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Foldon fusion of RBD and S1 fragments of SARS-CoV-2 to stabilize the structure of subunit protein as a vaccine candidate

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ABSTRACT The COVID-19 pandemic threatened public health around the world at the same time as highlighting the urgency of vaccine development. Subunit vaccines are safe and effective vaccine types that utilize parts of viruses to trigger the body's immune response. Previous research has shown that fusion of the spike protein with the foldon domain (fd) achieved the trimeric form to increase the protein stability of the recombinant subunit protein spike from SARS-CoV and MERS-CoV, thus exceeding the immune response in the body. The study aims to observe the expression of RBD-fd and S1-fd recombinant proteins from the spike protein of SARS-CoV-2 in CHO-K1 mammalian cells and investigate the binding activity of those proteins with hACE2 receptor, expressed in HEK293T cells using immunofluorescence staining. The plasmids were transiently transfected into the cells, followed by antibiotic selection using G418 as an initial stage to select the positive stable transformants. Protein expression was confirmed by Western blotting and showed an estimated size for monomeric RBD-fd of 35 kDa and S1-fd of 55 kDa. However, the trimeric form of the proteins was not observed. In addition, immunofluorescence staining showed the binding activity between the RBD-fd and S1-fd proteins and hACE2 expressing cell line, revealing binding and an internalization process.

KEYWORDS CHO-K1; Foldon; RBD; SARS-CoV-2; Spike

1. Introduction

The current pandemic of COVID-19 has caused the spreading of human coronavirus which is also known as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and widely menaces public health around the world (Dhama et al. 2020). Compared to the other coronaviruses, Middle-East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV), SARS-CoV-2 occurs to have a higher infection due to the high rate of human to human transmission (He et al. 2020). According to the World Health Organization (WHO), both MERS-CoV and SARS-CoV caused outbreaks in several countries. These include some regions in the Middle East, Africa, and South Asia for MERS-CoV and China for SARS-CoV, in 2012 and 2003, respectively. However, the total infection and the spreading are considered low, therefore, no specific developed vaccines and treatments were adjusted to the patient's conditions. Consequently, by comparing the case of SARS-CoV-2 to the other coronavirus cases, the urgency to develop vaccines as the ultimate prevention to the current pandemic has risen (Kashte et al. 2021).

Vaccine is one of the most used biopharmaceutical products, which is crucial to incite the immune system in the body in fighting against infectious diseases to protect the body from diseases in a long-term period. Instead of giving the instant cure to the disease, vaccines are able to reduce the risk and even prevent the disease from happening again in the future (Kashte et al. 2021). Therefore, vaccines are an effective approach to treat serious infectious diseases. The current development and improvement of the COVID-19 vaccine have been progressively on study all around the world. By investigating the type of available vaccines, the researchers are trying to construct a competent and reliable vaccine for the current COVID-19 pandemic. One of the types of vaccine that has great potential is the subunit vaccine, which is also known as recombinant protein vaccine. It has a clearer prediction in terms of the response, efficacy, and safety issues associated with the implementation of the vaccine (Yadav et al. 2020).

Previous studies showed that the fusion spike protein from SARS-CoV and MERS-CoV with foldon domain (fd) was found to achieve the trimeric form to increase the protein stability of subunit vaccines, thus exceeding immune response in the body (Mahmuda et al. 2023). In this study, we developed two subunit vaccines consisting of the SARS-CoV-2 receptor binding domain (RBD) and S1 fragment of spike protein which are fused with foldon protein to form trimeric structure. The proteins were then expressed in mammalian cells. This lab-scale research has been performed using one of the subclones of the native Chinese hamster ovary (CHO) cells, CHO-K1 cells, as the expression system of the RBD-foldon (RBD-fd) and S1-foldon (S1-fd) recombinant proteins. According to Kang et al. (2016), CHO-K1 is a stable cell line and suitable for therapeutic applications such as recombinant protein in high yield production. Hence, the hypothesis of this study was the RBD-fd and S1-fd recombinant proteins can be successfully expressed in the CHO-K1 cells by showing the binding activity towards the human angiotensin converting enzyme 2 (hACE2) receptor in the mammalian cells.

