble form of proteins was decreased than total target proteins in every condition tested duo to subtracting the insoluble fractions from the total target proteins. But the result showed the consistency with the result shown in **Figure 15** A.

As a conclusion of chapter 1, 1.0mM IPTG treatment at OD_{600} 0.5 would be the recommended induction condition for the 5BT protein production in *E. coli.*

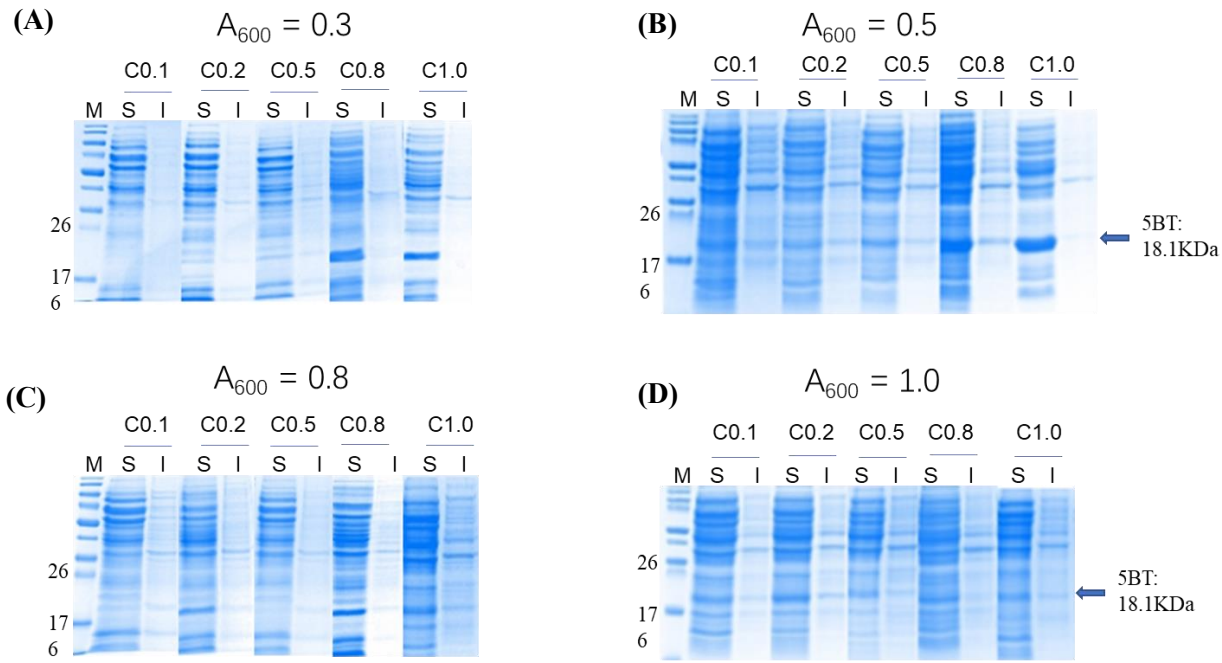


Figure 13. Determination of optimized expression conditions of 5BT with various induction time point and IPTG concentration

The different concentration of IPTG under the (A) induction time point OD_{600} 0.3, (B) induction time point 0.5, (C) induction time point 0.8, and (D) induction time point 1.0. C: concentration of IPTG; A_{600}: Absorbance at wave length 600 nm. M: marker; S: soluble form of protein; I: inclusion bodies. Molecular weight of 5BT: 18.1KDa. C: Concentration of IPTG; A_{600}: Absorbance at wave length 600 nm.

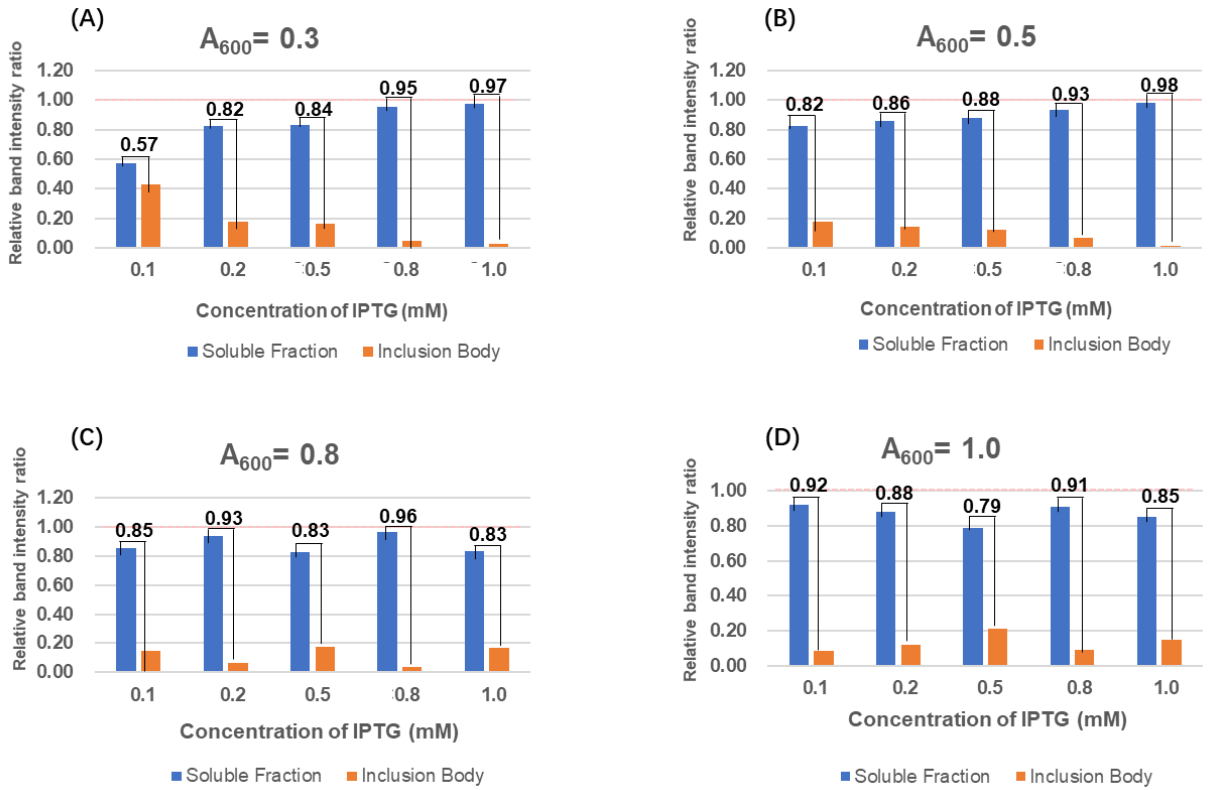


Figure 14. Solubility analysis of 5BT by densitometer from (A) set point density at A_{600}=0.3, (B) set point density at A_{600}=0.5, (C) set point density at A_{600}=0.8 and (D) set point density at A_{600}=1.0 with different concentration of IPTG, respectively. The data that come from band lane of SDS-PAGE gel was analyzed by Image J software. A_{600}: Absorbance at wave length 600 nm.

Table 8. The solubility of 5BT at different induction time point and concentration of IPTG

5BT protein	IPTG concentration	Solubility (%)
A_{600}=0.3	0.1	57
	0.2	82
	0.5	84
	0.8	95
	1.0	97
A_{600}=0.5	0.1	82
	0.2	86
	0.5	88
	0.8	93
	1.0	98
A_{600}=0.8	0.1	85
	0.2	93
	0.5	83
	0.8	96
	1.0	83
A_{600}=1.0	0.1	92
	0.2	88
	0.5	79
	0.8	91
	1.0	85

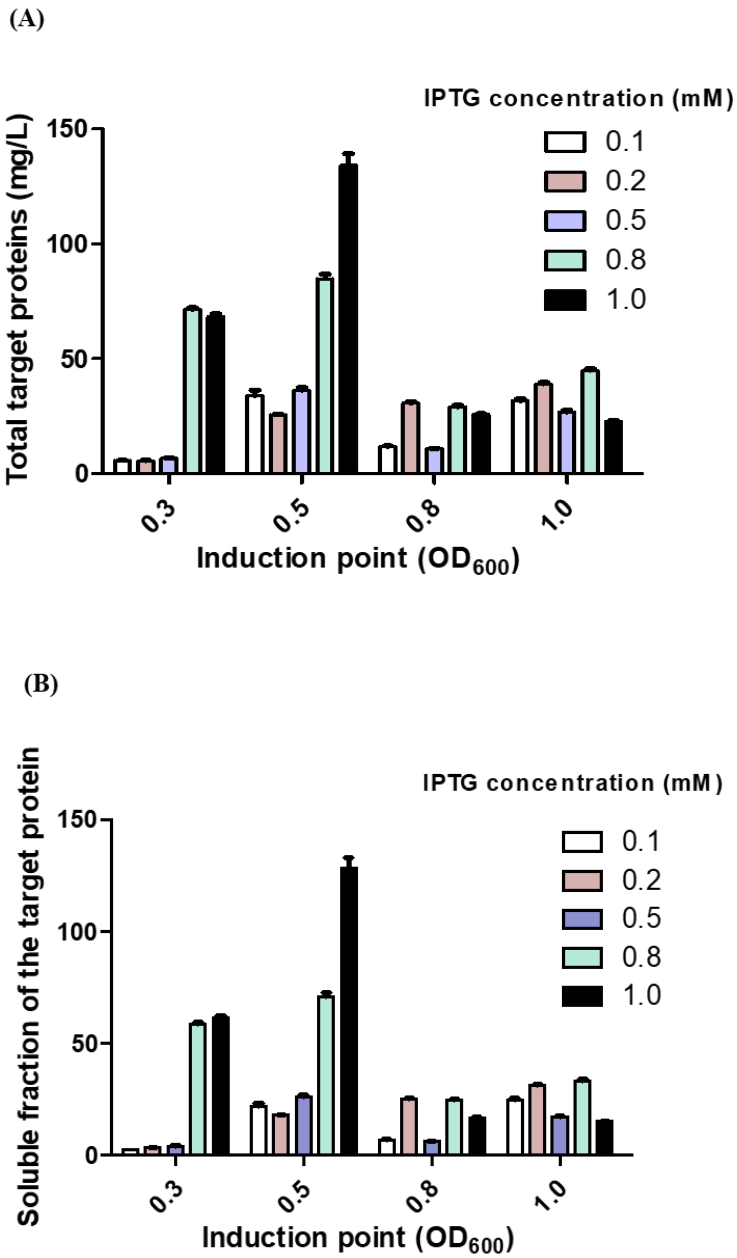


Figure 15. The expression of total protein and soluble form of protein.

The concentrations of 5BT target protein were analyzed by BCA assay. The 5BT target proteins were contained in soluble form and inclusion form. And the ratio of soluble and insoluble was detected by Image J software.

