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Research Article

The impact of N-glycans on the immune response of plant-produced SARS-CoV-2 RBD-Fc proteins

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ABSTRACT

Plant-based manufacturing has the advantage of post-translational modifications. While plant-specific N-glycans have been associated with allergic reactions, their effect on the specific immune response upon vaccination is not yet understood. In this study, we produced an RBD-Fc subunit vaccine in both wildtype (WT) and glyco-engineered (ΔXF) *Nicotiana benthamiana* plants. The N-glycan analysis: RBD-Fc carrying the ER retention peptide mainly displayed high mannose. When produced in WT RBD-Fc displayed complex-type (GnGnXF) N-glycans. In contrast, ΔXF plants produced RBD-Fc with humanized complex N-glycans that lack potentially immunogenic xylose and core fucose residues (GnGn). The three recombinant RBD-Fc glycovariants were tested. Immunization with any of the RBD-Fc proteins resulted in a similar titer of anti-RBD antibodies in mice. Likewise, antisera from subunit RBD-Fc vaccines also demonstrated comparable neutralization against SARS-CoV-2. Thus, we conclude that N-glycan modifications of the RBD-Fc protein have no impact on their capacity to activate immune responses and induce neutralizing antibody production.

1. Background

The SARS-CoV-2 coronavirus first appeared in China, and it ultimately caused the COVID-19 pandemic. Vaccination is considered the most viable option for preventing the serious illness and death associated with the novel virus. Notably, significant advancements have been achieved worldwide in the production of COVID-19 vaccines, with an array of technological platforms shaping the global vaccine landscape. These platforms encompass virus-derived vaccines, including live-attenuated and inactivated types, protein-based such as recombinant proteins and virus-like particles, along with nucleic acid vaccines and viral vectors [1]. Recombinant proteins have received a lot of attention as a basis for subunit vaccines, with examples of them conferring complete protection to vaccinated animals against SARS and MERS viral infections [2]. Moreover, the engagement of RBD to ACE2 is crucial for the spread and re-infection of SARS-CoV-2 [3,4]. RBD-Fc is an Fc fusion of RBD that has been used to develop subunit vaccines for COVID-19. Preclinical studies have shown that RBD-Fc-based vaccines are effective [5–7], and clinical research are currently underway. In addition, different platforms were used to generate RBD-based recombinant proteins, including yeast [8], mammalian cells [9,10], and plants [11,12]. Plant expression systems offer several advantages for recombinant protein biosynthesis. They are safe, cost-effective, scalable, and allow for easy product storage. Plant-derived proteins are correctly folded, assembled, and post-translationally modified, making them suitable for various applications [13–16]. Tobacco species have been extensively utilized as valuable platforms for therapeutic antibody production in plants [17].

Glycosylation is a vital modification in eukaryotic organisms that involves the conjugation of carbohydrates (glycans) with proteins and lipids. It is crucial for the proper conformation, functionality, and stability of both membrane-bound and soluble glycoproteins. This process

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is initiated in the endoplasmic reticulum (ER) and undergoes successive glycan processing in the Golgi apparatus [18]. Glycosylation can be categorized into two main types: N-glycosylation and O-glycosylation. This study focuses on protein modifications by N-linked glycosylation, which has been demonstrated to improve therapeutic protein stability, pharmacokinetics, and pharmacodynamics [19]. Plant glycosylation differs from mammalian glycosylation, with GnGnXF structures being the most common in plant-produced proteins. This typical complex plant N-glycan structure consists of β1,2-xylose and α1,3-fucose residues connected to the GnGn core. Additionally, paucimannosidic glycans (MMXF) are the second most abundant glycoform [20]. Carbohydrate moieties with xylose and fucose play a role in IgE recognition [21]. The presence of these non-human glycan species may induce allergic reactions and immunological responses [22,23]. Recently, researchers have successfully removed plant-specific sugars by deleting the genes that encode xylosyl- and fucosyltransferases. As a result, variety of plants can produce human-type glycans (GnGn) devoid of xylose and fucose residues with high uniformity (>90 %). To date, seven plant species, including *N. benthamiana*, have been glycoengineered in this manner [24].

The development of plant-based vaccine candidates has advanced since the COVID-19 pandemic. Yet, understanding the relevance of plant-specific glycan epitopes and recombinant RBD glycosylation in humoral immunogenicity remains limited. Hence, the purpose of this research is to investigate whether immune responses are affected by plant-derived RBD-Fc fusion proteins decorated with different N-glycan structures. The subunit vaccine RBD-Fc was generated in wildtype and glycoengineered *N. benthamiana* and the glycan profiles of each plant-produced glycovariant were characterized. Furthermore, the effect of plant glycosylation on immune response *in vivo* in female ICR mice was evaluated. To gain insight into the neutralizing ability of RBD-specific and total antibody titer, we conducted *in vitro* experiments with Vero E6 cells and a live SARS-CoV-2 isolate from a COVID-19 patient.

