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Efficient production of recombinant SARS-CoV-2 spike protein using the baculovirus-silkworm system

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ABSTRACT

In the case of a new viral disease outbreak, an immediate development of virus detection kits and vaccines is required. For COVID-19, we established a rapid production procedure for SARS-CoV-2 spike protein (S protein) by using the baculovirus-silkworm expression system. The baculovirus vector-derived S proteins were successfully secreted to silkworm serum, whereas those formed insoluble structure in the larval fat body and the pupal cells. The ectodomain of S protein with the native sequence was cleaved by the host furin-protease, resulting in less recombinant protein production. The S protein modified in furin protease-target site was efficiently secreted to silkworm serum and was purified as oligomers, which showed immunoreactivity for anti-SARS-CoV-2 S2 antibody. By using the direct transfection of recombinant bacmid to silkworms, we achieved the efficient production of SARS-CoV-2 S protein as fetal bovine serum (FBS)-free system. The resultant purified S protein would be useful tools for the development of immunodetection kits, antigen for immunization for immunoglobulin production, and vaccines.

1. Introduction

Coronavirus disease 2019 (COVID-19) was outbreaking in December 2019, and it rapidly spread worldwide in early 2020 [1]. The pathogen of COVID-19 is a novel coronavirus designated as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The genetic sequence of SARS-CoV-2 was immediately released in January 2020 [2], and many researchers started to develop detection kits and anti-COVID-19 vaccines [3–6]. Rapid and efficient production of target protein molecules is required to facilitate the development of these tools. For a laboratory-based expression of SARS-CoV-2 S protein, the expression systems using human cultured cell lines have been used [7–10]. Insect cell line Sf9 has also been used for the expression of SARS-CoV-2 S protein as a baculovirus expression vector system (BEVS) [9,11–13]. BEVS is known as an efficient protein expression system with eukaryotic protein folding and modifications. Indeed, structural studies for the recombinant SARS-CoV-2 S protein have been achieved with BEVS [11,12]. These indicate the potency of the BEVS for mass production of SARS-CoV-2 S antigen. However, BEVS using cultured cell lines sometimes requires fetal bovine serum (FBS) for its high protein production performance. Especially for the production of vaccines, the use of FBS should be avoided because it contains the risk of pathogen contamination, such as viruses or prions.

We have previously developed BEVS using *Bombyx mori* nucleopolyhedrovirus (BmNPV) and silkworm for the production of a variety of recombinant proteins [14–17]. By using the *E. coli*-based recombinant bacmid system and silkworm, it is possible to produce recombinant BmNPV without using FBS [18]. Therefore, we develop the BmNPV-silkworm expression system for the production of SARS-CoV-2 S protein without using cultured cell lines and FBS. Our data here presented the successful production of the ectodomain of SARS-CoV-2 S protein, which would facilitate developments of

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vaccines, virus detection systems, and immunogen detection systems.

2. Materials and methods

2.1. Recombinant virus construction

The cDNA of the SARS coronavirus-2 Spike (S) gene (Accession MN908947.3, position 1–1222) was chemically synthesized (Integrated DNA Technologies Inc.) then cloned into pFastBac1 vector DNA with Gibson-assembly method. In this process, we added the β-sheet domain of CELO long fiber knob (aa 583–590) [19], target sequence of Tobacco Etch virus (TEV) protease, His-tag, and Strep-tag to the C-terminal end of S gene instead of the transmembrane domain. The cloned gene was introduced into *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid (strain Qd04), then recombinant bacmid was extracted from cultured *E. coli* DH10Bac using Qiagen Midi kit (Qiagen). One microgram of extracted DNA was mixed with 1.6 μl of Avalanche-Everyday transfection reagent (Integrale), then injected to 4th-instar silkworm larvae (1 μg/larvae). The recombinant BmNPV (P1) was recovered from the larval serum at four days post-transfection (d.p.t.). For the test of the above procedure, we constructed the BmNPV bacmid carrying GFP under the control of cathepsin promoter.

