Expression of SARS-CoV-2 Spike Protein in *E. coli* and Purification

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A B S T R A C T

Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) is the virus responsible for causing COVID-19. The virus has a homotrimer S protein, which is a glycoprotein essential to the early stage of infection. The Spike protein has 1273 amino acids and a molecular weight of approximately 142.6 kDa. In this study, the Spike protein was expressed and purified from Escherichia coli using pGBW-m4046887, which was purchased from Addgene. Due to codon bias in the conventional Escherichia coli BL21 (DE3) strain, pGBW-m4046887 was transformed in Escherichia coli BL21-CodonPlus (DE3)-RP and grown in colonies with 50 µg/mL of chloramphenicol at 30℃. The Spike protein was induced using 0.5 mM IPTG overnight at 18℃, and the expressed protein was purified using immobilized metal affinity chromatography (IMAC) since it contains a 6xHis tag at the C-terminus end. After protein induction, samples were analyzed using SDS-PAGE, and predominant protein bands were observed between 100 and 130 kDa. However, protein at the expected molecular weight was not observed, which could be attributed to codon bias. In the future, protein expression could be performed in the eukaryotic expression system to overcome this.

Keywords:
Spike protein
pGBW-m4046887
Escherichia coli BL21 (DE3)
Escherichia coli BL21-CodonPlus (DE3)-RP

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1. Introduction

COVID-19 is caused by SARS-CoV-2, a highly contagious and virulent virus. Its spread threatens public health and safety. The virus was first reported in Wuhan, China in December 2019 (Florindo *et al.,* 2020). The World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern on January 30, 2020, and a pandemic on March 11, 2020 (Wang *et al*., 2020). As of April 6, 2023, the World Health Organization (WHO) announced 762 million confirmed cases and 6.89 million deaths globally. SARS-CoV-2 belongs to the family Coronaviridae and subfamily Coronavirinae. SARS-CoV-2 is related to Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Masuda *et al*., 2022). SARS-CoV-2 is an enveloped, positive-sense RNA virus. It has four main structural proteins, including spike (S) and membrane (M) glycoproteins, envelope (E), and nucleocapsid (N) protein (Mittal *et al*., 2020).

The homotrimer S protein, a glycoprotein, is essential to the early stage of infection (Yurkovetskiy *et al*., 2020). The main target of the S protein's binding activity is the Angiotensin-Converting Enzyme 2 (ACE2) receptor. The virus binds to the ACE2 receptor on the host cell, enabling viral binding, fusion, and subsequent pathogenesis leading to severe respiratory infection (Walls *et al*., 2020). Spike protein has 1273 amino acids and a molecular weight of around 140 kDa in its monomer form (Huang *et al*., 2020). It comprises two main functional subunits, S1 and S2 (Berger and Schaffitze, 2020; Guruprasad, 2020) (**Fig.1**). N-terminal (NTD) and receptor-binding domain (RBD) are present in the S1 subunit. The S2 subunit contains Fusion Peptide (FP), heptapeptide repeat sequences 1 & 2 (HR1 & HR2), Transmembrane Domain (TM), and CTD (Berger and Schaffitze, 2020; Walls *et al*., 2020). The NTD can identify carbohydrates like sialic acid necessary for viral attachment to the host cell surface. The RBD of the S1 subunit mediates the binding of Spike protein to the ACE2 receptor. FP is involved in the membrane-fusion machinery in the S2 subunit of the S protein. The HR1 and HR2 form the six helical bundles in an antiparallel format of the fusion core, and they are essential for the membrane-fusion machinery and subsequent entry of the S2 subunit. TM anchors the Spike protein to the SARS-CoV-2 membrane (Florindo *et al*., 2020; Huang *et al*., 2020).

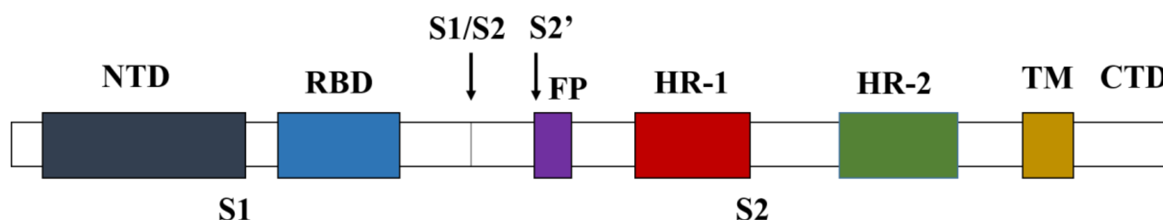

Fig. 1. Schematic representation of SARS-CoV-2 Spike Protein. S1: S1 Subunit; S2: S2 Subunit; S1/S2: S1/S2 Cleavage Site; S2’: S2’ Cleavage Site; NTD: N-terminal Domain; RBD: Receptor Binding Domain; FP: Fusion Peptide; HR-1: Heptad Repeat 1; HR-2: Heptad Repeat 2; TM: Transmembrane Domain; CTD: C-terminal Domain.

