

High-Yield Production and Antigenic Profiling of a Stabilized SARS-CoV-2 S2-Fc Fusion Protein Using GlycoSwitch *Pichia pastoris*

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Abstract

The continuous mutation of the SARS-CoV-2 virus drives the need for novel vaccine approaches that can effectively combat new strains. Although research has heavily focused on the spike glycoprotein's S1 domain, the S2 subunit—despite its highly conserved sequence—has received far less attention. Here, we detail the design, production, isolation, and immunological assessment of the recombinant SARS-CoV-2 S2 protein synthesized using *Pichia pastoris*. To maximize protein yield, a codon-adapted S2 sequence was integrated into a yeast vector driven by the *AOX1* promoter. Following affinity chromatography, SDS-PAGE and Western blot analysis confirmed the expression of a ~ 95–105 kDa glycosylated fusion protein peaking at 72–120 h post-induction. Bradford quantification demonstrated purified yields of 18.6 ± 0.72 µg/mL for the wild-type KM71H strain and 20.6 ± 0.85 µg/mL for the glyco-engineered SM10 strain ($p = 0.032$). Furthermore, ELISA and pseudovirus neutralization assays revealed that the human-like glycan profile of the SM10-derived S2-Fc exhibited significantly enhanced antigenicity (approximately $31.4 \pm 3.2\%$ higher binding affinity, $p < 0.001$) compared to the wild-type counterpart, confirming the preservation of native neutralizing epitopes. Ultimately, our results suggest that the S2 domain is a strong candidate for developing broad-spectrum COVID-19 vaccines capable of neutralizing diverse variants, highlighting the importance of subsequent preclinical evaluations to determine its full protective efficacy.

Introduction

The global spread of COVID-19, triggered by the SARS-CoV-2 virus, stands as a defining public health emergency of the current century (1). Since its emergence in December 2019, extensive efforts have focused on developing effective vaccines and therapeutics. Although current vaccines have contributed substantially to disease control, the continuous emergence of new viral variants has raised concerns regarding their long-term efficacy (2). Many of these variants harbor mutations within the spike (S) glycoprotein that facilitate partial immune escape, underscoring the need for next-generation vaccines capable of providing broader protection against diverse SARS-CoV-2 variants (3). The SARS-CoV-2 spike protein, the primary viral entry mediator, remains the principal target of current vaccine platforms. Structurally, this glycoprotein is divided into the S1 and S2 domains. Historically, vaccine design has primarily targeted the S1 region since it encompasses the receptor-binding domain (RBD); however, this segment is highly susceptible to frequent mutational changes. Conversely, the S2 domain, which facilitates the fusion process between the virus and the host cell, exhibits a much higher degree of structural conservation and minimal antigenic drift. Because of this genetic stability, the S2 subunit emerges as an excellent candidate for engineering broad-spectrum vaccines capable of neutralizing diverse viral lineages (4, 5).

Although S2 has received comparatively less attention as a vaccine antigen, emerging evidence demonstrates that conserved epitopes within this region can elicit functional antibody responses. Several studies have reported that S2-specific antibodies may retain activity against multiple SARS-CoV-2 variants, supporting the rationale for exploring S2-based vaccine candidates (6, 7). A major challenge

in S2-focused vaccine development arises from its structural characteristics. As a transmembrane domain containing a large hydrophobic region embedded within the lipid bilayer, the production of sufficient quantities of soluble, high-quality recombinant S2 protein is technically demanding. Various expression systems have been evaluated for producing recombinant viral proteins, each offering distinct advantages. Among them, the yeast *Pichia pastoris* has gained wide adoption due to its cost-effectiveness, scalability, robust secretion capacity, and ability to perform mammalian-like post-translational modifications (8). This platform is particularly suitable for generating glycosylated antigens and is already used for the commercial production of several licensed human vaccines, including HPV, influenza, and hepatitis B (9).

Given these advantages, the present study aimed to assess the feasibility of expressing and purifying the SARS-CoV-2 S2 subunit in *Pichia pastoris*. A codon-optimized construct was expressed under the control of the AOX1 promoter, and the purified protein was analyzed through SDS-PAGE, UV-Vis spectrophotometry, Western blotting, and Dot blot. The antigenicity of the recombinant S2 protein was evaluated by direct and indirect ELISA, as well as pseudovirus-based neutralization assays (5, 8). Additionally, engineering the human IgG1-Fc region with stabilizing mutations (T250Q/M428L) provided improved structural stability, resistance to proteolysis, and extended biological persistence, establishing a robust platform for scalable subunit vaccine manufacturing. The results indicate that *Pichia pastoris* can produce high-quality S2 protein with preserved antigenic properties, supporting further preclinical investigations (10–12).

Materials and Methods

Strains, Plasmids, and Reagents

For plasmid propagation, *Escherichia coli* DH5 α (Takara Biotech, Japan) was used as the host strain. Protein production was performed using *Pichia pastoris* strain KM71H (Invitrogen, USA) and a glyco-engineered strain, SuperMan5 (SM10; BioGrammatics, USA). The pPICZ α A plasmid (ProteoGenix, France) served as the expression vector. Bacterial cultures were grown in Luria-Bertani (LB medium) liquid and agar (Oxoid, UK). Yeast cultivation utilized tryptone, agar, and yeast extract (Merck, USA). Reagents for molecular analyses included zeocin (InvivoGen, USA), SacI restriction enzyme, DNA ladders, and protein markers (Cinagen, Iran). Yeast media (YNB, BMGY, and BMMY) were obtained from Invitrogen (USA). Protein G-Sepharose Fast Flow resin (Merck, USA) was used for affinity purification. Immune response evaluations utilized the subunit spike protein-based vaccine (RAZI CovoPars; RVSRI, Iran) and HRP-conjugated goat anti-human IgG (Booali Sina Biological, Iran). Hyperimmune human sera specific to SARS-CoV-2 were provided by Dr. Ashraf Mohammadi (Human Viral vaccine (HVV) Department, RVSRI). For capture antibody generation, a male Pirbright (Albino) guinea pig was immunized subcutaneously with the CovoPars vaccine on days 0 and 21, and serum was collected via the saphenous vein 14 days post-second dose.