3.3 Optimization of purification condition

3.3.1 Ion exchange purification

Optimal purification condition is very important to get a high yield of target protein for industrialization. Another, our 5BT protein as a vaccine needed to be more purity for immunization. Because the pI of the 5BT target protein is 10.27, the ion exchange purification was tried. However, shown in **Figure 16**, both anion and cation exchange chromatography didn't show any good performance. Therefore, it needed to find other purification methods to get pure 5BT target proteins.

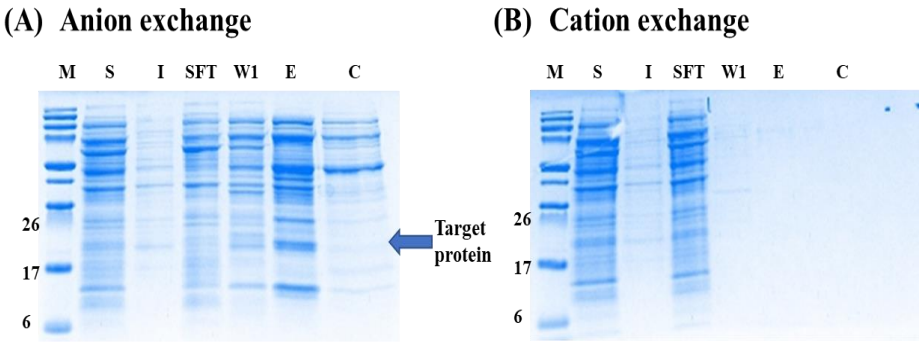


Figure 16. SDS-PAGE of each fraction was purified by ion exchange chromatography.

M: Marker; S: Sample; I: Inclusion bodies; SFT: Sample Flow Through;

W1: Washing fraction; E: Elution fraction; C: Cleaning fraction.

3.2.2 Ni-NTA His-Tag affinity chromatography

5BT protein could be expressed in pET21a vector as a fusion protein with His-Tag. His-Tag is a polyhistidine tag, an amino acid motif in protein that consists of at least six histidine residues. The His-Tag proteins can be purified by using Ni-NTA resin that contains nickel ion. And nickel can bind to His-Tag of the recombinant 5BT protein. The protocol and purification processes are described in detail in the materials and method section. Various purification conditions were tried during the optimization process. As seen in Figure 17**,** (A) and (B), the non-target proteins didn't be washed out. So, the concentration of imidazole in the washing condition should be increased gradually. When the concentration of imidazole was increased to 70mM (C), many target proteins were washed out. For the setting of the resin volume, compared with (C) and (D), 4ml resin was enough to do purification for 500ml culture. In (E), under the three steps of washing, many non-target proteins were washed out and the target proteins were eluted with 500mM imidazole. Therefore, it would be better to wash 3 times with 20, 30, 50mM imidazole in the washing condition. For the determination of the better elution condition, the effect of gradient elution from 0 to 500mM imidazole (D) and just a single elution with 500mM (E) imidazole were compared. However, both elution conditions showed the similar efficiency and purity for the target protein purification. There was a summary selection of purification conditions in washing step in Figure 18. Many target proteins were washed out with 70mM imidazole.

Finally, as a conclusion, in chapter 1, for protein purification optimization, it is better to wash 3 times with 20, 30, 50mM imidazole in washing step elution by 500mM

imidazole, and 4ml of the resin volume treatment 500ml of *E. coli* culture was determined for purification.

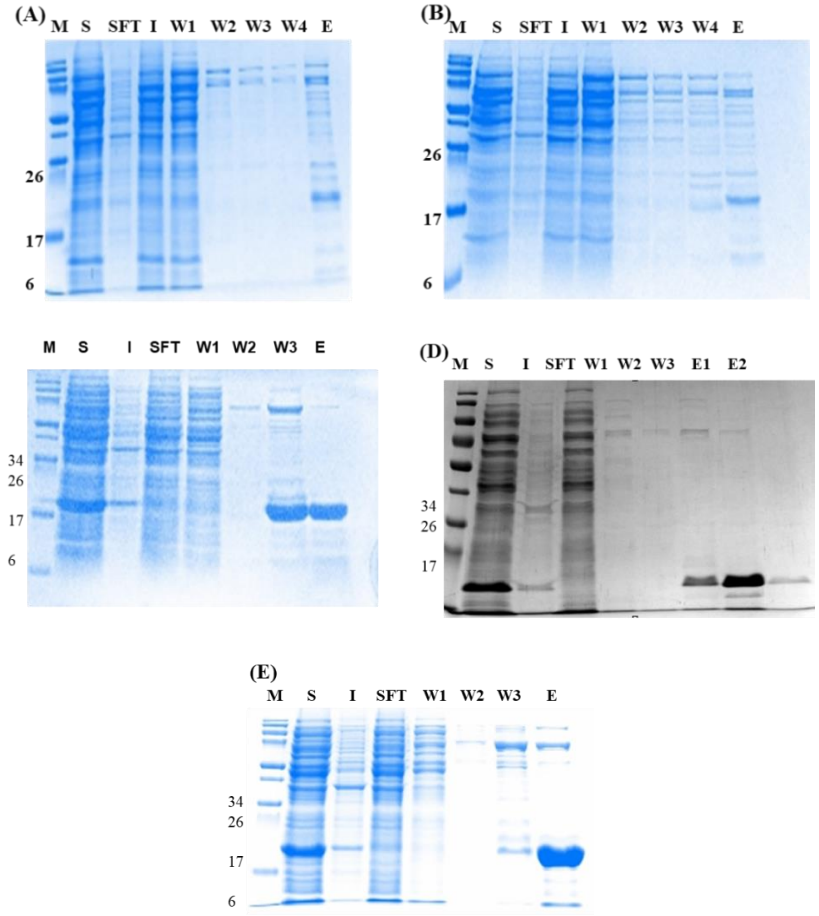


Figure 17. SDS-PAGE of different purification conditions.

M: Marker; S: Sample; I: Inclusion bodies; SFT: Sample Flow Through; W: Washed solution; E: Eluted solution. Molecular weight of 5BT protein, 18.1KDa. (A) Resin volume: 6ml; Washing condition: W1, W2, W3, W4 all are 20mM imidazole; Washing volume: 60ml; Elution condition: 500mM imidazole. (B) Resin volume: 6ml; Washing condition: W1, W2, W3, W4 all are 30mM imidazole; Washing volume: 60ml; Elution condition: 500mM imidazole. (C) Resin volume: 6ml; Washing step condition: W1, W2, W3: 20, 30, 70mM imidazole; Washing volume: W1, W2, W3: 36, 30, 18ml; Elution condition: 500mM imidazole. (D) Resin volume: 4ml; Washing condition: W1, W2, W3: 20, 30, 50, 0mM imidazole; Washing volume: W1, W2, W3: 32, 20, 12, 5ml; Elution condition: 0-500mM imidazole gradient concentration of (E) Resin volume: 4ml; Washing condition: W1, W2, W3: 20, 30, 50mM imidazole; Washing volume: W1, W2, W3: 32, 20, 16ml; Elution condition: 500mM imidazole.

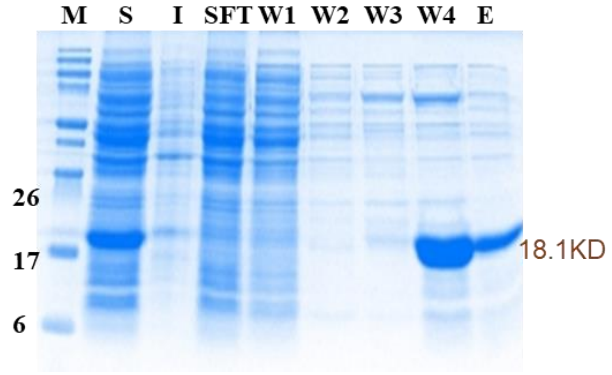


Figure 18. Selection for optimal washing condition during purification process.

M: Marker; S: Sample; I: Inclusion bodies; SFT: Sample Flow Through; W: Washed solution; E: Eluted solution. Molecular weight of 5BT protein: 18.1KDa. W1: Washing solution with 20mM imidazole; W2: Washing solution with 30mM imidazole; W3: Washing solution with 50mM imidazole; W4: Washing solution with 70mM imidazole; E: Elution solution with 500mM imidazole.

The optimal purification condition showed in **Figure 19** (A). The purity of 5BT, 23%, before purification was improved to 95% after purification by analyzing using image J software. And the total yield of the 5BT protein was achieved to 52.89mg in 1L culture volume. Compared to commercial protein Bovine Serum Albumin (BSA, Sigma Life Science), the purity of 5BT protein was 95% which is higher than 79% of BSA when analyzed by Image J software in **Figure 19** (B). So, it was considered that the purity of 5BT would be achieved as a level of commercialized protein by the purification procedure optimized in this study.

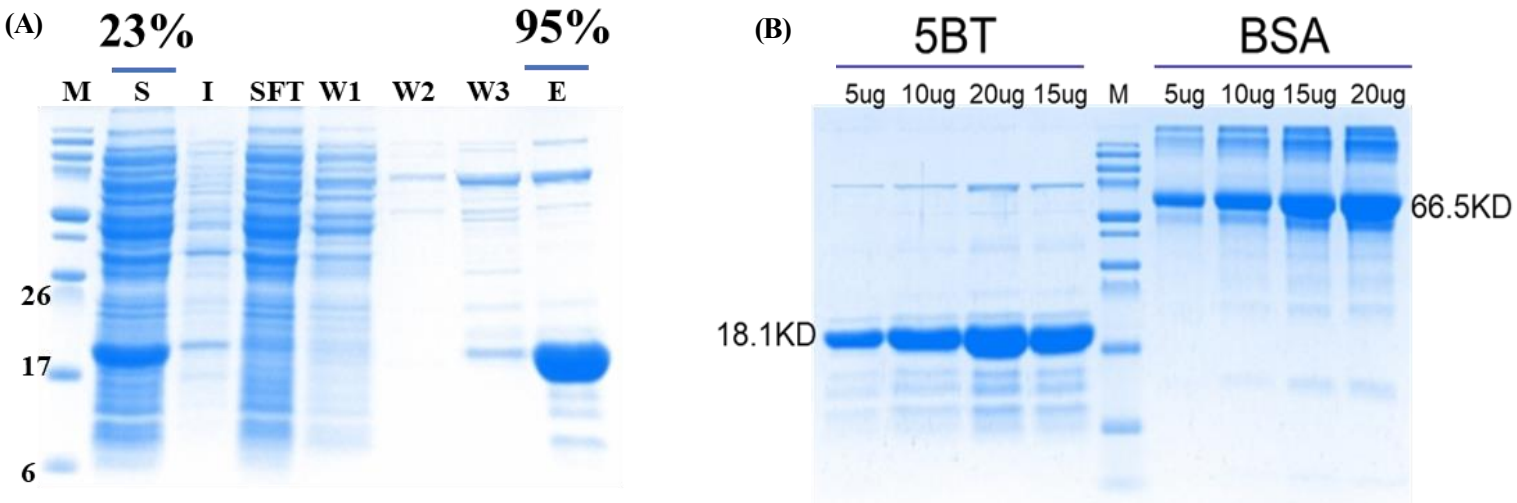


Figure 19. Evaluation of the optimized condition for 5BT purification (A) and purified 5BT protein (B) by SDS-PAGE

M: Molecular marker; S: Sample; I: Inclusion bodies; SFT: Sample Flow Through; W: Washed solution; E: Eluted solution. Molecular weight of 5BT protein: 18.1KDa. W1: Washing solution with 20mM imidazole; W2: Washing solution with 30mM imidazole; W3: Washing solution with 50mM imidazole; E: Elution solution with 500mM imidazole. Molecular weight of 5BT protein: 18.1KDa.