Spike protein has more immunogenicity compared to all other proteins. Therefore, one of the most sensible strategies for creating vaccines and therapeutic agents is to target the S protein (Masuda *et al*., 2022). mRNA-based and recombinant virus-based vaccines that

express spike protein in the human body are now in use. Vaccines against inactivated viruses and recombinant proteins have also been produced, although their production costs are expensive (Masuda *et al.*, 2022). Using the *E. coli* expression system, it can be made inexpensively. Recombinant protein expression in *E. coli* has the benefits of being quick, easy, and at a high level. However, the lack of tRNA molecules (codon bias) in *E. coli* BL21(DE3) impairs translation. This results in the expression of truncated or no proteins. *E. coli*. BL21-CodonPlus (DE3)-RP cells contain extra copies of arginine and proline tRNA genes which allows high-level expression of proteins that are difficult to be expressed in conventional BL21(DE3) strain.

2. Materials and methods

2.1. Plasmids, bacterial strains, and culture media

pGBW-m4046887, was a gift from Ginkgo Bioworks & Benjie Chen (Addgene plasmid #145730; http://n2t.net/addgene:145730; RRID:Addgene_145730). This plasmid has wild type DNA sequence of SARS-CoV-2 surface glycoprotein (Spike protein). The molecular size of pGBW-m4046887 is 5552 bp, and the insert (Spike gene) has a size of about 3815 bp. It has a bacterial origin of replication followed by the chloramphenicol-resistant marker gene. The spike gene with a 6xHis tag at the C-terminus end is located downstream of the lac operator.

Restriction digestion protocol was followed to confirm the presence of the pGBW-m4046887. EcoRI-HF and BamHI-HF were used to conduct a double digestion procedure as the plasmid consists of EcoRI and BamHI recognition sequences. The restriction mixture with a total volume of 10 µL contains Plasmid DNA (1 µg/ µL), rCutSmart Buffer (10X), Double distilled water, Restriction endonuclease EcoRI-HF (20,000 Units/mL), BamHI-HF (20,000 Units/mL) was prepared and incubated at 37℃ for 2 hours. The restriction mixture was loaded on 1% agarose gel, and electrophoresis was performed. pACYC, The molecular size of pACYC is 4245 bp. The vector pACYC has a bacterial origin of replication, chloramphenicol-resistant marker, and copies of proline and arginine tRNA genes. The pACYC plasmid was isolated from the *E. coli* BL21-CodonPlus (DE3)-RP cells. The plasmid was restricted with EcoRI-HF and resolved on 1% agarose gel.

Different media were used during the protein induction procedure, including Luria-Bertani (LB medium), Minimal medium (M9 medium), and Terrific medium. The LB medium contains tryptone, yeast extract, and NaCl. The Minimal medium (M9 medium) contains Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $MgSO_4$ and $CaCl_2$. The terrific broth contains Yeast extract, Tryptone, Glycerol, KH_2PO_4, and K_2HPO_4. The *E.coli* BL21 (DE3) competent cells were prepared by adding $CaCl_2$ (0.1 M). pGBW-m4046887 and pACYC vector were co-transformed into the conventional *E. coli* BL21 (DE3) cells by heat shock (42℃) method. To predict the Minimum Inhibitory Concentration (MIC), the transformed culture was inoculated in Luria-Bertani (LB) medium (broth & agar) containing different concentrations of chloramphenicol (20, 25, 50, 75, 100, and 125 µg/mL). The chemicals used in the media preparation were purchased from Sigma-Aldrich and Merck.

2.2. Cell growth and the expression of recombinant Spike protein in E. coli

After screening the transformed colonies on an agar plate containing 125 µg/mL of chloramphenicol, they were inoculated into 2 mL of LB broth with 50 µg/mL chloramphenicol. The tubes were incubated overnight at 30°C at 170 rpm for overnight. Plasmid was isolated, and restriction with EcoRI-HF was performed to confirm the presence of both plasmids in the colonies.