2. Materials and methods

2.1. Gene cloning

The vector containing the SARS-CoV-2 RBD fused with C-terminal human IgG1 Fc region was previously generated [7], and the same construct was used in this study. The RBD coding sequence was optimized for plant codons, synthesized (Genewiz, Suzhou, China), and linked to human Fc via G4S $(GGGGS)_3$ peptide. For construction, a murine leader sequence (SP3) [25] was incorporated into the N-terminus (SP3-RBD-Fc, Fig. 1A) and produced in WT or ΔXF *N. benthamiana* [26]. In order to induce fusion protein retention in the ER, a peptide comprising a retention signal (SEKDEL or short KD) was further added to the C-terminus (SP3-RBD-Fc-KD, Fig. 1B). The constructs were then ligated into pBYR2e geminiviral vector [19,27] using restriction sites of XbaI and SacI enzymes (Fig. 1C).

2.2. Production and purification of RBD-Fc in Nicotiana benthamiana

The expression constructs were introduced into competent GV3101 *Agrobacterium tumefaciens* via a MicroPulser electroporator system (Bio-Rad, United States). Transformed colonies were screened for positive gene integration using RBD gene-specific PCR primers. For infiltration, *Agrobacterium* harboring the recombinant RBD-Fc plasmids were cultured in selective LB medium at 28 °C overnight. Cells were pelleted, resuspended in MES buffer containing $MgSO_4$, and diluted to an OD_{600} of 0.2. Bacterial suspensions were then transfected into the leaves of *N. benthamiana* (6-week-old) from abaxial side, specifically SP3-RBD-Fc-KD in WT plants and SP3-RBD-Fc into WT and ΔXF plants. Finally, infiltrated tobacco plants were maintained in a growth room (28 °C, 16:8 h light:dark).

Four days after infiltration (4 dpi), leaves were collected and homogenized with 1X PBS (pH 7.4), and the resulting extract was centrifuged (15,000 rpm for 30 min) at 4 °C. Supernatant was collected and clarified prior to RBD-Fc purification using affinity column chromatography. Protein A resin (MabSelect SuRe™ LX, Cytiva, Sweden) was packed in-house and equilibrated with extraction buffer. Elution of bound proteins was performed with 0.15 M sodium citrate buffer (pH 3.0), followed by subsequent neutralization with Tris-HCl (pH 8.8). Then, RBD-Fc-containing purified fractions were concentrated on 30 kDa Amicon® and sterile filtered using 0.22 μm syringe filter. A schematic diagram for plant protein expression and purification is shown in Fig. 2.

The purity of recombinant RBD-Fc proteins was assessed using 8 % SDS-PAGE gel, followed by Coomassie staining. Meanwhile, peroxidase-linked anti-human IgG (Southern Biotech, United States) diluted 1:5000 and peroxidase-linked anti-RBD (Sino biological Inc., USA) diluted 1:2500 were employed for Western blot detection, as described in our previous study [7].

2.3. Glycan characterization

To examine the glycan patterns, each column-purified RBD-Fc was subjected to LC-ESI-MS. Briefly, SDS-PAGE gel bands corresponding to plant-produced RBD-Fc proteins (~150 kDa) were excised and in-gel digestion with endoproteinases Trypsin, LysC and GluC. Peptides were assayed, and intact protein analysis was carried out using a Q-TOF apparatus (Agilent Technologies 6230B LC-TOFMS) and an Orbitap MS (Thermo Fisher Exploris 480). LC-ESI-MS was performed using Agilent MassHunter BioConfirm B.08.00, and manual glycopeptide searches were done using Thermo Fisher FreeStyle 1.8. RBD-Fc protein generates four glycopeptides: three in the RBD domain (glycopeptides 1–3) and one in the Fc domain (glycopeptide 4), as shown in Table 1.

