STIMULATION OF IMMUNE RESPONSE IN MICE USING THE PLANT-PRODUCED SARS-COV-2 S1 SUBUNIT PROTEIN LINKED TO THE FC REGION

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การกระตุ้นการตอบสนองภูมิคุ้มกันในหนูโดยโปรตีน S1 ของไวรัส SARS-CoV-2 ที่เชื่อมกับ Fc ที่ผลิตจากพืช

น.ส.ชาลิสา พนาพิทักษ์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	STIMULATION OF IMMUNE RESPONSE IN MICE USINGTHE PLANT-PRODUCED SARS-COV-2 S1 SUBUNIT PROTEIN LINKED TO THE FC REGION
By	Miss Chalisa Panapitakkul
Field of Study	Pharmaceutical Sciences and Technology
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ชาลิสา พนาพิทักษ์กุล : การกระตุ้นการตอบสนองภูมิคุ้มกันในหนูโดยโปรตีน S1ของไวรัส SARS-CoV-2 ที่เชื่อมกับ Fc ที่ผลิตจากพืช. (STIMULATION OF IMMUNE RESPONSE IN MICE USINGTHE PLANT-PRODUCED SARS-COV-2 S1 SUBUNIT PROTEIN LINKED TO THE FC REGION) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

การระบาดของโรคติดเชื้อไวรัสโคโรนา 2019 (COVID-19) เกิดจากกลุ่มอาการของระบบทางเดินหายใจเฉียบพลันรุนแรงโคโรนาไวรัส 2 ที่ส่งผลกระทบร้ายแรงไปทั่วโลก และการควบคุมการติดเชื้อโคโรนาไวรัส 19 ยังคงมีความท้าทาย เนื่องจากขาดวิธีการรักษาที่เฉพาะทางต่อไวรัส และการเข้าถึงวัคซีนที่ใช้ป้องกันไวรัสในประเทศที่มีรายได้ระดับน้อยไปสู่ระดับปานกลาง เพราะฉะนั้นจึงเกิดความต้องการวัคซีนที่มีประสิทธิภาพในราคาต่ำเพื่อใช้ในการป้องกันการติดเชื้อไวรัสโคโรนา 19 ในการศึกษาครั้งนี้จึงผลิตรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1 จากพืชเพื่อใช้เป็นวัคซีนหน่วยย่อยในการตรวจการตอบสนองระบบภูมิคุ้มกัน ซึ่งรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1 ถูกเชื่อมต่อกับบริเวณของ Fc ที่ได้จากแอนติบอดี้ของมนุษย์ (Human immunoglobulin: IgG1) แล้วถูกแสดงออกในใบยาสูบที่มีชื่อสายพันธุ์ว่า *Nicotiana benthamiana* ผ่านกระบวนการแทรกซึมด้วยอะโกรแบคทีเรีย (Agroinfiltration) ซึ่งรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ถูกผลิตขึ้นภายในสี่วันหลังผ่านการแทรกซึมด้วยอะโกรแบคทีเรีย แล้วได้รับรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ในปริมาณ 30 ไมโครกรัมต่อน้ำหนักของใบที่ผ่านการแทรกซึมด้วยอะโกรแบคทีเรีย จากนั้นสารสกัดหยาบที่ได้จากการสกัดรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc จากใบที่ผ่านการแทรกซึมด้วยอะ-โกรแบคทีเรียถูกทำให้บริสุทธิ์ด้วยวิธีโปรตีน เอ ลิแกนด์ โครมาโทกราฟีแบบแอฟฟินิตี้ (Protein A Affinity Chromatography) แล้วรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ถูกวิเคราะห์หาโปรตีนด้วยวิธี Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) และ Western blot ซึ่งพบแถบของรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ที่ขนาด 100-150 กิโลดาลตัน (kDa) ภายใต้เงื่อนไขของสารรีดิวซ์ (Reducing condition) และขนาด 250 กิโลดาลตัน (kDa) ภายใต้เงื่อนไขของสารที่ไม่ถูกรีดิวซ์ (Non-reducing condition) จากนั้นรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ถูกเติม Alum ซึ่งเป็นสารเสริมฤทธิ์ (Adjuvant) ก่อนนำไปฉีดกระตุ้นภูมิคุ้มกันในหนู พบว่ารีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc สามารถกระตุ้นการตอบสนองของระบบภูมิคุ้มกันในหนูได้ และการวิเคราะห์ปริมาณเซลล์ที่ผลิตสารอินเตอร์เฟียรอนแกมมา (IFN-g) ด้วย Enzyme-Linked Immunospot (ELISPOT) พบว่า วัคซีนซับยูนิตรีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc สามารถกระตุ้นการตอบสนองของเซลล์ภูมิคุ้มกันชนิดที (T-lymphocyte) ดังนั้น รีคอมบิแนนท์โปรตีน SARS-CoV-2 S1-Fc ที่ผลิตจากพืชอาจเป็นกลยุทธ์ที่ใช้พัฒนาวัคซีนโคโรนาไวรัส 19 (COVID-19)

สาขาวิชา เภสัชศาสตร์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2565 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Chalisa Panapitakkul : STIMULATION OF IMMUNE RESPONSE IN MICE USINGTHE PLANT-PRODUCED SARS-COV-2 S1 SUBUNIT PROTEIN LINKED TO THE FC REGION. Advisor: Assoc. Prof. WARANYOO PHOOLCHAROEN, Ph.D.

Coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 made a devastating impact worldwide, and virus management is challenging owing to a lack of specific therapies or affordable vaccines, especially in low-and middle-income countries. Hence a low-cost and efficient vaccine is necessary. In the present study, the immunogenicity of the plant-produced S1 subunit protein of SARS-CoV-2 was investigated in order to utilize it as a subunit vaccine. The SARS-CoV-2 S1 subunit protein was fused with the Fc region of human immunoglobulin (IgG1) and expressed in tobacco plants in species of *Nicotiana benthamiana* plants by agroinfiltration. The SARS-CoV-2 S1-Fc protein was generated within 4 days of infiltration with 30 µg/gram of fresh-weight leaves. Afterward, plant-produced S1-Fc protein from the crude extract was purified using protein A chromatography. The S1-Fc fusion protein was observed in an expected band at 100-150 kDa under reducing conditions and 250 kDa under non-reducing conditions by analysis of SDS-PAGE gel and western blot. The S1-Fc fusion protein was formulated with alum as an adjuvant. The S1-Fc protein from plants induced a significant immune response in mice and an interferon-gamma (IFN-g) enzyme-linked immunospot test revealed that the S1-Fc protein vaccine enhanced antigen-specific T-lymphocyte responses. Hence the plant-derived SARS-CoV-2 S1-Fc may provide a candidate strategy for the development of a COVID-19 vaccine.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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Chalisa Panapitakkul

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CHAPTER 1

INTRODUCTION

1.1. Rationale and significant

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was discovered for the first time in Wuhan, China (1), and responsibility for the human pandemic across the world. For this reason, it has much research that develops the vaccine, antiviral drugs, and diagnostic reagents to delay this pandemic disease lives (2). Previous research has shown that the SARS-CoV-2 spike protein can elicit a potent immune response and neutralizing antibodies and it is thought to be useful for the recombinant vaccine development. (3-5) and it can be found on viral surface membranes and involved in binding and enter the host cell (3). Moreover, the spike protein contains the S1 subunit, which binds to the host cell receptor namely ACE2, and the S2 subunit is accountable for fusion and entry into the host cell (3).

For this reason, this study focused on the S1 subunit protein from spike protein to create the recombinant SARS-CoV-2 S1-Fc protein by linking the SARS-CoV-2 S1 with the Fc domain of human immunoglobulin G1 (IgG1) for producing as a subunit vaccine. The subunit vaccine has a limitation of exhibiting immunogenicity, thus it has to be delivered by the adjuvant (6). This study used the alum adjuvant to improve the potentiality of immunogenicity because this adjuvant can induce the antibody response and use in the human vaccine. Moreover, the recombinant SARS-CoV-2 S1-Fc protein in this study also uses the fusion tag to make the stabilized protein and can select the suitable protein purification method. The fusion protein in this study is the Fc tag, which can purify the protein in one step by using the protein A affinity chromatography and increase the target antigen immunogenicity (7). Moreover, it has some research indicated the recombinant of S1-Fc fusion protein has a strong virus-neutralizing activity and antibody titers (8).

There are several hosts for recombinant protein production such as insect cells, yeast, and mammalian cells. They have different advantages and disadvantages, which are essential for selecting the suitable organism to produce the recombinant protein. Therefore, this study used the plants to produce the recombinant protein because it has many advantages such as low-cost manufacturing, high scalability, fast rate of production, and low contamination by human pathogens (9). In addition, the efficient method to produce the recombinant protein by plants is a transient expression method using *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation is a method that has a DNA transgene of the interested protein gene, which insert into the plants using the syringe or vacuum infiltration and express the recombinant protein and therapeutics (10). Furthermore, the suggested plant host to produce the biopharmaceutical is *Nicotiana benthamiana* because it can express the sequences of the heterologous gene as a natural capability, which makes this plant suitable for producing the recombinant protein (10). Therefore, it has much research about plants production of the SARS-CoV-2 vaccine, which reported it can elicit high antibody titers with a potent virus-neutralizing activity (11).

1.2. Research Objectives and Hypotheses

The objectives

1) To produce recombinant SARS-CoV-2 S1-Fc in *N. benthamiana*
2) To determine the efficacy of plant-derived SARS-CoV-2 S1-Fc protein *in vivo*

The Hypotheses

1) The recombinant SARS-CoV-2 S1-Fc can be produced in *N. benthamiana*
2) The purified plant-derived SARS-CoV-2 S1-Fc protein can induce immunogenic response in animals

1.3. Literature Review

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Coronavirus disease 2019 (COVID-19) is named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which occurred in late December 2019 and first identified in human in Wuhan, China (12). The coronaviruses (CoVs) are a large virus groups that cause respiratory disease in human for example, severe acute respiratory syndrome (SARS) was detected in 2003 and has a high mortality rate (12). The CoVs can divide into four genera: alphacoronavirus, betacoronavirus, deltacoronavirus, and gamma-coronavirus, however the genus of CoVs that can infect in human is belong to the alphacoronavirus and betacoronavirus (12). Moreover, the alpha coronavirus has two types namely HCoV-229E and HCoV-NL63 that cause a few symptoms of cold, and the betacoronavirus has five types, divided to two types: HCoV-OC43, HCoV-HKU1, which cause a few cold symptoms and three types: SARS-CoV, MERS-CoV, and SARS-CoV-2, that severe symptoms and death (3, 12).

SARS-CoV-2 belongs to the *Coronaviridae*, is an enveloped, positive single-strand RNA virus, and the largest size of the enveloped RNA virus because it has genome size around 26.2 to 31.7 kb (13), which has significantly size lager than the other RNA viruses (14). The SARS-CoV-2 can infect in human because it belongs to the coronaviruses, which has an origin from bats and can be spread from bats to the other wildlife species (15). Including, it causes a chronic illness in the respiratory, hepatic, gastrointestinal, and neurological systems (16). The coronaviruses have a genome of open reading frames (ORFs), that can be estimated 67% all the genome of ORFs encoded the non-structural proteins, and the remain of ORFs was encoded the structural and accessory proteins (17).

The SARS-CoV-2 structural protein is composed of the nucleocapsid protein (N) formed as a helical capsid to pack the genome and is surrounded by the envelope. Associated with the envelope has three structural proteins: the membrane protein (M) and the envelope protein help virus assemble, and the spike protein (S) helps the virus enter host cells (**Figure 1**) (18).

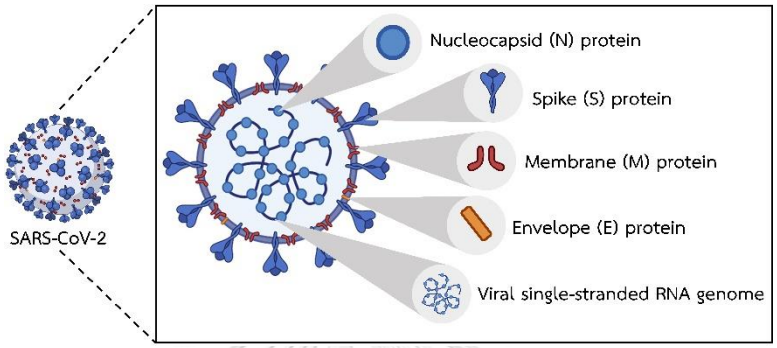

Figure 1: The scheme of SARS-CoV-2 structure

The SARS-CoV-2 has a glycosylated S protein, that cover the surface, and bind to the angiotensin-converting enzyme 2 (ACE2) as a host cell receptor, and mediate viral cell entrance (19, 20). While the S protein adhered to the host cell receptor, the TM protease serine 2 (TMPRSS2), that located on the host cell membrane was activating the virus to entry the host cell. Then the virus releases the viral RNA into the host cell before translation the RNA genome to create the polyprotein, replication, and transcription the RNA virus genome. After that, the cell occurs the replicase–transcriptase complex assembly and protein cleavage then the host cell synthesizes the structural protein, replicated the viral RNA, and packaged it before releasing the viral particles (**Figure 2**) (20, 21).