Design and Construction of the S2-Fc Chimeric Gene

A sequence alignment of SARS-CoV-2 genomes identified the Wuhan-Hu-1 reference sequence (NC_045512.2) as the optimal template. The recombinant construct incorporated residues 686–1213 of the S2 subunit, fused to the human IgG1 Fc region (227 aa) via a 18-residue Gly-Ser linker (GGGSGGGGSSGGGGGS). Two point mutations, T250Q and M428L (EU index numbering, corresponding to T30Q and M208L in the truncated sequence), were introduced into the Fc region to enhance FcRn affinity at acidic pH. The codon-optimized S2-Fc gene was synthesized by ProteoGenix (France) and inserted into the pPICZαA vector at XhoI and EcoRI sites. Following transformation into *E. coli* DH5α and selection on low-salt LB agar containing 25 µg/mL zeocin, plasmids were validated via restriction digestion, PCR, and whole-gene sequencing.

Pichia pastoris Transformation and Clone Selection

The pPICZαA-S2Fc plasmid was linearized using SacI (Fermentas, USA). Competent *P. pastoris* KM71H and SM10 strains were transformed via electroporation using a Bio-Rad Gene Pulser Xcell. Electroporation parameters were set to 2000 V, 25 µF, and 200 Ω using a 0.2 cm cuvette. Following the pulse, cells were immediately recovered in 1 mL of cold 1 M sorbitol and incubated in YPD broth at 30 °C for 1–2 hours. Transformants were initially screened on YPD agar with 100 µg/mL zeocin and subsequently enriched at higher concentrations (500, 1000, and 2000 µg/mL) to select multi-copy integrants. Genomic integration was verified by PCR.

Protein Expression, Extraction, and Affinity Purification

Selected colonies were cultured in BMGY medium at 28 °C and 250 rpm until the OD₆₀₀ reached 2–6. Cells were harvested and resuspended in BMMY medium to an initial OD₆₀₀ of 1.0 for induction, adding methanol to a final concentration of 0.5% every 24 hours for up to 144 hours. Culture supernatants were collected and prepared for purification. Prior to chromatography, the supernatant was dialyzed, which effectively reduced the conductivity from 3.8 mS/cm to 1.7 mS/cm. The pH of the clarified solution was adjusted from 5.6 to 7.0 before loading onto Protein G-Sepharose columns pre-equilibrated with PBS [17]. Elution was performed using 0.1 M glycine buffer (pH 2.8), and fractions were immediately neutralized by adding 0.1 mL of 1 M Tris-HCl (pH 7.5) to each collection tube. Protein concentrations were precisely measured using the Bradford assay kit (DNAbiotech, Iran).

Dot Blot Immunoassay

To confirm initial antigenic reactivity, 10 µL of crude and purified samples were spotted onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in TTBS buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) for 1 hour at room temperature. The blots were probed with human

hyperimmune sera, followed by an HRP-conjugated secondary antibody, and visualized using a diaminobenzidine (DAB) substrate solution (50 mg/100 mL TTBS with 10 μ L H₂O₂).

SDS-PAGE and Western Blot Analysis

Samples were boiled in a loading buffer containing DTT and resolved on 10% polyacrylamide gels, followed by Coomassie Brilliant Blue or silver nitrate staining [10]. For Western blotting, proteins were electroblotted onto nitrocellulose membranes, blocked with 5% skim milk in TTBS, and probed with human hyperimmune sera followed by HRP-conjugated goat anti-human IgG.

ELISA Antigenic Evaluations

Double Direct Sandwich ELISA: Plates were coated with 0.25 μ g/mL of the generated polyclonal guinea pig anti-SARS-CoV-2 antibodies. After blocking with 2% skim milk, samples were added, followed by detection using human hyperimmune sera (1:4000) and HRP-conjugated goat anti-human IgG (1:4000). Standard curves were generated concurrently using serial dilutions (10^{-1} to 10^{-11}) of the RAZI CovoPars vaccine and a heat-inactivated SARS-CoV-2 viral suspension (65°C for 45 min, starting at a 10^{-5} titer).

Indirect ELISA: Wells were directly coated with 0.2 μ g/mL of purified S2-Fc, blocked, and incubated with human hyperimmune sera (1:100) before secondary detection and colorimetric readouts at 450 nm using TMB substrate.

Pseudovirus Neutralization Assay

SARS-CoV-2 pseudotyped lentiviral particles expressing spike glycoproteins of the Wuhan-Hu-1 reference strain and variants were generated. Briefly, HEK293T-hACE2 cells (1×10^4 cells/well) were seeded in 96-well plates. Serial four-fold dilutions of human hyperimmune sera pre-incubated with purified recombinant S2-Fc (0.2 μ g/well) or control antigens were added to the pseudovirus suspension and incubated at 37°C for 1 h. The mixture was then transferred onto target cells. After 48 h of incubation, luciferase activity was determined using a Luciferase Assay System (Promega) on a microplate luminometer. The half-maximal inhibitory concentration (IC₅₀) was calculated using a non-linear four-parameter logistic regression model.