2.4. Mice immunization

The experimental procedures involving animals were approved by

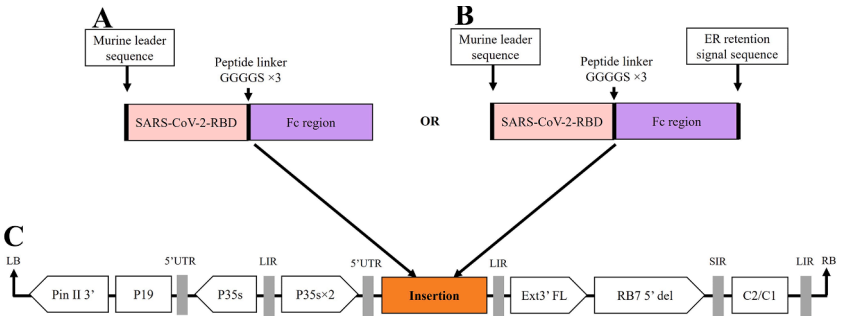


Fig. 1.. Illustration of expression vector constructs used in this study. (A) gene of choice tagged with murine leader sequence at the N-terminus (labeled SP3-RBD-Fc). (B) gene of choice containing both murine leader sequence and ER retention signal sequence (SEKDEL) (labeled SP3-RBD-Fc-KD). (C) The major regulatory components of pBYR2e plant expression vector along with the RBD-Fc gene inserts (labeled Insertion) are depicted.

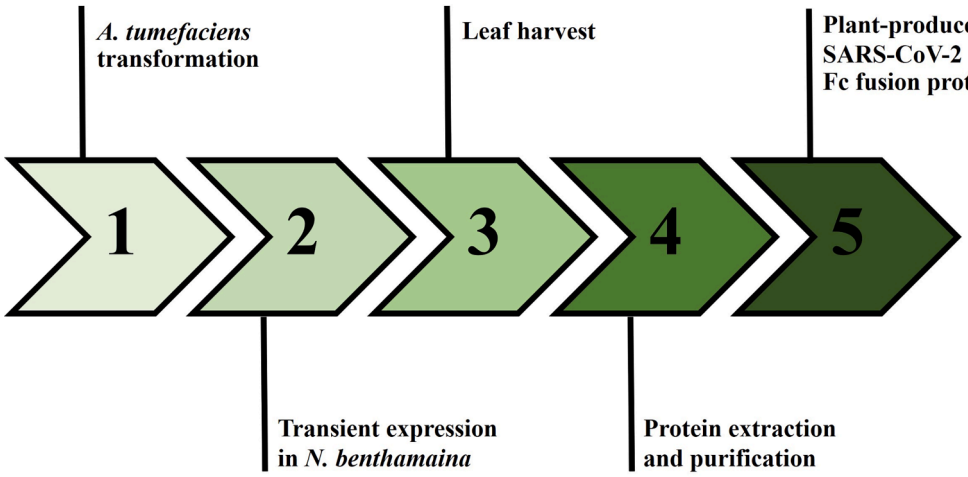


Fig. 2.. Diagram of the RBD-Fc production process in *N. benthamiana* plants using transient expression.

Table 1.
RBD-Fc protein generates four glycopeptides.

Glycopeptide	Sequence
Glycopeptide 1	FPNITNLCPFGE
Glycopeptide 2	VFNATR
Glycopeptide 3	ITPCSFGGVSVITPGTNTSNQVAVLYQDVNCDISGGGGSGGGGSPPCPAPE
Glycopeptide 4	EEQYNSTYR

the IACUC of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Protocol review No. 23-33-004). 20 ICR mice of seven-week-old were randomly allocated to four groups (n= 5/group) and given either the plant-produced subunit vaccines or PBS for the control group. Before immunization, each RBD-Fc protein (10 μg) was mixed with 3M-052-AF (10 μg) and alum (2 μg) adjuvants [28]. Mice were immunized via i.m. route with the following vaccine formulations at weeks 0 and 3. Blood samples were drawn on day zero (pre-bleed) and two weeks after each vaccination to prepare serum, which was then analyzed in the next experiment.

2.5. Evaluation of total antibody responses

The anti-RBD IgG titers in mouse sera were determined using ELISA [7], with 2 μg of Sf9-derived RBD (GenScript, United States) as the coating antigen. Plates were washed with 1X PBS-T, then blocked with 5 % nonfat dry milk. After three washes, serum samples in twofold serial dilution (from 1:100) were assayed for 2 h at 37 °C. Bound RBD-specific IgGs were probed with HRP-linked anti-mouse IgG (1:2000) (Jackson ImmunoResearch, Pennsylvania, United States) for detection with peroxidase substrate TMB (Promega, United States). The binding antibody responses were measured at 450 nm wavelength (A_{450}) using a S-100 Nano Scan microplate reader (Hercuvan, United Kingdom). Endpoint titer against RBD-Fc was reported as the highest reciprocal dilution of vaccinated sera with a calculated A_{450} twofold greater than the A_{450} threshold value of pre-immune sera in 1X PBS (1:100) [29].