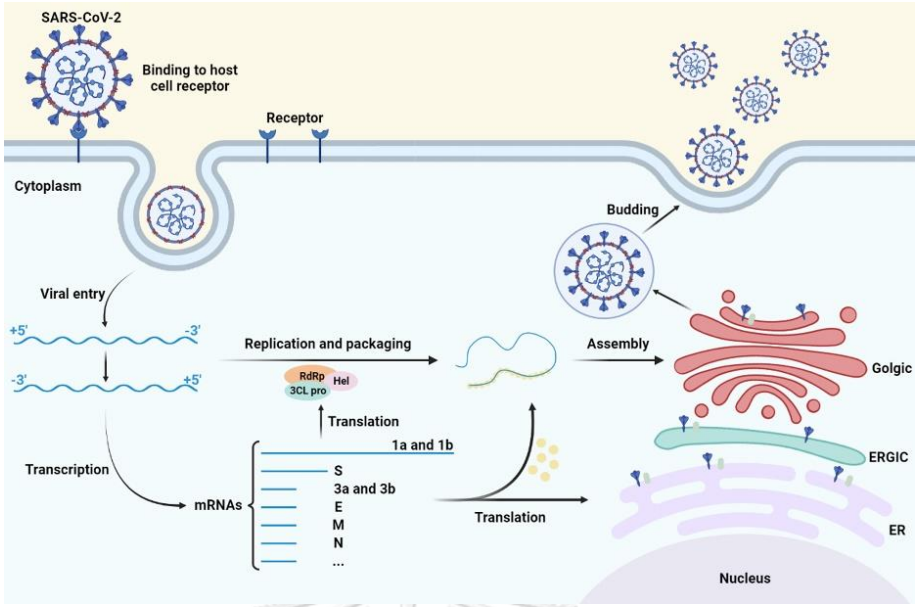

Figure 2: The scheme of SARS-CoV-2 life cycle (modified from (3))

Therefore, the life cycle of SAR-CoV-2 has a necessary compound that is the structural proteins in the virus, which can be used for a therapeutic target. For instance, the SARS-CoV-2 S protein is used as a base for developing the vaccine because the SARS-CoV-2 S protein involves in the recognition of receptors, viral attachment, and enter host cells. For these reasons, the SARS-CoV-2 S protein is essential target for the vaccine and research of COVID-19.

Spike (S) protein structure

The S glycoprotein is a transmembrane which has the large multipurpose and role in the attachment of viral, fusion and entry the host cell. The S protein is general found in a prefusion, metastable form and composes of S1 subunit and S2 subunit which play the viral entrance and fusion the host cell, respectively (3). For this reason, the S protein can rearrange the structure for combining with the cell membrane of the host cell, when the virus interacts with the host cell. As, the S protein covered by polysaccharide molecules, that helps the S protein avoid the detection of immune system from host cell when the virus entry cell (22). The S protein contains the signal peptide, the S1 subunit composes an N-terminal domain

and a receptor-binding domain (RBD), that perform receptor binding of the host cell, and the S2 subunit consists of the fusion peptide (FP), heptapeptide repeat sequence 1 (HR1), HR2, TM domain, and cytoplasm domain, which carry out the membrane fusion between the virus and the host cell (23). The S protein trimeric structure was derived from the S1 and S2 subunit, which formed bulbous head and stalk region, respectively, so the characterization of the S protein trimer was formed like a crown-like halo surrounds the particle of the virus (24) (**Figure 3A**).

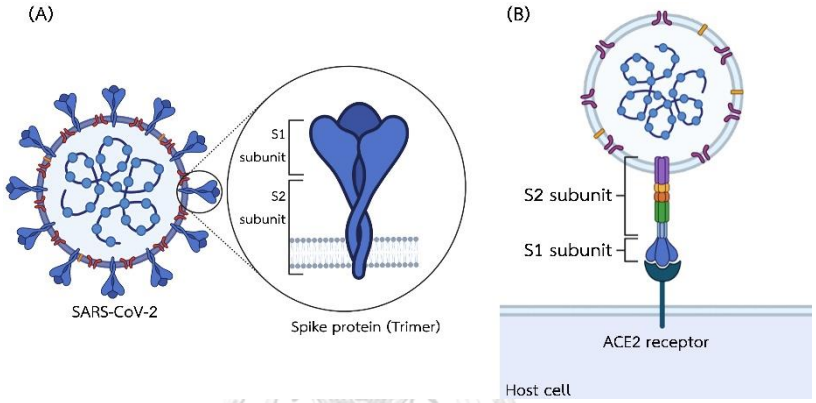

Figure 3: The spiker protein in trimeric structure **(A)** and binding between the S protein and the ACE2 receptor **(B)**

The S protein in trimeric form was identified with the cryo-electron microscopy at the atomic level, demonstrated that the conformation of S protein trimeric form was different when the S RBD domain opened and closed conditions (25, 26). In general phase, the CoV S protein was inactive condition before infecting the host cell. When the virus infects the target cell, the S protein will become in the active phase by the proteases because this enzyme will activate the S protein to cleavage into the S1 and S2 subunits, which is essential for activating the fusion of cell membrane when the viral entry the target cell (27, 28). The SARS-CoV-2 S protein is like the other coronaviruses, that can cleavage the S protein to S1 and S2 subunit and this cleave was recognized by furin and other proteases such as trypsin-like proteases, and cathepsins. Therefore, the entrance of SARS-CoV-2 was depended on

lysosomal cathepsin and the type II transmembrane serine protease (TTSP) TMPRSS2, which express on the cell surface (28, 29).

S1 subunit structure

The beginning of the viral infection involves a utilization of cell receptor, located on the host cell surface because it can help the virus particles enter the host cell. For this reason, this structure plays a crucial role in viral entry and target for medication design (3).

The S1 subunit has an RBD region, located on aminopeptidase N region, which can bind to the ACE2 receptor as a host cell receptor (**Figure 4**). The S1 subunit comprises NTD and CTD, which can demonstrate the interface of binding by the atomic information that SARS-CoV-2 CTD has a critical replacement of residue. In addition, the SARS-CoV-2 S CTD has a surface, is complex bury in ACE2 receptor, and the binding interface of SARS-CoV-2 S CTD has residues that can directly connect to the ACE2 receptor more than the SARS S RBD. Therefore, the mutation of the critical residues is necessary for improving the interaction of ACE2 receptor. For example, the I472 in SARS-RBD and F486 in SARS-CoV-2 can indicate that the F486 in SARS-CoV-2 can form an aromatic-aromatic interaction with ACE2 stronger than the I472 in SARS-RBD (25, 26, 30, 31). Hence, the target for neutralization antibody of SARS-CoV-2 is the RBD region because the sequence of SARS-CoV-2 RBD is as same as the sequence of SARS-CoV RBD around 73%–76% (3), and the SARS-CoV RBD already has a detail that can be a safe and effective vaccine candidate (32). Moreover, the most residues of SARS-CoV S protein that is necessary for binding to ACE2 are conserved in the SARS-CoV-2 S protein reported by the analysis of receptor-binding motif, which is a RBD portion that directly connects to ACE2. On the other hand, it has several studies found that the polyclonal and murine monoclonal antibodies are unable to bind with the SARS-CoV-2 S protein, indicating antigenic differences between SARS-CoV and SARS-CoV-2 (31). Including, the SARS-CoV RBD-specific antibody could not to prevent infection caused by the SL-CoV-SHC014 S protein (33), indicated that the S1

subunit and RBD region may not a treatment target because it has a highly natural change of anti-CoV drugs (3).

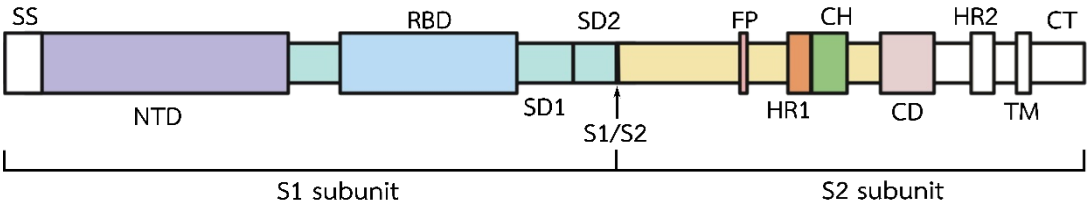

Figure 4: The scheme of SARS-CoV-2 spike protein consist of S1 and S2 subunits (modified from (34))

S2 subunit structure

The entrance and fusion of the virus is involved in the S2 subunit, which consists of a FP, HR1, HR2, TM domain and cytoplasmic domain fusion (CT) (**Figure 4**).

The fusion peptide (FP) is a short segment amino acid conservation of the viral family, and the main composing is hydrophobic residue. The FP can adhere to the membrane of the target when the S protein forms the prehairpin. Moreover, the FP can break and link to lipid bilayers in the host cell membrane because the FP is an important compound for fusion membrane (35).

The heptad repeats 1 and 2 (HR1 and 2) contain the hydrophobic as H, hydrophilic as P, and another charges substance as C, which is a repeated heptapeptide like HPPHCPC (36). When the S2 subunit fusion and entrance to the host cell membrane, the HR1 and HR2 will combine and create the six-helical bundle (6-HB) (23). The HR1 can be found in hydrophobic FP of the C-terminus, while the HR2 can be found in the transmembrane (TM) domain of the N terminus (37). The attachment of S protein of the virus to the host cell membrane, which use the downstream TM region and the CT tail of the ends of S2 subunit (24).

The binding between the virus and the host cell starts at the RBD binds to ACE2 of the host cell. Then the S2 subunit of the virus will insert the FP domain into the host cell membrane in order to change the structure and expose the HR1 domain into the prehairpin coiled-coil form before initiating a link the HR2 domain and HR1

trimer to produce the 6-HB before allowing the virus to fusion and entry into the host cell (38). The HR1 forms a homotrimeric assembly on the surface with three substantially conserved hydrophobic grooves that bind to HR2. When the HR2 domain interacts with the HR1 domain, it will form the stiff helix and a flexible loop, which is much strong interaction between the HR1 and HR2 domains within the helical area namely, fusion core region in the conformation of the CoVs post-fusion hairpin (3).

The S protein can use as a target for developing therapeutic, but S1 and RBD region is not suitable for the creation of antiviral inhibitors because they are in the region that has a lot of change the residue (39). On the other side, the HR domain from the S2 subunit is a significant for infecting HCoV, and this part is conserved in HCoVs include the interaction between the HR1 and HR2 mechanism (40). In addition, the research *in vitro* about the peptide synthetic obtained from the ZIKV envelope protein stem region in 2017 demonstrated that it could prevent the infection of ZIKV and other flaviviruses (41), and the peptides derived from conserved viral protein areas are antiviral (33). Consequently, HR1 is suitable fusion inhibitors target for developing against the infection of SARS-CoV-2.

The S protein functions

The S protein is an important structure that plays role of virus infection because the S protein is located on the surface, and has the S1 and S2 subunit, which is responsible for binding and fusing, respectively. Moreover, the S protein has a trimeric class I TM glycoprotein, that can be found in all types of HCoVs and other viruses such as HIV, influenza virus, and Ebola (42). The SARS-CoV-2 S protein is as same as the other coronaviruses because it can fusion during viral infection, mediates receptor recognition, and cell attachment (26, 30, 31, 43-45). The trimer structure of S protein is found on the surface of the viral envelope, which can bind to the host cell receptor (26, 45). The S1 subunit of S protein comprise the RBD region that is a

necessary structure for binding to the host cell. While the S2 subunit of S protein contains the HR1 and 2 regions, that is a primary of the virus fusion (46).

Receptor binding

The SARS-CoV-2 S protein can recognize the ACE2 receptor on the host cell and binds it (45). The ACE2 is a homolog of ACE, can convert angiotensin I to angiotensin 1-9 (47). The ACE2 is the most mainly express cell in lung, kidney, heart, and intestine with the alveolar epithelial type II cells (48). In general, the SARS-CoV uses the ACE2 receptor because the SARS-CoV S protein has the S1 subunit to bind ACE2 receptor for inducing the formation of endosome, which cause the virus fusion activity under the low pH (**Figure 3B**) (20). The different species of the ACE2 can be utilized for identification of the SARS-CoV-2 intermediate hosts because it has a preservation of the ACE2 primary structure. For this reason, we can identify the ACE2 species, when the S protein connects with the ACE2 receptor.

The S1 subunit from S protein contains the RBD region, that binds ACE2 receptor to mediate viral for attaching the host cell with the trimer conformation (25). The SARS-CoV-2 S can bind to human ACE2 receptor with the response more than the SARS-CoV S (49) because the dissociation constant (K_D) of the binding between the SARS-CoV-2 S and human ACE2 is 14.7 nM, while the binding between the SARS-CoV S and human ACE2 is 325.8 nM (25). In addition, several details indicated that the different RBD domain between SARS-CoV-2 and SARS-CoV estimate around 23%, whereas the S protein is around 24% after identifying SARS-CoV-2 protein (49).

Fusion of virus

The fusion of virus is a fusion between the host and viral cell membrane to release the viral genome into the host cell. The fusion system starts from cleavage the S1 and S2 subunits of the SARS-CoV-2 S protein using the protease from the host cell. Then the subunit of the S protein will exist in a noncovalent state before occurring the fusion (50). According to the researcher revealed the cleavage location site of SARS-CoV-2 was found a cleavage site of specific furin, while the other SARS-

like CoVs did not find (51, 52). The mutation cleavage site of SARS-CoV-2 or SARS-like CoVs demonstrated the S protein in other SARS-like CoVs can cleavage, but in SARS-CoV-2 S protein cannot cleavage. Nevertheless, it has a few of the furin cleavage sites in SARS-CoV-2 S protein, which assist the protease like furin to increase the opportunity of cleavage sites in order to boost the infection of the virus (3, 53, 54). Furthermore, the transmembrane serine protease 2 (TMPRSS2) from the host cell is used for activating the entry of SARS-CoV into the host cell (28, 55). Furthermore, the the SARS-CoV-2 S protein has a structure as same as the SARS-CoV S protein, and the important for stimulate the cleavage of the S protein in both SARS-CoV-2 and SARS-CoV must utilize the proteases from the host cell. Therefore, the furin cleavage site on the SARS-CoV-2, which is preserved can use for explaining why SARS-CoV-2 can be spreader than the SARS-CoV (3).

The conformation of 6-HB is a significant structure for viral fusion include the FP domain in N-terminus and the HR1 and 2 domains on SARS-CoV-2 S2 subunit (56). When the S protein cleavages, the SARS-CoV-2 FP domain will disclose and mediates virus and host cell interaction. Then the fusion protein will change the conformation and entry into the host cell membrane (57), which makes the viral cell membrane close to host cell membrane, and the HR1 domain from S protein is close to the membrane of the host cell, but the HR2 domain is close to the viral cell membrane. After that, the HR2 domain will return to the HR1 domain and the HR1 and 2 domains will create the conformation of the antiparallel six-helix in the fusion core. Before, the viral cell membrane connects to the host cell membrane and binds it very tightly before both membranes fuse (3, 58).