4. Conclusion

As is well-known, FMDV has a high mutation rate, subunit vaccine is one of the best choices to prevent FMD, unlikely with the inactivated vaccine with many problems such as high cost in production, safety consideration and so on. A new recombinant protein, 5BT, that consists of five B cell epitopes derived from the GH loop in VP1 of various type O mutant FMDV and one T cell epitope in one of NSPs of FMDV was developed (Lee et al., 2017).

Then the recombinant proteins expressed in *E. coli* production system efficiently. Usually, during the recombinant protein production, it is limited by the formation of inclusion bodies. To overcome the problem of inclusion body formation, the culture condition for producing protein should be controlled strictly (Schein, 1989).

In this chapter 1, to get more soluble recombinant protein, culture condition of recombinant *E. coli* was optimized during the protein production including induction time and concentration of inducer, IPTG, at the temperature of 37°C. To improve the yield of pure recombinant protein, proper purification conditions should be set to remove unrelated protein contaminants from the target protein. And their properties were analyzed via SDS-PAGE by expression pattern and BCA assay to test the yield of recombinant protein.

As a conclusion, the optimal conditions for IPTG induction and His-Tag affinity purification were achieved with 1.0mM IPTG treated with OD_{600} 0.5 for 4 hours at 37°C and subsequent 3 time-washing by 20, 30, 50mM imidazole after binding the samples to the resin, respectively. Moreover, 5BT protein has shown 98% in solubility and 95%

in purity by the application the optimized production and purification methods. Finally, the yield of recombinant 5BT protein was achieved to 52.89 mg in 1L of *E. coli* culture volume. Our recombinant protein 5BT has the potential to be developed in a commercial scale production in the future.

Chapter 2. *In vivo* evaluation of 5BT protein for the FMD subunit vaccine

1. Introduction

Already known, the FMD is difficult to control due to the high mutation rate. In chapter 1, the multi-epitope recombinant protein, 5BT, five B cell epitopes from the GH loop of VP1 on each strains of serotype O FMDV and one T cell epitope originated from the FMDV NSPs, was produced with *E. coli* system. The high yield of pure 5BT protein was prepared under the optimal induction condition and optimal purification condition.

In chapter 2, the efficiency of multi-epitope recombinant 5BT protein as an FMDV vaccine was evaluated. Compared with previous research (Lee et al., 2017), the various doses of 5BT with combination of commercial adjuvants (alum, MF59, MPLA and AS04) were administered intramuscularly into SD-rats. In order to validate the effect of the recombinant 5BT protein as an FMDV vaccine, it was compared with commercial FMDV vaccine for its immune response in this study.

There are strong correlations between protective efficacy of the vaccine and the administered dose of antigen. Low dosage of vaccine will decrease the effect, however, higher dosage of vaccine showed not always better effect (Aagaard et al., 2009). Hyun Ju In reported that 10μg dose of EV71 vaccine showed the most effective for induction of humoral and cellular immune responses against EV71 compared with dose group of PBS as control, 5μg, 10μg and 20μg EV71 vaccine (In et al., 2017).

Besides, in order to induce antigenicity, the adjuvant is necessary to be used together with recombinant antigen as vaccine. In general, adjuvants can make antigen more potent with less dose and improve the immune response for long-lasting protection. Until now, several different classes of adjuvants have been developed. CFA or IFA is commonly used in the laboratory. Alum is the most widely used adjuvant and is found in numerous vaccines today, including HAV, HBV, HPV, and Diptheria and Tetanus (Mbow et al., 2010). Moreover, alum enhances the Th1 type immunity (Marrack et al., 2009). MF59, oil in water emulsion, induces both Th1 and Th2 type responses (Calabro et al., 2013). Monophosphoryl lipid A from *S. Minnesota* R595 (MPLA), TL4- based adjuvant, induced Th1 response (Rhee et al., 2010). AS04 is a novel adjuvant and a combination adjuvant composed of MPLA absorbed to alum. It elicited local NF-kB activity and cytokine production (Didierlaurent et al., 2009).

In chapter 2, efficiency of the 5BT as a subunit vaccine against FMD has been evaluated *in vivo* using SD-rat model. Dose-dependent protective serum IgG induction of 5BT has been monitored by comparison with commercial vaccine. Effective adjuvants which could maximized the vaccine efficacy of 5BT also been explored with various commercially available adjuvants. Finally, we have instigated the subtypes of IgG and cytokines from the serum of each immunized group to analyze which type of immune response, Th1 or Th2, would be induced by 5BT immunization.

2. Materials and methods

2.1 SD-rat immunization and blood sampling

Sprague-Dawley (SD) rats were used for the immunization following the policy and regulations for the care and use of laboratory animal (Laboratory Anima Center, Seoul National University, Korea) under the approval of the animal ethics committee at Seoul National University (SNU-181011-2). After one week for adaption, the SD-rats were immunized with vaccine via the intramuscular route for 6 weeks. The immunization schedule consisted of a primary injection and two booster injections at two-week-intervals on day 0, 14, 28. Also, the blood samples were collected at before priming (day 0), 2, 4, 6 weeks after immunization (**Figure 20**). Each blood sample was collected by syringe (Korea vaccine, KOREA) from the tail vein before immunization and delivered into serum separating tube (BD, USA), immediately. Serum was isolated from blood via centrifuge at 3,000rpm for 15 minutes, and then it was divided into several equal parts and was stored at -80°C until used later. After blood sampling finished, the rats were sacrificed at days 42 by the method of euthanasia with CO_2.

2.2 The plans of immunization in SD-rat

To evaluate the efficiency of 5BT as FMD vaccine with various aspects, three individual *in vivo* immunizations with SD-rats were performed with same immunization and sampling schedule which is described in **Figure 20.**

2.2.1 1st animal experiment: Investigation of dose-dependent immune response of 5BT

For vaccines, the dose of antigen affects the efficiency of vaccine protection. 30 female SD-rats (8-week-old) were immunized with 5BT proteins with 4.5μg/μl concentration emulsified in Complete Freund's Adjuvant (CFA, priming) or Incomplete Freund's Adjuvant (IFA, boosting) as antigen. And the rats were randomly divided into one control and five test groups (5 rats per group): control (PBS), 50μg, 100μg, 150μg, 200μg and 250μg of 5BT proteins. And each test group was injected the designated amount of 5BT proteins into the tail vein with same volume (0.2ml) (**Table 9**).

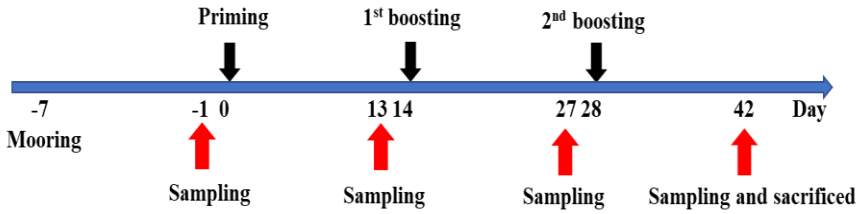


Figure 20. The immunization schedule of SD-rat *in vivo*

Before starting the animal experiment, the rats should adapt the environment for one week. The SD-rats were immunized intramuscularly at days 0, 14 and 28. Blood samples were collected at before priming and 2, 4, 6 weeks after vaccination from vein of tail.

Table 9. SD–rat *in vivo* immunization with different doses of 5BT protein as antigen

Group	Con of 5BT(μg)	Animals	Adjuvants	Vaccination route
Control	**0**	5	CFA/IFA	IM
T1	**50**	5	CFA/IFA	IM
T2	**100**	5	CFA/IFA	IM
T3	**150**	5	CFA/IFA	IM
T4	**200**	5	CFA/IFA	IM
T5	**250**	5	CFA/IFA	IM

CFA: Complete Freund's Adjuvant; IFA: Incomplete Freund's Adjuvant; IM: Intramuscular; each group 5 rats. Each rat was injected commercial vaccine 200μl.

2.2.2 2nd animal experiment: Investigation of dose-dependent immune response of the commercial FMD vaccine

To evaluate the efficacy of 5BT as an FMD vaccine, dose-dependent immune response of a commercial FMD vaccine currently available in Korea (Green Cross Veterinary Products Co., Ltd, Republic of Korea, O Manisa + O 3039 + A 22 Iraq) was investigated and the results were compared with 1st animal experiment to find out the dose level of 5BT including equivalent immune response with commercial vaccine. 25 female SD-rats (8-week-old) were immunized with commercial vaccines. And the rats were randomly divided into five test groups (5 rats per group): 20, 40, 60, 80 and 100μl of commercial vaccines. Here, the 2nd animal experiment was used commonly control group with 1st animal experiment. Each test group was injected the designated amount of commercial vaccine into the tail vein with same volume (0.2ml) (**Table 10**).

Table 10. SD–rat *in vivo* immunization with different doses of commercial vaccine

Group	Con of Ag(μl)	Animals	Vaccination route
T1'	**20**	5	IM
T2'	**40**	5	IM
T3'	**60**	5	IM
T4'	**80**	5	IM
T5'	**100**	5	IM

Note: Each rat was injected commercial vaccine 200μl.

2.2.3 3rd animal experiment: Exploration of effective adjuvants for 5BT

Generally, it is well known that the immunogenicity of a commercial antigen as vaccine could show different efficacy by the combination of different adjuvants. Thus, various commercially available vaccine adjuvants were investigated to maximize the immunogenicity of 5BT. The injection dose of the 5BT protein antigen depends on the result of the optimal dose in 1st animal experiment. The different commercial adjuvants, which including CFA/IFA, Alum, MF59, MPLA and Alum + MPLA, were selected for the study. 40 rats were divided into 8 groups (5 rats per group), and each group was injected the designated vaccine with different adjuvants into the tail vein with same volume (0.2ml) (**Table 11**).