Statistical Analysis

All experiments were conducted in at least three independent biological replicates ($n = 3$), and quantitative data are presented as Mean $\pm$ Standard Deviation (SD). Statistical differences between two groups were determined using a two-tailed unpaired Student's t-test. Comparisons among multiple groups, clones, or time-points were performed by one-way or two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc test. Linear regression and goodness

of fit (R^2) were determined for standard curves, and correlations were assessed using Pearson's correlation coefficient (r). Statistical significance thresholds were set at * $p < 0.05$, $p < 0.01$, and *** $p < 0.001$. All analyses were performed using GraphPad Prism software (version 9.4.1)

Results

Design of Constructs and Bioinformatic Analysis

To ensure optimal solubility, secretion, and structural accessibility of relevant epitopes in *Pichia pastoris*, the recombinant construct was engineered to incorporate residues 686–1213 of the SARS-CoV-2 spike protein. The C-terminal membrane-proximal and transmembrane region (residues 1214–1273) was intentionally excluded to prevent hydrophobic aggregation and misfolding while preserving the immunologically exposed core containing the HR1 and HR2 domains. The S2 gene was fused to a human IgG1-Fc domain using a 16-amino acid Gly-Ser linker (GGGSGGGGSSGGGGGS), and the codon optimized gene was cloned into the pPICZαA vector under the control of the AOX1 promoter (Fig. 1A). The 3D structural modeling of the full-length spike protein and the isolated S2 subunit confirmed the stable spatial configuration of the target domain (Fig. 1B, C). The final recombinant construct measured approximately 5.6 kb, as confirmed by *in silico* evaluation using SnapGene.

In Silico Structural and Functional Analysis of Stabilized S2-Fc

Computational modeling via PyMOL and UCSF Chimera verified that the engineered S2-Fc chimera adopted a stable, unhindered 3D conformation. Sequence analysis identified a 10-amino acid N-terminal spacer extension (VSLEKREAEA) derived from partial α-factor prepro-peptide processing (Fig. 2A). The persistence of this extension reflects inherent limitations in yeast secretory processing specifically reduced accessibility of the Kex2 cleavage site and partial Ste13 trimming. Rather than constituting a design flaw, structural evaluation confirmed that this short extension acts as a flexible spacer. Its distal position relative to major S2 epitopes prevents steric constraints during translocation and minimizes premature aggregation within the ER without impacting antigenicity. Furthermore, the incorporation of the T250Q mutation in the CH2 domain and the M428L substitution in the CH3 domain of the Fc tag was modeled (Fig. 2B). These mutations did not perturb the native Fc fold or interfere with the S2 core architecture. The M428L substitution was introduced to replace an oxidation-sensitive methionine residue with leucine at the hydrophobic dimerization interface, improving hydrophobic packing. Concurrently, T250Q removed a potential non-functional yeast glycosylation site while maintaining hydrogen-bonding capacity. Together, this synergistic engineering enhances folding kinetics, secretion efficiency, and structural robustness of the fusion protein.

P. pastoris Transformation and Multi-Copy Enrichment

The pPICZαA-S2Fc plasmid was introduced into *E. coli* DH5α and validated through restriction enzyme analysis (Fig. 3A), PCR, and whole-gene sequencing (Fig. 3B). Following authentication, the purified plasmid was linearized and electroporated into competent *P. pastoris* strains KM71H and SM10. Primary

transformants on YPD agar with 100 µg/mL zeocin were subcultured onto escalating doses (500, 1000, and 2000 µg/mL) to isolate high-yielding multi-copy clones, and genomic integration was further validated by PCR (Fig. 3C).

Recombinant S2-Fc Expression and Time-Course Analysis

SDS-PAGE revealed a distinct band between 90–105 kDa, consistent with glycosylated S2-Fc. *In silico* prediction estimated the unglycosylated S2-Fc at approximately 75 kDa, with the ten predicted N-glycosylation sites in the S2 domain contributing roughly 3 kDa each, strictly matching the observed molecular weight range. Recombinant S2-Fc expression accumulated progressively post-methanol induction, reaching peak target accumulation between 72 h and 120 h in both strains (Fig. 4A, B). Dot blot and Western blot analysis of crude supernatants further confirmed S2-Fc expression and antigenic detectability using human anti-SARS-CoV-2 hyperimmune sera across the time-course (Fig. 4C, D).

S2-Fc Immunoreactivity Study and Affinity Purification

S2-Fc purification via Protein G-Sepharose chromatography yielded a single 90–105 kDa band on SDS-PAGE (Fig. 5A, B). Western blotting with anti-S2 antibodies confirmed the strong antigenic integrity of the purified S2-Fc (Fig. 6A). Furthermore, time-course stability analysis revealed that the purified S2-Fc proteins from both KM71H and SM10 strains robustly maintained their structural and antigenic stability over 144 hours (Fig. 6B, C). Bradford quantification demonstrated final purified yields of 18.6 ± 0.72 µg/mL for KM71H and 20.6 ± 0.85 µg/mL for SM10 ($n = 3$). A two-tailed unpaired Student's t-test indicated that the yield enhancement in the glyco-engineered SM10 strain was statistically significant ($t = 3.12$, $p = 0.032$).

Recombinant S2-Fc Antigenic Evaluation by ELISA

The antigenicity and conformational integrity of the recombinant S2-Fc were assessed using robust double-direct and indirect ELISA formats against heat-inactivated virus (Fig. 7A, B) and CovoPars standard curves (Fig. 7C, D). Two-way ANOVA followed by Tukey's post-hoc test demonstrated a highly significant difference between the two expression hosts ($p < 0.0001$). The glyco-engineered SM10-derived S2-Fc exhibited an average $31.4 \pm 3.2\%$ increase in absorbance values compared to KM71H at equivalent concentrations. Standard curves showed excellent linearity ($R^2 = 0.988$ for inactivated virus and $R^2 = 0.991$ for CovoPars). Binding reactivity strongly correlated with antibody capture titers (Pearson's $r = 0.994$, $p < 0.0001$), supporting the superior antigenicity of the GlycoSwitch variant.

Confirmation of Antigenicity via Pseudovirus Neutralization

Pseudovirus neutralization assays demonstrated that antibodies elicited against recombinant S2-Fc were capable of neutralizing pseudotyped viral particles representing multiple SARS-CoV-2 variants.

Neutralization activity significantly correlated with the ELISA binding strength, supporting the functional integrity and proper folding of the recombinant S2 antigen generated in the yeast platforms.