Potential drugs targeting the spike (S) protein

The S protein has a role in viral infection, so it is a target for development of therapy and vaccine, which can explain below.

The SARS-CoV-2 S protein-based antibodies

The structural protein of SARS-CoV-2 that is used for the antigen component is the S protein because the S protein can use to induce the host immunological response neutralizing antibodies (nAbs) to protects the infection of the virus. The research of SARS-CoV-2 nAbs will focus on monoclonal antibodies (mAbs), single-domain antibodies (Nbs), fragments of antigen-binding, and single-chain variable regions, which point at the S1 and S2 subunits, RBD domain, and NTD domain so as to block the viral fusion by the S2 subunit like the SARS-CoV and MERS-CoV (3, 59, 60). Moreover, the structure of SARS-CoV-2 S protein has structure similar to the SARS-CoV S protein, which is the significant structure for target to treat with antibody (61). When comparison the interaction between hACE2 and SARS-CoV-2 RBD and CTD (C-terminal region) can demonstrate the SARS-CoV-2 RBD and CTD has a great affinity with the receptor binding. Including, the RBD region of SARS-CoV-2 can stimulate the potent nAbs responses and has an ability for developing the safe and effective subunit vaccine to treat the SARS-CoV-2 (31). Furthermore, it has a few mAbs in SARS-CoV-2, which can induce the neutralizing such as CR3022 is a specific human mAb of SARS-CoV, which can bind potently to SARS-CoV-2 and has an ability to develop as a candidate therapy for the prevention and treatment of SARS-CoV-2 infection using CR3022 mAb alone or CR3022 mAb combination with the other nAbs (45).

The infection of SARS-CoV-2 and SARS-CoV can be found, it can neutralize by mAb targeting the S1 subunit, which derived from vaccinated transgenic mice express the human Ig variable heavy and light chains via an unknown mechanism unrelated to the blockage of RBD-hACE2 interaction (62). As a result, the specific mAbs can efficiently neutralize infection and bind to SARS-CoV-2. The cross-neutralization of

SARS-CoV-2 by serum from SARS patients undergoing restitution or vaccinated animals with S1 of SARS-CoV may inhibit S protein-mediated SARS-CoV-2 entrance (28).

Inhibitors of fusion

The SARS-CoV-2 S protein has a stability lower than the SARS-CoV S protein (52). The mapping of S sequence conversation of sarbecovirus subgenus indicated the S1 subunit less conserved than S2 subunit, but the S1 subunit is more expressed on the viral surface (3, 26). Moreover, the SARS-CoV S2 subunit is significant to fusion and entry into the host cell, which has the conformation of 6-HB, derived from combination of HR1 and 2 domains and permits the virus bind and fuse the host cell membrane (40). According to the alignment of sequence, the SARS-CoV HR2 has the sequence same as the SARS-CoV-2 HR2 so the SARS-CoV-2 HR2P will create to prevent SARS-CoV-2 fuse and entrance the host cell. Furthermore, the mediated SARS-CoV-2 S protein and pseudovirus of SARS-CoV-2 was inhibited by the HR2P with 0.18 and 0.98 M of IC_{50}, respectively (3, 23).

Furthermore, the pancoronavirus fusion inhibitor is EK1 that is a specific target in the HR1 region of HCoV S (3, 33). The X-ray crystal of 6-HB core structure from SARS-CoV-2 S2 subunit HR1 and 2 indicated that the multiple mutant residues in the HR1 area may be linked to improved HR2 interaction (63). Hence, the lipopeptide that derived from EK1 namely EK1C4 was created and tested for its ability to prevent the mediate cell-cell fusion of SARS-CoV-2 S. EK1C4 can block the SARS-CoV-2 S pseudovirus when it enters the cell with the high value of IC_{50}, which is more than the original peptide of EK1 149-fold. Moreover, it has a fusion inhibitor of the sequence-based lipopeptide which effectively blocks the cell-cell fusion and pseudo-virus infection by mediating SARS-CoV-2 S namely IPB02 (64). Including, the nelfinavir mesylate (Viracept) is a protease inhibitor of anti-HIV, which can inhibit the mediate between cell-cell fusion in SARS-CoV-2 S and SARS-CoV S, and may decrease the activity of TMPRSS2, which is implicated in S protein activation (65). For

these reasons, it makes the way for applying in clinical to anti-SARS-CoV-2 in the initial stages of infection.

Protease inhibitors which target SARS-CoV-2 S cleavage sites

The S protein is involved in the entrance of SARS-CoV-2 because the S protein will cleavage into the S1/S2 and S2 sites to prime for entry the host cell using the proteolysis of TMPRSS2 and cathepsin B and L. On the other hand, the camostat mesylate is effective protease inhibitor for TMPRSS2 serine because it has some studies about the mechanism of SARS-CoV and SARS-CoV-2 cell entry indicated camostat mesilate can inhibit SARS-CoV-2 cellular entry (28, 66). Furthermore, lysosome cathepsins are required for SARS-CoV entrance by endocytosis such as E-64d is a cathepsin L inhibitor that can prevent SARS-CoV and SARS-CoV-2 PsV infection (67, 68), which may be had a successful treatment in the future experiment with COVID-19 patients. In early endosomes, phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is the primary enzyme that produces PI(3,5)P2 (69), and it has a strong inhibitor of apilimod, that can reduce the entrance of SARS-CoV S pseudovirus in 293/hACE2 cells by early endosomes (3, 70). YM201636 is a different PIKfyve inhibitor, which can exhibit a similar impact on 293/hACE2 cells (71). Furthermore, the two-pore channel subtype 2 (TPC2) is a significant downstream effector of PI (3,5)P2 (72), which is necessary for entry of SARS-CoV-2, and tetrandrine (a TPC2 inhibitor) can inhibit the pseudovirus activity of SARS-CoV-2 S.

Furin belongs to the secretory proprotein convertase (PC) family, which can catalyze the peptide hydrolysis and protein substrates with paired basic residues (3, 73, 74). At the S1/S2 interface, the SARS-CoV-2 S possesses a furin cleavage site, which could improve SARS-CoV-2 transmission efficiency (75). The furin-like cleavage site in SARS-CoV-2 S protein involve in the virus life cycle and pathogenesis, so Furin inhibitors can utilize for a treatment of SARS-CoV-2 (51). Furin has a patent literature, that described about using furin as a treatment inhibitor such as -1-PDX (one-

antitrypsin Portland), hexa-D-arginine (D6R), serpin proteinase inhibitor 8 (PI8), and a peptidomimetic furin inhibitor (3, 76-78).

Hence, the target of SARS-CoV-2 S protein for development of therapy can summarize the antibodies, small molecules, and peptide inhibitors can use for developing the vaccine to prevent the infection of SARS-CoV-2, which can conclude the mechanism of all the target in the S protein in **Figure 5**.

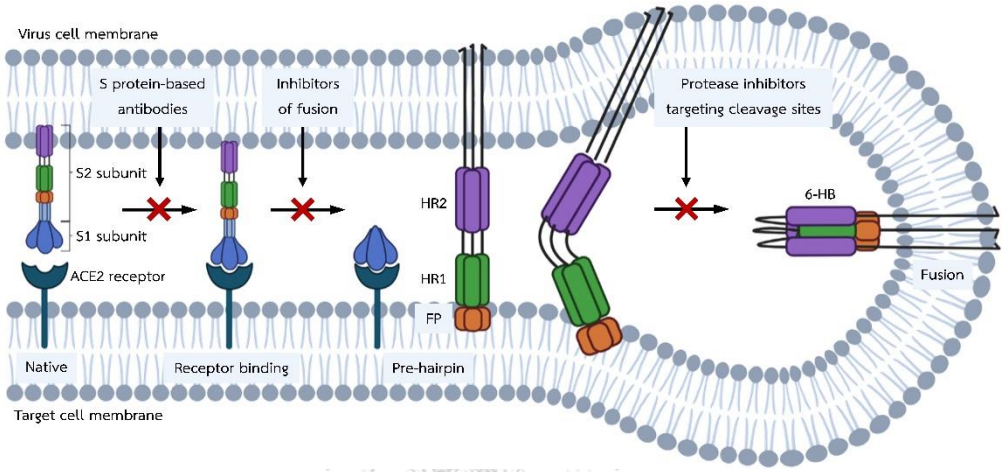

Figure 5: The mechanism that inhibit the virus fusion in S protein (modified from (79))

Vaccine types

The prevention of this disease by vaccine, which has many types of vaccine can use for developing to reduce the mortality and morbidity, as indicated below and the summary of the feature and limitations of the vaccine show in **Table 1**.

1.) Inactivated virus vaccine derived from the whole virus, which is inactivated by chemical or physical. The inactivated virus vaccine indicates the multiple proteins of the virus, that help the immune system recognize, including its safe and inexpensive because it does not involve the manipulation of genetics and is easy to produce on a large scale (80, 81).

2.) Live-attenuated vaccine is a vaccine like a natural infection because it derived from the virus gene, removed the part of the gene that can lead to virulence. The live-attenuated vaccine is not necessary to use the adjuvant for high

immunogenicity, but it causes the unwished risk, such as the virulent mutation of the strain and infection of opportunistic (81).

3.) Subunit vaccine is a vaccine base on recombinant protein or synthetic peptides. The subunit vaccine is essential for inducing the immune response in the long term to protect and therapy, but it exhibits low immunogenicity. Therefore, the subunit vaccine has to use the adjuvant to help the vaccine has the potential to induce the immune response, and the adjuvant can improve the weakness of the subunit vaccine, such as improve the immunomodulatory cytokine response or improve the weakness of the subunit vaccine. In addition, the subunit vaccine has a few components of the virus that are not complex. For these reasons, the subunit vaccine may have a limitation on the protection efficacy (6, 80, 81).

4.) Virus-like particles (VLPs) vaccine is a vaccine that based on the protein or nanoscale particle related to the native virus particle, which has not the genetic of infection. Moreover, the VLPs vaccine is as same as the whole-virus inactivated vaccine, but the VLPs vaccine does not need to use the structure of high containment because it utilizes genetic material that does not infect. As a result, the VLPs vaccine is safer than the other platforms and can induce high immune responses (80, 81).

5.) DNA vaccine derived from the recombinant plasmid, encodes the immunogen of the virus, which expresses on the host cell that infected the virus and can stimulate the cellular and humoral response. The DNA vaccine can stimulate the T-cell response, humoral immune response and enhance the production of

antibodies, including low-cost and easy to produce (80, 81).

6.) Messenger RNA (mRNA) vaccine is a nucleic acid platform like a DNA vaccine, which uses the genetics information to encode the immunogen of the virus. The mRNA vaccine is a platform, that has high potency, and is non-infectious. Including, it can decrease the production cost and has the rapid ability to develop the vaccine (6, 80, 82).

7.) Viral vector vaccine is a vaccine, uses a virus vector to deliver the specific component, that can induce the immune response. The viral vector vaccine can specifically deliver the compound to the target cell and has a high potential to stimulate an immune response (6, 81, 82).

Table 1: The feature and limitations of the vaccine types[83]

Vaccine platform	Advantages	Limitations	Human-approved vaccines
Inactivated virus	- Stable and no risk of reversion - Strong antibody response - Cost-effective	- Biosafety issue - Usually requires adjuvants - Weak cellular immune response	Influenza (injection), polio (injection), hepatitis A
Live attenuated virus	- Strong immune responses - No adjuvant required - Cost-effective	- Biosafety issue - Risk of reversion to virulence - Time-consuming development	Influenza (nasal), polio (oral), measles
Recombinant protein subunit	- No risk of infection and reversion - Fewer side effects - Easy antigen modification	- Low immunogenicity - Requires adjuvants - High cost	Hepatitis B, influenza (injection)
Virus-like particle (VLPs)	- No risk of infection and reversion - Fewer side effects - Good antibody response	- Complicated manufacturing process - Requires adjuvants - High cost	Cervical cancer by human papillomavirus
DNA	- Rapid development and production - Stable in room temperature - High producibility	- Low immunogenicity - Requires a delivery device (electroporator or jet-injector)	-
mRNA	- Cell-free - Rapid development and production - Good immunogenicity	- Unstable - High cost - Requires low temperature storage	-
Viral-vectored	- Strong immune responses - Various viral vectors - Large-scalable	- Pre-existing immunity against the vector	Ebola

However, this study focuses on the subunit protein because the subunit vaccine has a few side effects and is a non-infectious vaccine when compared with other vaccines, including it can use in immunocompromised patients (84). Therefore, it is necessary to know-how is the mechanism of the vaccine.

Subunit vaccine

As previously indicated, the subunit vaccine can use for the booster vaccine. Therefore, the mechanism of the vaccine starts with entering of viral antigen by vaccination and then the Antigen-presenting cell (APC) is activated and presents the antigen to the B cell for activating the humoral immunity. After that, the B cells separate into memory B cells and plasma cells to prevent the infection and produce the specific neutralizing antibodies of the virus, respectively. Moreover, the viral antigens are activated the naive T cell to divide into cytotoxic T cells ($CD8^+$ T cells) and helper T cells ($CD4^+$ T cells), which the helper T cells secrete interferon-gamma for inducing the $CD8^+$ T cell to kill pathogens. Nevertheless, the immune system can be seized by the virus that will boost the Antibody-dependent enhancement (ADE), and the helper T cell 2 (Th-2), which is related to interleukins will stimulate the eosinophil that is accountable for Th-2 immunopathology (**Figure 6**) (85).