Table 11. SD–rat *in vivo* vaccine immunization with different adjuvants

Group	Adjuvants	5BT dose	Animals	Vaccination route
Control	**PBS**	200μg(100μl)	5	IM
T1	**IFMDV**	0	5	IM
T2	**CFA/IFA**	200μg(100μl)	5	IM
T3	**Alum**	200μg(100μl)	5	IM
T4	**MF59**	200μg(100μl)	5	IM
T5	**MPLA**	200μg(100μl)	5	IM
T6	**Alum + MPLA**	200μg(100μl)	5	IM
T7	**Alum (other dose)**	50μg(50μl)	5	IM

The rats were injected 5BT protein emulsified with different adjuvants; 1:1 means optimal dose of antigen: adjuvant; 1:3 means 5BT protein: alum. Each rat was injected 200μl of various of vaccine.

2.3 Serological analysis

2.3.1 Determination of 5BT-specific serum IgG by indirect ELISA

The induction of 5BT-specific serum IgG level was measured from each immunized rat blood sample by indirect ELISA. 96 well immune-plates (SPL life science, Korea) was coated with purified 5BT (0.1μg/well) in the 0.05M carbonate-bicarbonate buffer (CBB) at 37°C for 1 hour. After washed by 200μl of PBS with 3 times, the wells were blocked by 200μl of blocking buffer that consists of PBS and 0.5% skim milk at room temperature for 1hour and washed 3 times by PBS. Then, a series of five-fold dilution of each serum was prepared, starting at 1/50 and volume adjusted to 100μl with PBST (0.5% Tween 20 in PBS) at room temperature for 2 hours. After washed 3 times with 200μl of PBST, HRP conjugated rabbit anti-rat IgG, IgG subtypes (IgG1, IgG2a) diluted 1: 5000 in PBST containing 0.5% skim milk was used as a secondary antibody (100μl/well) at room temperature for 1 hour. After washing with PBST, 100μl of TMB substrate solution (Sigma, USA) was added to incubate at room temperature for 30 minutes without light interference. Then, 100μl of 0.5M H_2SO_4 was added to stop the reaction and the solution color turns from blue to yellow. The plate was read at absorbance of 450nm in a spectrophotometer. ELISA results were expressed as the endpoint titer. The titer of specific antibody was calculated by Softmax Pro 5.4.1 (Molecular Devices).

2.3.2 Determination of type O FMDV-specific serum IgG by FMDV ELISA kit

Efficacy of the 5BT as an FMD vaccine was validated by measuring type O FMDV-specific serum IgG using commercial FMDV ELISA kit (PrioCHECK FMDV type O ELISA kit, TheroFisher Scientific). The protocol was followed by the manufacturer's instruction. In brief, each plate was coated with non-infectious FMDV type O antigen in the kit. 10μl of test serum samples, blank, negative control and a positive control were diluted in 90μl of ELISA buffer. After incubating at room temperature for 1 hour, the plates were washed 6 times by 300μl of washing solution. Then, 100μl of the diluted conjugates were added at room temperature for 1 hour and the plates were washed 6 times by 300μl of washing solution. The color was developed with 100μl of TMB substrate and incubate at room temperature for 15 minutes. Finally, 100μl of stop solution was added for stopping the reaction. All reagents were provided in the kit. And the plates were measured in a spectrophotometer at the absorbance of 450nm. The percentage inhibition (PI) was calculated followed by this formula:

$$PI\ (\%) = 100 - (\text{corrected } OD_{450} \text{ test sample} / \text{corrected } OD_{450} \text{ max}) \times 100$$

If PI is less than 50%, it was considered as negative, meaning FMDV type O antibodies were not induced sufficiently in the test serum. If PI showed more than 50%, it was considered as positive, meaning FMDV type O antibodies were induced enough to neutralize the FMDV in the test serum.

2.3.3 Cytokine ELISA

Cytokine levels in serum collected from immunized rats, associated with development of acquired immune responses, were investigated using ELISA kits (Rat IL-4 ELISA kit, Catalog No. K0332133; Rat IFN-gamma ELISA kit, Catalog No. K0331209). Briefly, 200μl of washing solution was added into pre-coated 96 well ELISA microplate and plates were washed 3 times by 300μl of washing solution. 100μl of the standard, blank and samples were added and incubated at room temperature for 2 hours. After the plates were washed, 100μl of the diluted detection antibody was added and washed again. The color developed by 100μl of TMB substrate and incubate at room temperature for a proper color. Then, 100μl of H_2SO_4 was added for stopping reaction. The plates were read in a spectrophotometer at the absorbance of 450 nm. ELISA results were expressed as the endpoint titer.

2.4 Statistical analysis

All of the results were expressed as mean ± standard deviation (SD). The data were analyzed by GraphPad PRISM software 5.0 (GraphPad) with ANOVA method. Antibody titers were reported as log 10 of the reciprocal of the highest dilution. And the levels of cytokine were reported as log 2. Difference between experimental measures were considered to be statistically significant with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control groups.

3. Results and Discussion

3.1 Immunogenicity evaluation of 5BT protein as an FMD vaccine

3.1.1 Dose-dependent immunogenicity of 5BT in rat model

The efficacy of 5BT protein as an antigen was determined by different doses of antigen via intramuscular injection to SD-rat. PBS was used as a negative control in this experiment. Our 5BT protein as antigen could induce humoral immune response to produce 5BT-specific serum IgG by reaction to T-helper cells to activate B cells that were differentiated to induce plasma cells. Different doses of 5BT protein antigens were injected with one time priming with CFA as adjuvant and two times boosting with IFA as an adjuvant into rats. The antibodies would be detected by indirect ELISA in the serum collected at 0, 2, 4 and 6 weeks after vaccination with 5BT protein antigen. As seen in Figure 21, after priming vaccination, anti-5BT IgG levels of all treatment groups increased as the immunization time went and maintained a constantly increasing trend until the end of the experiment. Also, all treatment groups of IgG titers were higher than the control group. In Figure 21 (B), interestingly, at 2^{nd} week, IgG level of some groups were increased quickly, such as 100μg, 150μg, 200μg, and 250μg dose groups. Especially, at the 4^{th} week, compared to the control group, the group of 200μg dose had a significant difference ($P < 0.01$) in anti-5BT IgG levels. In Figure 21 (D), at the 6^{th} week, the IgG titers to 5BT protein antigen were significantly higher in all groups of SD-rats than in control group. The 250μg dose of IgG level was lower than the 200μg dose group, it means that a higher dose of antigen could inhibit the production of IgG. This tendency was already reported from other research group in a similar dose-

dependency analysis of an antigen with this study (In et al., 2017). Even though both the group of 150μg ($P < 0.01$) and 200μg dose ($P < 0.001$) were significant difference in IgG levels compared with control group treated with PBS, the 200μg dose of group showed highest serum IgG levels than other groups, and on 2nd week, the IgG levels were varied with five rats in 150μg dose group because one of the rats of IgG level was most low than other four rats. Therefore, for 5BT protein antigen dose dependence, the 200μg group was the optimal dose for SD-rat to induce the highest IgG titers in the humoral immune response.

It is known that the Fc portion of IgG2a has high affinity with Fc receptor, which can activate Fc receptor mediated effector function and stimulate the antibody-dependent cell-mediated cytotoxicity. IgG2a is produced during the Th1 type immune response or cellular immune response. Nevertheless, the Fc portion of IgG1, which is produced by Th2 immune response, has no ability to stimulate the Fc receptor-mediated immune response. It is still very important for the humoral immunity to defense foreign antigens (Huber et al., 2006). We investigated the ability of 5BT peptides induce helper T cell type 1 (Th1) and type 2 (Th2) responses, anti-5BT IgG2a and IgG1 were analyzed (Blanco et al., 2013; Lee et al., 2017). In Figure 22, IgG1 or IgG2a level of all treatment groups was higher than control-treated only with adjuvant CFA/IFA. Also, our 5BT protein antigen would induce both Th1 and Th2 immune responses. In Figure 22 (A), at 6-week, anti-5BT IgG2a titers were higher in both 150μg dose and 200μg dose groups than other dose groups. And there was no difference between 150μg and 200μg antigen dose groups. For the IgG1 level, like Figure 22 (B), there was a difference ($P < 0.05$) between in 150μg dose of the antigen group and control group.

However, 200μg dose of antigen group was a significant difference ($P < 0.01$) compared to the control group. Consequently, 5BT protein antigen emulsified into CFA/IFA adjuvant has shown significant induction of both Th1 and Th2 immune responses as shown in **Figure 22** (A) and (B), respectively. However, the ratio of IgG2a/IgG1 after immunization with 5BT showed more dominant induction in Th1 immune response as shown in **Figure 22** (C).

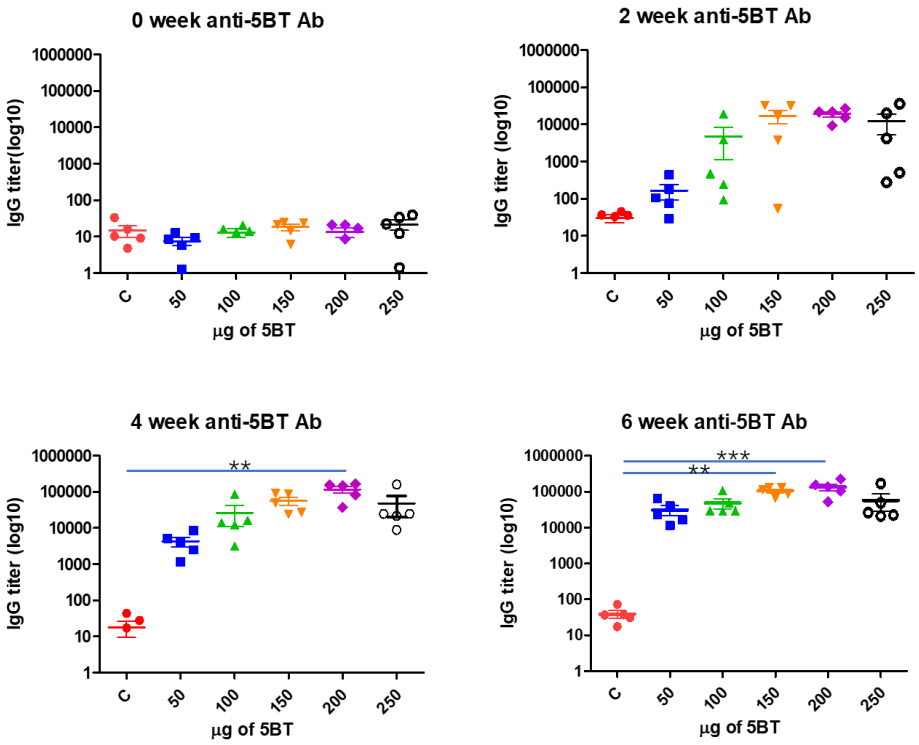


Figure 21. Detection of 5BT specific IgG antibodies from SD–rats immunized with different doses of antigens.