2.6. In vitro neutralization ability

The microneutralization assay was conducted in a biosafety level 3 facility at the Department of Microbiology in Mahidol University, Bangkok, Thailand, as previously described [7]. Vero E6 cells were seeded with 10,000 cells per well for overnight at 37°C. Meanwhile, convalescent serum from COVID-19 patient and immunized mice sera were heat-inactivated (30 min at 56 °C), twofold serially diluted in DMEM, and then incubated with 100$TCID_{50}$ of the live SARS-CoV-2 virus (SARS-CoV-2/01/human/Jan2020/Thailand) for 1 h at 37 °C. Additionally, virus control (100$TCID_{50}$) and uninfected cells were tested in parallel. The mixtures of virus and diluted sera were added to the Vero E6 cell monolayer and incubated for two days at 37 °C. Post incubation, cells were washed once with 1X PBS, immersed in ice cold methanol: acetone (1:1) for fixation and permeabilization, and then blocked with a 2 % BSA blocking solution. To detect viral nucleocapsids, anti-SARS-CoV-2 N-specific IgG (SinoBiological, United States) diluted 1:5000 was used as the primary antibody and incubated on cells for 1 h at 37 °C. Secondary antibody incubation with peroxidase-linked goat anti-rabbit polyclonal antibody (Dako, Denmark) diluted 1:2000 followed and was performed in the same manner as the primary incubation step. Finally, the KPL Sureblue™ TMB substrate was added (SeraCare, United States), and enzyme reaction was quenched with 1 M HCl. UV absorbances were measured using the Sunrise™ microplate reader (Tecan, Switzerland) at 450 nm and 620 nm as a reference.

The effective neutralization titers of vaccinated sera were calculated using the average absorbances at 450 and 620 nm (A_{450}-A_{620}), and the endpoint was estimated by 50 % specific signal of the cut point [7]. The neutralizing anti-RBD IgG titer was reported to be the highest reciprocal serum dilution, with an absorbance value twofold greater than the cut-off threshold.

2.7. Statistical Analysis

The graphs were built with GraphPad Prism 8.0 (GraphPad Software). Comparative analysis of immunological responses and neutralizing antibody titers among each experimental groups used the Kruskal Wallis test, followed by Dunn-Bonferroni post hoc test. Data analysis was performed by SPSS Statistics 23 (IBM), and a value of $p< 0.05$ was considered statistically significant.

3. Results

3.1. Production and purification of RBD-Fc fusion protein

To express the recombinant fusion protein in plant leaves, we linked RBD to human IgG1 Fc and inserted the RBD-Fc coding sequence into the pBYR2e expression vector (Fig. 1). In this study, two RBD-Fc protein constructs were synthesized: SP3-RBD-Fc and SP3-RBD-Fc-KD. With a murine leader peptide at the N-terminus, the SP3 signal sequence enables entry to the secretory pathway for glycosylation process of proteins in the ER and Golgi complex [30]. Moreover, the SEKDEL peptide fused to the C-terminus, enables protein retrieval to the ER to prevent glycan processing in the Golgi [31]. The pBYR2e plasmids encoding the RBD-Fc proteins were infiltrated into wildtype and glycoengineered (ΔXF) plants using the Agrobacterium-mediated method, and the expression period was obtained at 4 dpi, as previously described [7,32].

After leaf harvest, extraction, and pellet removal, the resulting supernatant underwent purification using protein A column to capture the Fc region containing proteins. SDS-PAGE and immunoblotting results showed the presence of RBD-Fc in the purified fractions (Fig. 3). The yields of SP3-RBD-Fc-KD (WT), SP3-RBD-Fc (WT), and SP3-RBD-Fc (ΔXF) proteins after purification were 10.56, 13.82, and 7.00 μg/g fresh leaf weight, respectively. Protein bands of approximately 75 kDa and 150 kDa were observed in stained gels under reduced and non-reduced conditions, respectively (Fig. 3A,B). These bands correspond to the monomeric (75 kDa) and dimeric (150 kDa) states of RBD-Fc. Homodimer formation is likely caused by disulfide bonds between RBD-Fc molecules [33,34]. The purified plant-produced RBD-Fc proteins were then examined using either anti-RBD (Fig. 3C) or anti-human IgG (Fig. 3D,E) antibodies. Similar band patterns from stained SDS gels were detected with RBD-Fc proteins in the dimeric state when probed with RBD-specific (Fig. 3C) and Fc fragment-specific (Fig. 3E) antibodies under non-reducing condition. In contrast, monomeric RBD-Fc was observed when proved with anti-human IgG (Fig 3D) under reducing condition. These results confirm the presence of both RBD and Fc in the fusion protein, as indicated by distinct bands of expected molecular sizes. The data presented here demonstrate the succesful production in *N. benthamiana* plants and purification of RBD-Fc glycovariants.