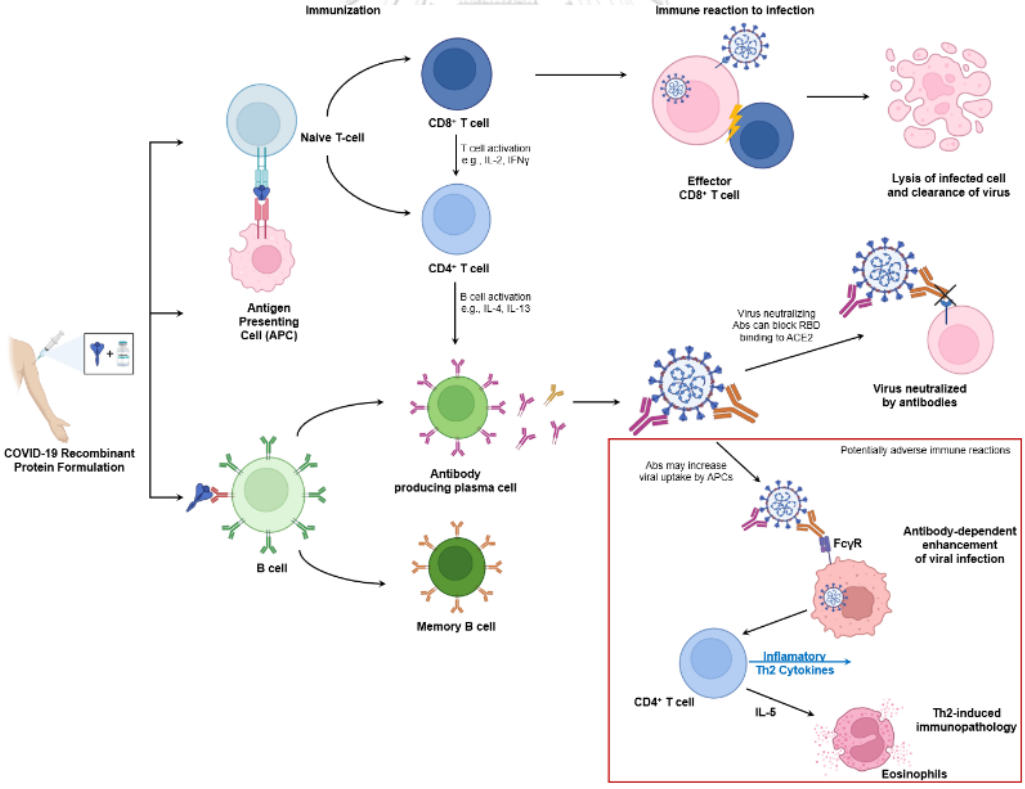

Figure 6: The description of immunological responses elicited by recombinant proteins vaccine (modified from (86))

In addition, the main target antigen of the subunit vaccine for SARS-CoV-2 is an S protein, including the component of the S protein such as the S1 and S2 subunit, NTD, RBD may also be the target antigen for developing the subunit vaccine. The S protein can be the target antigen because it can stimulate the antibodies that neutralize virus infection by prevent the virus bind and fusing. In general, the subunit vaccine expresses in eukaryotic cells and uses different systems of expression (84). Hence, the subunit vaccine has to delivery with an adjuvant because it can increase the potential of the subunit vaccine for inducing the immune response better, and it may increase the antigen biological half-life or improve the response of immunomodulatory cytokine. Consequently, it can help the subunit vaccine overcome its weak point (6) and has to choose the suitable adjuvant for developing the subunit vaccine. Therefore, the summary of the mechanisms action of adjuvants shows in **Table 2** and the example of target antigens for subunit vaccine show in **Table 3**.

Table 2: Mechanisms action of adjuvants[87]

Adjuvant	Proposed mechanisms of action	Immune response activated	Licensed vaccines
Alum	No depot effects NLRP3 activation in vivo? Independent of TLR signaling ↑ Local cytokines and chemokines ↑ Cell recruitment (eosinophils, monocytes, macrophages) ↑ Ag presentation	↑ Ab responses ↑ Th2 responses Poor Th1 responses	Many human vaccines (e.g., DTap, Hib, Hepatitis A, Hepatitis B)
MF59	No depot effects NLRP3 independent but ASC-dependent Independent of TLR signaling but MyD88-dependent for Ab responses ↑ Local cytokines and chemokines ↑ Cell recruitment (neutrophils, macrophages, and monocytes) ↑ Ag uptake Activate muscle cells ↑ Ag-loaded neutrophils and monocytes in dLNs	Balanced Th1 and Th2 responses	Licensed for influenza vaccine (Fluad®), H5N1 pre-pandemic vaccine (Aflunov®), H1N1 pandemic vaccines (Focetria® and Celtura®)

Table 2: Mechanisms action of adjuvants[87]

Adjuvant	Proposed mechanisms of action	Immune response activated	Licensed vaccines
AS04	MPL signals through TLR4 to activate APCs ↑ Local cytokines and chemokines ↑ Cell recruitment (DCs and monocytes) ↑ Ag-loaded DCs and monocytes in dLNs	↑ Ab responses ↑ Th1 responses	Licensed for human papilloma virus (HPV) (Cervarix™), hepatitis B virus (Fendrix®)
AS03	Spatio-temporal co-localization with Ag Transient ↑ cytokines locally and in dLNs ↑ Cell recruitment (granulocytes and monocytes) ↑ Ag-loaded monocytes in dLNs	↑ Ab responses ↑ Immune memory	Licensed for pandemic flu vaccine (Pandemrix®)
Virosomes	Ag delivery vehicle Bind APCs and induce receptor-mediated endocytosis Escape endosomal degradation Ag presentation via MHC class II and MHC class I to CD4+ CT cells and CD8+ CT cells respectively Immunopotentiator	↑ Ab responses ↑ CTL responses	Licensed for Inflexal® V and Invivac® influenza vaccine and hepatitis A vaccines (Epaxal®)

Ab, antibody; Ag, antigen; CTL, cytotoxic T lymphocytes; dLNs, draining lymph nodes

Table 3: Example of target antigens for subunit vaccine

Antigen	Platform	Adjuvant	Result	Reference
Full-length S-protein based vaccines				
Trimer	Insect cells	Matrix M	- Safe and elicit immune responses	(88)
SCB-2019 trimer	CHO cells	Alum+CpG 1018 or AS03	- Induced high-level of neutralizing antibodies and Th1-biased cellular immune responses	(89)
S-2P (MVC-COV1901)	CHO cells	Alum+CpG1018	- Most potent immunogen - Induced high titer of neutralizing antibodies	(90)
Full-length spike ectodomain (S: S1–S2)	baculovirus insect cells	AS01-like adjuvant (QS-21 + MPL)	- More immunogenic when compare with S1 subunit in low dose (0.8 μg)	(91)
SARS-CoV-2 ectodomain with furin cleavage site removed	Expi293 cells	Addavax or Monophosphoryl Lipid A liposomes	- Highly immunogenic in mice - Increased the neutralization titer	(92)
S-2P (prefusion trimer-stabilized form (S-2P))	Sf9 insect cells	Aluminium adjuvant	- Elicited high neutralizing antibodies after three rounds of immunization	(93)
Prefusion form of SARS-CoV-2 S protein (S-2P)	ExpiCHO cells	Aluminum hydroxide (Alum) or Emulsion-type adjuvant (SWE)	- S-2P with SWE elicited the immune response higher than the S-2P with alum - S-2P with SWE elicited high neutralization antibodies	(94)

Table 3: Example of target antigens for subunit vaccine

Antigen	Platform	Adjuvant	Result	Reference
Full-length S-protein based vaccines				
CMP trimerization domain of SARS-CoV-2 S protein	Silkworm larvae serum	Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, Aluminum hydroxide, and Paramylon	- Trimeric form of S protein induced high antigen-specific antibodies but low neutralizing antibodies - Trimeric form of S protein adjuvanted with alum induced strong neutralizing antibodies	(95)
S1 subunit-based vaccines				
S1 with RBD	baculovirus insect cells	AS01-like adjuvant (QS-21 + MPL)	- Highly effective in stimulating neutralizing and receptor-binding inhibition antibodies in 4 μg vaccine dose	(91)
SARS-CoV-2 S1	*E. coli*	Alum	- SARS-CoV-2 S1 domain is more immunogenic than the RBD domain, inducing higher IgG and IgA antibodies and efficient virus neutralization antibodies	(96)
Recombinant SARS-CoV-2 S1-Fc fusion protein	CHO cells	AD11.10, AD20Gold+, and Freund's complete adjuvant	- Strong antibody titers and virus neutralizing activity - Developed higher virus neutralizing titers than a recovered COVID-19 patient in a live SARS-CoV-2 infection assay	(8)
Recombinant SARS-CoV-2 S1 protein	Expi293 cells	Aluminum hydroxide	- Induced high level of S-specific IgG responses more than DNA vaccine	(97)
SARS-CoV-2 S1 protein	Mammalian cells	TriAdj	- Induced neutralizing antibodies - Preventing effects from the pathophysiology of lung disease	(98)
S2 subunit-based vaccines				
S2 with fusion domain	baculovirus insect cells	AS01-like adjuvant (QS-21 + MPL)	- Highly effective in stimulating neutralizing and receptor-binding inhibition antibodies in 4 μg vaccine dose	(91)
Spike S2 protein which has an important antigenic epitope (P2 protein)	*E. coli* BL21	Aluminum hydroxide gel and combination of aluminum hydroxide gel plus MPLA	- P2 protein adjuvanted with alhydro gel plus MPLA induced higher IgG and IgG2a antibody titer than adjuvanted with alum only - Induced high virus neutralizing activity and cell-mediated immunity	(99)
HR121 protein contains HR1–linker1–HR2–linker2–HR1 from HR1 and 2 domains of S2 subunit in S prtoein	*E. coli* BL21	CFA and IFA, or aluminum adjuvant	- Induced high potent cross-neutralizing antibodies - Efficient anti-infection against Omicron BA.2 - Elicited strong and broad antibodies against SARS-CoV-2	(100)

Table 3: Example of target antigens for subunit vaccine

Antigen	Platform	Adjuvant	Result	Reference
RBD-based vaccines				
SARS-CoV-2 RBD	Expi293 cells	Addavax or Monophosphoryl Lipid A liposomes	- Low immunogenicity in mice - Increasing the neutralization titer	(92)
Recombinant fusion protein RBD-mFc	Expi293 cells	Alum, and Freund's	- Induced highly potent neutralizing antibodies - Induced humoral and cellular immunity in mice	(34)
Recombinant SARS-CoV-2 RBD-Fc fusion protein	*N. benthamiana* (Plants)	Alum	Elicited high neutralization titers Induced a mixed Th1/Th2 immune responses and vaccine-specific T-lymphocyte responses	(101)
Recombinant SARS-CoV-2 RBD in a glycosylated and deglycosylated form	*N. benthamiana* (Plants)	0.3% Alhydrogel	- Elicited high titers of antibodies with a potent virus-neutralizing activity	(11)
Monomeric and Dimeric RBD proteins	*P. pastoris* (Yeast)	Alum	- Elicited neutralizing antibodies and long-term immunization against SARS-CoV-2 infection	(102)
Recombinant RBD219-WT and RBD219-N1C1	*P. pastoris* (Yeast)	Alhydrogel® (aluminum oxyhydroxide)	- Induced high binding of IgG antibodies after second dose and high neutralizing antibody titer	(103)
Trimeric and monomer form of RBD protein	HEK293 cells	Aluminum hydroxide or Alum-3M-052	- Trimeric form of RBD induced higher neutralizing antibodies response than RBD monomer - alum-3M-052 adjuvant induced higher neutralizing antibodies response than alum adjuvant	(104)

Expression system for recombinant protein

The expression system for the recombinant protein has many systems to use, but they have different advantages and disadvantages which can choose to produce the recombinant protein, as suitable show in **Table 4**.

Table 4: The comparison of advantages and disadvantages of the expression system for the recombinant protein[105]

Expression system	Advantages	Disadvantages
Bacteria	- Low cost and Easy to manipulate - High expression and Ease of scale up - Short turnaround time - Established regulatory procedures and approval	- Improper folding - Lack of post-translational modifications which may affect the protein function - Endotoxin accumulation
Mammalian cells	- Proper folding and authentic post-translational modifications - Existing regulatory approval	- High production cost - Expensive media and culture condition requirements
Yeast	- Easy to manipulate, Rapid growth, and scalable - Simple and inexpensive media requirements and culture conditions - Post-translational modifications of recombinant proteins	- Difficulty in cell disruption due to the thick and hard cell walls - Hyperglycosylation of proteins - Limited glycosylation capacity
Insect cells	- High expression levels - Ability to produce complex proteins including secreted, membrane, and intracellular proteins - Proper folding and post-translational modifications	- High cost and time consuming - Expensive media and culture condition requirements
Plant	- Economical, Rapid, and affordable - Optimized growth conditions - Free from pathogen and bacterial toxin contaminants - Post-translational modification somewhat similar like mammalian system	- Regulatory compliance - Limited glycosylation capacity

As a result, this study chooses the molecular farming because it is a platform that uses plants for recombinant protein products or diagnostic reagents for over 30 years (2, 106). The utilization of the plants has many advantages, for example, low cost to grow the plants, agricultural output has enormous scalability, and the intrinsic safety of plants as hosts for pharmaceutical manufacture (2, 106). For this reason, this study uses this platform to produce the recombinant protein that is tobacco in the species of *N. benthamiana*. This plant is from Australia and has many characteristics; for example, can harvest in all seasons, has a fast growth rate, and can express sequences of heterologous gene, which is why this plant is suitable for biopharmaceuticals product (9, 10). However, efficient strategies for temporary expression of recombinant proteins in *N. benthamiana* have been developed, which involve agrobacteria that have a DNA transgene of the interested protein using the infiltration method into the leaf. Therefore, it has many diagnostic proteins or

therapeutics that produce from *N. benthamiana* plants by using agroinfiltration method (10).

The genetic transformation of the plant uses the *Agrobacterium tumefaciens* as equipment to insert the gene of interest into the plant because utilization of the *A. tumefaciens* can show a transient expression in high level (107). The process of plant genetic transformation mediated by *A. tumefaciens* requires two genetic elements discovered on the bacterial Ti-plasmid. T-DNA is the first element, determined by border sequences, which are preserved 25-base pair incomplete repetitions at the T-region extremities. The virulence (vir) region is the second element, which contains at least seven mains namely *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, and *virG*, that code for elements of the bacterial protein equipment that mediates the processing and transfer of T-DNA. The two-component regulators VirA and VirG stimulate the additional *vir* genes to express on the Ti-plasmid, and T-DNA from *A. tumefaciens* is processed, transferred, and integrated into a plant cell by the VirB, VirC, VirD, VirE, and maybe the VirF (108) as show the process of plant transformation mediated by *A. tumefaciens* in **Figure 7**.