Discussion

SARS-CoV-2 S2 Subunit as a Broad-Spectrum Vaccine Target

The SARS-CoV-2 S2 subunit represents a promising target for broad-spectrum vaccine research due to its relatively high sequence conservation across viral variants, in contrast to the highly variable S1 subunit. In this study, we successfully expressed the stabilized S2 glycoprotein in *Pichia pastoris*, achieving high yields using the GlycoSwitch strain. The recombinant S2 protein demonstrated strong antigenicity, supported by ELISA and pseudovirus neutralization assays (13–15). The deliberate engineering of the Fc domain (T250Q/M428L) provided a dual advantage during *P. pastoris* fermentation. Beyond extending biological persistence via enhanced FcRn interactions, these substitutions critically minimized host proteolytic degradation and oxidative stress during prolonged methanol induction phases (16).

Comparison with S1-Based Approaches and Yield Profiles

The structural stability of the S2 subunit provides a key advantage over S1, especially the receptor-binding domain (RBD), which is highly mutation-prone in emerging variants (17). In contrast to S1-Fc-based constructs—which may exhibit reduced activity against heavily mutated variants our findings are consistent with prior reports that S2-directed responses can contribute to variant-resistant immunity (18, 19). Our achieved expression yield of 20.6 ± 0.85 $\mu\text{g/mL}$ for the glyco-engineered S2-Fc demonstrates superior yield compared to previously reported *P. pastoris* derived S1-Fc constructs (14.6–18.9 $\mu\text{g/mL}$), illustrating that S2-Fc truncation effectively overcomes the expression bottlenecks typically associated with large coronaviral envelope glycoproteins (20, 21). *Pichia pastoris* offers a scalable, cost-efficient platform for recombinant S2 production, particularly when compared with mammalian systems (Table 1).

Table 1

Comparative yield and glycosylation profiles of recombinant SARS-CoV-2 spike subunits expressed in yeast and mammalian platforms.

Expression Platform	Target Antigen	Glycosylation Profile	Expression Yield (µg/mL)	Scalability & Cost-Efficiency	Reference
<i>P. pastoris</i> (GlycoSwitch SM10)	S2-Fc (residues 686–1213)	Human-like core Man ₅ GlcNAc ₂	20.6 ± 0.85	High / Highly cost-efficient	Present study
<i>P. pastoris</i> (wild-type KM71H)	S2-Fc (residues 686–1213)	Hyper mannosylated Man _{30–150} GlcNAc ₂	18.6 ± 0.72	High / Highly cost-efficient	Present study
<i>P. pastoris</i> (wild-type and engineered strains)	S1-Fc / RBD	Hyper mannosylated / Human-like glycans	14.6–18.9	High / Cost-efficient	Majidi et al. (18), Ng et al. (19)
Mammalian cells (HEK293 / CHO)	S1, S2, or full-length Spike	Native complex mammalian glycans	Variable / Lower specific volumetric yields	Moderate / High production cost	Jiang et al. (11), Walls et al. (30).
Note: Comparison of expression yields, glycosylation patterns, and economic feasibility across different recombinant production platforms. The glyco-engineered <i>P. pastoris</i> SM10 strain utilized in this study not only circumvents the hyper-mannosylation issue typical of wild-type yeast—thereby exposing neutralizing epitopes—but also achieves superior volumetric yields for the S2-Fc fusion protein compared to previously reported S1-Fc constructs. Mammalian systems provide native-like complex glycans but present significant challenges regarding industrial scalability and cost of goods.					

Role of Glycosylation in Immunogenicity

Glycosylation is a critical determinant of SARS-CoV-2 spike antigenicity (22). Unlike wild-type *P. pastoris* strains (such as KM71H) which impart hyper-mannosylated N-glycans (Man_{30–150}GlcNAc₂) that can sterically hinder antigenic epitopes or induce undesirable immunogenic clearance, the SuperMan5 (SM10) strain carries an α-1,2-mannosidase gene disruption combined with heterologous α-1,2-mannosidase catalytic targeting (23, 24). This restricts N-glycosylation predominantly to human-like Man₅GlcNAc₂ core structures, thereby exposing critical conserved neutralizing epitopes on the S2 core while avoiding steric interference. Our findings suggest that glycan quality rather than quantity is the dominant factor influencing antigenicity, directly evidenced by the 31.4 ± 3.2% higher binding affinity observed in the GlycoSwitch strain (25, 26).

Limitations and Future Directions

Although Fc fusion has been shown to enhance stability and immune responses, our use of the S2-Fc construct was focused primarily on improving expression, stability, and purification in yeast. The present study did not evaluate *in vivo* Fc-specific functions such as half-life extension or Fc-mediated effector

activity (27, 28). Future work should include *in vivo* testing against live SARS-CoV-2 variants, formulation optimization using adjuvants or nanoparticle delivery systems, and cross-protection studies involving SARS-CoV and MERS-CoV. Integrating the recombinant S2 construct with next-generation mucosal adjuvants will be critical for determining its translational potential (29, 30).

Conclusion

This study demonstrates the successful production and structural characterization of the SARS-CoV-2 S2 glycoprotein using the *Pichia pastoris* GlycoSwitch system, resulting in a properly folded, glycosylated, and antigenically competent recombinant protein. The GlycoSwitch expression platform enabled efficient secretion and human-like glycosylation, supporting its strong immunoreactivity in ELISA and neutralization assays. These findings reinforce the notion that optimized glycan quality plays a central role in shaping the antigenicity of coronavirus spike-derived proteins, establishing a robust glycoengineered platform for the development of durable countermeasures against future coronavirus threats.

Declarations

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Author Contributions

MA contributed to formal analysis, investigation, writing—original draft, formal analysis; KAK contributed to conceptualization, methodology, validation, formal analysis, resources, data curation, writing—review & editing, supervision, project administration, and funding acquisition; AA writing, editing.

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Conflict of interest: 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.