2. Materials and Methods

2.1. Plasmid design, construction and isolation

RBD-fd and S1-fd synthetic genes sequences refer to registered patent (P00202211076) were synthesized by ATUM (https://www.atum.bio). We engineered the protein-based subunit vaccine of SARS-CoV-2 containing RBD and S1 fragments of spike protein to be expressed in mammalian cells. We introduced signal peptide for recombinant protein secretion into media at the N-terminus. The RBD or S1 fragments was fused with foldon domain using flexible linker containing GS sequence (Figure 1). The insert genes were ligated into mammalian expression vector (pcDNA-3) containing G418 resistance gene sequence to clone the gene of interest (Addgene, #141183). Plasmid preparation and confirmation were carried out by double digestion using restriction enzymes XhoI and NheI (New England Biolabs, #R0146S and #R3131S). The restriction mixture was incubated at 37 °C for 2 h. The confirmation result can be seen by carrying out gel electrophoresis to visualize the digested plasmid. After the successful confirmation of digestion, the insert genes (RBD-fd and S1-Fd) were ligated to the plasmid in separate ligation reaction using Quick Ligation™ Kit (New England Biolabs, #M2200S) at room temperature for 1 h. For the transformation process, 50 µL of TOP10 competent cells were added each with 2 µL of ligation reaction and incubated on ice for 15 min, then quickly heat-shocked at 42 °C for 50 s, and incubated again on ice for 15 min. Two hundred milliliters of SOC medium were added to the mixture and incubated in a shaker incubator for 1 h. Then, the mixture was grown overnight in the LB agar medium with 50 µg/mL ampicillin and in the LB agar without ampicillin as the control by using the spread plate method. The single colonies were observed after overnight incubation at 37 °C. The plasmid minipreparation was performed by following manufacturer's instruction using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, #K210010). The positive transformants were checked using restriction enzymes method, then visualized by gel electrophoresis.

2.2. Cell cultures

The cryopreserved CHO-K1 for RBD-fd and S1-fd expression and HEK-293T cells for ACE-2 expression were thawed in a 37 °C water bath for 30 s. Several reasons

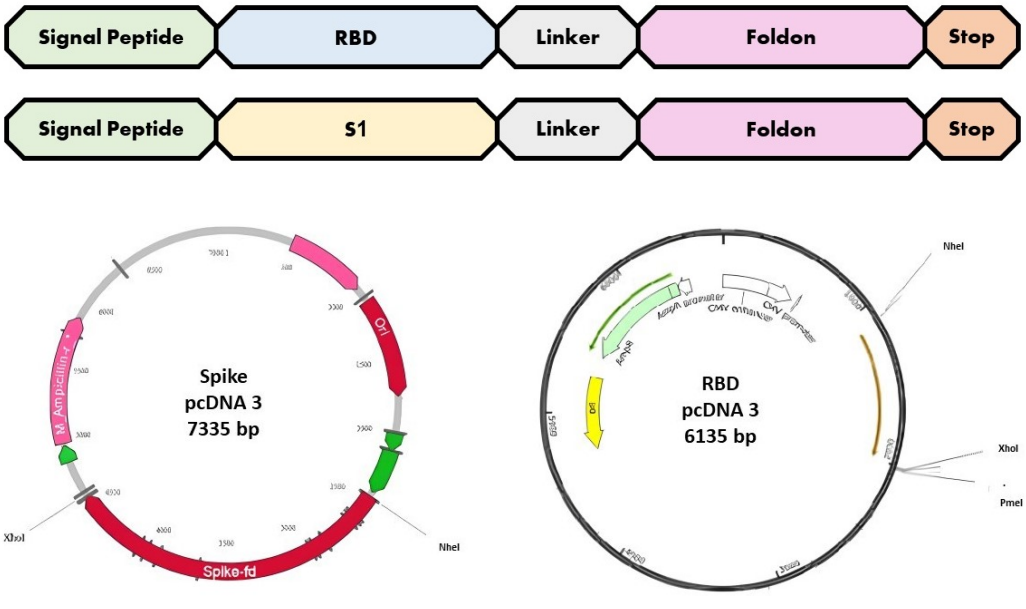

FIGURE 1 Gene constructs representing RBD or S1 fusion foldon (a) and the illustration of the plasmid DNA mapping (b).