2.2. Recombinant protein purification

All blood was extracted from recombinant BmNPV-infected silkworm larvae (strain d17 or f38) at four days post-infection (d.p.i.). The blood was separated into serum and blood cells by centrifugation (9000 rpm, 30 min). The serum was diluted in a 5-times volume of T-buffer (10 mM Tris-HCl (pH7.5), 250 mM NaCl), and pass through a 0.45 μm filter. The filtrated sample was loaded into the HisTrap excel column (Cytiva) then washed with T-buffer containing 30 mM imidazole. The recombinant protein was eluted out by 2-step with T-buffer containing 100 mM and 500 mM imidazole. The buffer of resultant eluates was replaced with phosphate-buffered saline (PBS), loaded into the Strep-Tactin Superflow column (IBA lifesciences). The column was washed with PBS, and the recombinant proteins were eluted out with PBS containing 2.5 mM desthiobiotin. The concentration of purified proteins was determined in the PAGE image with a diluted series of control bovine serum albumin.

2.3. SDS-PAGE and western blotting

The serum samples were recovered as described above. The fat bodies of infected larvae were homogenized in buffer (20 mM Tris-HCl (pH7.5), 500 mM NaCl), then sonicated for 10 min. The homogenized samples were centrifuged (9000 rpm, 30 min, 4 °C), and the supernatants and pellets were recovered as soluble and insoluble fractions, respectively. For the analysis of pupae, the homogenates were prepared at four d.p.i. Briefly, three pupae were homogenized in lysis buffer (20 mM Tris-HCl (pH7.5), 500 mM NaCl, 0.5% CHAPS), sonicated, and centrifuged with the same protocol for fat bodies.

The sample was separated in 8%, 7.5%, or 5–20% SDS-PAGE. The gels were stained by the reverse stain method. Briefly, the gels were rinsed in distilled water (DW) then incubated in the solution I (200 mM imidazole, 0.1% SDS) for 10 min. The gel was then incubated with solution II (200 mM $ZnSO_4$) for 2 min, then rinsed with DW. After imaging, the gel was destained by rinsed in 100 mM ethylenediaminetetraacetic acid (ETDA) for 15 min, then used for western blotting.

The proteins in the gel were blotted on PVDF membrane, then incubate with Tris-buffered saline with 0.1% Tween 20 (TBST) containing 4% skim milk. The membrane was rinsed in TBST then incubated with anti-SARS-CoV-2 Spike S2 antibody (Sino Biological Inc.). Anti-rabbit IgG HRP-conjugated antibody was used as a secondary reaction, and the signals were visualized using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

2.4. Native PAGE

The samples were mixed with 1/5 volume of native sample buffer (250 mM Tris-HCl (pH 6.8), 0.1% BPB, 40% glycerol), and loaded on the 5–20% gradient gel without heat-denaturing. The proteins were separated in PAGE with native running buffer (25 mM Tris and 192 mM glycine) at 4 °C. The gel was subjected to reverse staining and western blotting, as described above.

3. Results

For the production of candidate recombinant vaccine molecules for SARS coronavirus 2, it is desirable to use the eukaryote system without using fetal bovine serum (FBS). Therefore, we decided to apply the BmNPV-silkworm expression system without using cultured cells [18]. We first determined the efficiency of progeny production of recombinant BmNPV in our method (Fig. 1). The recombinant bacmid carrying GFP gene was transfected to 4th-instar larvae of silkworms. At 4-days post-transfection, the silkworms showed GFP expression in the whole body, especially strong fluorescence in head and tail leg regions (Fig. 1B). The progeny viruses (P1 viruses) were recovered from 4 d.p.t. (approximately 100 μl serum from one larva), then ten μl of the diluted series of the P1 viruses were inoculated into day 2 of 5th-instar larvae (6-days after the first transfection). At 2-days post-infection, all the infected larvae showed GFP fluorescence even with the 1×10^5 dilution of P1 viruses (Fig. 1B). Thus, our method achieved efficient production (more than 1×10^5 shots from one larva) of recombinant BmNPV before the same reared series of silkworms reached to the 5th-instar stage.

We then constructed recombinant BmNPV bacmid carrying the extracellular domain of the SARS-CoV-2 spike gene. The spike protein of coronavirus has been known to form the trimeric structure on the surface of the virion. We introduced the β-sheet domain of CELO long fiber knob with an expect to facilitate oligomerization [19]. The expected molecular size of the designed recombinant nCoV-S protein was 141 kDa, including peptide tags (Fig. 1C).