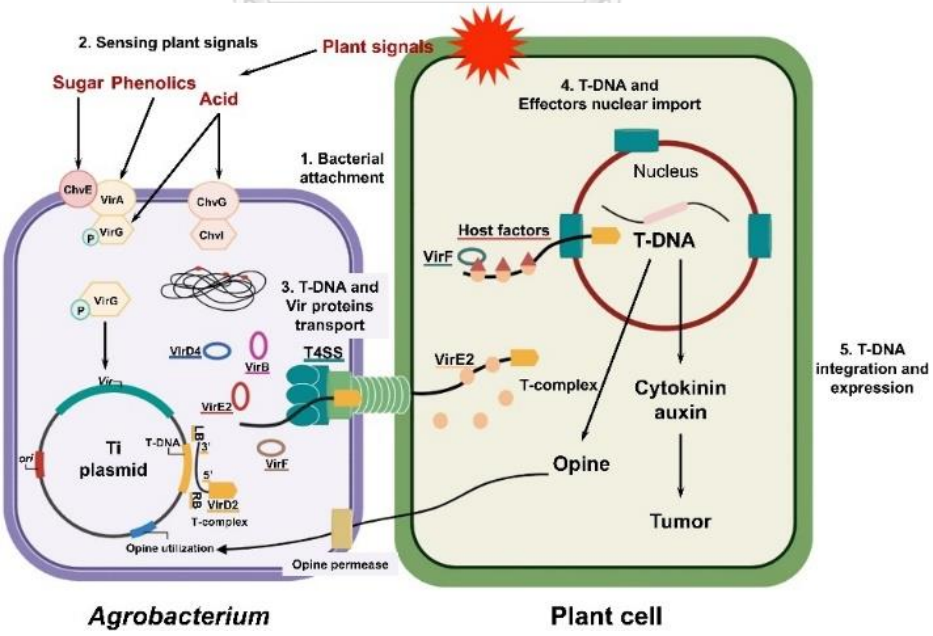

Figure 7: the process of plant transformation mediated by A. tumefaciens (modified from (108))

In addition, genetic transformation has two types: stable transformation and transient transformation (109).

1) Stable transformation is a time-consuming procedure that frequently requires the use of proven tissue culture methods to support full plant development from changed cells or tissues including the T-DNA must integrate into the genome of the host cell in order to be passed on to the following generation (108).

2) Transient transformation is a method that expresses the T-DNA lasts for a few days, and T-DNA incorporation into the genome of host is not necessary and has an advantage of the experimental outcomes therapies to be evaluated in a short duration of time (108).

For these reasons, this study uses transient transformation because it is a faster, easier, and less time-consuming way of analyzing specific genes (107). The transient transformation procedure uses agroinfiltration works on the concept that foreign DNA can stay in the nucleus for a short time before integrating into the plant genome. Moreover, agroinfiltration has two methods; syringe and vacuum infiltration, which have fast and notable the protein expression (110).

Protein purification

Protein purification methods are used in three types of proteins: natural protein, recombinant protein, and protein complex mixtures (111). In addition, the purification method for the protein has to use a method that is suitable for the type of protein, which in this study uses the recombinant protein. Therefore, the suitable purification method for the recombinant protein is affinity chromatography purification because this method can isolate the target protein from other proteins that extract from expression cells by capturing the beads that pack into a column, then the proteins are washed past through the column, and the target protein is attached to the bead by the affinity matrix before eluting the target protein and identify the target protein by mass spectrometry or the other proper method (112). However, using this method

has to use the tag that fusion with the protein to purify the recombinant protein with the high purity and efficient recovery from the expression host (113).

The fusion tags are used for making protein stabilized, chromatography purification of the protein, or other purposes (113). For example, the dual hexahistine (His6)-MBP tag can design to get the protein production quickly in one step, or MBP or glutathione-S-transferase is a fusion tag that can function in affinity and solubility state (114). However, this study is interested in the Fc tag because it makes the purification method faster, has a somewhat lengthy half-life, and can increase the target antigen immunogenicity. Moreover, the Fc tag can encourage the fusion protein to have right folding and improve the antigen-presenting cell binding (7). The Fc tag is from a human IgG1 Fc fragment, which is an antibody composed of the same two light chains and the same two heavy chains, which have a variable and constant region. Each chain consists of an antigen-binding variable region in a Fab region (fragment antigen-binding region) of the N-terminal, and the constant region in C-terminal is the Fc region, which in two chains adhere with a disulfide bond in the hinge region (**Figure 8**) (115).

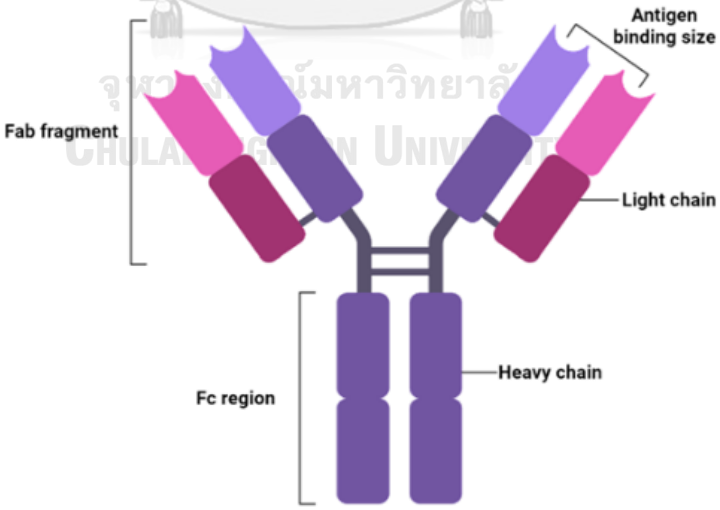

Figure 8: The IgG1 molecule structure

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials and Equipment

2.1.1. Genetic materials

- A geminiviral-based plant expression vector (pBYR2eK2Md; pBYR2e)[116]
- The gene of SARS-CoV-2 S1 subunit protein
  (Genbank accession number: YP_009724390.1)
- The Fc region of human immunoglobulin G1 (IgG1)
  (GenBank accession number: 4CDH_A)

2.1.2. Biological materials

- *Agrobacterium tumefaciens* strain GV3101
- *Escherichia coli* strain DH10B
- Four-week-old female ICR mice from Faculty of Medicine, Chulalongkorn University
- Tobacco plants (*N. benthamiana*)
- SARS-CoV-2 spike peptides (Mimotopes, Australia)

2.1.3. Equipment

- ELISpot reader (ImmunoSpot® Analyzer, USA)
- GENESYSTM UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA)
- Hettich® Universal 320/320R centrifuge (Andreas Hettich GmbH & Co. KG, Germany)
- Micropipette 2-1000 µL (Pipetman®, USA)
- Microplate incubator (Hercuvan Lab systems, Malaysia)
- MicroPulser Electroporator (Bio-Rad®, USA)
- Mini Centrifuge (Bio-Rad®, USA)
- Mini-PROTEAN® Tetra handcast systems (Bio-Rad®, USA)
- Mini Trans-Blot® cell (Bio-Rad®, USA)
- MJ Mini Thermo Cycler Machine (Bio-Rad®, USA)

- Multichannel pipette (Cleaver scientific, UK)
- Mupid-EXu Electrophoresis (Mupid Co., Ltd, Japan)
- OTTO® Blender (OTTO, Thailand)
- SpectraMax® M5 Microplate Reader (Molecular Devices LLC, USA)
- TOMY Autoclave sx series (Amuza Inc., Japan)
- WIS-20 Precise Shaking Incubator (WiseCube®, Korea)

2.1.4. Materials

- 0.22 µm Syringe filter (MilliporeSigma, USA)
- 0.45 µm Membrane filter (MilliporeSigma, USA)
- 0.45 µm Nitrocellulose Membrane (Bio-Rad®, USA)
- 1.5 mL graduated microcentrifuge tube (Molecular BioProducts, USA)
- 1 mL Pipet tips (Axygen, China)
- 1-200 µL Pipet tips (Molecular BioProducts, USA)
- 0.1-20 µL Pipet tips (Molecular BioProducts, USA)
- Centrifuge tube 0.2-50 mL (Axygen®, USA)
- Cuvette
- DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc., Korea)
- High binding microplate-96 well (Greiner bio-one, Germany)
- MEGA quick-spinTM Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Inc., Korea)
- Nitrocellulose membrane plate (96 wells) (Merck, USA)
- NIPROTM Disposable Syringe 1, 10, 20, and 50 mL and needle (Nipro, Thailand)
- PCR tubes/strips (Axygen®, USA)
- Petri-dish (Corning, USA)
- Protein-A beads (Expedeon, UK)
- Purification column

2.1.5. Proteins and antibodies

- Anti-mouse IFN-γ (AN18) monoclonal antibody (mAb) (Mabtech, Sweden)
- Anti-mouse IFN-γ-biotinylated mAb (Mabtech, Sweden)
- Goat anti-mouse IgG-HRP conjugated antibody (Jackson ImmunoResearch, USA)
- Goat anti-mouse IgG1-HRP conjugated antibody (Abcam, UK)
- Goat anti-mouse IgG2a-HRP conjugated antibody (Abcam, UK)
- Plant-produced anti-SARS-CoV-2 (H4) monoclonal antibody
- SARS-CoV-2 spike peptides (Mimotopes, Australia)
- SARS-CoV-2 RBD-His tag from Sf9 cells (Genscript, USA)

2.1.6. Immunoadjuvants

- Alhydrogel® 2% (Aluminium hydroxide gel adjuvant) (CRODA, Denmark)

2.1.7. Chemical reagents

- 1x Phosphate Buffer Saline (PBS) (Cytiva, USA)
- 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) (Bio Basic Inc., Canada)
- 2-mercaptoethanol (Sigma-Aldrich, USA)
- 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; BCIP/NBT substrate (Mabtech, Sweden)
- 50X Tris-Acetate-EDTA (TAE) Buffer, pH8.0, Ultra-Pure Grade (Vivantis, Malaysia)
- β-merceptoethanol (Applichem, Germany)
- Acrylamide/Bisacrylamide 40% (HiGenoMB®, India)
- Agar powder (Titan Biotech Ltd., India)
- Agarose powder (Vivantis, Malaysia)
- Amersham ECL prime western blotting detection reagent (GE Healthcare, UK)
- Ammonium Chloride (NH_4Cl) (HIMEDIA®, India)
- Ammonium Persulfate (APS) (HIMEDIA®, India)
- Antibiotic-Antimycotic (100x) (GIBCO, USA)
- Antibiotics

  - Ampicilin (Panreac AppliChem®, USA)
  - Gentamycin (Panreac AppliChem®, USA)
  - Kanamycin sulfate (Panreac AppliChem®, USA)
  - Rifampicin (Bio Basic Inc., Canada)
- Concanavalin A (ConA) (Sigma, USA)
- Deoxynucleoside triphosphate (dNTP): dATP, dCTP, dGTP, dTTP
- Di-Sodium hydrogen phosphate (Na_2HPO_4) (Merck, USA)
- Dimethyl Sulfoxide (DMSO; C_2H_6OS) (Sigma-Aldrich, USA)
- Ethanol (EMSURE®, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (HIMEDIA®, India)
- Gel Loading Dye-purple 6x (New England Biolabs, USA)
- Glycerol (HIMEDIA®, India)
- Glycine (HIMEDIA®, India)
- Magnesium sulfate ($MgSO_4$) (KEMAUS®, Australia)
- Peptone (HIMEDIA®, India)
- Phosphate Buffer Saline (Sigma, USA)
- Potassium chloride (KCL) (Merck, USA)
- Potassium dihydrogen phosphate (KH_2PO_4) (Merck, USA)
- Protein ladder (Bio-red®, USA)
- Restriction enzymes: *Xba*I, *BamH*I, *Sac*I (New England Biolabs, UK)
- RPMI 1640 media with phenol red (GIBCO, USA)
- Skim milk (BD DifcoTM, USA)
- Sodium chloride (NaCl) (Merck, USA)
- Sodium dodecyl sulfate (SDS) (Carloerbareagets, Italy)
- Streptavidin-alkaline phosphatase (ALP) (Mabtech, Sweden)
- Sulfuric acid (H_2SO_4) (RCI labscan, Thailand)
- T4 DNA ligase (New England BioLabs, UK)
- Taq DNA polymerase (Vivantis, Malaysia)
- Tetramethylethylenediamine (TEMED) (Affymetri®, USA)
- TMB stabilized substrate (Promega, USA)
- Tris-base (Vivantis®, Malaysia)

- Tween -20 (Vivantis®, Malaysia)
- Tween-20 (Sigma, USA)
- VC 1 kb DNA Ladder (Vivantis®, Malaysia)
- ViSafe Green Gel Stain (Vivantis®, Malaysia)
- Yeast Extract (Himedia Laboratories Pvt. Ltd., India)

2.1.8. Software and database

- ClustalOmega Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/)
- ExPAsy Bioinformatics Resource Portal (https://web.expasy.org/translate/)
- GenBank NCBI: NIH genetic sequence database (https://www.ncbi.nlm.nih.gov/genbank/)
- GeneArt Gene Synthesis Portal (https://www.thermofisher.com/order/geneartgenes/projectmgmt)
- GraphPad Prism software version 9.0.
- National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/)
- NEBcutter V2.0 (New England Biolabs, UK) (http://nc2.neb.com/NEBcutter2/)

2.1.9. Facilities

- Certified biosafety level III facility, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand
- Greenhouse for *N. benthamiana*, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
- Hygienic conventional mouse housing system, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

2.2. Experimental Procedures

2.2.1. Gene cloning of SARS-CoV-2 S1-Fc

The SARS-CoV-2 S1 (Genbank accession number: YP_009724390.1) was cloned along with a peptide linker at the C-terminus and linked with the Fc region of human immunoglobulin G1 (IgG1) (GenBank accession number: 4CDH_A) (**Figure 9**).