of the utilization of CHO-K1 cells were the clear regulation and standardization on its biosafety and manufacturing practices. CHO-K1 also has high tolerance and easily adapts to the environmental changes. Aside from that, its capability to develop in serum-free media and high productivity yield is also intriguing for further vaccine development (Omasa et al. 2010). While HEK293-T cells was used due to its common usage as viral vectors, high transfection efficiency and good protein processing capability (Tan et al. 2021). One milliliter of each cell suspension was transferred into a tube containing 4 mL of F12 complete media for CHO-K1 cells, and 4 mL of DMEM (Sigma, #D7777) complete medium for HEK-293T cells, respectively. Both of the complete media were supplemented with 10% fetal bovine serum albumin (Sigma, #12103C) and 1% penicillin-streptomycin (Sigma, #P4333). The tube was centrifuged at 3000 rpm for 3 min and the supernatant was discarded. Ten milliliters of complete fresh complete media were added to the tube to resuspend the pellet and transferred to a 10 cm cell culture dish. The culture was incubated at 37 °C with 5% CO_2 concentration for 3 days to obtain approximately 3×10^6 cell density. The cell was observed to ensure 80-90% confluency prior to the subculture. The growth media was decanted and the cell was washed with 3 mL phosphate-buffered saline (PBS) three times. Five hundred microliters of trypsin was added and incubated at 37 °C for 3 min. The cells were passage into 2 different 10 cm cell culture dishes and were incubated at 37 °C with 5% CO_2 for 2-3 days.

2.3. Transient transfection

The transfection method of CHO-K1 cells in a 6-well plate was modified from the method developed by Cheung et al. (2018) and Saifudin et al. (2011). The cells that achieved the desired seeding density and confluency were washed using 1× PBS and a new fresh complete medium was added into the plate. After overnight culture, RBD-fd or S1-fd were transfected into the cells using Lipofectamine™ LTX (Invitrogen, #A12621). Both plasmid DNA and Lipopectamine were diluted in Opti-MEM™ I Reduced Serum Medium (Gibco, #31985062) and were prepared according to the manufacturer's instruction. Mock transfection as the negative control was done in a different well by adding 1× PBS instead of protein at the same volume as samples. Then, the complete transfection media was added into the CHO-K1 cells in the 6 well-plate and incubated at 37 °C and 5% CO_2 for 24-48 h. The medium was collected one day and two days after the transfection for observation. After 72 h of transfection, 0.05% G418 antibiotic (Sigma-Aldrich, #G8168) was added as the transfection selection approach and was incubated at 37 °C with 5% CO_2 concentration to grow until its maximum confluency.

2.4. Selection of transfected cells

Transformants selection was performed by scaling up and down the cell culture into different sizes of the well plates, initiated by 6-well plate, followed by 24-well plate, 6-well plate, 6 cm-cell culture dish, and 10 cm-cell culture dish, respectively. Cell culture in every stage of scaling was incubated at 37 °C with 5% CO_2 concentration and the addition of 0.05% G418 antibiotic. The survived and more stable cells were managed and maintained every two days. The medium from each stage of cell culture scaling was obtained and stored at -20 °C for further analysis and observation. The culture medium was further concentrated by adding 500 µL of cell culture medium to Amicon® Ultra 0.5 mL Centrifugal Filters (MWCO: 10 kDa, Millipore) and centrifuged at 14,000 ×g for 30 min at room temperature. The filtrate was collected and stored at -20 °C for further usage.