We then transfected the recombinant bacmid into 4th-instar larvae and infected P1 progeny viruses to 5th-instar larvae of silkworms as described above. In the SDS-PAGE and western blotting analysis, we could see the expression of nCoV-S in the silkworm serum as a secreted protein, however, the amount of expressed recombinant protein was not so much in comparison of other background proteins (Fig. 2A, left). In the fat bodies of the infected larvae, the recombinant protein appeared as an insoluble fraction with a smeared pattern (Fig. 2A, center). It was also the same in the analysis of the infected pupae (Fig. 2A right). Thus, the recombinant nCoV-S was secreted to blood but seemed to form aggregates with some degradation in the fat bodies and pupal cells.

It has been known that the S protein of SARS-CoV-2 possesses the furin protease-recognition site [20]. Silkworm *Bombyx mori* encodes some putative furin protease genes in its genome (Accession: PRJNA205630). We considered that the expressed SARS-CoV-2 S protein was targeted by endogenous furin proteases, which worsened the recombinant protein secretion. Therefore, we constructed another recombinant virus with modification in the furin protease-recognition site according to Wrapp's method [7]. We

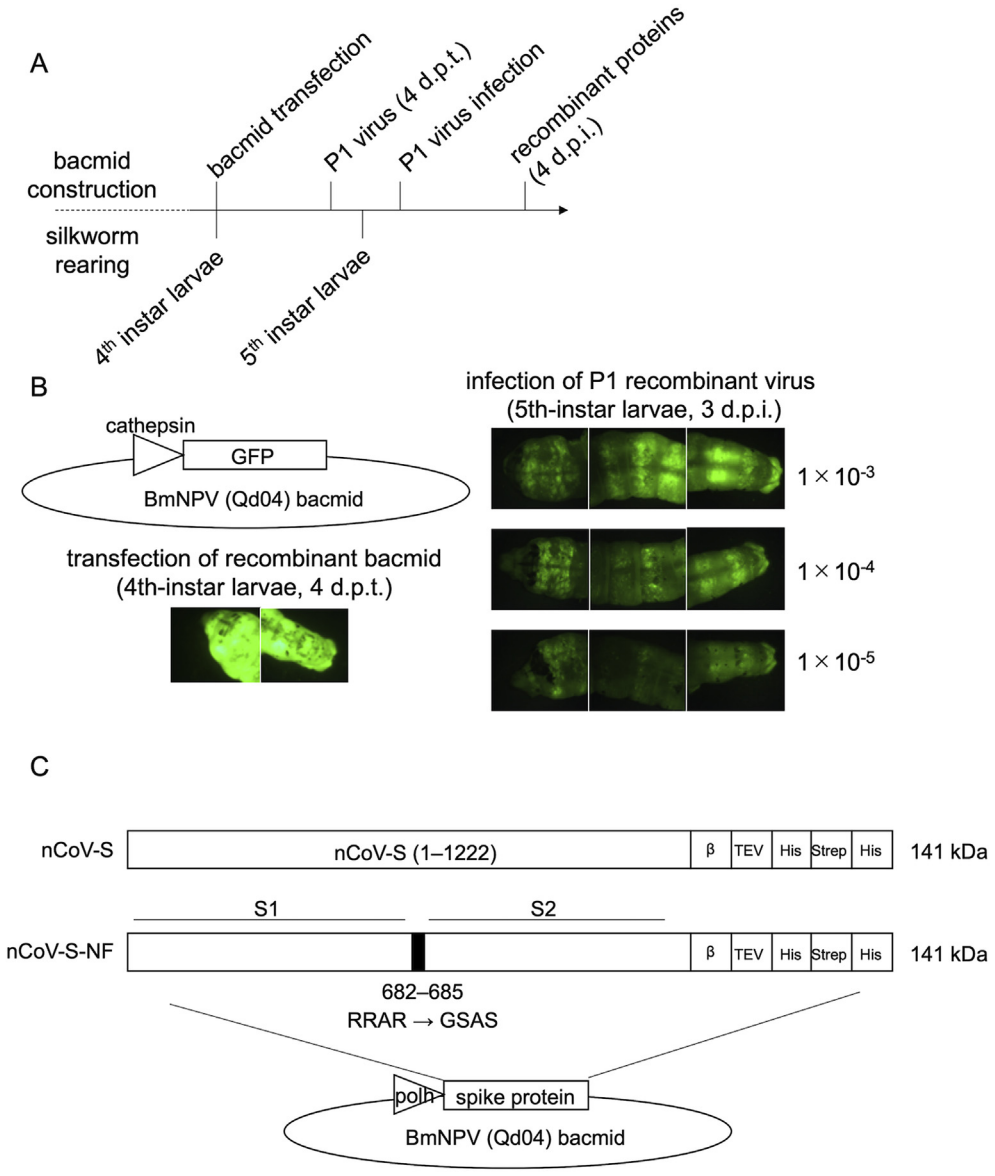

Fig. 1. Construction of recombinant baculovirus and the procedure for protein production. **A:** Schedule for the production of the recombinant viruses and the proteins. Extracted bacmid DNA was transfected to early stages of 4th-instar larvae, and recombinant viruses were recovered four days post-transfection. The viruses were infected to the same rearing series of 5th-instar larvae, and the recombinant proteins were extracted at four days post-infection. **B:** BmNPV bacmid harboring GFP gene under the control of cathepsin promoter was used to monitor the recombinant virus infection. Ten micro litter of the recovered viruses were infected to 5th-instar larvae with the dilution concentration indicated. **C:** Construction of the recombinant BmNPV. The ectodomain of the SARS CoV-2 S gene was fused with the β-sheet domain of CELO knob (β), Tobacco Etch virus protease targeting site (TEV), His-tags (His), and Strep-tag (Strep). The amino acid sequence of 682–685 was replaced with GSAS not to be target by furin protease (nCoV-S-NF).