3.2. Plant-derived RBD-Fc proteins contain different N-glycan patterns

To determine N-linked glycosylation on plant-derived RBD-Fc proteins, SDS gels containing purified proteins (~150 kDa) were digested by proteases prior to analysis by LC-ESI-MS. According to its amino acid sequence, RBD-Fc has four N-glycan positions: three on the RBD site (Fig. 4A–C) and one on the Fc site (Fig. 4D). Expression of SP3-RBD-Fc-KD in WT plants generated mannose-type glycans at both RBD and Fc N-glycosylation sites. As shown in the mass spectrum of RBD-KD_WT, purified RBD-Fc carries oligomannosidic N-glycans (Man5-Man9), specifically Man8. Meanwhile, purified RBD-Fc from WT (RBD-Fc_WT) contains high levels of complex-type glycans with β1,2-xylose and α1,3-fucose (GnGnXF) and lower levels of GnGn or oligomannosidic glycans. In contrast, the mass spectra of SP3-RBD-Fc expressed in ΔXF (RBD-Fc_XF) almost completely lacked plant-specific complex glycans and showed a predominance of human-type complex N-glycans (GnGn).

3.3. Vaccination elicited similar anti-RBD and neutralizing antibody responses

The immunogenicity of RBD-Fc glycoproteins produced in plants was assessed in vivo in ICR mice using three vaccine formulations: one 10 μg of SP3-RBD-Fc-KD with adjuvants, 10 μg of SP3-RBD-Fc (WT) with adjuvants, and 10 μg of SP3-RBD-Fc (ΔXF) with adjuvants. In the case of formulations with adjuvants, 10 μg of 3 M was mixed with 2 μg of Alum (3M-Alum). A negative control group received PBS with 3M-Alum adjuvants. A two-dose regimen was employed between days 0 and 21, and mouse sera were collected on days 0 (pre-dose), 14, and 35 (Fig. 5A). Anti-RBD antibodies were detected using ELISA as captured by the RBD-His antigen. Compared to the control group, plant-based RBD-Fc-vaccinated mice sera induced high titers of total IgG after the second immunization. RBD-specific titers increased after the second RBD-Fc dose, with the geometric mean titers (GMT) increasing from 2425.15

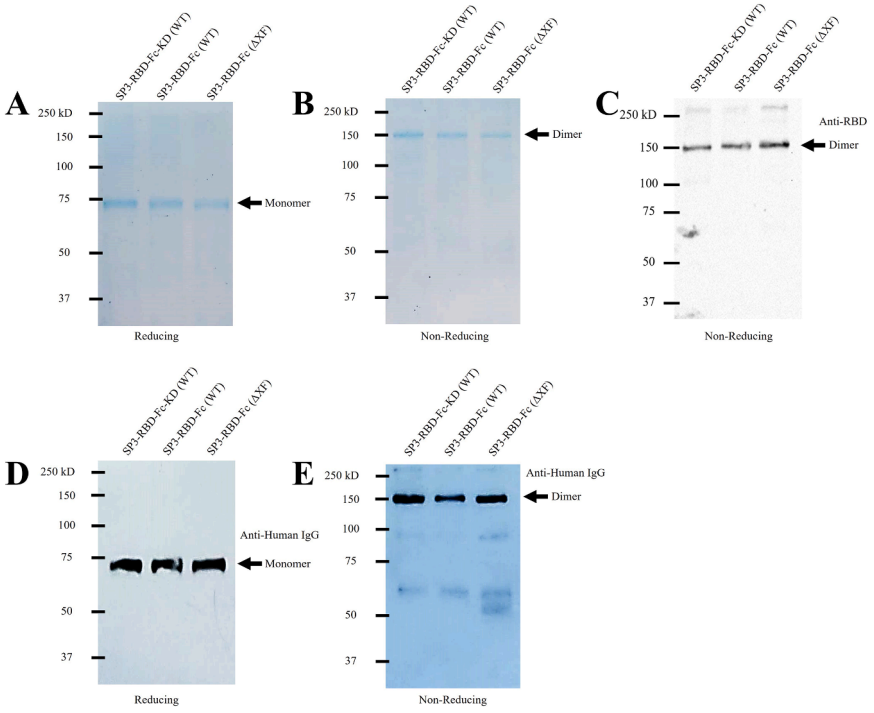


Fig. 3.. Purification of plant-produced Fc-tagged RBD. (A, B) Coomassie stained acrylamide gels of purified RBD-Fc fusion proteins under (A) reduced and (B) non-reduced conditions. (C-E) Immunoblotting of purified RBD-Fc proteins against (C) HRP-conjugated anti-RBD and (D, E) HRP-conjugated anti-human IgG.