2.5. Western blot

The expression of RBD-fd and S1-fd recombinant proteins in the transfected cells were observed by using Western blot. Thirty microliters of each protein sample in reducing sample buffer were boiled for 5 min. The protein samples were then loaded into 4-15% precast polyacrylamide gel (Biorad, #4561084) with a running buffer 1× Tris/glycine/SDS (Biorad, #1610732) at 100 V for 1 h. The SDS-PAGE gel was removed from the tank assembled into the sandwich Turbo Mini 0.2 PVDF Transfer Packs (Biorad, #1704156) and run with Trans-Blot Turbo Transfer System apparatus (Biorad) at 25 V, 1.3 A for 7 min. Afterward, the membrane was incubated with blocking solution (10% skim milk in 1× tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST)) for 1 h. The primary antibody, SARS-CoV-2 Spike RBD Antibody (R&D Systems, #MAB10540) was added into the blocking solution with a 1:3000 ratio and incubated for 2 h in the gentle shaker. The secondary antibody, mouse alkaline phosphatase antibody (R&D Systems, #AF2910) was added into the blocking solution at 1:2000 ratio and incubated for 1 h. In between the steps of blocking, primary antibody and secondary antibody, the membrane was washed three times by using 1× TBST. At the end, the membrane was visualized by adding the One Step NBT-BCIP substrate (Thermo Scientific Pierce, #34042).

2.6. Binding and internalization into ACE-2 receptor

The binding activity of RBD-fd and S1-fd recombinant proteins and ACE2 receptor were observed using immunofluorescence staining. HEK-293T cells were transfected with the hACE2 gene, pcDNA3.1-hACE2 (Addgene, #145033) containing plasmid. A half microgram (0.5 µg) purified recombinant plasmid was diluted in 50 µl of Opti-MEM I Reduced Serum Medium (Gibco, #31985062). The diluted DNA solution was further added with 1 µL of polyethylenimine (PEI) salt (Sigma, #408727) in 50 µL of Opti-MEM I Reduced Serum Medium, mixed gently and incubated overnight at 37 °C with 5% CO_2 concentration. The growth medium was replaced with a new 500 µL of DMEM. These ACE2 transfected HEK-293T cells were grown in the chamber slides and added with 20 µL of each concentrated supernatant

from the RBD-fd and S1-fd transfected CHO-K1 cell culture. Over expressed of ACE-2 receptor were then confirmed by immunofluorescence assay using anti h-ACE-2 antibody as described in section 2.7 below. Both samples were then incubated at 37 °C and 5% CO2 for 2-3 h (Shang et al. 2021).

2.7. Immunofluorescence (IF) assay

The IF process covered fixing, permeabilization, blocking, primary and secondary antibody incubation, and visualization. The chamber slide containing cells were fixed by adding 100 µL of 3,7% formaldehyde solution on top of the cells and incubated for 10 min at room temperature. The formaldehyde solution was discarded and the cells were washed three times using 500 µL PBS. One hundred microliters (150 µL) 0.2% Triton-X was added for permeable process and incubated for 10 min at room temperature. The cells were washed two times using 500 µL followed by the adding of 150 uL 1% BSA (Thermo Scientific, #B14) to the cells and incubated for 30 min at room temperature for blocking non-specific binding. The primary antibodies (anti-RBD-mouse and anti-human ACE2-rabbit antibodies) (R&D Systems, #MAB105808 and #MAB10823) to detect RBD-fd or S1-Fd, and human ACE-2 respectively, were diluted in blocking solution at a ratio of 1:250. The diluted antibodies were added into each chamber slide and incubated for 1 h at room temperature. The secondary antibodies (AF488-conjugated anti-mouse and AF594-conjugated anti-rabbit) (Abcam, #ab150113 and #ab150080) at ratio of 1:2000 and 1:1000 in blocking solution, respectively were added to chamber slide and incubated overnight at 4 °C. After the primary and secondary antibodies incubation, the cells were washed three times using 500 µL PBS. The mounting of the slides was done by using Fluoroshield™ with DAPI (Sigma, #F6057) and dried overnight. The cells in each chamber slide were observed under the fluorescence microscope (Olympus) with magnification of 20×.