IgG titers expression of all groups with 0, 2, 4 and 6 weeks. Specific antibody titers against 5BT was measured by ELISA in serum samples collected at 0, 2, 4 and 6 weeks after vaccination. Each point means the value of individual rat. All values represent the means ±SD (n=5). Horizontal lines indicate the mean of each group of animals.

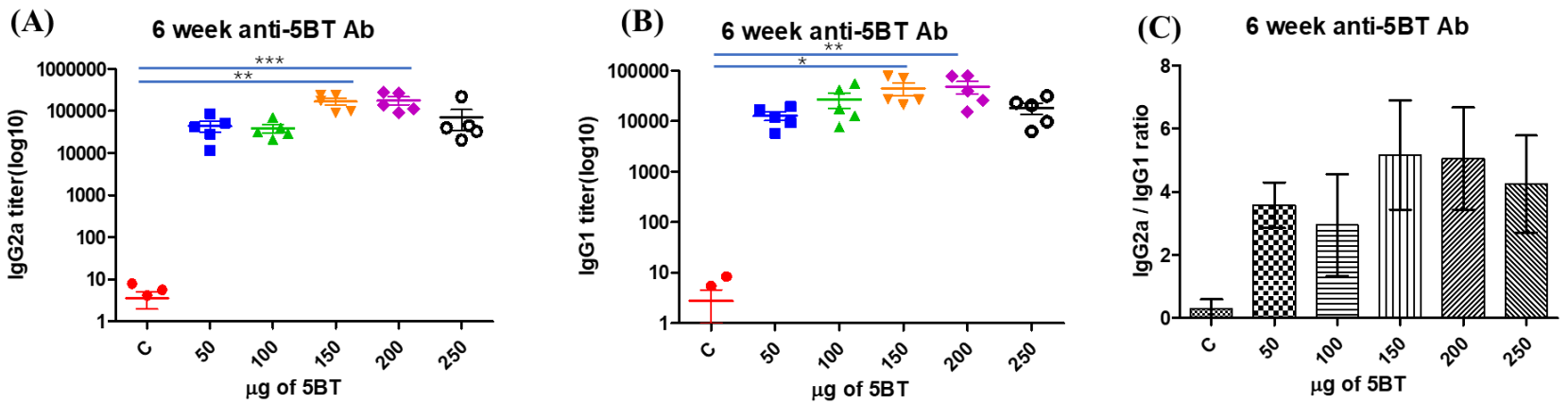


Figure 22. Detection of anti- 5BT IgG1 and IgG2a from SD–rats immunized with different doses of antigens at 4 weeks post immunization.

Anti- 5BT serum IgG subtype titers were measured using ELISA assay. (A) IgG2a titers, (B) IgG2a titers and (C) the ratio of IgG2a and IgG1. Error bars represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA.

3.1.2 Dose-dependent immunogenicity of commercial FMD vaccine in rat model

To compare with the efficiency of the 5BT protein antigen, the commercial vaccine (type O + type A) was immunogenicity into the SD-rats with different doses to get high production of antibodies. The method was followed as the part of materials and methods. The Figure 23 showed a significant difference ($P < 0.001$) between 100µl group and the control group. Besides, on 6th week (4 weeks after vaccination), 80µl and 100µl groups had little differences ($P < 0.05$) with control group. However, there was a significant difference ($P < 0.001$) between 20µl and control groups. The small dose like 20µl of dose could be enough to induce a high level of antigen-specific IgG titers and high humoral immune response. Thus, the dose of 20µl commercial vaccine was selected as an optimal group. Moreover, to be surprised, our 200µg of 5BT protein antigen and 20µl of commercial vaccine were an equivalent level of serum IgG titers even at six-week.

The induction levels of IgG isotypes, IgG1 and IgG2a, after immunization of commercial FMD vaccine have shown in **Figure 24**. Both IgG2a and IgG1 have induced significantly compared with control group even in the lowest dose of test group. Also, the tendency of IgG2a and IgG1 induction was lower in the 5BT protein antigen than the commercial vaccine. Here it also made sure that 20µl dose of commercial vaccine was optimal choice for immunizing SD-rats. For the commercial vaccine, the IgG1 levels of all doses of groups were slightly higher than IgG2a titers. That means that commercial vaccine preferred to induce Th2 immune response. However, the ratio of IgG2a and IgG1 was almost near one, therefore, it would be considered as a balanced

induction between Th2 type humoral immune response and Th1 type cellular immune response.

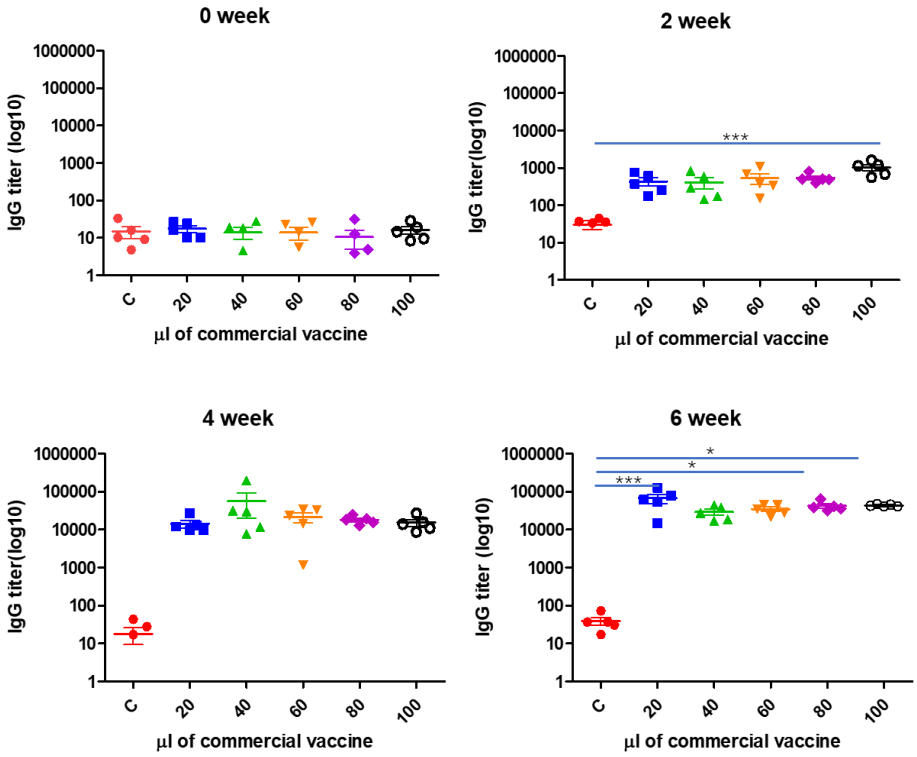


Figure 23. The IgG induction by different dosages of commercial FMD vaccine in SD-rats.

IgG titers expression on 0, 2, 4 and 6 weeks. Specific antibody titers against 5BT was measured by ELISA in serum samples collected at 0, 2, 4 and 6 weeks after vaccination. The two animal experiments of 5BT protein antigen dose dependence and commercial vaccine dose dependence were commonly used same control group. The antibody titers were expressed as the log10 of the last dilution. Each point means the value of individual rat. All values represent the means ± SD (n=5).

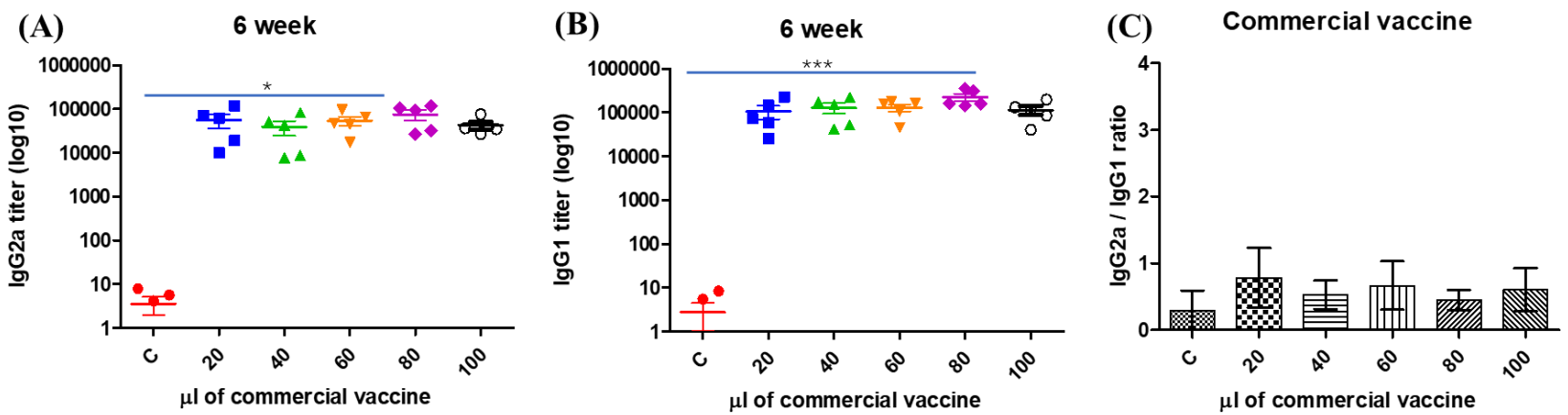


Figure 24. The IgG1 and IgG2a productions by different dosages of commercial vaccine in SD-rats.

(A) IgG2a titers, (B) IgG2a titers, and (C) the ratio of IgG2a and IgG1.

3.1.3 Selection of effective adjuvant for 5BT

According to the above results that 200μg of 5BT protein antigen and 20μl of commercial vaccine were equivalent doses to maximize induction of antigen-specific serum IgG level. Adjuvants can help antigen to improve the immunogenicity. So, various commercial adjuvants have been investigated by combinatory immunization with 5BT in SD-rat via intramuscular injection. The method was followed in **Table 12**. And the antibodies were detected by ELISA in the serum.