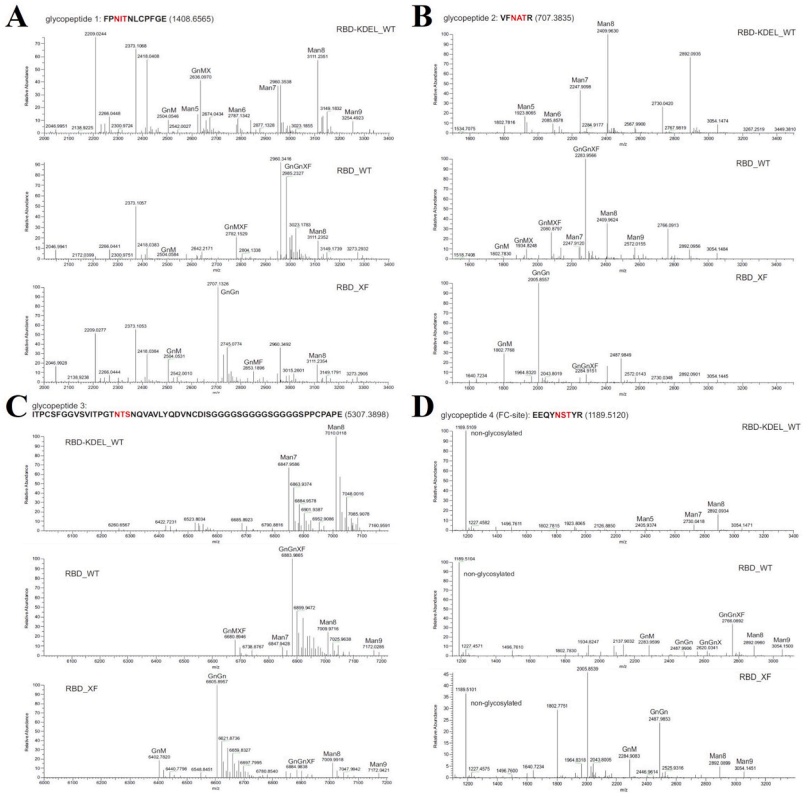
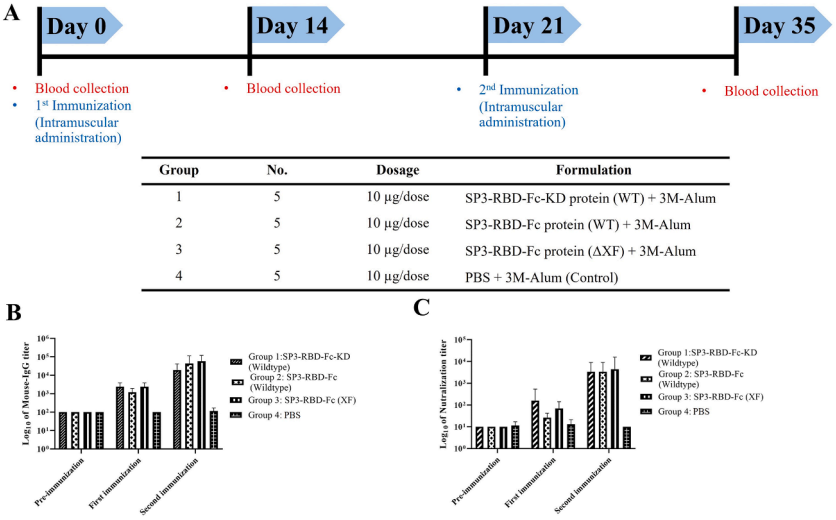


Fig. 4.. N-glycan analysis of plant-based RBD-Fc glycoforms. Purified fractions from infiltrated *N. benthamiana* WT (RBD-KDEL_WT and RBD_WT) and ΔXF plant (RBD_XF) were analyzed by LC-ESI-MS. The spectra show RBD-Fc glycopeptides carrying four N-glycosylation sites. The labeled peaks indicate $(M + Na)^+$ ions, while other peaks represent potassium adducts. Cartoon representations of main peaks are provided.