3. Results and Discussion

3.1. Engineering and production of subunit vaccines

The recombinant plasmids were confirmed before transiently transfected into CHO-K1 cells. The plasmid obtained from miniprep was tested by double digestion using NheI and XhoI restriction enzymes and visualized using gel electrophoresis. The result of double digestion can easily be observed by comparing bands between undigested and digested plasmids (Table 1). The successfully digested plasmid showed favorable results by showing two bands representing insert gene either RBD-fd or S1-fd (lower) and vector pcDNA3 (higher) as shown in Figure 2. The purity and concentration of plasmids were measured before transiently transfected into the cells. We used Lipopectamine-LTX to enhance the efficiency of transfection. The expressed protein was observed 24 h and 48 h after transfection by slot blotting using antibody specific into RBD spike glycoprotein SARS-CoV-2 (data not shown). On day 3, the transfected cells were selected by using G418 treatment. The treated cultures were scaled down to a 24-well dish and then gradually scaled up again until in a 10 cm-dish to increase the protein expression. The protein expression in every single dish were confirmed by slot blotting (data not shown). Western blot analysis was performed only in the medium of 10 cm-dish, and thick bands at approximately 35 kDa for RBD-fd and 55 kDa for S1-fd. As a comparison, the negative control (mock transfection) showed no band as expected (Figure 3).

TABLE 1 Results of the *P. aeruginosa* protease clear zone test.

Plasmid and Insert	Length (bp)
pcDNA3 vector	5,335
pcDNA3 + S1-fd	7,335
pcDNA3 + RBD-fd	6,135
Cut S1-fd with Restriction Enzyme	2,000
Cut RBD-fd with Restriction Enzyme	800

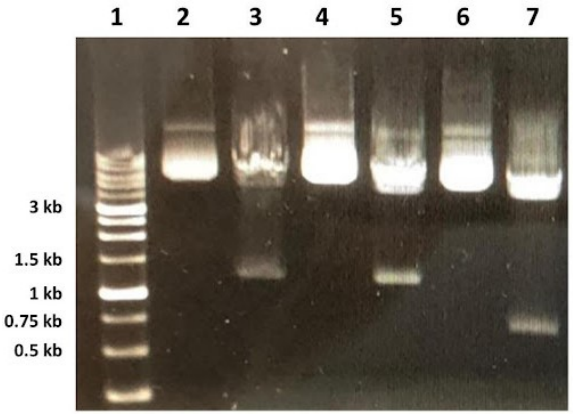

FIGURE 2 Agarose gel electrophoresis of RBD-fd and S1-fd. Lane 1: DNA marker (Gene Ruler 1 kb DNA Ladder); 2 and 4: undigested plasmids containing S1-fd; 3 and 5: digested plasmids containing S1-fd; 6: undigested plasmids containing RBD-fd; 7: digested plasmids containing S1-fd.

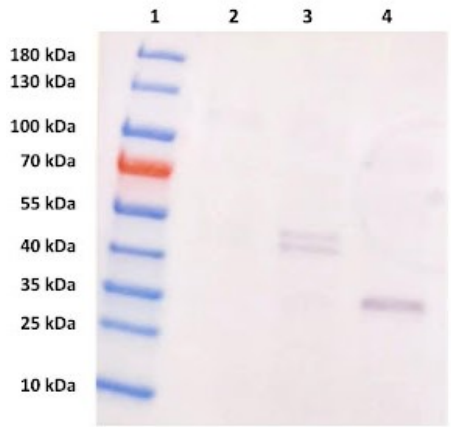

FIGURE 3 Western blotting of RBD-fd and S1-fd protein. Lane 1: protein marker; 2: negative control; 3: S1-fd; 4: RBD-fd.

3.2. In vitro binding analysis of recombinant subunit vaccines into ACE2 receptor

To investigate the biological effect of the subunit vaccines, we utilized immunofluorescence staining for hACE2 receptors and recombinant protein RBD-fd or S1-fd was performed. The HEK 293T was used to over expressed the ACE2 protein using transient transfection technique. Before adding the concentrated supernatant containing RBD-fd or S1-fd into ACE-overexpressed HEK 293T, it was confirmed that the HEK 293T cells has already expressed ACE-2 protein, which can be shown on Figure 4a. The red color showed the ACE2 protein expressed in membrane HEK 293T cells. Furthermore, the proteins binding to the ACE2-expressing HEK 293T cells was confirmed. The RBD-fd or S1-fd protein from concentrated supernatant of the 10 cm-dish was added into transiently transfected ACE2-expressing HEK 293 cells. The cells were incubated with mouse anti-RBD primary antibody, followed by secondary antibodies (AF488-conjugated anti-mouse). The successful binding showed a green color in membrane cells area, which represents the interaction of the recombinant RBD-fd or S1-fd with the hACE2 receptor in the cell membrane (Figure 4b, c). Finally, we investigated the binding and internalization of RBD-fd and S1-fd to ACE 2-expressing cells by using two primary antibodies, which are rabbit anti-ACE2 and mouse anti-RBD spike SARS-CoV-2 antibodies, followed by two secondary antibodies (AF594-conjugated anti-rabbit and AF488-conjugated anti-mouse). The result showed that both RBD-fd and S1-fd protein successfully bound and internalized into the hACE2 protein expressing cells, which were shown by yellowish-orange color inside the cells. Therefore, the RBD-fd and S1-fd proteins were determined to be able to bind with the hACE2 protein (Figure 4d, e).