could detect the significant signal in the serum fraction with the expected molecular size in the expression analysis for the larval blood (Fig. 2B). However, the recombinant protein still formed an insoluble structure in the fat bodies and the pupal cells.

So far, the recombinant proteins were secreted in serum as soluble proteins. We conducted an affinity chromatography purification for these proteins. In the purification nCoV-S with Ni-sepharose column, some ladder pattern was found around 140 kDa molecular size, especially in elution-1 fraction (100 mM imidazole) (Fig. 3, left). The ladder pattern was moderate in elution-2 (500 mM imidazole), suggesting that the ladder pattern around 140 kDa was the background proteins. Therefore, we carried out subsequent Strep-Tactin column purification for elution-1 and -2 of His-tag purification, respectively. The results showed that the purified protein bands were not visible in the SDS-PAGE image but found in western blotting using anti-SARS-CoV-2 spike S2 antibody (Fig. 3, center and right). In the Strep-tag purification of His-tag column elution-1, we could see two bands (approximately 140 kDa and 70 kDa). The bands of 140 kDa seemed to be intact recombinant nCoV-S protein, whereas the 70 kDa protein was considered a furin-digested S2 domain. We could not find a significant signal in the purified fraction from the His-tag elution-2 sample.

We then tried to purify the nCoV-S NF protein, which was expected not to targeted by furin protease. In the Ni-sepharose purification, we could detect the possible recombinant protein bands around 140 kDa molecular weight, although other background proteins appeared (Fig. 4A, left). In the subsequent Strep-Tactin sepharose column purification, we could see the fine bands in elution fractions with expected molecular size (Fig. 4A; center and