Ethical Approval: Human hyperimmune serum samples were obtained with informed consent under institutional ethical approval from the Ethics Committee of Islamic Azad University and Razi Vaccine and Serum Research Institute (RVSRI). All animal procedures (guinea pig immunization) were conducted strictly following institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Iranian Veterinary Organization (Approval No. IR.IAU.TM.REC.1400-05).

Data Availability: Data will be made available on request.

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Figures

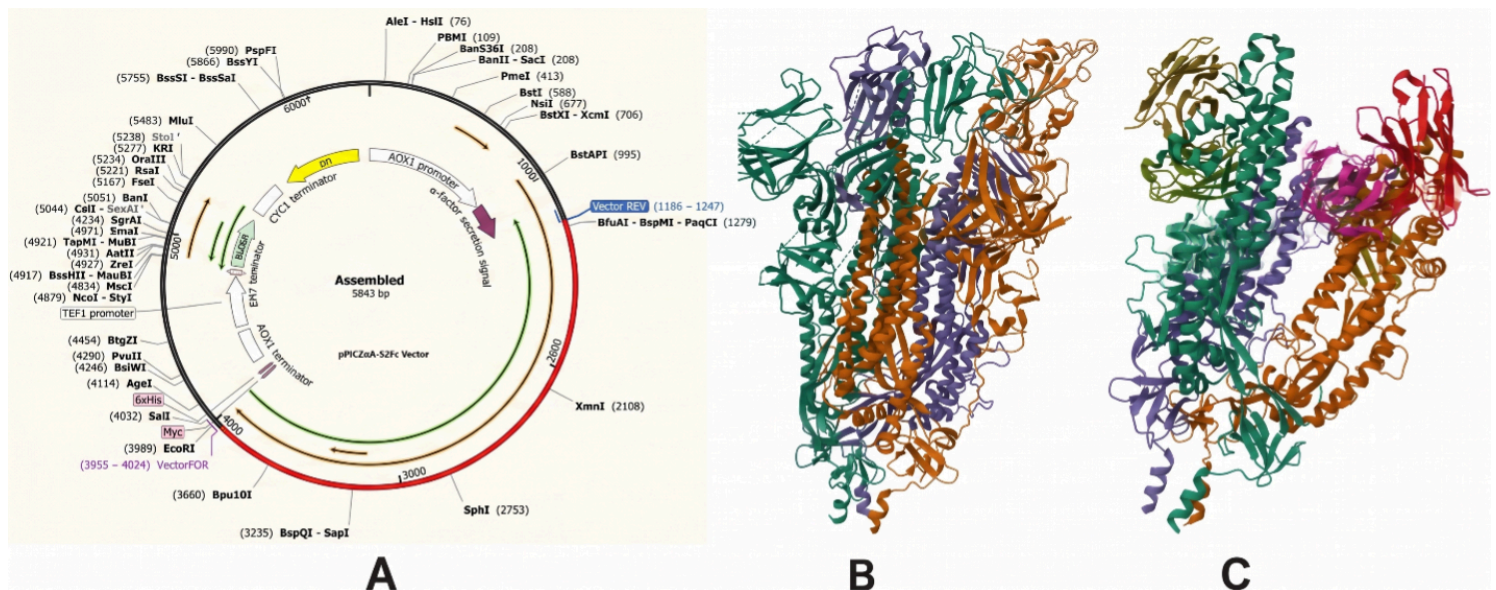
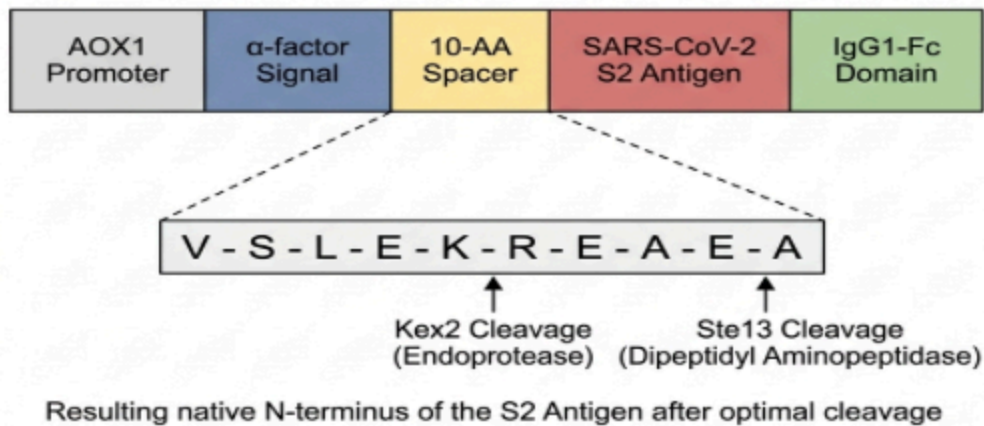


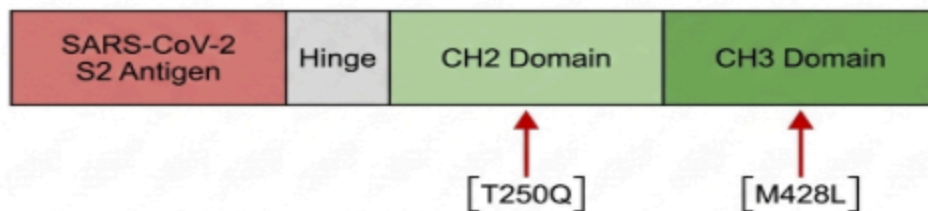
Figure 1

***In silico* design and structural modeling of the recombinant pPICZαA-S2Fc construct.** A) Map of the pPICZαA-S2Fc vector designed using SnapGene version 7.2.1. B) Three-dimensional structure of the SARS-CoV-2 spike (S) protein. C) Three-dimensional model of the S2 subunit without the other spike subunits, showing its spatial configuration. Structural visualization was performed using PyMOL version 2.7 and UCSF Chimera version 1.2.2.