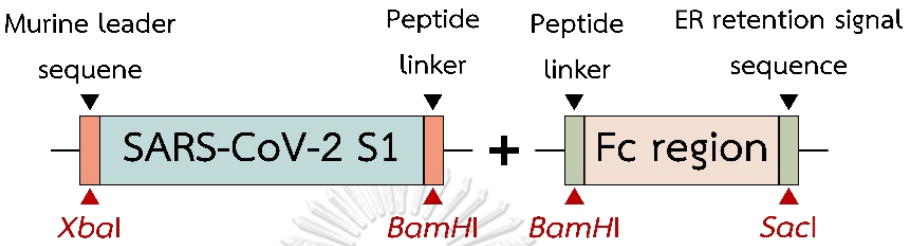

Figure 9: Schematic gene design of SARS-CoV-2 S1 and Fc region

The nucleotide sequence of SARS-CoV-2 S1 was amplified from total DNA of S1-His protein plasmid by the Polymerase chain reaction (PCR) method with the specific primer pairs, which contains *Xho*I S F/*BamH*I GS R (U2Bio, Thailand; **Table 5**) as a primer to generate cDNA for the S1 gene with the PCR mixture show in **Table 6**.

Table 5: The primer pairs use for the construction of SARS-CoV-2 S1-Fc gene

Name	Primer sequence
*Xba*I-SP F	5'-TCTAGAACAATGGGCTGG-3'
*BamH*I-GS R	5'-CGGGATCCACCACCACCAGAGATATCTCTAGCCCTTCTAGGAG-3'
*Xho*I S-plant-F	5'-GGGCTCGAGGGGATGTTCGTGTTCCTTGTGCTGCTTCCGCTTGTGTCATCTCAGTGCG-3'

Table 6: The PCR component for construction of SARS-CoV-2 S1-Fc

The PCR mixture	Volume (µL)
pfu enzyme	0.5
$MgCl_2$	1
10 mM primer	1
dNTPs	3
5X pfu Buffer	6
template	0.5
pure water	18
Total	**30**

The amplification conditions show in **Table 7** before resolving the PCR product by electrophoresis in a 0.8% agarose gel and visualized under UV illumination. Then the constructed S1 sequence and Fc region was ligated into pGEMT-SP vector with the component of the ligation mixture on **Table 8** then incubated overnight at 4 ℃.

Table 7: Conditions of PCR reaction

Reaction steps	Temperature	Time
Initial denaturation	94 ℃	5 min
Denaturation	94 ℃	30 sec (28 cycles)
Annealing	63 ℃	30 sec
Extension	72 ℃	2 min (1 min/1kb)
Final extension	72 ℃	5 min
Storage	4 ℃	10 min

Table 8: The component of ligation mixture

The ligation mixture	Volume (µL)
2X ligation buffer	5
50 ng pGemT-Easy vector	1
T4 DNA ligase	1
PCR product	3
Total	**10**

After ligation, the pGEMT-SP-S1-Fc plasmid was artificially transformed into the *E. coli* competent cells (DH10B strain) by heat shock method. Briefly, start from dissolving the competent cell of *E.coli* in ice and use 100 µL into the ligation then incubated 20 – 30 minutes in ice before bringing the ligation to heat shock at 42 ℃ for 1 minute after that bringing the ligation on ice immediately 2 minutes then add the media 500 µL and incubated at 37 ℃ for 1 hours then spread plate with the 100 mg/mL ampicillin (ITW Reagents, Darmstadt, Germany) media and incubated at 37 ℃ overnight. After that, the colonies were selected and verified by Colony PCR using the specific S1 gene primers (**Table 5**) then grown in Luria Bertani broth medium with 100 mg/mL ampicillin overnight at 37 ℃. The clone of S1-Fc fragment

in pGEMT-SP vector was obtained, and nucleotide sequences of S1-Fc plasmids was confirmed by DNA Sanger sequencing (U2Bio, Thailand).

2.2.2. Generation of pBYR2-SARS-CoV-2 S1-Fc expression vector

The nucleotide sequences coding for SARS-CoV-2 S1-Fc was excised from the pGEMT cloning vectors by *Xba*I/*Sac*I as a restriction enzyme (New England BioLabs, Ipswich, MA, USA) and inserted into geminiviral pBYR2e expression vector containing the similar restriction endonucleases (**Figure 10**), which contains the ligation mixture in **Table 9** and incubated overnight at 4 °C.

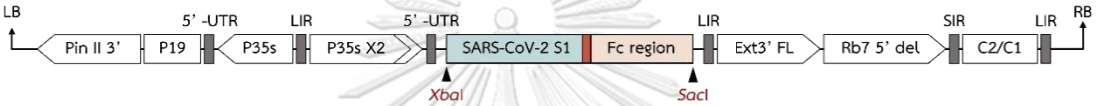

Figure 10: Schematic construction of SARS-CoV-2 S1-Fc in expression vector for expression in *N. benthamiana*

Table 9: The ligation mixture for ligation into the geminiviral vector pBYR2eK2Md (pBYR2e)

The ligation mixture	Volume (µL)
10X ligation buffer	1
enzyme ligase	1
pBYR2e vector that digest with *Xba*I/*Sac*I	1
The SARS-CoV-2 S1 gene with the *Xba*I/*BamH*I digestion	3
the Fc region gene with the *BamH*I/*Sac*I digestion	3
Total	**10**

The ligation of pBYR2e-SP-S1-Fc plasmid was transformed into *E. coli* competent cells (DH10B strain) by heat shock method. Then, the transformed competent cells were spread on Luria Bertani agar plate comprising 50 µg/mL kanamycin (Bio Basic, Markham, ON, Canada) and incubated at 37 °C overnight. After that, the colonies were selected and verified by Colony PCR using the specific primer pairs (**Table 5**) to detect presence of S1-Fc gene. The PCR positive transformants was cultured in 5 mL of Luria Bertani broth medium with 50 µg/mL kanamycin and incubated at 37 °C on

rotary shaker at 250 rpm overnight. Finally, the plasmid was extracted from the bacterial culture by using the AccuPrep Nano-Plus Plasmid Mini Extraction kit protocol (Bioneer, Korea) and stored in -20 ℃

2.2.3. Gene transformation into *A. tumefaciens* GV3101 strain

The recombinant plasmids of the SARS-CoV-2 S1-Fc gene was cloned into the geminiviral expression vector pBYR2eK2Md (pBYR2e) and transformed into *A. tumefaciens* (GV3101 strain) using the electroporation method with MicroPulser (Bio-Rad, USA). The transformed *A. tumefaciens* cells were spread on a Luria Bertani agar plate contained with 50 μg/mL kanamycin, 50 μg /mL rifampicin (Thermo Fischer Scientific, Waltham, MA, USA), and 50 μg/mL gentamicin (ITW Reagents, Darmstadt, Germany) and incubated for 48 hours at 28 ℃. After that, the colonies were selected and verified by Colony PCR to check the success of gene insertions. Then, the positive transformants were cultured in a 5 mL Luria Bertani medium with 50 μg /mL kanamycin, rifampicin, and gentamicin for 16 hours at 28 ℃. The *A. tumefaciens* suspension containing the recombinant of SARS-CoV-2-S1-Fc plasmid was inoculated into a 1:100 freshly culture and propagated on a rotary shaker at 250 rpm for 16 hours at 28 ℃. Finally, the growth of bacterial cells is blocked to prepare for plant transfection.

2.2.4. Modified Agroinfiltration of *N. benthamiana* leaves

Transformation on *N. benthamiana* leaves were used agroinfiltration by syringe and vacuum. *A. tumefaciens* suspensions were centrifuged at 8,000 rpm for 5 minutes at 4 ℃ to obtain the cell pellet. Then, the cell pellet was resuspended with optimal density at 600 nm (OD_{600}) of 0.2 in 1X infiltration buffer (10mM 2-N-morpholino-ethanesulfonic acid (MES) and 10mM $MgSO_4$, pH 5.5). The seedlings of *N. benthamiana* were transferred into sterile soil and grown in pots for 6 weeks at a constant temperature of 28 ℃ with 16 hours of daily illumination. For day optimized infiltration was injected *Agrobacterium* solution (OD_{600} = 0.2) containing the SARS-

CoV-2 S1-Fc gene using a 1 mL syringe without a needle onto the underside of *N. benthamiana* leaves and collected on 2-, 4-, 6-, 8-, and 10 days post-infiltration (dpi). After day optimization, the infiltration for producing the recombinant SARS-CoV-2 S1-Fc protein was used 2 liters of *Agrobacterium* solution that contained SARS-CoV-2 S1-Fc gene (OD_{600} = 0.2) to submerge *N. benthamiana* leaves at an invert position. Then, the vacuum was applied for 2 minutes at 600 - 760 mmHg and drawn out the pressure very gently to atmospheric pressure. The infiltrated *N. benthamiana* leaves were maintained in an indoor plant room with the regulated growing condition. The leaves were collected on 4 days post-infiltration (dpi) to quantify the optimization of SARS-CoV-2 S1-Fc protein expression and was stored in -80 °C.

2.2.5. SARS-CoV-2 S1-Fc Protein Extraction

The day optimization infiltrated leaves were ground with a ratio of 1:2 of leaves weight and cold 1X PBS extraction buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4) using a Homogenizer pestle. Before, suspend the crude by using the microcentrifuge for 15 minutes. In addition, the infiltrated leaves were homogenized by a blender containing cold 1X PBS extraction buffer for at least 2 minutes. Following, crude extracts were precipitated by using a centrifuge at 13, 000 g for 1 hour and 30 minutes, 4 °C. The resulting supernatant was filtered by a sterile 0.45 µm membrane filter (Merck, Massachusetts, United States).

2.2.6. SARS-CoV-2 S1-Fc Protein Purification

The plant-produced SARS-CoV-2 S1-Fc purification was used with Protein A affinity chromatography. Firstly, the column was packed with Protein A bead (GE healthcare) and equilibrated by loading three column volumes of 1X PBS buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4). The total filtered plant extract was loaded onto the column. The bead was washed with 40 mL of 1X PBS buffer as a binding buffer to simplify the efficient elution of weakly bound nonspecific proteins. Finally, the plant-produced SARS-CoV-2 S1-Fc was eluted with

100mM glycine at pH 2.7 and neutralized with 1.5M Tris-HCl at pH 8.8. The protein fraction of purified protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration of purified protein was measured by the sandwich ELISA method.

2.2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

The purified plant-produced protein was analyzed by SDS-PAGE and Western Blot. The total soluble protein from crude extraction and purification was mixed with non-reducing loading buffer (125mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue) and reducing loading buffer (125mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue), heated at 95 ℃ for 5 minutes and separated on 4 – 15% SDS-PAGE gel. The separation of 4 – 15% SDS-PAGE gel under non-reducing and reducing conditions was conducted in 1X running buffer (25mM Tris, 192mM glycine, and 1% SDS) for 1 hour. The plant-produced protein was either visualized by InstantBlueTM (Expedeon, UK) staining solutions and transferred from gel to nitrocellulose membrane (Bio-Rad, USA) 2 hours in 1X transfer buffer (25mM Tris, 192mM glycine and 15% methanol) by using the constant voltage at 100V. The membrane was blocked with 5% skim milk (BD Difco, USA) in 1X PBS for 30 minutes. Then, the membrane was probed with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Southern Biotech, Alabama, USA) diluted 1:10,000 in 3% skim milk for 2 hours. The membrane was washed and developed by enhanced chemiluminescence (ECL) plus detection reagent (Thermo Fisher Scientific, USA) before exposed with Chemiluminescent ImageQuant^{TM} LAS500.

2.2.8. SARS-CoV-2 S1-Fc Quantification by Enzyme-linked immunosorbent assay (ELISA)

The production of plant-produced SARS-CoV-2 S1-Fc was quantified by sandwich ELISA. A 96-well microplate (Greiner Bio-One, Germany) was coated with 50 µL of 2 µg/mL monoclonal antibody H4 diluted in 1X PBS buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4) and incubated overnight at 4 ℃. The microplate was washed three times with 200 µL of PBST buffer (0.05% Tween 20 in 1X PBS). Besides, the 96-well plate was blocked with 200 µL of 5% skim milk (BD Difco, USA) in 1X PBS for 2 hours at 37 ℃. Then, the plate was washed three times with PBST before being loaded with the preparation of SARS-CoV-2 spike protein RBD from Sf9 insect cells (GenScript, USA) as a standard and sample into the well and incubated for 2 hours, 37 ℃. Afterward, the plate was washed three times and incubated with SARS-CoV-2 Spike antibody (HRP) (Sino Biological, Beijing, China) diluted 1:1000 in 1X PBS for 1 hour at 37 ℃. Finally, the plate was washed three times with PBST before being developed by a TMB One Solution (Promega, USA) and incubated for at least 2 minutes. The reaction was stopped by 50 µL of 1M H_2SO_4 and measured at 450 nm with a 96-well plate reader (BMG Labtech, Germany)

2.2.9. Immunization of Mice with SARS-CoV-2 S1-Fc protein

The protocol for immunization of mice was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chulalongkorn University On days 0 and 21, immunized the four-week-old female ICR mice (*n* = 5 per group) by intramuscularly (IM) with 10 µg of SARS-CoV-2 S1-Fc protein prepared with 0.1 mg aluminum hydroxide gel adjuvant (CRODA, Denmark). Mice serum were obtained prior to the immunization (pre-bleed) and 14 days after each vaccination to estimate the antigen-specific immune response and quantitative analysis of SARS-CoV-2 RBD-specific T-cell responses. Then mice were euthanized 14 days after the second dose (day 35).