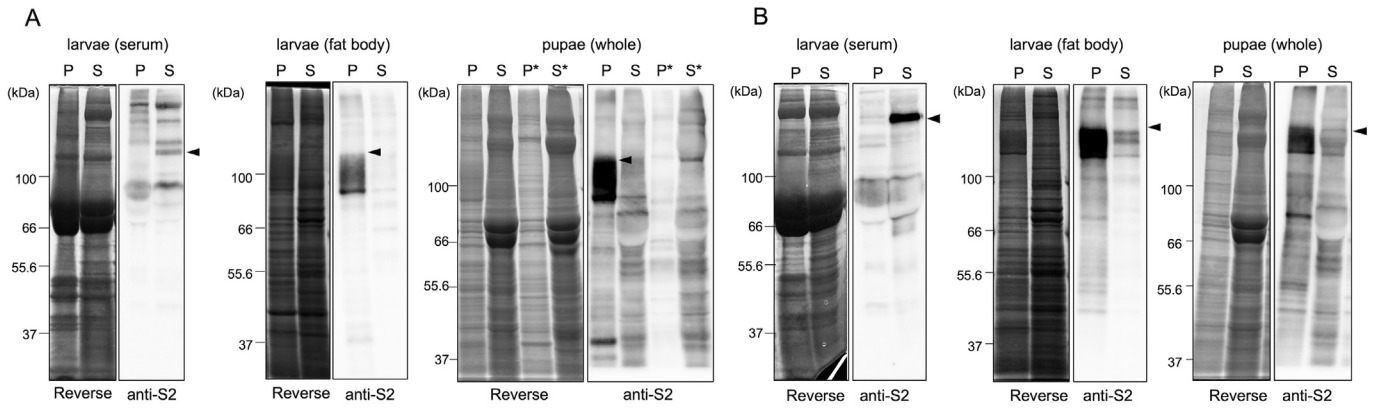

Fig. 2. Expression of recombinant SARS-CoV-2 S proteins in silkworms. **A:** The expression of nCoV-S was analyzed in silkworm larvae and pupae at four days post-infection. The extracted serum and homogenate of fat body and pupal body was separated in pellets (P) and supernatants (S). The left side of each image shows the reverse staining image of SDS-PAGE, and the right sides indicate the Western blot using the anti-SARS-CoV-2 S2 antibody. Black arrowheads indicate the expected size of the expressed proteins. Asterisks indicate the sample from mock-infected pupae. The positions of the molecular marker are also indicated. **B:** The expression analysis of nCoV-S-NF protein.

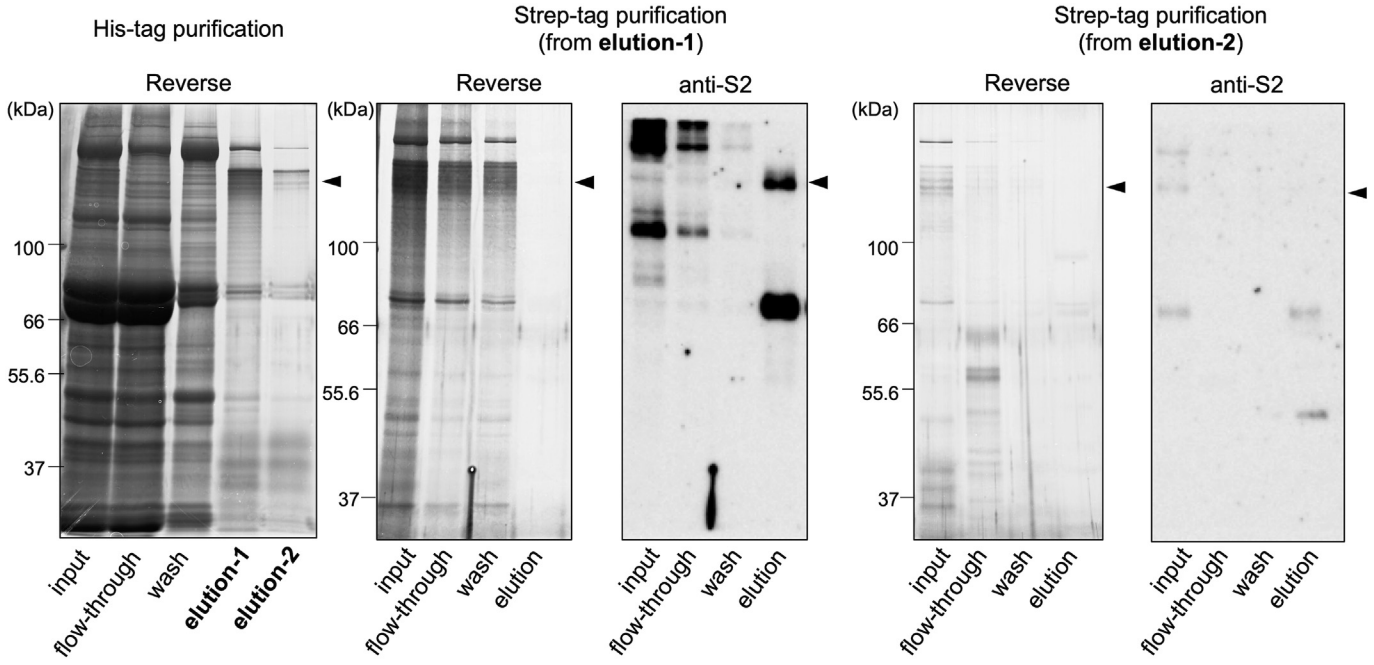

Fig. 3. Purification of nCoV-S protein expressed in silkworms. The images are the reverse staining of SDS-PAGE and Western blot for the fractions of His-tag purification (left) and the subsequent Strep-tag purification (center and right). The fractions of elution-1 (100 mM imidazole) and elution-2 (500 mM imidazole) were independently applied for Strep-tag purification. Black arrowheads indicate the expected size of nCoV-S. The positions of the molecular marker are also indicated.

right). The western blotting analysis revealed that the eluates were surely the nCoV-S recombinant proteins. Interestingly, we still found the anti-SARS coronavirus S2 antibody-reactive protein with the size of 70 kDa, although the furin-target site was not in the recombinant protein.