Group	No.	Dosage	Formulation
1	5	10 μg/dose	SP3-RBD-Fc-KD protein (WT) + 3M-Alum
2	5	10 μg/dose	SP3-RBD-Fc protein (WT) + 3M-Alum
3	5	10 μg/dose	SP3-RBD-Fc protein (ΔXF) + 3M-Alum
4	5	10 μg/dose	PBS + 3M-Alum (Control)

Fig. 5.. Immunogenicity study of adjuvanted RBD-Fc formulations in ICR model. (A) Schematic illustration depicts immunization schedule and sample collection. (B) Reciprocal mouse RBD-specific total IgG titers from immunized sera against SP3-RBD-Fc-KD (WT), SP3-RBD-Fc (WT), SP3-RBD-Fc (ΔXF), and PBS (negative control) collected at days 0 (pre-immunization), 14 (first immunization) and 35 (second immunization). (C) Calculated neutralizing IgG titers of mice sera against SP3-RBD-Fc-KD (WT), SP3-RBD-Fc (WT), and SP3-RBD-Fc (ΔXF) collected at pre-dose, week 2, and week 5. The anti-RBD total IgG and neutralizing IgG titers were represented as GMT.

(SP3-RBD-Fc-KD WT), 1212.57 (SP3-RBD-Fc WT), and 2425.15 (SP3-RBD-Fc ΔXF) to 19,401.17, 44,572.19, and 58,813.36, respectively (Supplementary Table S1). Moreover, the anti-RBD IgG levels were comparable among mice given different adjuvanted-vaccine formulations of SP3-RBD-Fc-KD (WT), SP3-RBD-Fc (WT), and SP3-RBD-Fc (ΔXF) ($p > 0.05$) (Supplementary Table S2 and Fig. 5B).

The neutralizing antibody response of RBD-Fc-immunized sera was evaluated through an in vitro microneutralization assay. Mice sera were incubated with the live SARS-CoV-2 virus before infection to Vero E6 cells. Neutralization titers were then calculated and reported as the highest sera dilution greater than the negative control. The results showed that neutralizing antibody responses from RBD-Fc-vaccinated sera were significantly induced after boost injections ($p < 0.05$) (Supplementary Table S3,S4). Meanwhile, the levels of neutralizing

antibodies were similar across all adjuvanted-vaccine formulations ($p > 0.05$) (Fig. 5C). After the second immunization, the neutralizing GMT for SP3-RBD-Fc-KD (WT), SP3-RBD-Fc (WT), and SP3-RBD-Fc (ΔXF) were 3377.94, 3377.94, and 4457.22, respectively. All analyzed data are provided in Supplementary Material.

Several COVID-19 subunit vaccines contain RBD antigens, which are widely recognized as highly effective targets for specific immune reactions to the SARS-CoV-2 virus. Their specificity and critical function in virus entry make them a prominent focus in COVID-19 vaccine research and development [1,35]. In particular, the SARS-CoV-2 infects the host cell via RBD and receptor ACE2 binding. Previously, recombinant RBD-based vaccines have demonstrated their effectiveness in protecting rodents and monkeys [7,36,37]. In this study, the RBD-Fc was a partial sequence of the spike protein from residues 317–617, which includes segments of the RBD as well as the furin cleavage site [7]. Moreover, the spike protein contained over 20 N-linked glycosylation sites [38,39]. For glycan analysis, we used the glycosylation sites N331, N343, and N603 at RBD and N297 at Fc that were included in the RBD-Fc protein. The RBD sequence was synthesized by fusing the amino-terminal murine signal peptide or SP3 for protein translocation across the ER membrane [40] and the carboxy-terminal signal peptide (SEKDEL) for retrieval from the Golgi complex or effective retention within the ER. One advantage of ER-associated glycosylation via SEKDEL tagging is the generation of oligomannose glycans that are identical across all species, including humans, yeasts, and plants. The high-mannose structures are then processed in the Golgi, where they undergo extensive modifications to form a diverse array of hybrid and complex glycan subtypes [41]. Herein, we examined the effect of N-glycans on the plant-produced RBD-Fc in the context of different N-glycosylation patterns and their impact on mouse immune response. The goal of the present research was to improve the efficacy of RBD-Fc immunotherapy in humans.

Plant-based manufacturing processes have been advantageous in producing the RBD-Fc protein owing to their high yield capacity, with roughly 22 mg/kg of *N. benthamiana* leaves [11]. Conversely, the use of ExpiCHO-S cells obtained a comparatively lower yield of approximately 4.2 mg/L [6]. This disparity highlights the apparent benefits of using plants to generate higher quantities of the RBD-Fc protein, which could increase its availability for therapeutic applications and reduce productions costs. Nonetheless, plants continue to face challenges in their role in manufacturing methods due to differences in glycan processing with humans. For example, a characteristic plant-specific complex glycoform (GnGnXF) attaches β1,2-xylose and α1,3-fucose to protein linked N-glycans [24,42]. This variation in glycosylation patterns represents a fundamental distinction between human protein counterparts, and it may influence the protein's functional properties and immunogenicity.