A. Recombinant Construct Design & N-terminal Secretion Processing



B. Fc Domain Engineering (Stability & Half-life Extension via Dual Mutations)



Synergistic effect of T250Q & M428L:

1. High Affinity to FcRn at pH $\approx$ 6.0 (Endosomal Rescue).
2. Rapid Dissociation from FcRn at pH $\approx$ 7.4 (Bloodstream Release).
3. Prevention of non-functional yeast glycosylation and enhanced structural stability.

Figure 2

Molecular engineering and secretion processing map of the stabilized S2-Fc construct. **A)** Schematic of the N-terminal α -factor signal peptide cleavage, showing the expected Kex2 and Ste13 endoprotease sites and the resulting 10-AA retained spacer. **B)** Schematic of the C-terminal IgG1-Fc domain highlighting the targeted T250Q (CH2 domain) and M428L (CH3 domain) mutations engineered to optimize FcRn binding affinity and minimize host proteolytic degradation.

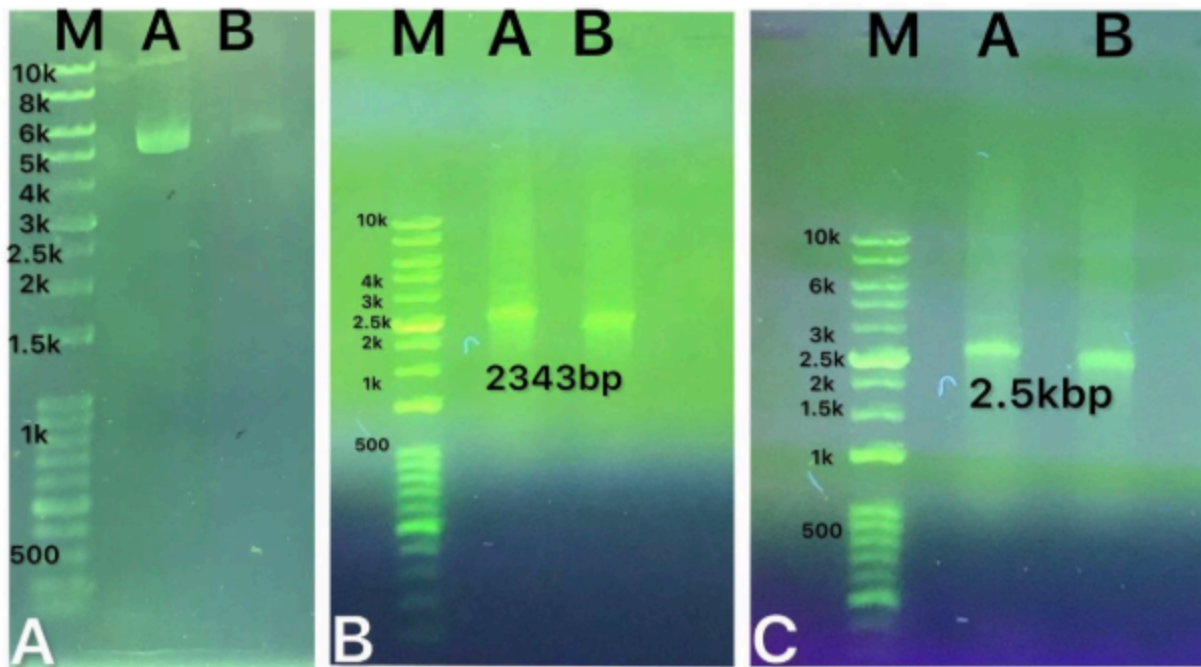


Figure 3

Molecular validation of the pPICZαA-S2Fc construct and recombinant *Pichia pastoris* transformants.

A): Agarose gel electrophoresis of the pPICZαA-S2Fc plasmid extracted from transformed *E. coli* DH5α cells. M, 1-kb DNA ladder; lane A, SacI-linearized pPICZαA-S2Fc; lane B, supercoiled pPICZαA-S2Fc before linearization. **B):** PCR amplification of the S2Fc fragment. M, 1-kb DNA ladder; A, S2Fc fragment amplified from *E. coli*-extracted pPICZαA-S2Fc; B, synthesized pPICZαA-S2Fc used as the positive control; C, no-template negative control. **C):** PCR amplification of the S2Fc gene from transformed *Pichia pastoris*. M, 1-kb DNA ladder; A, KM71H strain; B, SM10 strain; C, no-template negative control.