The recombinant nCoV-S protein without furin-target sequence was found to be purified in our method with possessing the reactivity for the SARS-CoV-2 spike S2 antibody in denatured PAGE. The purified nCoV-S NF (elution-A and -B in Fig. 4A) were subjected to native PAGE analysis (Fig. 4B). The proteins appeared as broad bands around 700 kDa, and those were immunoreactive for anti S2 antibody. On the other hand, no significant signals were found around the molecular size marker of 146 kDa, which was considered to be the size of the monomeric form of the recombinant protein. These results suggested that the recombinant ectodomain of SARS-CoV-2 spike protein without furin-target sites expressed in the BmNPV-silkworm system can be purified as an oligomer. Finally, we determined the amount of purified protein in comparison with the dilution series of BSA standards from the PAGE image. The estimated value of the purified protein from 10 ml serum (corresponding to 30–40 larvae) was about 100 μg.

4. Discussion

For a rapid and efficient production of recombinant proteins using the BmNPV-silkworm system, steps of the production of recombinant viruses and the silkworm rearing schedule are sometimes to be a bottleneck. To overcome this issue, we designed the plan to generate the recombinant BmNPV in 4th-instar larvae and express recombinant proteins in the following 5th-instar larvae

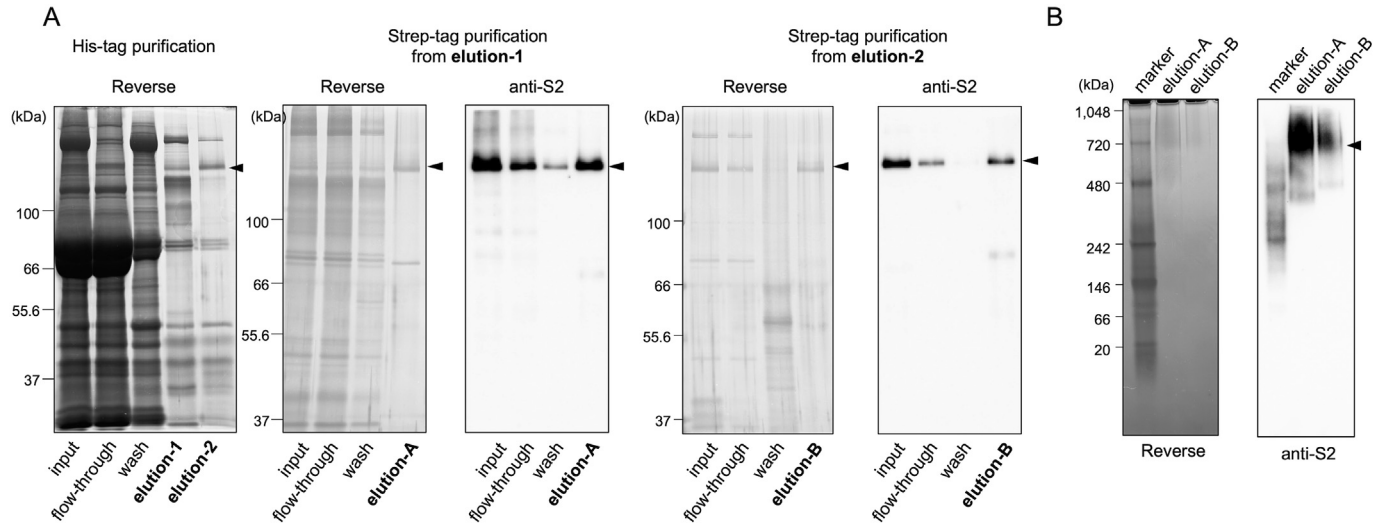

Fig. 4. Purification of nCoV-S-NF protein expressed in silkworms. **A:** The reverse staining images of SDS-PAGE and Western blot for the fractions of His-tag purification (left) and the subsequent Strep-tag purification (center and right). The fractions of elution-1 (100 mM imidazole) and elution-2 (500 mM imidazole) were independently applied for Strep-tag purification. Black arrowheads indicate the expected size of nCoV-S-NF. **B:** The reverse staining images of Native PAGE and Western blot. The sample elution-A is the protein of Strep-tag purification from the His-tag elution-1 fraction (panel A, center), and elution-B is that from the elution-2 fraction (panel A, right). The positions of the molecular marker are also indicated.