The colonies have both plasmids that were further used for protein expression. 1 mL of the overnight culture was inoculated into 100 mL LB medium with 50 µg/mL chloramphenicol. The culture was incubated at 30°C at 170 rpm until the OD^{600nm} values reached 0.6-0.8. Cell growth was monitored by measuring the OD^{600nm} with a spectrophotometer (Hitachi, Japan). Once the absorbance reached 0.6, the temperature was decreased to 18°C, and the cells were incubated at 90 rpm for 30 min. 1 mL of culture was collected before protein induction as a negative control. Then the culture was induced by adding IPTG (final concentration of 0.5 mM) and left overnight in the shaker incubator at 18°C. The culture was centrifuged at 8000 rpm for 10 min at 4°C. The cell pellet was collected and stored at -20°C.

2.3. Purification of recombinant Spike protein

After harvesting, the pellet was suspended in 3 mL of buffer1 (50% Sucrose, 1M Tris-HCl, 0.5M EDTA). 60 µL of lysozyme was added to the sample to remove the bacterial cell wall debris. It was incubated at room temperature for 30 min. After the centrifugation (12000 rpm, 10 min, 4℃), 4 mL of lysis buffer (20 mM Phosphate buffer (pH 8.0), 500 mM NaCl, and 2 mM PMSF) was added to the pellet. The cells were lysed by homogenization at 5 kPsi, twice. Ten minutes of incubation in ice (4℃) between the processes were carried out. The crude protein sample (supernatant) was centrifuged at 12000 rpm, for 10 min, at 4°C.

The crude protein sample was purified in a column containing 1 mL of Ni-IDA - agarose resin (GE HealthCare, USA). Initially, the column was equilibrated with wash buffer (20 mM Phosphate buffer (pH 8.0), 500 mM NaCl and 2 mM PMSF). 6 mL of cell lysate were added to the column by washing with 10 mL of wash buffer. The purified Spike protein was eluted with 2 ml of buffer containing different concentrations of imidazole (50, 100, 150, 200, 250, 300, and 350 mM). The purified Spike protein was quantified by Bradford assay and analyzed using SDS-PAGE.

2.4. Analytical methods

The total protein concentration was quantified using the Bradford method. The bovine serum albumin was used as a standard protein (Bradford, 1976). The recombinant protein expressed and accumulated in cell lysates was analyzed using SDS-PAGE. The sample pellets were resuspended in phosphate buffer saline (PBS) and boiled for 10 min (Laemmli, 1970). The protein samples were resolved on 10% SDS-PAGE gel. The gel was stained with a Stainer containing 0.25% Coomassie Brilliant Blue R-250, 50% methanol, 7% acetic acid, and 43% double distilled water. Then it was destained with the detainer (50% methanol, 7% acetic acid, 43% double distilled water).

3. Results and discussion

3.1. Plasmid isolation and confirmation

The pGBW-m4046887 was received as a bacterial stab of *E. coli* DH5α from Addgene. The vector map of the plasmid is shown in **Fig.2**.