3.3. Discussion

Subunit vaccines are the vaccine using some parts of the germs or viruses in the structure construction to incite the immune response in the body, without causing any infection risks (Chong et al. 2015). Therefore, the improvement can be made for constructing COVID-19 vaccine by utilizing the essential remnant of the virus, such as the spike protein, or even smaller remnants such as RBD that located in the spike protein of the virus. Several previous studies also used RBD-based or spike-based recombinant protein as the vaccine development in other coronaviruses (MERS-CoV and SARS-CoV), which showed great immunogenicity effects (Li et al. 2020). However, in the native state, spike protein formed in prefusion state as a trimeric glycoprotein mediating both binding to host cell receptors and fusion of virus and host cell membranes. Therefore, some improvements in developing structure-based design of recombinant subunit vaccine to form a trimeric prefusion state of spike protein can be promoted by fusing with other protein fragments. Most approved COVID-19 vaccines use prefusion stabilized structure of spike protein, including mRNA, viral vector and subunit vaccines (Creech et al. 2021). In this study, we combined

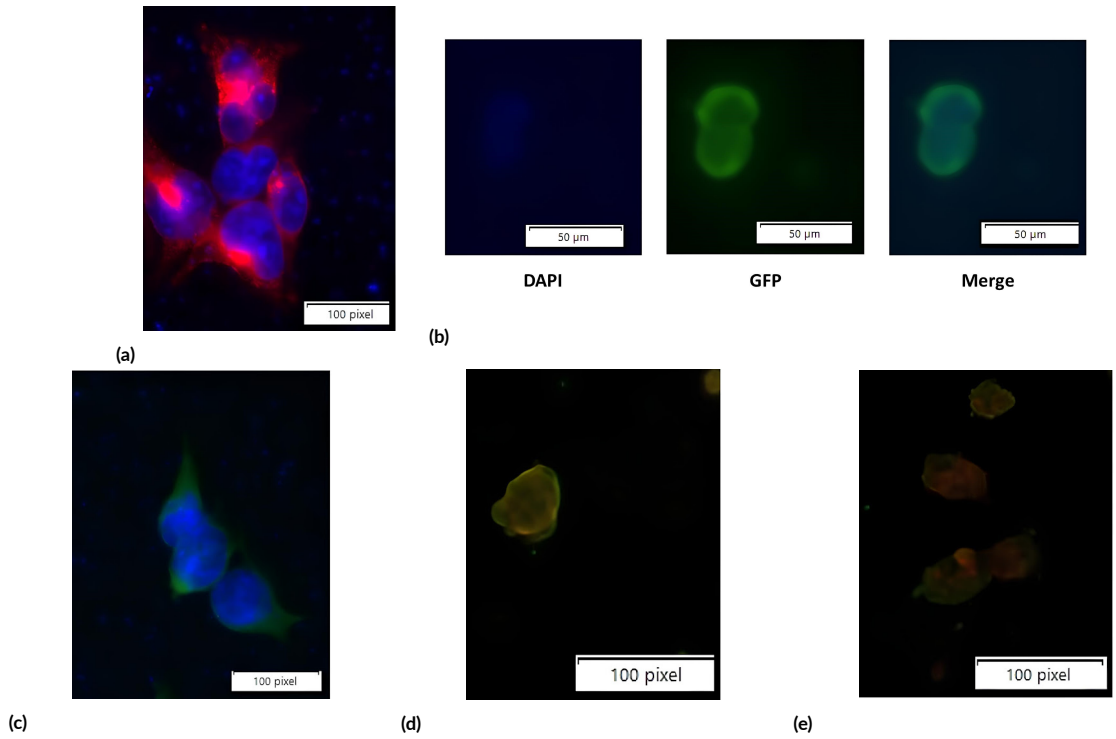

FIGURE 4 Binding and internalization of RBD-fd and S1-fd into hACE2 expressing cells. (a) hACE2 protein was transiently transfected into HEK 293T cells, expressed in membrane cells exerting red color. Blue color indicated the nucleus which was stained with DAPI. (b, c) The binding of RBD-fd and S1-fd proteins in hACE2 expressing cells, respectively. (d, e) The binding and internalization of RBD-fd and S1-fd proteins in hACE2 expressing cells, resulting the yellowish colors.

the RBD or S1 fragment with foldon domain using GS flexible linker without any protein tags. Foldon protein consists of 27 amino acids sequences that can help to increase the length of the RBD in the recombinant protein and achieve the trimeric form (Tai et al. 2016). A signal peptide utilizing hemagglutinin sequence was used as stated in the registered patent (P00202211076) for protein secretion.

Mammalian cells are widely used as the expression system of recombinant proteins. About 85% of the recombinant proteins that are available in the market are expressed in mammalian cells, due to the capability to express the complex recombinant proteins (Tripathi and Shrivastava 2019). One of the most commonly used mammalian cells is CHO cells that well known for its stability, and the available cell lines is CHO-K1. In addition, the stable cell lines are able to produce a high yield recombinant protein product, flexible to grow in a serum-free medium, and less affected by the infections of human viruses (O'Flaherty et al. 2020). Therefore, this cell has a great advantage in developing biopharmaceutical products such as a vaccine. In lab-scale research, the expression of recombinant protein is essential for proceeding to larger-scale production.