(Fig. 1A). The method enables us to purify the recombinant proteins within ten days from the construction of recombinant bacmid, which is completed in the same rearing series of silkworms. Because the production of the recombinant P1 virus reached to more than 1×10^5 shots from 1 μg of the extracted bacmid DNA (containing helper plasmids and pFastBac plasmids), it is easy to expand the production scale with the same schedule. Our method presented here was an FBS-free method, which could keep cost low and avoid pathogen contamination from FBS.

We first tried to express the ectodomain of SARS-CoV-2 S protein with the native sequence; however, the purified protein was less, and the S protein seemed to be cleaved. Recent studies demonstrated that SARS-CoV-2 possesses a furin protease-cleavage site in its spike gene [7–9,11,20]. In the BEVS using Sf9 cells, the expressions of the ectodomain of SARS-CoV-2 S protein seemed to be successful, although the native sequence of S protein was partially affected by furin proteases. On the other hand, most of the recombinant nCoV-S protein in the silkworm seemed to be processed by furin protease (Figs. 2A and 3). Because the cleavage by furins is a proprotein processing, the expressed nCoV-S was digested in the cells, resulting in the loss of N-terminal signal peptide from the C-terminal half of S protein (S2 domain). According to this idea, the truncated form of S2 protein could not be secreted to silkworm serum, so we could not find enough nCoV-S in the extracted silkworm serum. Indeed, S protein without furin cleavage site (nCoV-S-NF) was successfully appeared in the silkworm serum (Fig. 2B), supporting the above idea. Although the intact form of nCoV-S-NF was purified, we also found the digested protein (about 70 kDa), including the S2 domain (Fig. 4). The S protein of SARS-CoV-2 possesses another proteolytic site called S2′ (aa 814–817), which is conserved among human-related coronaviruses [20,21]. Therefore, the digested protein found in the purified nCoV-S-NF fraction with the size of 70 kDa was considered to be an S2′-cleaved protein. It was also possible that the digested protein was a furin-cleaved protein with less digestion activity, although the details have not been clear so far. Because S protein of SARS-CoV-S is considered to be a glycoprotein and has multiple glycosylation sites in its amino acid sequence [22], the size of nCoV-S and its cleaved protein would appear at the position higher than the estimated sizes (intact nCoV-S = 141 kDa, furin-cleaved S2 = 64 kDa, S2′-cleaved S2 = 50 kDa). The furin-cleavage site of S protein is considered to be a unique feature of SARS-CoV-2 and might function in its infectivity [8,9,21]. For the production of mature S2 protein in silkworm, one possible idea is a construction of S2 protein with directly connecting signal peptides in its N-terminal region. For the production of the entire ectodomain of S protein without mutation in the furin-cleavage site, it is required to repress the furin activity in silkworms, although it has been unclear which and how many furin proteases function in silkworms.

In the affinity chromatography of nCoV-S-NF using the Ni-sepharose column, the recombinant proteins were separated in elution-1 (containing 100 mM imidazole) and elution-2 (containing 500 mM imidazole) (Fig. 4). The denatured PAGE showed that the purified proteins that came from those two fractions showed almost the same in size, suggesting that the components of those fractions consist of the same molecules. On the other hand, the Native PAGE image showed that the significant signals appeared in almost the same position, but the size of lower bands was different. These implied that the secreted nCoV-S-NF formed some different complexes. It might include heterooligomer with a cleaved S2 domain.

In the fat body of infected larvae and the infected pupal cells, nCoV-S was insoluble and appeared as the smeared bands in the PAGE and Western blot images (Fig. 2A). The estimated size of the smeared patterns ranged around 100 kDa, suggested that the nCoV-S was degraded and formed an insoluble complex with other host proteins in the larval fat body and pupal cells. The furin protease would contribute to this degradation because the size of the insoluble protein was larger in nCoV-S-NF, although they still showed smeared pattern in the Western blot images (Fig. 2B).

We successfully purified the ectodomain of SARS-CoV-2 S protein as a secreted oligomer protein. Because the purified nCoV-S-NF was immunoreactive even in the native page, it will be a useful tool for the development of immunodetection kits, antigen for immunization, and also vaccines.

Declaration of competing interestCOI

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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