Biotech Studies 33(2), 82-90
http://doi.org/10.38042/biotechstudies.1497521

BIOTECH STUDIES

RESEARCH PAPER

Aspergillus oryzae as a host for SARS-CoV-2 RBD and NTD expression

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How to cite:
Karaman, E. & Uysal, S. (2024). *Aspergillus oryzae* as a host for SARS-CoV-2 RBD and NTD expression. *Biotech Studies*, *33*(2), 82-90. https://doi.org/10.38042/biotechstudies.1497521

Article History
Received 22 January 2024
Accepted 22 May 2024
First Online 07 June 2024

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Keywords
Receptor binding domain
N-terminal domain
SARS-CoV-2
Aspergillus oryzae
Recombinant

Abstract

The COVID-19 pandemic has increased demand for effective diagnostics, and extensive research has been conducted on the N-terminal domain (NTD) and the receptor-binding domain (RBD) of the SARS-CoV-2 spike glycoprotein, which are critical for viral binding. This study focuses on the expression of NTD and RBD in *pyrG* auxotrophic *Aspergillus oryzae* for the first time. Recombinant NTD and RBD were expressed as glucoamylase-fusion proteins and purified using metal affinity chromatography. Size-exclusion chromatography was used to confirm the correct folding and purity of the recombinant proteins. Employing an enzyme-linked immunosorbent assay, the binding ability of the fusion proteins to human anti-IgG antibodies in serum samples was evaluated. The results indicated a significant and concentration-dependent interaction, affirming the functionality of the NTD and RBD fusion proteins and establishing their efficacy in antigen-antibody interactions. This study not only elucidates the usage potential of the fusion proteins in immunoassays but also addresses the suitability of the *A. oryzae* expression system as a biotechnological platform to produce SARS-CoV-2 proteins. Furthermore, this study lays the foundation for scalable and cost-effective mass production of effective NTD and RBD proteins in *A. oryzae*, opening up a new era of COVID-19 research, vaccine development, and immunoassay design.

Introduction

Coronavirus disease 2019 (COVID-19) poses a serious threat to human health as a pandemic disease. One of the most significant characteristics of COVID-19 is its ability to spread quickly from the beginning to produce a variety of variant forms that have impacted millions of people worldwide (Hassanin et al., 2021; Tay et al., 2020). As a result, rapid solution recommendations are becoming increasingly important in the diagnosis and treatment of pandemic diseases.

SARS-CoV-2, the COVID-19 virus, penetrates host cells via epithelial cells and interacts with the angiotensin-converting enzyme-2 (ACE2) receptor of the host respiratory system (Lan et al., 2020; Walls et al., 2020). The virus has high pathogenicity with its single-stranded, enclosed, positive-sense RNA genome size of 30 kb. It encodes four structural proteins: spike glycoprotein (S), envelope glycoprotein, membrane glycoprotein, and nucleocapsid, which are responsible for infection initiation, assembly, release, and packing, respectively (Hassanin et al., 2021; Zhu et al., 2020). S protein consists of two subunits: S1 and S2. The N-terminal region (NTD) (aa 14–305) of the S1 subunit is followed by a receptor binding domain (RBD) (aa 319–591) (Balasubramaniyam et al., 2022; Cao et al., 2021; Huang et al., 2020; Wrapp et al., 2020).

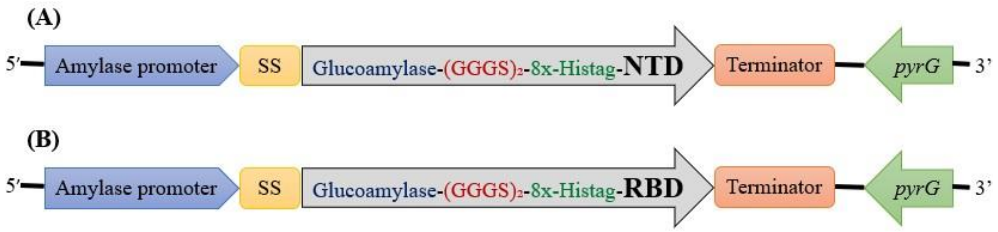

Figure 1. Representation of the linear expression vectors containing the amylase promoter, signal sequence (SS), gene-encoding fusion proteins, terminator, and *pyrG* gene for the transformation of *pyrG* auxotrophic *A. oryzae*. For Gla-NTD, the glucoamylase fusion protein is depicted as glucoamylase, linker $(GGGS)_2$, 8x-Histag, and gene-encoding NTD (A). For Gla-RBD, the glucoamylase fusion protein is depicted as glucoamylase, linker $(GGGS)_2$, 8x-Histag, and the gene-encoding RBD (B).

An easy-to-use, affordable, and straightforward immunoassay, useful diagnostic kits, and therapies, including vaccine development, are required in order to promptly combat COVID-19 cases. It has attempted the expression of SARS-CoV-2 proteins and their fragments in several expression systems for these purposes and has studied the efficacy of the expressed proteins as target antigens for COVID-19 detection in immunoassays (Conzentino et al., 2022; Márquez-Ipiña et al., 2021; Tozetto-Mendoza et al., 2021). Because the RBD of the S protein interacts directly with ACE2, it is the most crucial component to consider as a target (Cao et al., 2021; Shang et al., 2020; Zhu et al., 2020).