2.2.10. Evaluation of RBD-Specific antibody responses

A 96-well microplate (Corning, USA) was coated with 50 µL of 2 µg/mL SARS-CoV-2 spike protein RBD from Sf9 insect cells (GenScript, USA) diluted of in 1X PBS buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4) and incubated overnight at 4 ℃. The microplate was washed three times with 200 µL of PBST buffer (0.05% Tween 20 in 1X PBS). Besides, the 96-well plate was blocked with 200 µL of 5% skim milk (BD Difco, USA) in 1X PBS for 2 hours at 37 ℃. Subsequently, the microplate was washed three times with 200 µL of PBST buffer before being loaded on the wells of the mice sera were diluted in 1X PBS with a two-fold serial condition and starting at 1:100 with a volume of 50 µL and incubated for 2 hours at 37 ℃. Then, the plate was washed three times with PBST before being loaded 50 µL of goat anti-mouse IgG HRP conjugate antibody (Jackson ImmunoResearch, USA) diluted 1:2000 in 1X PBS and incubated for 1 hour at 37 ℃. Finally, the plate was washed three times with PBST before being developed by using a TMB stabilized substrate (Promega, USA) and incubated for 5 minutes. The reaction was stopped by 50 µL of 1M H_2SO_4 and measured at 450 nm with a microplate reader (Molecular Devices, USA). For analysis of mouse IgG1 and IgG2a, the mice sera were diluted in 1X PBS with a two-fold serial condition and starting at 1:100 in the same method and loaded 50 µL of goat anti-mouse IgG1 (HRP) and goat anti-mouse IgG2a heavy chain (HRP) (Abcam, UK) that diluted 1:2000 in 1X PBS, respectively.

The endpoint titers were calculated as the highest dilution of vaccinated sera with A450 greater than the cutoff computed from A450 of pre-immunized sera in a 1:100 dilution in 1X PBS using the equation below (117).

$$Cut\ off = \bar{X} + SDf$$

$$SD = \sqrt{\frac{\sum(x_i - \bar{X})^2}{n-1}} \qquad f = t\sqrt{1 + (1/n)}$$

When $\bar{X}$ is mean of independent control pre-immunized sera reading from ΔA450

SD is standard deviation

x_i is the individual ΔA450 independent control sample

n is the number of independent controls

f is standard deviation multipliers, tis the $(1-\alpha)^{th}$ percentile of the one-tailed t-distribution with v = n−1 degrees of freedom, and α is significance level.

The statistical analyses of immunogenic data were plotted as geometric mean titer (GMT) by GraphPad Prism software version 9.0. Two-way analysis of variance (ANOVA) was used to determine statistical significance. Tukey multiple comparisons test was used to compare all results in each group, and $p< 0.05$ (*: <0.05, **: <0.01, ***: <0.001 and ****: <0.0001) was considered statistically significant.

2.2.11. Mouse IFN-γ ELISpot assay

The IFN-γ ELISpot assay (Mabtech, Stockholm, Sweden) was used to determine the cells secreting mouse IFN-γ by SARS-CoV-2-specific cells. The splenocytes were resuspended at $5x10^6$ cells/ml in R5 medium. 96-well nitrocellulose membrane plates (Millipore, Bedford, MA, USA) was coated with 10 μg/mL anti-mouse IFN-γ (AN18) monoclonal antibody (mAb) (Mabtech, Sweden) in phosphate buffer saline (PBS) 50 μL/well with 5% CO_2 for 3 hours at 37 ºC. After that, the plates were washed with 200 μL/well of PBS six times and blocked with R10 medium for 1 hour at room temperature (RT). The quantity of $5x10^5$ splenocytes/well was cultured with 2 μg/mL of SARS-CoV-2 peptide pools with 5% CO_2 for 40 hours at 37 ºC. The negative was cultured only in the medium and the positive control was used concanavalin A (ConA). After incubation, the plates were washed with 200 μL/well of PBST (1X PBS contained 0.05% tween20) six times and three times with 1X PBS. Then, the plates were incubated with anti-mouse IFN-γ-biotinylated mAb at a final concentration 1 μg/mL (R4-6A2 biotin; Mabtech, Sweden) in PBS for 3 hours at RT.

After washing was added the streptavidin-alkaline phosphatase (ALP; Mabtech, Stockholm, Sweden) and incubated at RT for 1 hour. Then, the substrate solution (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; BCIP/NBT) was added 100 µL into each well after washing the plates. The spots were developed until distinct spots appeared. The reaction was stopped by washing extensively in tap water and rinsing the underside of the membrane. Then, the plates were dried before counting the spots by the ELISpot reader (ImmunoSpot® Analyzer, USA). After subtracting the negative controls, the results are reported as spot-forming cells (SFCs)/10^6 splenocytes. A positive response was defined as more than three standard deviations above the background wells, i.e., more than 50 SFCs/10^6 splenocytes. Moreover, each sample had a negative control of culture media alone and positive control of ConA to assess the quality and quantity of the splenocytes using the mouse IFN-γ ELISpot assay.

2.2.13. Statistical analysis

All statistical analyses were performed by using GraphPad Prism 9.0. In total IgG and IgG subclasses studies were used two-way analysis of variance (ANOVA), Tukey test, multiple comparison to calculate the results. In IFN-γ ELISpot assay were used Mann-Whitney test to calculate the result. All p values < 0.05 were defined as significant.

CHAPTER 3

RESULTS AND DISCUSSION

The emergence of SARS-CoV-2 makes the world concerns about the development of vaccines to decrease the mortality and the infection of SARS-CoV-2 (3, 118). For this reason, there are many studies to develop COVID-19 vaccines, which have many types of vaccines emergent in the present such as mRNA vaccine, viral vector-based vaccine, and protein subunit vaccine (119-121). Especially, these vaccines almost use the spike protein to develop the COVID-19 vaccines because this structure is easy to find on the surface of the virus particles and involve in the entry into the host cell (3). Moreover, the spike protein has two components that involve in binding to the host cell receptor and fusing to the host cell namely, the S1 subunit and S2 subunit, respectively (3, 80).

Since the widespread of the virus, it requires platform for producing antigens in a short time to make a recombinant protein vaccine. Plants are a suitable platform to use in this situation because plants have an advantage over other expression systems in terms of cost, safety, and speed of production and it has been used for producing biopharmaceuticals for more than 30 years ago (2, 122-125). In addition, plants are used for producing the recombinant antibodies, antibody fragments and antibody fusion proteins because they both have a significant commercial value as pharmaceuticals, are easy to characterize, and are stable (126). Therefore, many studies indicated the plant-derived biopharmaceuticals and vaccines such as taliglucerase alfa (ELELYSO^{TM}), H5N1 influenza virus-like particle, and Chimeric anti-Ebola IgG cocktail (127, 128).

For these reasons, this study interested in the S1 subunit because the S1 subunit has many expose epitopes that can be neutralized. For example, the receptor binding domain (RBD) from S1 subunit, which has many results reveal that it can induce an immune response and neutralizing antibodies (11, 34) and the full length

of S1 subunit also can stimulate the immune response and the neutralization antibodies (91, 96, 129). As a result, the S1 subunit in S protein could be a target for developing the SARS-CoV-2 vaccine.

Hence, this study produced the SARS-CoV-2 S1 which fused with Fc region of human IgG1 from *N. benthamiana* plant before evaluating the immune response in mice that demonstrated the results below.

3.1. Gene cloning and Construction of SARS-CoV-2 S1-Fc expression vector

The gene sequence of SARS-CoV-2 S1 was amplified from total DNA of S1-His protein plasmid by the Polymerase chain reaction (PCR) method with the specific primer pairs and got the size around 2,000 base pairs by electrophoresis in agarose gel shown in **Figure 11A**. Then the SARS-CoV-2 S1 was fused with the Fc region of human IgG1 at the C terminus of the S1 gene construct by ligating into pGEMT-SP vector, which confirmed by electrophoresis in agarose gel, that obtained the size around 2,700 base pairs (**Figure 11B**).

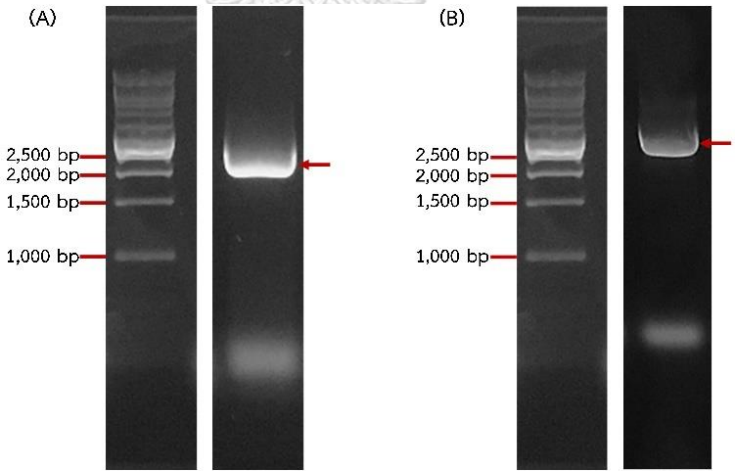

Figure 11: The SARS-CoV-2 S1 gene in 1% agarose gel. **(A)** SARS-CoV-2 S1 gene and **(B)** pGEMT-SARS-CoV-2 S1-Fc gene from transformant *E. coli* by PCR colony

After obtaining the SARS-CoV-2 S1-Fc sequence in the pGEMT vector, the SARS-CoV-2 S1-Fc sequence was excised from the pGEMT cloning vectors by *Xba*I/*Sac*I as a restriction enzyme (**Figure 12A**) and inserted into geminiviral expression vector

(pBYR2e) containing the similar restriction endonucleases before confirmation the SARS-CoV-2 S1-Fc sequence by electrophoresis agarose gel, which gained the size around 2,700 base pairs shown in **Figure 12B**.

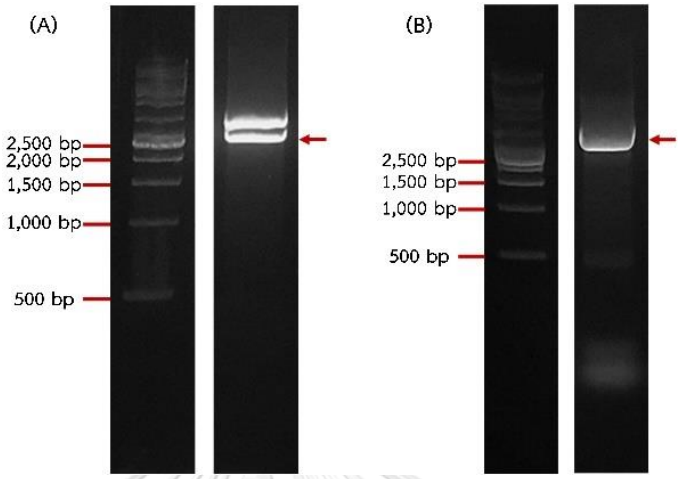

Figure 12: The pGEMT-SARS-CoV-2 S1-Fc vector in 1% agarose gel. **(A)** digestion of pGEMT-SARS-CoV-2 S1-Fc cloning vector by *Xbal/Sac*I and **(B)** ligation of pBYR2e-SARS-CoV-2 S1-Fc expression vector

3.2. Transformation of SARS-CoV-2 S1-Fc expression vector in *A. tumefaciens* and Agroinfiltration of *N. benthamiana* leaves

The pBYR2e containing the SARS-CoV-2 S1-Fc was confirmed the gene of SARS-CoV-2 S1-Fc by excising the gene from expression vector with the restriction enzyme, which has size around 2,700 base pairs shown in **Figure 13A**. The transformation of *A. tumefaciens* GV3101 strain was used an electroporation method then culture the *A. tumefaciens* containing the SARS-CoV-2 S1-Fc expression vector before confirming by PCR method, that has size around 2,700 base pairs shown in **Figure 13B**.

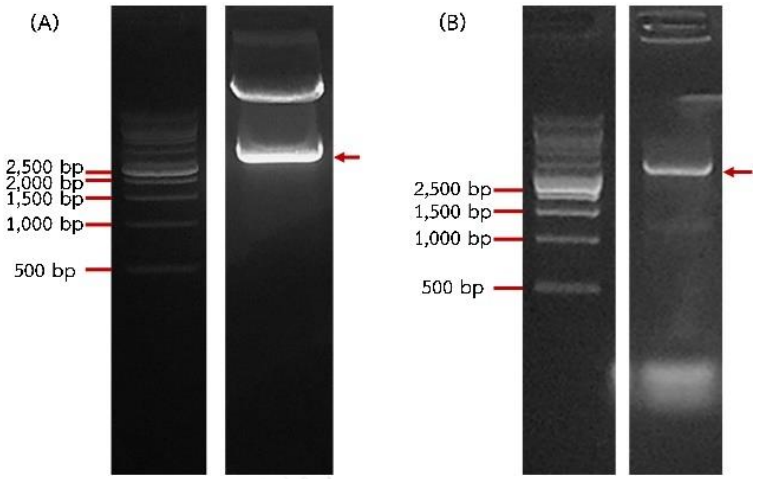

Figure 13: The pBYR2e-SARS-CoV-2 S1-Fc expression vector in 1% agarose gel. **(A)** digestion of pBYR2e-SARS-CoV-2 S1-Fc expression vector by *Xba*I/*Sac*I and (**B)** transformant of pBYR2e-SARS-CoV-2 S1-Fc in *A. tumefaciens* GV3101 strain

After that, this *Agrobacterium* was infiltrated into the *N. benthamiana* by agroinfiltration method for the time-course experiment, which harvested on 2-, 4-, 6-, 8-, and 10-days after infiltration. Then the day optimization infiltrated leaves were ground by homogenizer pestle with the extraction buffer. After extraction the yield of SARS-CoV-2 S1-Fc protein was quantified by ELISA indicated that the SARS-CoV-2 S1-Fc has the optimization time for expression on four days after infiltration with necrosis leaves compared to control (**Figure 14A**), and the amount of SARS-CoV-2 S1-Fc protein was found to be ~30 µg/g of fresh leaf weight (**Figure 14B**). These results showed that the SARS-CoV-2 S1 subunit fused with Fc domain can be expressed in *N. benthamiana* plants.