From Figure 25, in the control group treated with only the 200μg of 5BT protein antigen without any adjuvant, the total IgG titers were increased slightly on 0, 2, 4 and 6 week of serum. It revealed antibodies produced with 5BT protein antigen alone. On 2^{nd} week and 4^{th} week of serum, IgG level of CFA/IFA group was increased quickly and had significant difference ($P < 0.001$) compared with rest groups (Figure 25). Interestingly, on the 2^{nd} week and 4^{th} week, compared to the commercial vaccine, our 5BT protein antigen induced an early antibodies production. It could be a beneficial characteristic for a vaccine to control the disease at early period. However, on the 2^{nd} week, except for CFA/IFA group, other adjuvants groups showed no early induction. Usually, the CFA/IFA can be only used in the laboratory and has some side effects at the application site to animals (Liebermann et al., 1994). On the 6^{th} week (after 4 weeks of immunization), CFA/IFA group had a significant difference ($P < 0.001$) with control, alum, MPLA and MPLA + Alum groups and had little difference ($P < 0.05$) with the commercial vaccine group and MF59 group. And there was no difference between commercial vaccine and MF59 groups. Thus, it appeared that MF59 would be the best adjuvant for 5BT antigen. In **Figure 26,** in 200μg of 5BT protein antigen and 50μg of

5BT protein antigen emulsified with alum groups, there were no difference, and the anti-specific IgG titer levels were similar after 4 weeks of immunization. Therefore, if alum as adjuvant emulsified to 5BT protein antigen, the small amount (50μg) of 5BT protein antigen was enough for production of total IgG titers.

To investigate the induction of Th1 and Th2 type immune response, the IgG2a and IgG1 were analyzed by ELISA assay. On the production of IgG2a (**Figure 27** A), after 4 weeks immunization, both CFA/IFA with 5BT protein group ($P < 0.001$) and MF59 group ($P < 0.01$) had significant difference compared to control group treated with only 200μg 5BTprotein. Every group had a significant difference ($P < 0.001$) compared to the CFA/IFA group. However, there were litter difference ($P < 0.05$) between CFA/IFA and MF59 groups. And there was no difference between the commercial vaccine and MF59 groups. For IgG1 production (**Figure 27** B), commercial vaccine group and alum- adjuvanted with 5BT protein groups produced a higher amount of IgG1 than the control group. However, all of the vaccines induced highly both IgG1 and IgG2a. It elicited that they have a balanced induction ability to the Th1 type and Th2 type immune response. Specifically, MF59–adjuvanted vaccine induced higher immune responses in both IgG1 and IgG2a levels than other test groups. In Figure 27 C**,** for the commercial vaccine, the production of IgG1 was higher than IgG2a. Also, it means the commercial vaccine was dominantly preferred to induce Th2 type response. For the control group, the Th1 response was induced higher than Th2 response in the 5BT protein antigen alone. For alum-adjuvant, as known as Th2 type inducer (Coffman et al., 2010), was emulsified with 5BT protein as a vaccine that was dominant Th2 type immune response. Interestingly, MF59 adjuvant group showed a decreased tendency in IgG2a/IgG1 ratio

like commercial vaccine group compared with CFA/IFA group. It showed that selection of adjuvant could be change the immune response characteristics of the antigen.

Therefore, the optimal adjuvant that emulsified with 5BT protein antigen was CFA/IFA. But, CFA/IFA was just used in the laboratory, because of safety issues. For commercial adjuvants, MF59 was selected as an optimal adjuvant for immunizing SD-rat for the efficient induction of the antigen-specific serum IgG.

Table 12. The method of immunization in SD-rat with different adjuvants.

Group	Adjuvants	Adjuvants volume (μl)	5BT dose volume (μl)	Vaccination route
Control	**PBS**	0	200	IM
T1	**IFMDV (**20 ul)	0	0	IM
T2	**CFA/IFA**	100	100	IM
T3	**Alum**	100	100	IM
T4	**MF59**	100	100	IM
T5	**MPLA**	100	100	IM
T6	**Alum + MPLA**	(50+50)	100	IM
T7	**Alum**	50	150	IM

Note: T3 and T7 are same adjuvant (Alum) and different 5BT protein antigen dose. It can compare the different efficiency of vaccine.

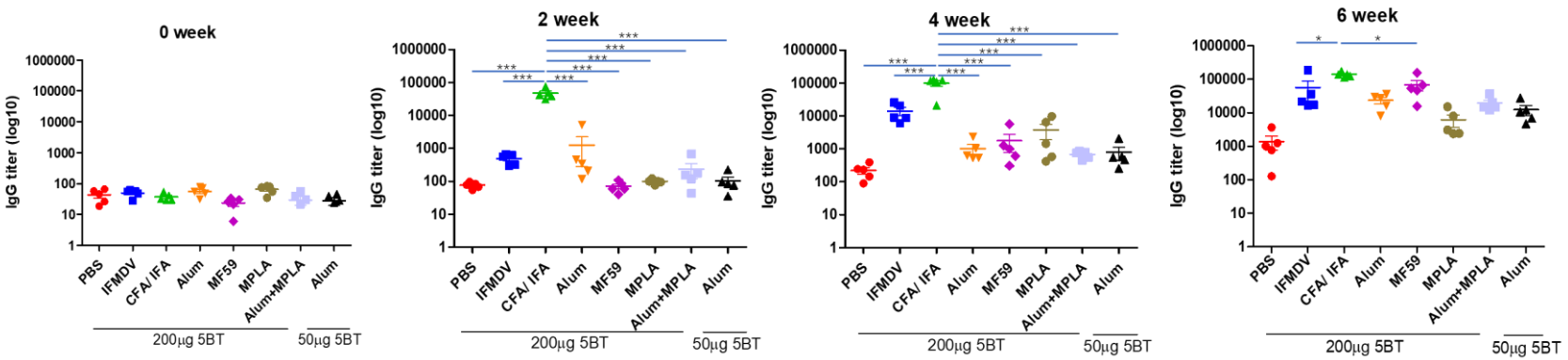


Figure 25. The total IgG titers with different adjuvants emulsified by 5BT protein antigen.

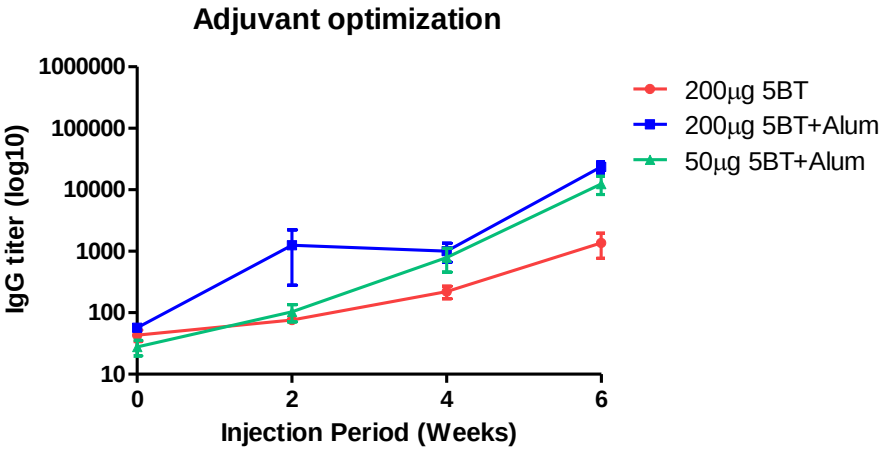


Figure 26. The comparation of vaccine efficiency with same adjuvant and different amount of 5BT protein antigen.

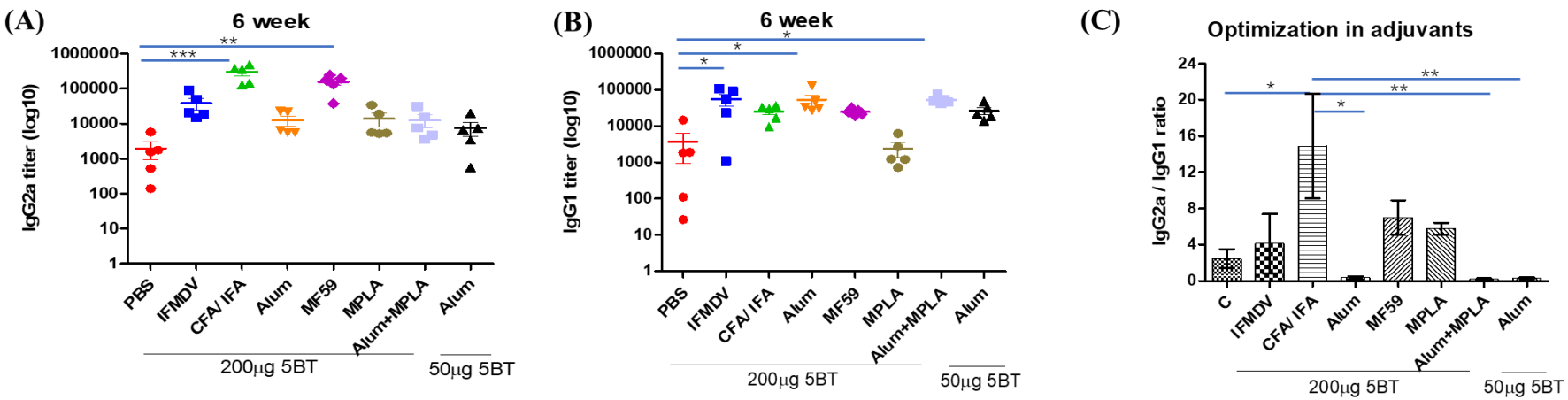


Figure 27. The IgG1 and IgG2a induction by different adjuvants emulsified with 5BT protein as vaccine in SD-rats.

(A) IgG2a titers, (B) IgG2a titers and (C) the ratio of IgG2a and IgG1.

3.2 Efficacy validation of 5BT as an FMD vaccine

To validate the efficacy of 5BT as an FMD vaccine, the rat serum samples immunized by 5BT with various adjuvants were examined by commercial FMDV ELISA kit which included non-infectious FMD type O antigen-coated plates. According to the manufacturer's instruction, if PI showed more than 50%, it could be considered as positive that FMDV type O-specific IgG were induced enough to neutralize the FMDV in the test serum.

In **Figure 28** (A), immunization of 200μg of 5BT alone without any adjuvant showed low PI under 50%. However, same amount of 5BT with CFA/IFA or MF59 adjuvant showed PI over 50%, meaning that 5BT would have a potential to be an FMD vaccine which could induce neutralizing IgG against FMDV sufficiently. Compared to commercial vaccine (PI = 52%), the groups of CFA/IFA (PI = 72%) and MF59 (PI = 66%) as adjuvants emulsified with 200μg 5BT protein antigen showed higher vaccination efficacy. From **Figure 28** (B) and (C), it is identified that the optimal dose was so important (less or more dose of vaccine will affect the vaccination efficacy). Except for the control group (animals injected with CFA/IFA plus PBS), the PI values were more than 50% in the showed doses of 5BTprotein antigen and commercial vaccine. Moreover, it was no efficacy in control group treated with CFA/IFA alone. In **Figure 28** (D), among the commercial adjuvants tested in this study, only MF59 group showed over 50% of PI (66%) when emulsified with 200μg of 5BT. Although CFA/IFA group showed higher PI value than MF59 group. MF59 could be selected for the best adjuvant for the 5BT protein antigen because CFA/IFA is just available for laboratory use only. When SD-rats injected with the same alum and the different doses of 5BT

protein antigen, a high amount (200μg) of 5BT protein produced a high immune response than 50μg of 5BT protein antigen.