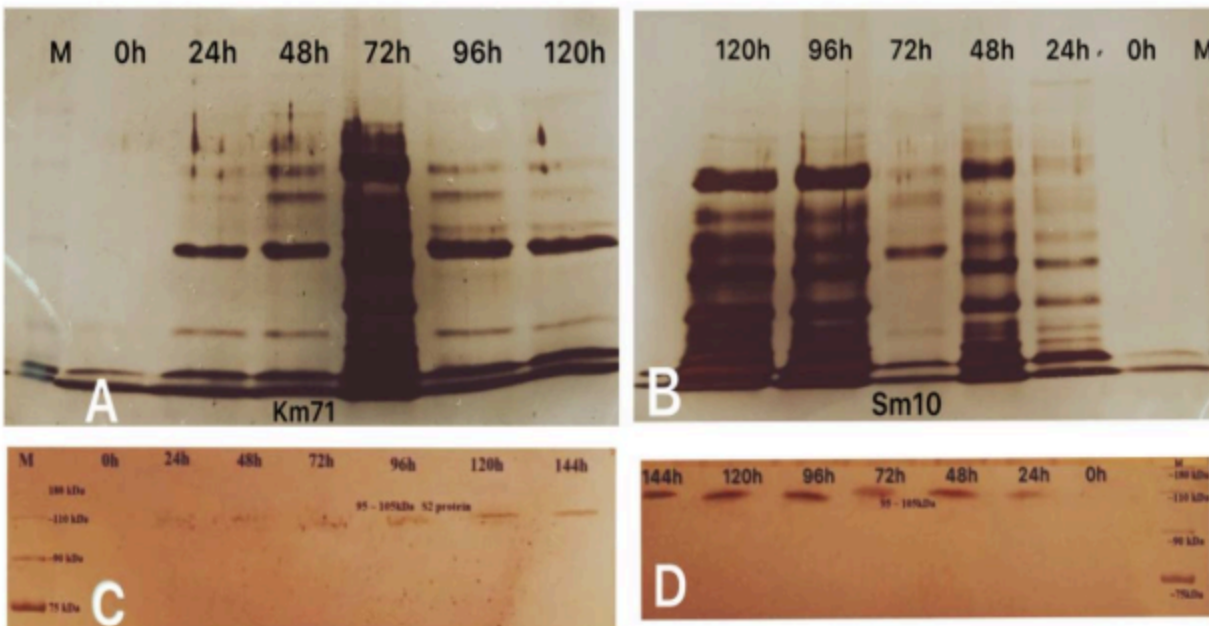


Figure 4

Time-course analysis of recombinant S2Fc expression in *Pichia pastoris*. **A, B)** Silver nitrate staining of recombinant S2Fc expressed by the KM71H and SM10 strains, respectively. M, prestained protein ladder; lanes 1–7, culture supernatant samples collected at 0, 24, 48, 72, 96, 120, and 144 h after methanol induction. **C, D)** Western blot analysis of the corresponding samples from the KM71H and SM10 strains, respectively. Human anti-SARS-CoV-2 hyperimmune sera were used as the primary antibody, and goat anti-human IgG conjugated to horseradish peroxidase (HRP) was used as the secondary antibody.

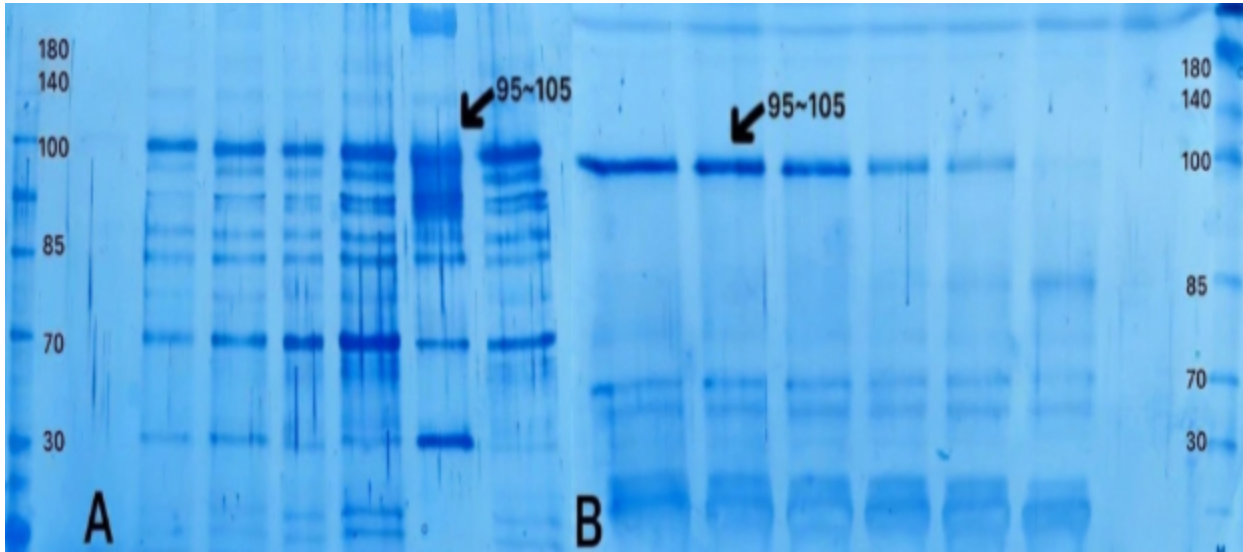


Figure 5

SDS-PAGE analysis of recombinant S2Fc purification by Protein G-Sepharose chromatography. **A)** Purification profiles of S2Fc expressed by the KM71H strain and **B)** the SM10 strain. M, prestained protein ladder; lane 1, sample before diafiltration; lane 2, sample after diafiltration; lanes 3–6, selected fractions with high absorbance at 280 nm containing purified S2Fc: lane 3, second fraction; lane 4, fourth fraction; lane 5, sixth fraction; lane 6, eighth fraction.