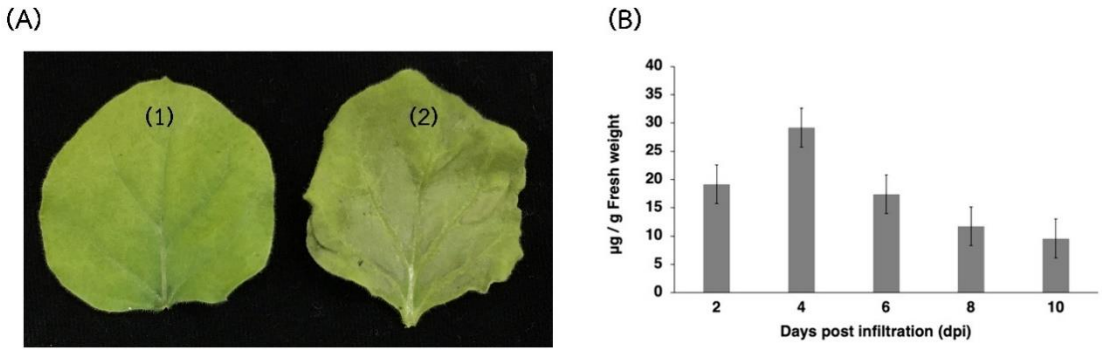

Figure 14: (A) the necrosis and the control leaves. **(B)** the optimization time of SARS-CoV-2 S1-Fc protein

3.3. The extraction, purification, and characterization of SARS-CoV-2 S1-Fc

The infiltrated leaves were homogenized by a blender with extraction buffer and precipitated by a centrifuge, then the crude extract was filtered before purification of plant-produced SARS-CoV-2 S1-Fc protein using Protein A affinity chromatography. The purified plant-produced SARS-CoV-2 S1-Fc protein was characterized by SDS-PAGE and western blot analysis (130) and the concentration of purified protein. The plant-produced SARS-CoV-2 S1-Fc protein was observed at 100–150 kDa under reducing condition (**Figure 15A; lane 1**) and 250 kDa under non-reducing condition in the InstantBlue-stained SDS gel (**Figure 15A; lane 2**). Western blot analysis with anti-human gamma chain-HRP conjugate antibody confirmed the molecular weight of SARS-CoV-2 S1-Fc at 100–150 kDa and 250 kDa under reducing and non-reducing conditions, respectively (**Figure 15B; lane 1 and lane 2**). The result showed the some of the proteins were degraded inside the plant cell, and the degraded protein can be combined together that can affect the immunogenicity response such as IgG, IgM, IFN-γ, and IL-6 expression (131). Therefore, optimization is required to enhance the protein accumulation and to prevent protein degradation. The yield of plant-produced SARS-CoV-2 S1-Fc was quantified by ELISA and observed to be 3.9 mg/ml.

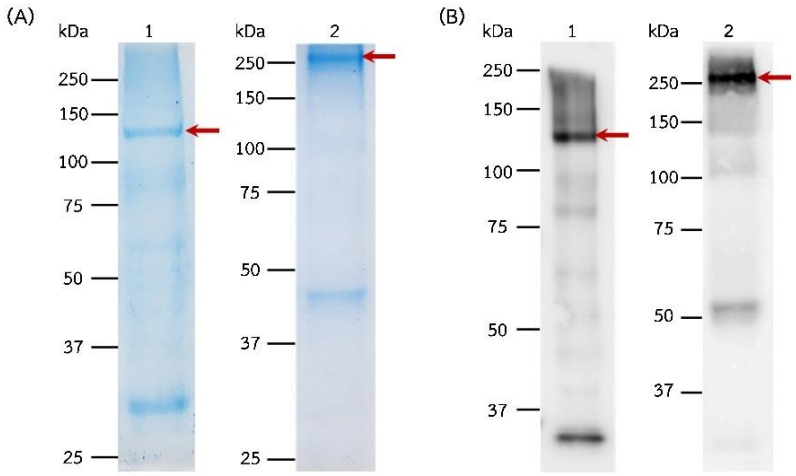

Figure 15: The expression of SARS-CoV-2 S1-Fc protein in plants. The purified SARS-CoV-2 S1-Fc protein expression was evaluated by **(A)** SDS-PAGE analysis stained with InstantBlue™ and **(B)** Western blot analysis was probed with anti-human gamma-HRP conjugate antibody. The purified SARS-CoV-2 S1-Fc protein was prepared in Lane 1 and 2 under reducing and non-reducing conditions, respectively.

3.4. The immunogenicity of plant-produced SARS-CoV-2 S1-Fc protein

Four-week-old female ICR mice (*n* = 5) was intramuscularly (IM) immunized with 10 µg of SARS-CoV-2 S1-Fc with alum and alum alone as a control. Mice serum were obtained on Day 0, Day 14, and Day 35 for measuring IgG response and collect the spleen on day 35 for evaluating the IFN-γ secretion (**Figure 16**).

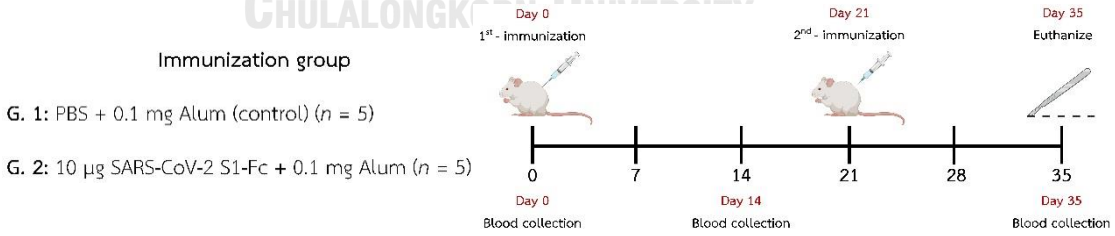

Figure 16: The schematic demonstration of immunization group and samples collection protocol on day 0, 14, and 35

SARS-CoV-2 RBD-specific response was analyzed by ELISA using Sf-9 produced SARS-CoV-2 RBD-His as a capture antigen. The SARS-CoV-2 RBD-specific total IgG antibody of S1 -Fc mouse (GMT = 9 1 9) was increased after 1 4 days of second

immunization and significantly higher than control (GMT = 115) with $p < 0.01$, but not in 1 4 days after first immunization (**Figure 17A**). Moreover, the mice sera were evaluated for the IgG subtypes. The RBD-specific-IgG1 (**Figure 17B**) and -IgG2a (**Figure 17C**) titer indicated the SARS-CoV-2 S1-Fc protein was not significantly increased the immunization higher than the control group, but RBD-specific-IgG1 titers (GMT = 1,600) was found to be higher than SARS-CoV-2 RBD-specific IgG2a titers (GMT = 459.47). In addition, the analysis of T cell helper-1 (Th-1) (mouse IgG2a) versus Th-2 (mouse IgG1) antibody responses showed it was not significant difference with the control group (**Figure 17D**).

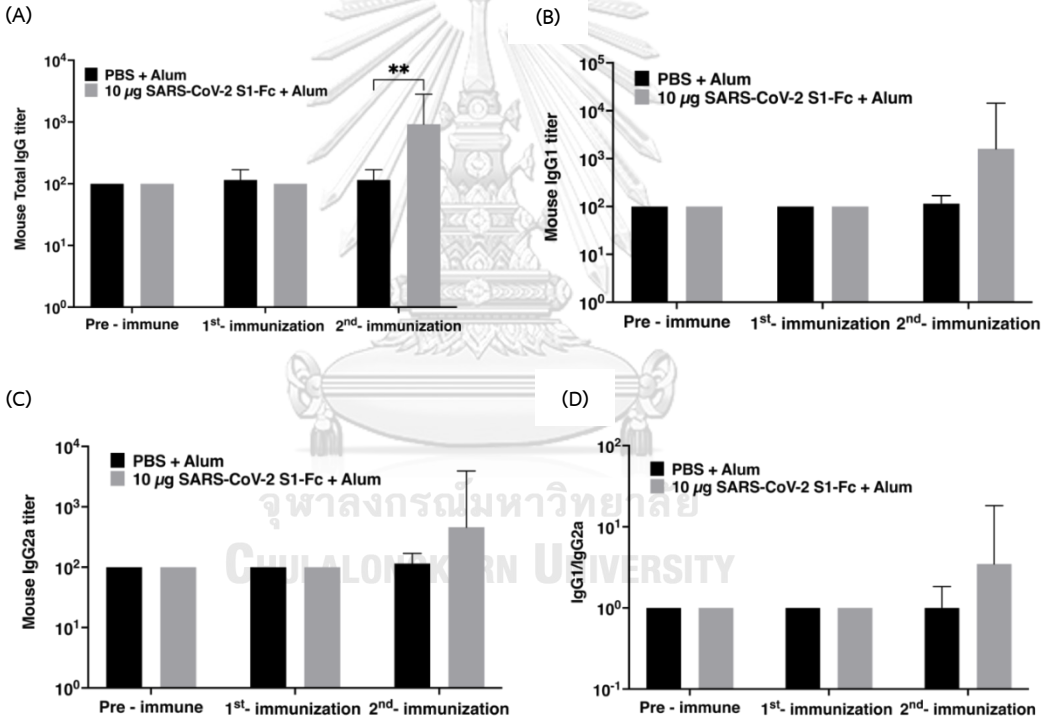

Figure 17: Titers of SARS-CoV-2 RBD-specific mouse of **(A)** Total IgG, **(B)** IgG1, **(C)** IgG2a, and **(D)** the ratio of SARS-CoV-2 RBD-specific mouse IgG1/IgG2a titer at indicated time-point. Data presented as geometric mean ± 95% CI of the endpoint titers in each group, $n = 5$. Two-way ANOVA, Tukey test, was used (**: $p < 0.01$).

In consistent with this study used the plant-produced SARS-CoV-2 RBD-Fc protein to immunize in mice and test the SARS-CoV-2 RBD-specific antibody and the result indicated that the plant-produced SARS-CoV-2 RBD-Fc protein can induce the SARS-CoV-2 RBD-specific antibody higher than the plant-produced SARS-CoV-2 S1-Fc in this study (101). It corresponding to the study from Yunfei Wang and his team demonstrated that used the different coating antigen protein can affect the analysis of enzyme-linked immunosorbent assay of antibodies titer because using the SARS-CoV-2 S1 protein by HEK293K cell as a coating antigen can stimulate the SARS-CoV-2 S1-specific IgG titer more than used the SARS-CoV-2 RBD protein as a coating antigen (96). This study was used the alum adjuvant to formulate with the SARS-CoV-2 S1-Fc, which can stimulate the Th-2 (IgG1) immune response better than the Th-1 (IgG2a) immune response (87). Whereas it has research indicated some adjuvant can stimulate the Th-1 (IgG2a) such as AS01 (QS-21 +MPL) liposome adjuvant and AS04 adjuvant (87, 91). For these reasons, it has many factors that affect to immunize the immune response such as the type of SARS-CoV-2 protein specific antibody, adjuvant, and recombinant protein, which can differ from other studies.

On the other hand, the splenocytes isolated from mice was used to evaluate the IFN-γ secretion using IFN-γ ELISpot assay. The result indicated that the plant-produced SARS-CoV-2 S1 -Fc elicited the IFN-γ secretion that is significantly higher than the control group with $p < 0.05$ (**Figure 18**). For this reason, it means that alum adjuvant is not significant elicit the T-cell response in mice, while the plant-produced SARS-CoV-2 S1-Fc formulated with Alum as adjuvant can induce T-cell response in mice.

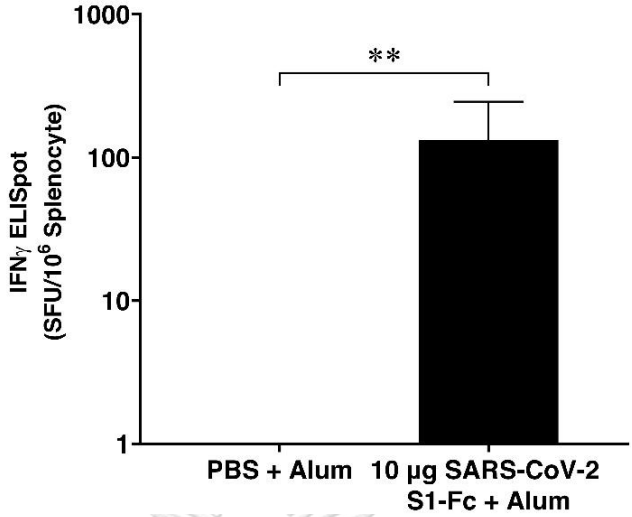

Figure 18: The SARS-CoV-2 RBD-specific T-cell responses in immunized mice. Mouse splenocytes were stimulated with RBD peptide pools and analyzed by mouse IFN-γ ELISpot assay. Data presented as mean ± SD (*n* = 5). Mann-Whitney test was used by compared with control (**: $p < 0.01$)

CHAPTER 4

CONCLUSION

Since the outbreak of SARS-CoV-2 in 2019, scientist and medical concern about the safe and efficient vaccine for defensive use. Nowadays, it has few vaccines that using for preventing infection of SARS-CoV-2 such as mRNA-1273 vaccines from BioNTech/Moderna (132, 133), BNT162b2 mRNA vaccine from BioNTech/Pfizer (134, 135), ChAdOx1 nCoV-19 vaccine from Oxford–AstraZeneca (136, 137), and recombinant protein subunit vaccine from Novavax (138, 139). The most compound in these vaccines are the spike (S) protein of SARS-CoV-2 because the S protein of SARS-CoV-2 can stimulate the immunogenicity and neutralize antibody (3-5). In addition, the S protein has two parts of the S1 subunit and the S2 subunit, which involve in the binding and fusing to the host cell, and they have the epitopes that can neutralize antibody (140).

Therefore, it requires an expression system that can produce antigens in a short time to make the recombinant vaccine. Plants are suitable platform to use during emergency situation, because plants have several benefits over alternative expression methods such as low cost, safety, and manufacturing speed (2, 122-125). Moreover, plants is used for producing the antibodies, vaccines, and replacement human proteins such as human serum albumin (126). Moreover, the plant-produced biopharmaceutical that has approved for human use by the Food and drug administration (FDA), such as taliglucerase alfa (ELELYSO^{TM}) is used as a replacement therapy of enzyme to treat Gaucher disease in adults (127).

This study demonstrated that the S1 subunit of SARS-CoV-2 fused with Fc region can be expressed in *N. benthamiana* within 4 days post infiltration and the infiltrated leaves showed necrosis compared to the control. Further, the plant-produced vaccine can induce antigen specific antibodies and T cell responses in mice. Hence, the plant-produced SARS-CoV-2 S1-Fc may provide a candidate strategy for the development of COVID-19 vaccine.

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