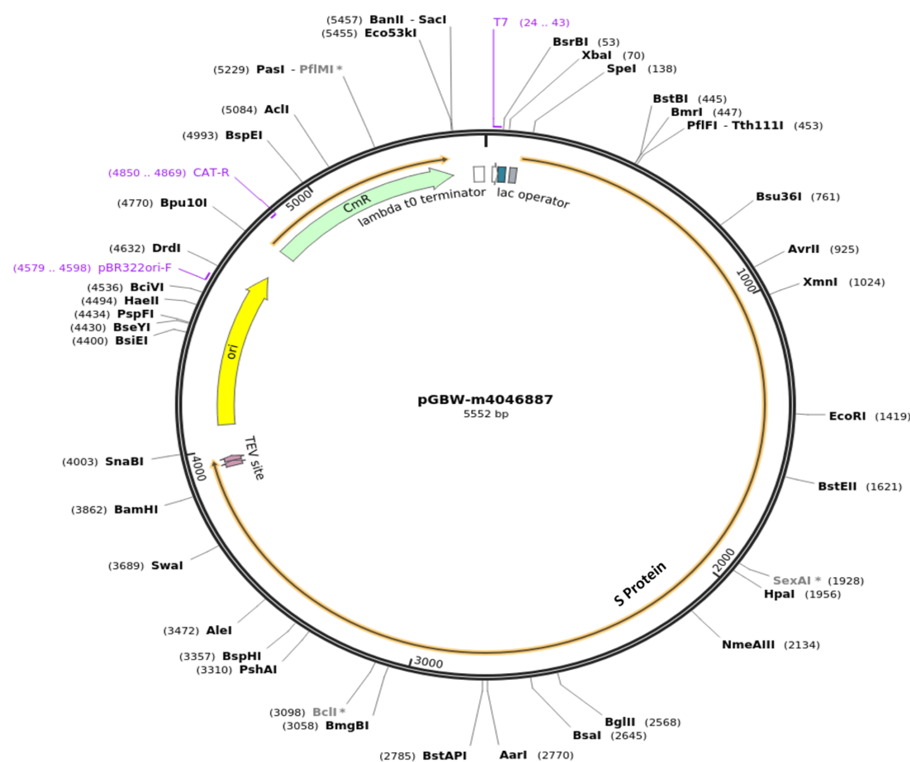

Fig. 2. Plasmid map of pGBW-m4046887. CmR: Chloramphenicol acetyltransferase confers resistance to chloramphenicol; S Protein: SARS-CoV-2 Spike Protein (Surface glycoprotein); TEV site: tobacco etch virus protease recognition and cleavage site; ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication

The plasmid was isolated using the alkaline lysis method. Double digestion was done using restriction endonucleases (EcoRI and BamHI), as each has single restriction sites. Restricted samples were then resolved on 1% agarose gel. The DNA bands near 3100 and 2500 bp (**Fig.3a**) were observed on the UV platform, which equals the molecular weight of the plasmid pGBW-m4046887. Thus, the presence of the plasmid pGBW-m4046887 was confirmed.

E. coli BL21-CodonPlus (DE3)-RP is a modified *E. coli* BL21 (DE3) strain with pACYC derived plasmid of 4245 bp. This pACYC plasmid harbors extra copies of arginine and proline tRNA genes that enhance protein expression. *E. coli* BL21-CodonPlus (DE3)-RP was used as a source of the pACYC plasmid. The pACYC plasmid was isolated using the alkaline lysis method. The plasmid was restricted with EcoRI-HF enzyme and resolved on 1% agarose gel. The DNA band near 4200 bp was seen on the gel (**Fig.3b**).

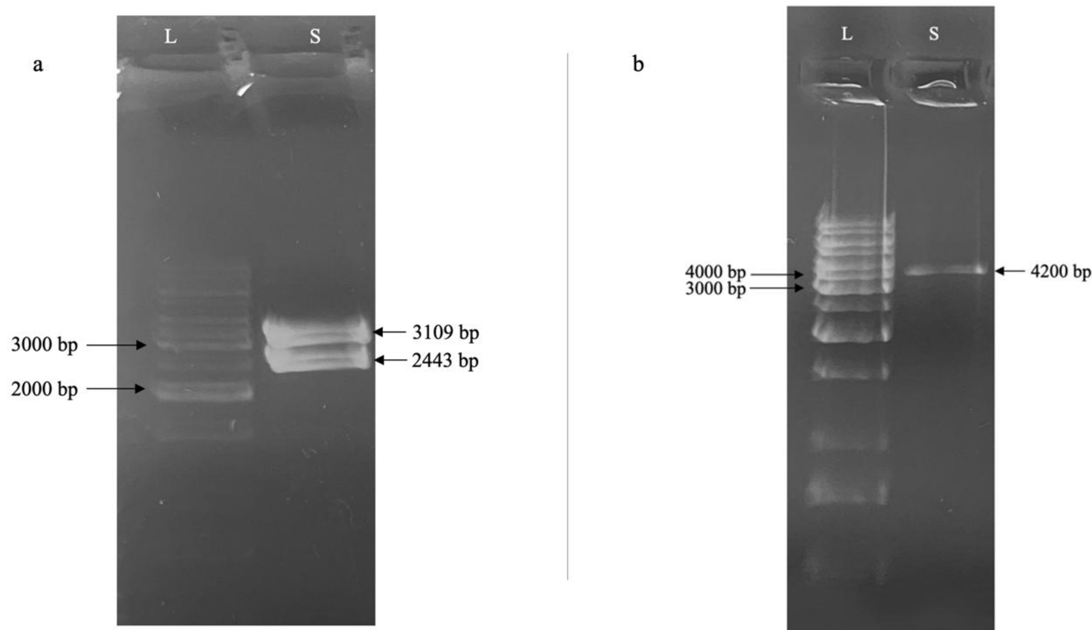

Fig. 3. (a) Double digestion of pGBW-m4046887. Legend: L: 1kb DNA ladder; 1: pGBW-m4046887 digested with EcoRI and BamHI; (b) Single digestion of pACYC. Legend: L: 1kb DNA ladder; S: pACYC restricted with EcoRI