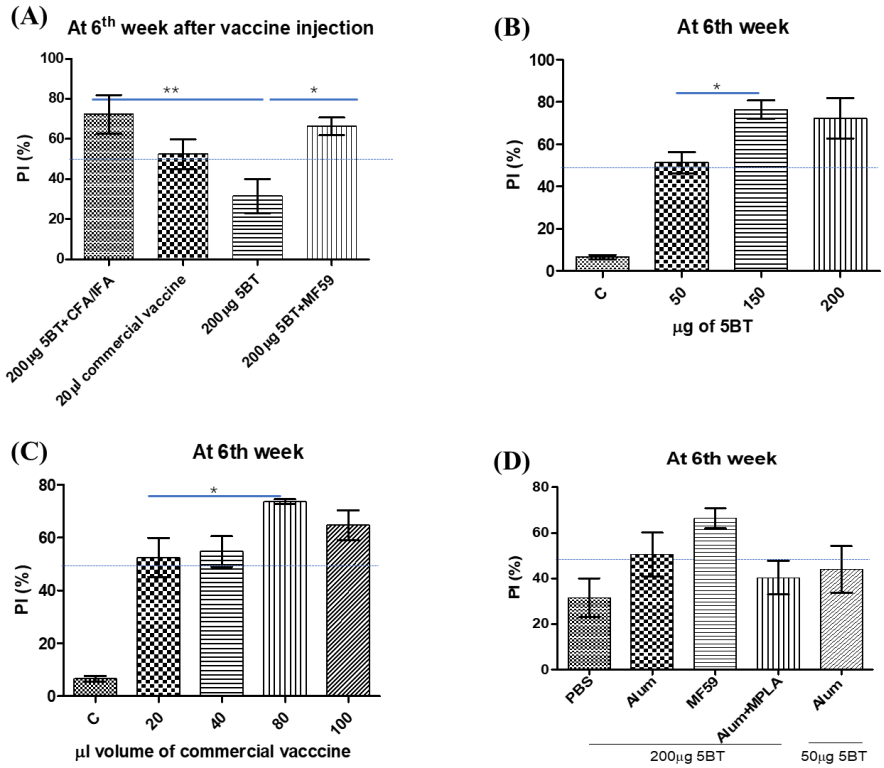


Figure 28. FMDV serotype O specific antibody using ELISA assay.

(A) The groups vaccinated optimal condition as vaccine; (B) The groups vaccinated with different dosage of 5BT protein antigen; (C) The groups vaccinated with different dosage of commercial vaccine; (D) The groups vaccinated with different adjuvants emulsified with 5BT protein antigen. The test serum 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 PI 50%. If PI is more than 50%, it is positive, and it means FMDV type O antibodies were present in the test serum. Otherwise, FMDV type O antibodies were absent in the test serum.

3.3 Investigation of cytokine profile associated with Th1 and Th2 immune response

Cytokines are key protein molecules which regulate the immune responses in various ways including vaccine-mediated immunity. When a vaccine is administered through the intramuscular route, the antigen is transported from the muscle tissue to the regional lymph nodes, where immune responses occur. All effective vaccines activate innate immunity with inflammatory responses. Also, they could induce not only immunogenicity but also immunotoxicity (Nakayama, 2016). In the immune response, the helper T cells, Th1 and Th2 cells, play a major role in humoral and cellular immune responses. A cytokine, interferon-γ (IFN-γ), is induced by Th1 cells and is capable of promoting cytotoxic T cells. Another cytokine, interleukin-4 (IL-4), is secreted by Th2 cells and induces the B cell differentiation (In et al., 2017). Therefore, the level of cytokines is important to index to estimate the Th1 or Th2 immune response.

In **Figure 29** (A), for commercial vaccine, the level of IL-4 was much higher than IFN-γ, which induced dominantly Th2 type immune response. Compared to the commercial vaccine, the level of IL-4 in the groups of 5BT alone and 5BT with CFA/IFA showed similar or little lower induction of IFN-γ level. The result showed that it was consistent with the data of IgG1 and IgG2a and these two groups induced dominantly Th1 type immune response in **Figure 27**. Hae- Mi Nam also reported that the Th1- type cytokine IFN-γ was more prominently expressed than IL-4 vaccinated with VLPs vaccine. For inactivated commercial vaccine was expressed much higher IL-4 level than vaccinated with the VLPs vaccine that containing the GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) (Nam et al.,

2013). Strangely, when 200μg 5BT protein emulsified with MF59 as a vaccine was administered to rats, there was no production of anti-inflammatory cytokine IL-4 and only pro-inflammatory cytokine IFN-γ. In **Figure 29** (B), on the group of 50μg 5BT protein emulsified with CFA/IFA, the level of IL-4 was higher than IFN-γ. Other groups showed the IFN-γ level was little higher than IL-4. For commercial vaccine (**Figure 29** C), all of the groups with the different doses of commercial vaccine showed the IL-4 was higher than IFN-γ. However, the tendency of difference between IL-4 and IFN-γ level in 20μl of commercial vaccine is bigger than other dose groups of the commercial vaccine. In **Figure 29** (D), except for MF59-adjuvanted group, other groups showed the dominant Th2 type immune response because the level of IL-4 was higher than IFN-γ. Besides, alum-adjuvanted with different dose of 5BT protein as vaccine showed a similar tendency of the cytokine differences between IL-4 and IFN-γ levels. But, the vaccine with a high amount of 5BT protein (200μg) group showed higher levels in cytokines than 50μg of 5BT protein group.

Briefly, all the combinations of adjuvants and 5BT showed similar induction level of a Th1 type of cytokine, IFN-γ. Interestingly, the commercial FMDV vaccine also showed the similar level of IFN-γ with 5BT combinations, but strikingly induced a Th2 type of cytokine, IL-4. Moreover, 5BT with MF59 showed no induction of IL-4. 20μl of commercial FMD vaccine and 200μg of 5BT with MF59 showed similarly the highest induction of antigen-specific IgG in the serum after vaccination among the test group. However, their cytokine profiles appeared markedly different. So, it needs further research to interpret the result.

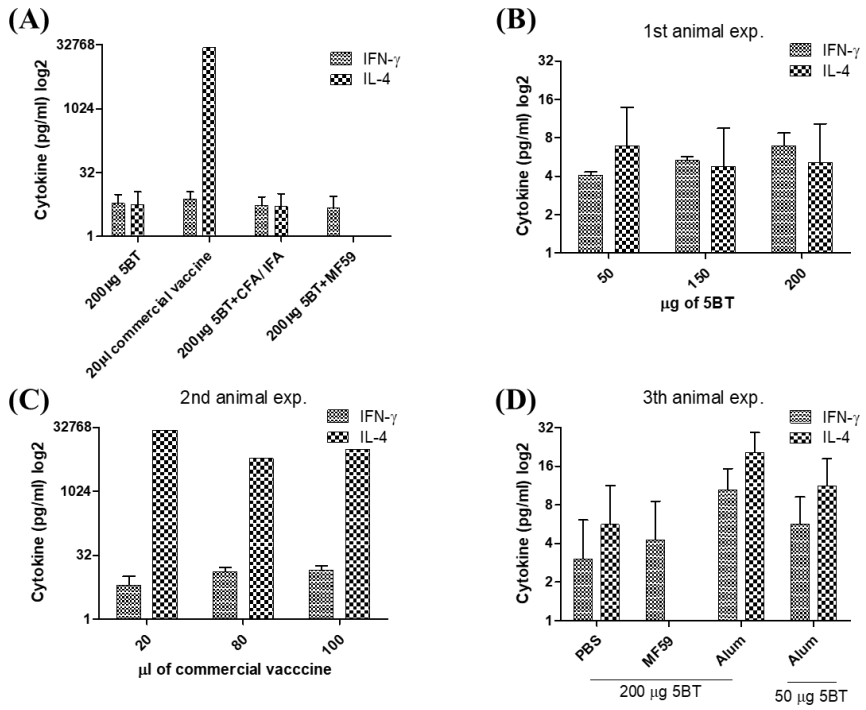


Figure 29. Cytokine profiles of IFN-γ and IL-4 after immunization of each vaccine formula

(A) The groups vaccinated optimal condition as vaccine; (B) The groups vaccinated with different dosage of 5BT protein antigen; (C) The groups vaccinated with different dosage of commercial vaccine; (D) The groups vaccinated with different adjuvants emulsified with 5BT protein antigen. The test serum was collected at week 4 post- immunization.

4. Conclusion

To control and prevent FMD, vaccination is one of the options. However, the methods of vaccination have many sorts. In this research, a subunit vaccine, multi-epitope recombinant 5BT protein, was developed. After production of 5BT from *E. coli*, its efficacy as an FMD vaccine was evaluated. Various doses of 5BT with combination of commercial adjuvants were administered intramuscularly into SD-rats. The vaccinal characteristics of 5BT protein antigen were identified by ELISA using the rat serum samples.

In chapter 2, firstly, for a dose of 5BT protein antigen, the group treated with 200μg 5BT protein emulsified with CFA/IFA produced highest antibodies compared to other dose groups. Secondly, to compare the efficacy between 5BT protein antigen and commercial vaccine (type O + type A), the doses of commercial vaccine were optimized for rat model. Twenty microlitter of commercial vaccine induced higher level of antibodies. It showed equivalent antigen-specific IgG level between 200μg of 5BT protein emulsified with CFA/IFA and 20μl of the commercial vaccine. In a surprise, 200μg of 5BT protein group emulsified with CFA/IFA (PI 72%) had higher vaccination efficacy than 20μl of commercial vaccine (PI 52%) against type O FMDV. Moreover, our 5BT protein antigen could induce an early induction after 2 weeks post priming injection. Usually, the vaccine consists of antigen and adjuvant. The adjuvant can help to improve the immunogenicity of the vaccine. On adjuvants optimization experiment (3rd animal experiment), the data have consistency with 5BT protein antigen dose dependence experiment (1st animal experiment) and commercial vaccine dose-dependence experiment (2nd animal experiment) when rats injected with 200μg 5BT

protein emulsified with CFA/IFA at different environment. Also, it means the results are not occasional. Besides, expect for CFA/IFA, the MF59 adjuvant group emulsified with 5BT protein antigen can produce the highest level of antibodies against the type O FMDV antigen than commercial vaccine containing strain O and A types to against FMDV. Thus, our 5BT protein antigen has the potential to achieve a level of the commercial vaccine. Interestingly, although the multi-epitope 5BT protein vaccine does not contain the O Manisa + O 3039 + A 22 Iraq stains that are the source of commercial vaccine, the sera of commercial vaccine groups showed specific binding to 5BT probably through the antibodies recognizing the conserved region. It would be preliminary evidences that the 5BT protein vaccine may protect other strains and has the possibility of cross-immunity. However, the limitation is that the neutralizing assay and the other FMDV variants should be challenged.