Our investigation revealed the variability of N-glycans produced by each plant-based RBD-Fc protein [43]. The expressed RBD-Fc with SEKDEL peptide (SP3-RBD-Fc-KD) harbors oligomannosidic glycans, as expected [44]. These findings confirm that the SEKDEL motif successfully retains RBD in the ER and inhibits maturation to complex glycans. Then, we expressed the SP3-RBD-Fc lacking the SEKDEL peptide in WT *N. benthamiana* (SP3-RBD-Fc WT), and the results showed the presence of both xylose and fucose residues (GnGnXF) as major glycan structures. These findings support previous research indicating that RBD-Fc is directed to the Golgi complex, where mannose-type subtypes are converted to complex-type glycans [43]. Lastly, the same SP3-RBD-Fc was expressed in ΔXF plant and contained complex human-like N-glycans (GnGn). The effect of these N-glycans decorated on plant-produced RBD-Fc fusion proteins on immune system activation was investigated further, with all three of them found to elicit a mouse-specific immune response and SARS-CoV-2 neutralizing antibodies. Nonetheless, no significant difference in immune response or enhanced immune induction ability was observed between plant-specific glycans and glycoengineered human-like glycans.

Previous studies have shown that plant allergens contain critical IgE-binding carbohydrate determinants, particularly on paucimannosidic N-glycans (MMXF) with xylose and/or fucose [46,47]. These specific glycostructures have been identified as pivotal triggers for IgE-mediated immune responses and can induce other antibody (IgG or IgM) production in the human immune system, elucidating their dual role in allergic reactions as well as immune responses in individuals exposed to such allergenic plant components [45]. The GnGnXF complex N-glycan present on plant-produced RBD-Fc also represents a carbohydrate epitope that could be detected by anti-carbohydrate IgGs in human sera, potentially causing false-positive results in serological assays [46]. Other studies have shown that glycoengineered ΔXF plants can modify the N-glycans at one glycosylation site located in the Fc domain [47], potentially influencing the effectiveness of antibodies [48,49]. The abundance of specific GnGn glycan structure has been associated with the potency and safety of recombinant monoclonal antibodies derived from plants [50]. Hurtado et al. [51] evaluated the efficiency of anti-CHIKV antibodies from both WT and ΔXF plants in a murine model, with the ΔXF-produced mAbs exhibiting marginally higher efficacy. However, no prior studies has been carried out to assess the effect of glycan variants of plant-produced RBD-Fc antigens on immune response or virus neutralization activity.

5. Conclusions

In conclusion, this study presents an *in vivo* potency data of three plant-derived RBD-Fc fusion proteins composed of different N-glycans obtained via expression in wildtype and glycoengineered *N. benthamiana*. The RBD-Fc subunit vaccines for SARS-CoV-2 are as follows: (i) RBD-Fc with an ER retention motif SEKDEL was produced in WT, while RBD-Fc without SEKDEL was produced in (ii) WT and (iii) ΔXF. All three have a high abundance of either mannose, GnGnXF, or GnGn structures. In a murine model, it was shown that the presence of a specific N-glycan profile had no effect on the vaccine-induced protective immunity of the glyco-modified SARS-CoV-2 RBD-Fc vaccine antigens.

Ethics approval

The study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed. and was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Protocol review No. 23-33-004; Date of Approval: 01-Jul-23).

CRediT authorship contribution statement

Theerakarn Srisangsung: Writing – review & editing, Writing – original draft, Methodology. **Thareeya Phetphoung:** Writing – original draft, Methodology. **Suwimon Manopwisedjaroen:** Writing – original draft, Methodology. **Kaewta Rattanapisit:** Writing – review & editing, Writing – original draft, Methodology. **Christine Joy I. Bulaon:** Writing – review & editing, Writing – original draft, Methodology. **Arunee Thitithanyanont:** Writing – original draft, Methodology. **Vudhiporn Limprasutr:** Writing – original draft, Methodology. **Richard Strasser:** Writing – review & editing, Writing – original draft, Methodology. **Waranyoo Phoolcharoen:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Waranyoo Phoolcharoen is a co-founder and shareholder of Baiya Phytopharm Co., Ltd. Kaewta Rattanapisit and Christine Joy I. Bulaon are employees of Baiya Phytopharm Co., Ltd. The remaining authors have no conflicts to disclose.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2024.e00847.

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