3.2. Spike protein expression in E. coli

The pGBW-m4046887 plasmid was chemically transformed into the conventional *E. coli* BL21 (DE3) competent cells. These cells were grown on an agar plate containing 25µg/ml of chloramphenicol and incubated at 30℃ overnight. The growth of a few bacterial colonies were seen on the agar plate. To predict the optimum antibiotic concentration, the transformed bacterial colonies were grown in LB broth containing different chloramphenicol concentrations (5, 10, 15, and 20 µg/ml). The optimum growth was seen at 10 µg/ml of chloramphenicol concentration. The same protocol was carried out in LB agar plates containing different chloramphenicol concentrations (5 µg/ml, 10 µg/ml, 15 µg/ml, and 20 µg/ml). The optimum growth of the transformed bacterial colonies was seen at 10 µg/ml. During the initial stage, the induction procedure was carried out overnight using 1 mM of IPTG at 18°C. But no significant band was seen at 142 kDa, corresponding to the Spike protein's molecular weight. Considering that, the pH could have been a problem, the transformed colonies were induced in a terrific broth containing phosphate buffer. The protein induction procedure was also carried out using M9 media. The samples were induced at different IPTG concentrations (0.2, 0.4, 0.5, 0.6, and 0.8 mM) and incubated at different temperatures (30℃, 37℃, 42℃).

Table 1

Conditions used for Protein Expression and Purification in *E. coli* BL21 (DE3)

PARAMETERS	CONDITIONS	PROTEIN EXPRESSION

Chloramphenicol concentration in LB Broth	10 µg/mL and 25 µg/mL	NIL
IPTG Concentration	0.2 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.8 mM, and 1mM	NIL
Induction Temperature	18°C, 30°C, 37°C, and 42°C	NIL
Induction Medium	LB, M9, and Terrific Broth	NIL
Buffers for Protein purification	Tris-HCl and Phosphate	NIL
Buffer components	The buffer contains PMSF (2 mM), 2-mercaptoethanol (10mM)	NIL
Sample Preparation	Lysed by using Lysozyme	NIL
Cell Disruption	Homogenization, Ultrasonication	NIL

Escherichia coli has been widely used to produce recombinant proteins due to the enormous knowledge about its genetics and metabolism that already exists. In many circumstances, a few hours after induction, the expression of the target protein reaches a high level. There are several *E. coli* strains are available. It can be employed to get around any difficulties that can arise during the expression of recombinant proteins, depending on the characteristics of the target protein. Rare codons can be a significant drawback for the expression of recombinant protein *E. coli* due to the low availability of some tRNAs during the translation. This can significantly decrease the protein yield or lead to truncated protein expression. One of the most commonly used *E. coli* strains for the expression of genes containing rare codons is BL21-CodonPlus (DE3)-RP. BL21-CodonPlus cells allow high-level expression of proteins that are difficult to express in conventional *E. coli.* These *E. coli* BL21-CodonPlus (DE3)-RP cells contain a chloramphenicol-resistant marker gene in the pACYC plasmid. The isolated plasmids were transformed into *E. coli* BL21 (DE3). The optimum growth of co-transformed cells was seen at 50 µg/mL chloramphenicol concentration. Presence of both the plasmids was confirmed.

3.3. Spike protein expression purification using a nickel affinity column

IPTG induction was performed, and the induced sample was purified using a nickel affinity column. The crude protein, eluent with different concentration of imidazole, were loaded on SDS PAGE gel. The uninduced cell pellet was kept as the negative control. A protein band between 130 and 170 kDa was seen (**Fig. 4**) in the crude sample. Multiple bands were observed in eluent with 50, 100, 150, and 200 mM imidazole concentrations.