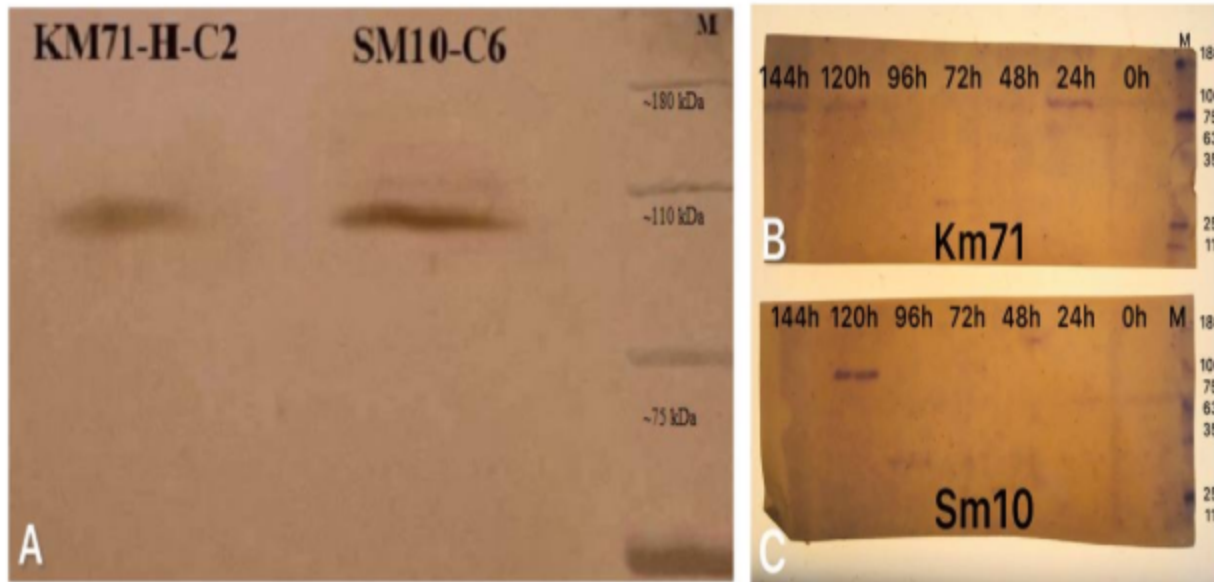


Figure 6

Western blot confirmation and time-course analysis of purified recombinant S2Fc. A): Western blot analysis of purified S2Fc expressed by the KM71H and SM10 strains. Human anti-SARS-CoV-2 hyperimmune sera were used as the primary antibody, and goat anti-human IgG conjugated to HRP was used as the secondary antibody. DAB was used as the chromogenic substrate. Lane 1, prestained protein ladder; lane 2, S2Fc purified from the SM10 strain; lane 3, S2Fc purified from the KM71H strain. **B, C):** Time-course analysis of purified S2Fc from the KM71H and SM10 strains, respectively, at 0, 24, 48, 72, 96, 120, and 144 h. Samples were analyzed by SDS-PAGE followed by Western blotting. M, prestained protein ladder. Human anti-SARS-CoV-2 hyperimmune sera were used for detection, followed by goat anti-human IgG-HRP and DAB development

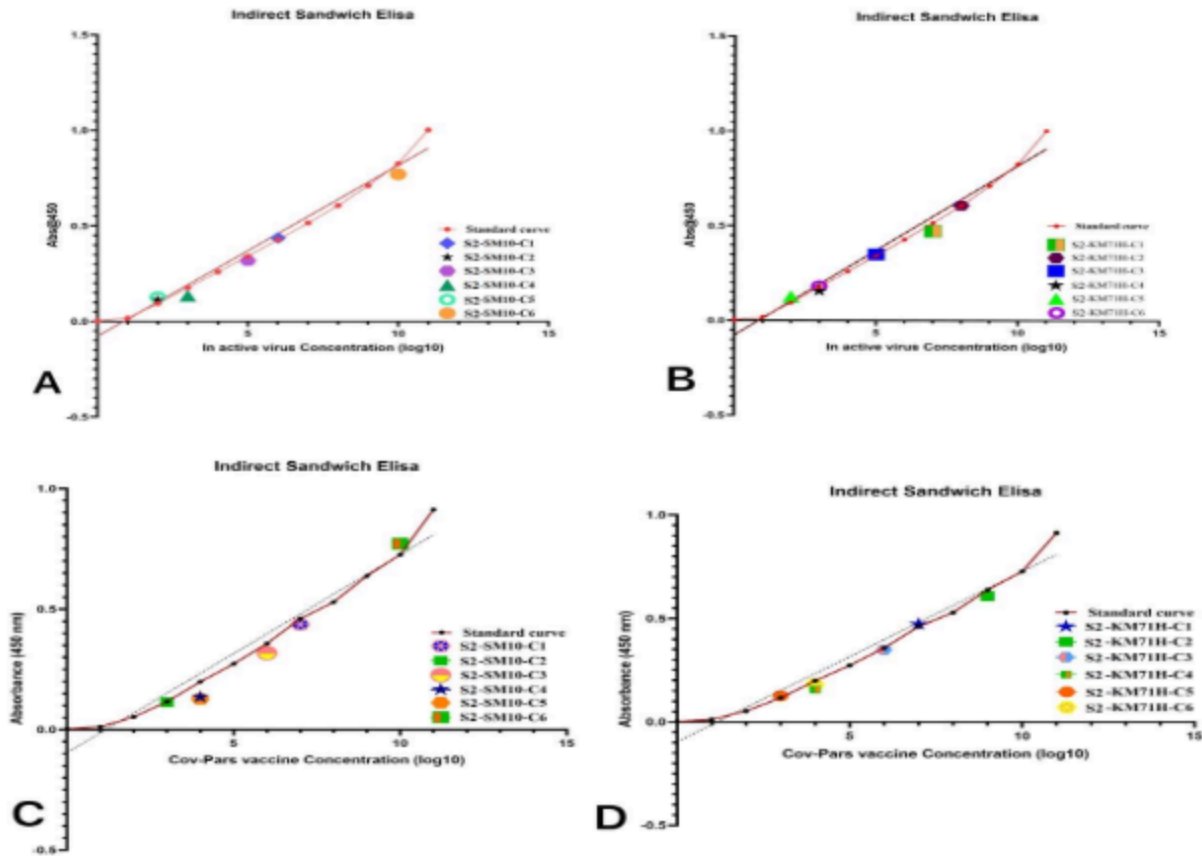


Figure 7

Antigenic evaluation of recombinant S2Fc glycoprotein by indirect sandwich ELISA. **A, B):** Binding reactivity of different S2Fc-producing clones from the SM10 and KM71H strains, respectively, against inactivated SARS-CoV-2 antigen. **C, D):** Reactivity of S2Fc produced by the SM10 and KM71H strains, respectively, against the RAZI CovoPars vaccine antigen. Absorbance was measured at 450 nm and plotted against antigen concentration on a logarithmic scale. The standard curves were generated using serial dilutions of the reference antigen. Each data point represents the mean $\pm$ standard deviation of three independent measurements. The dose-dependent increase in absorbance indicates specific antigen–antibody interaction and confirms the immunoreactivity of recombinant S2Fc.