In this study, we used transient transfection for protein production, followed by selection of G418 as the initial stage for stable cell line production. The protein expressed were confirmed by Western blot analyses using antibody specific into RBD spike glycoprotein of SARS-CoV-2. The proteins were analyzed in reduced SDS-PAGE. Therefore, the size of both proteins shown as a monomeric form. The two bands of S1-fd protein were observed in the blot, but not for the RBD-fd. There are several causes that makes S1-fd sample had two bands in the Western blot. First, it may be caused by protease degradation (Mahmood and Yang 2012), that cleaved the peptide in S1 peptide but not at RBD sequence. Second, it might be due to a glycosylation difference (Semaan et al. 2012). The S1 subunit has 13 putative N-glycosites, and 3 of them in RBD sites (Gong et al. 2021). Therefore, the hyperglycosylated S1 protein expressed in CHO cells may be produced in heterogeneity glycoforms, resulting different size of protein. The binding activity of expressed proteins were also confirmed using *in vitro* assay using ACE2 expressing transgenic cells. The proteins were bound and internalized into hACE2 receptor showing the biological effect of those recombinant proteins. However, we have not managed to determine the trimeric form of both foldon fusion proteins. For the next study, the trimeric form might be investigated using non reducing protein sample buffer before SDS-PAGE or using native PAGE and size exclusion chromatography. Further study, the sophisticated instrument such as cryo-electron microscopy can be used to investigate the trimeric structure of these antigens design. The confirmation of trimeric form is essential to ensure the antigens will have a great immunogenicity as vaccine candidates (Hsieh et al. 2020).

4. Conclusions

In summary, both RBD-fd and S1-fd have been successfully expressed in CHO-K1 adherent cells and characterized using specific antibody. Moreover, both proteins have a binding activity with receptor hACE2 based on *in vitro* studies. However, further studies must be performed to purify the proteins and characterize trimeric structure of these protein design.

Acknowledgments

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Authors' contributions

AW, WK, RAN, EPS designed the study. GCLP, FID, DFA, PHW, ATF, YR, RDR, MKLS, PWP, AW, DS carried out the laboratory work. AW, GCLP, FID, ITP analyzed the data. AW, GCLP, FID, ATF wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare there are no competing interests.

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