Besides, inflammatory response is also essential for the induction of immunity after vaccination. The balance between Th1 and Th2 cells play a major role in immunity and pathogenesis. Th1 cell secretes IFN-γ and induces cell-mediated immune response and virus-neutralizing antibody response of the IgG2a isotype. In contrast to Th1 cell, Th2 cell secretes IL-4 and stimulates B- cell to produce IgG1 antibody. Although the vaccine with 5BT protein induced the balance between Th1 and Th2 type immune responses, our 5BT protein alone can induce a higher level of IgG2a than IgG1 production and a higher level of IFN-γ than IL-4. It means 5BT protein antigen alone is preferred to induce Th1 type immune response. For commercial vaccine, all doses groups of commercial vaccines produced higher IgG1 level than IgG2a and higher IL-4 level than IFN-γ. This result illustrated that commercial vaccine (type O + type A)

dominantly induced type Th2 immune response. For type Th1 or Th2 immune response, it depends on the adjuvant characteristics. Finally, the MF59 adjuvant can help 5BT protein antigen to enhance the immunogenicity of the vaccine.

In conclusion, the multi-epitope recombinant 5BT protein as a vaccine could induce a high and strong neutralizing antibody against type O FMDV antigen compared to commercial vaccine. In the future, the neutralizing assay should be performed with target animals

Overall conclusion

FMD is a highly infectious disease of cloven-hoofed animals in the livestock industry. In recent years, the world-wide animal production has been suffered from large scale of economic loss by the FMD. The government tried to prevent and control FMD, but it is still hard to eradicated. Because the FMDV has a high rate of mutation and various variants, it is difficult to solve the cross-immunity for controlling FMD. It is widely used to control and prevent FMD with commercial vaccines, inactivated FMD vaccine, which lacks cross activity among serotypes. Nowadays, the multi-epitope recombinant vaccine is interested in the vaccine research field.

In this study, the recombinant 5BT protein antigen was studied. 5BT is composed of five B cell epitopes and one T cell epitope. Five B cell epitopes derived from the GH loop (amino acid residues 130 to 160) in VP1 of FMDV that is important to produce neutralizing antibodies. One T cell epitope (amino acid residue 21 to 35 of 3A) is from NSPs of FMDV that can improve the immune response of the recombinant vaccine by activating T cells (Lee et al., 2017).

The artificial recombinant 5BT protein is expressed using the *E. coli* system. However, recombinant protein also forms inclusion bodies. To form more soluble proteins, the culture conditions and purification conditions were optimized in chapter 1. The large amounts of soluble 5BT proteins was induced at OD_{600} 0.5 using 1.0mM IPTG and culture 4 hours at 37°C. To get a high yield of 5BT proteins, it needs to find a proper condition in His-tag affinity purification. The 5BT protein could be obtained

52.89mg/ L yield and 95% of purity by the 3 times washing with 20, 30, and 50mM imidazole to remove the non-target proteins in purification.

To evaluate the efficacy of 5BT protein as an FMD vaccine, the immunogenicity of 5BT protein antigen was evaluated *in vivo* immunization via intramuscular injection with SD-rat. The highest total IgG level could be got when SD-rats were immunized with 200μg of 5BT protein that emulsified with CFA/IFA. Also, 20μl of the commercial vaccine showed the equivalent IgG level with 200μg of 5BT protein. Compared with the commercial vaccine, CFA/IFA adjuvant group emulsified with 200μg of 5BT protein as the vaccine only has early antibody induction after 2 weeks of vaccination. For vaccine adjuvants, the MF59 adjuvant appeared to be the best choice to emulsify with 5BT protein as a vaccine in this study. Besides, 200μg of 5BT protein combinations with MF59 adjuvant as vaccine can produce higher vaccination efficacy of specific type O antibodies against FMDV than 20μl of the commercial vaccine. The vaccines with 5BT protein or commercial vaccine induced the balance between humoral and cellular immune responses. But, the level of IL-4 in the group of 5BT protein antigen alone showed similar or little lower induction level of a Th1 type of cytokine, IFN-γ. The commercial vaccine was dominantly preferred to induce Th2 immune response.

In this study, even though some characteristics of 5BT protein as a potential FMD vaccine were identified, the evidence is not enough. The neutralizing assay needs to be performed in target cloven-hoofed animals such as pigs and cows in the future. Moreover, further analysis is to challenge the cross-immunity against various variants.

Another, T cell epitope also is a key role to immune response. It is worth considering the other T cell epitopes to make new recombinant protein.

In conclusion, the recombinant 5BT protein has a potential to be an FMD vaccine to control and prevent FMD. This also provides a foundation for researching another novel recombinant vaccine.

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Abstract in Korean

FMD (구제역)는 가축 산업에서 가축, 양 및 돼지와 같은 발굽이 달린 동물의 전염병으로 심각한 생산성 손실과 대규모 경제 피해를 초래한다. 예방 접종이 FMD를 예방하는 신뢰할 수 있는 솔루션이지만 FMD 바이러스의 다양한 하위 유형과 높은 돌연변이로 인해 FMD를 제어하고 예방하기는 여전히 어렵다. 재조합 단백질 백신은 백신 생산을 위한 바이러스가 없는 안전 문제를 포함하여 여러 방식으로 약독화된 바이러스 백신에 대한 좋은 대안 중 하나이다. 5BT는 다양한 O형 FMDV로부터 VP1의 GH루프로부터 유래된 5개의 B세포 에피토프 (아미노산 잔기 130 내지 160개) 및 NSP 중 하나인 3A의 1 개의 T세포 에피토프 (아미노산 잔기 21 내지 350개)로 구성된다. FMDV.

적절한 IPTG 유도 및 정제 조건의 결정에 의해 대장균 BL21 (DE3)로부터 5BT 단백질의 생산을 최적화하였다. IPTG 유도 및 히스티딘-태그 친화도 정제를 위한 최적 조건은 OD_{600} 0.5에서 4 시간 동안 1.0mM IPTG로 처리한 후, 샘플을 수지에 결합시킨 후 각각 20, 30 및 50mM 이미다졸로 3 회 세척하여 달성하였다. 5BT는 조건 최적화에 의해 52,89mg/L의 수율 및 95%의 순도를 갖는 가용성 단백질로써 수득 되었다.

FMD 백신으로써 5BT 단백질의 효율을 평가하기 위해, 5BT 단백질의 혈청 항체 유도 수준을 상용 백신 (타입 O + 타입 A)과 비교하였다. 20, 40, 60, 80 및 100μl의 상용 구제역 백신, 또는 50, 100, 150, 200, 및 250μg의 상이한 용량의 5BT 단백질을 8주령의 SD-rat의 근육 내에 투여함으로써 면역반응을 유도하였다. 면역화 개시 후 6주 째에, 5BT 단백질에 대한 다양한 투여 그룹 중에서 200μg으로 처리된 그룹이 가장

높은 혈청 IgG 수준을 나타냈다. 상용 백신은 20μl로 처리된 최적 그룹에서도 200μg의 5BT와 동등한 수준의 혈청 IgG를 나타냈다.

일반적으로 어쥬번트는 백신 내에 포함되어 있는 항원의 면역원성을 향상시키는 것을 돕는다. 본 연구에서는 CFA/IFA와 같은 실험실용 어쥬번트 외에 5BT 단백질 항원과 Alum, MF59, MPLA와 같은 다양한 상용 어쥬번트와의 조합을 통해 5BT 단백질 항원의 구제역에 대한 아단위 백신으로써의 효능 향상 여부를 SD-rat을 이용한 면역시험을 통하여 평가하였다. 그 결과, 5BT와 MF59의 조합은 CFA/IFA와의 조합이나 상용 구제역 백신과 유사한 백신 효과를 나타내었다. 다음으로는 구제역백신으로써 5BT 단백질 항원의 중화항체 형성능력을 평가하기 의하여 O혈청형의 특이적 FMDV 항원을 포함하는 상용 구제역 검정 키트를 활용하여 각각의 항혈청 내의 항원 특이적 IgG 수준을 조사하였는데, 5BT와 MF59의 조합은 앞서 결과와 일치하게 상용 구제역 백신보다 높은 중화항체 형성능력을 보였다,

본 연구에서 5BT와 다영한 어쥬번트의 조합물은 대체로 체액성 면역과 세포성 면역 간에 균형 잡힌 반응을 보여주었으나, 상용 구제역 백신의 경우 IL-4 및 IgG1의 발현이 우세한 Th2 유형의 면역반응을 나타낸 반면, 5BT와 MF59의 조합의 경우에는 IFN-γ 및 IgG2a의 발현이 우세한 Th1 유형의 면역반응을 나타내었다.

결론적으로, 재조합 5BT 단백질은 가용성 형태의 높은 수율 및 순도로 대장균 숙주에서 생산 가능하며, MF59과 같은 상용 어쥬번트와의 조합을 통해 SD-rat 모델레서 상용 구제역 백신을 상회하는 중화항체 형성능력을 보여주었다. 따라서 5BT 단백질 항원은 향 후 효과적이고

경제적인 아단위 구제역 백신으로써 활용될 잠재력이 높은 것으로 평가되었다.

주요어: 5BT, 다중 항원결정기, 구제역 백신, 어쥬번트, 생산최적화, 면역반응, 히스티딘 친화성 컬럼 크로마토그래피, 중화항체

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junior and friend, 남혜린, the same age as me, is a nice and cute person. It is surprising that she like Chinese food so much. She also is interested people in our lab and popular with Chinses people. We always kidding her, but she never was angry with us and smile to us every day. During this year, we work, play and eat together. If I need her help, she would help me at any time. Our four girls, 은비 언니, 왕신, 혜린, 나, are named 'blood sampling sisters' on the second floor during animal experiment. At that time, even it is a hard time, we finished perfectly and spent an interesting time in animal center. Thanks for you to help me. Then, my another second junior, 조영화 오빠, is a so kind and interested people. Even he has already married, he also plays with us well and make fun with us. If I ask him for help, he will help without any hesitation. 오빠, 화이팅! And take it easy in a master degree. And before lab number 김민중 오빠 is a so kind person to me. He cared of me so much and treated me like a true sister. When I was a hard time, he would make me happy. I am so glad to meet all of you in the same lab to make the warm family.

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호리나 올림