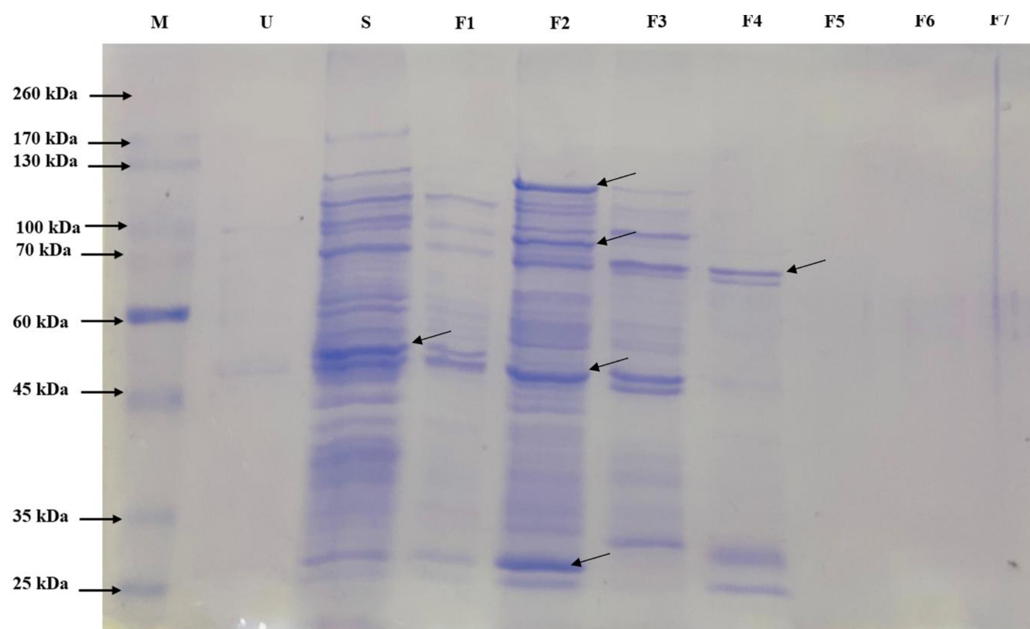

Fig. 4. SDS-PAGE analysis of Spike protein purified using Ni-IDA affinity chromatography. M: pre-stained protein MW marker; U: The Uninduced Sample; S: The crude extract (supernatant); F1: Eluent with 50 mM imidazole mM; F2: Eluent with 100 mM imidazole; F3: Eluent with 150 mM imidazole; F4: Eluent with 200 mM imidazole; F5: Eluent with 250 mM imidazole; F6: Eluent with 300 mM imidazole; F7: Eluent with 350 mM imidazole.

No band was seen in the negative control. Protein n with molecular r weight 122 kDa was predominantly seen in all eluted samples. Around 80, 65, 45, 42, 28, and 26 kDa bands were observed.

3.3. Prediction of the rare codon in spike wild-type gene sequence

The rare codons present in wild-type gene sequence and their occurrence frequency were predicted using the expasy bioinformatics tool. The Codon usage bioinformatics tool predicted the frequency of codon occurrence in wild-type Spike gene sequence. The result is shown in **Table 2.**

Table 2.

Number spike rare codons in E. coli

Amino acid	Rare codon	Number of codons in spike sequence
Arginine	CGA	0
	CGG	2
	AGG	10
	AGA	20
Glycine	GGA	17
	GGG	3
Isoleucine	AUA	18
Proline	CCC	4
Threonine	ACG	3

The rare codons AGG (arginine) and CCC (proline) encoding tRNA gene copies exist in *E. coli* BL21-CodonPlus (DE3)-RP, which are rare in conventional *E. coli* BL21 (DE3) strains. Another rare codon, CGA (arginine), is absent in the wild-type Spike gene sequence. But the rare codon GGA (glycine) occurs 17 times in the wild-type Spike gene sequence. As GGA encoding tRNA gene copies are absent in *E. coli* BL21-CodonPlus (DE3)-RP, the protein expression may be hindered due to codon bias, and truncated proteins were produced.

4. Conclusions

The Spike protein of SARS-CoV-2 was expressed and purified from *E. coli* BL21 (DE3) and *E. coli* BL21-CodonPlus (DE3)-RP. The same was analyzed using SDS PAGE and western blot. But there was, no significant protein bands were seen near 142 kDa. Non-specific bands were observed with different molecular weights. Codon bias could be a reason for the truncated protein production, which can be overcome by the expression of spike protein in the eukaryotic protein expression system.

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