Aspergillus oryzae (*A. oryzae*) is a filamentous fungus used in fermentation technologies and has been given the GRAS (Generally Recognized as Safe) classification by the FDA. It is a safe microorganism for producing primary and secondary metabolites and industrial enzymes due to its high secretory capability (He et al., 2019). It is also a useful expression system due to its low-cost media, ability to produce complex proteins, and tolerance to various environmental conditions (Ntana et al., 2020).

In this study, the NTD and RBD of S protein were produced for the first time in *A. oryzae*, and their binding ability to detect anti-IgG antibodies in human serum was investigated utilizing a low-cost indirect enzyme-linked immunosorbent test (ELISA) as a straightforward immunoassay.

Materials and Methods

Materials

Chemicals and reagents used in this study were purchased from Sigma (MO, USA) and Biofroxx (Germany). The Gangnam-Stain Protein Ladder (24052) was purchased from Intron Biotechnology (South Korea). The plasmid midiprep kit (12143, Qiagen) was purchased from Qiagen (12143, Valencia, CA). 96-well ELISA plates were purchased from NEST (514201, Wuxi NEST Biotechnology Co., Ltd., China). The nickel resin (88221, HisPurTM Ni-NTA Resin), TMB ELISA Substrate (#34021), and 10K molecular weight cut-off (MWCO) of SnakeSkinTM Dialysis Tubing (68035) were purchased from Thermo Fisher Scientific (MA, USA). Amicon® Ultra-15 centrifugal filters (10K MWCO) were purchased from Merck Millipore (MA, USA). The Superdex 75 Increase 10/300 GL column was purchased from GE Healthcare (IL, USA). Restriction enzymes were purchased from New England Biolabs (MA, USA). The yatalase enzyme was purchased from Takara Bio Inc. (Japan).

Escherichia coli (E. coli) TOP10 (C404010) was purchased from Thermo Fisher Scientific (MA, USA). The *pyrG* auxotrophic *A. oryzae* strain was obtained in our previous study (Karaman et al., 2023) by using *A. oryzae* RIB40 (42149), which was purchased from the American Type Culture Collection (VA, USA).

Construction of expression vectors

In this study, the NTD and RBD regions of SARS-CoV-2 were selected for expression in the *pyrG* auxotrophic *A. oryzae*. The amino acid sequences of NTD (aa 14–305) and RBD (319–591 aa) were obtained from the NCBI database (accession number UBE87647.1). The DNA sequences encoding NTD and RBD were codon-optimized, and their constructs were separately prepared as fusions into the glucoamylase gene. For purification, a linker site consisting of two consecutive GGGS amino acid sequences and an 8x-Histag was inserted between the glucoamylase and the protein of interest. The nucleotide sequences were synthesized at GenScript Biotech PTE. LTD. (NJ, USA) and inserted into the separate expression vectors under the control of the amylase promoter (Figure 1).

Transformation of expression vectors into *pyrG* auxotrophic *A. oryzae*

The expression vectors were amplified by transforming into chemically component *E. coli.* 1% tryptone, 0.5% yeast extract, and 1% NaCl supplemented with 100 µg/mL ampicillin were used as growth medium for the bacteria. The amplified expression vectors were isolated with the plasmid midiprep kit and linearized for *A. oryzae* transformation by using *HindIII* and *EcoRI* restriction enzymes.

The transformation was carried out according to Sakai et al. (2012). Briefly, the *pyrG* auxotrophic *A. oryzae* was grown in a medium containing 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (pH 5.5), 20 mM uridine, and 0.2% uracil at 30 °C and 180 rpm. After overnight incubation, mycelia were incubated in a lysis solution containing 50 mM malate buffer, 0.6 M $(NH_4)_2SO_4$, and 1% yatalase (pH 5.5) at 30 °C and 80 rpm for 4 h. The obtained

spheroplasts were washed with a solution containing 1.2 M sorbitol and 50 mM $CaCl_2$. Following that, spheroplasts and the linearized expression vectors belonging to glucoamylase fusion NTD (Gla-NTD) and glucoamylase fusion RBD (Gla-RBD) were combined in the presence of Polyethylene glycol 4000 and spread on minimal media consisting of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_20$, 0.05% KCl, 0.001% $FeSO_4.7H_2O$, 3% sucrose, 5% NaCl, and %2 agar (pH 5.5). For the control group, no DNA was added to spheroplasts. Incubation was carried out at 30 °C and 180 rpm for 5-7 days.

Expression of recombinant Gla-NTD and Gla-RBD proteins

Transformant *A. oryzae* colonies were inoculated into 15 mL of growth medium containing 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4.7H_2O$ (pH 5.5) and incubated overnight at 30 °C and 180 rpm. Following that, culture suspensions were diluted in 75 mL of growth medium containing 4% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4.7H_2O$ (pH 5.5) at a ratio of 1:10. Incubation was performed at 30 °C and 180 rpm for 7 days. Supernatant samples taken from cultures were analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gels.

Purification of recombinant Gla-NTD and Gla-RBD proteins

Expression cultures were filtered through Whatman filter paper to obtain a medium containing the recombinant Gla-NTD and Gla-RBD proteins secreted by *A. oryzae*. Following collection of the culture medium, recombinant 8x-Histagged Gla-NTD and Gla-RBD proteins were purified by metal affinity chromatography under native conditions. In this method, the harvested medium was loaded into a nickel-nitrilotriacetic acid (Ni-NTA) affinity column containing HisPur™ Ni-NTA resin, and then the column was washed with 50 mM NaH_2PO_4 and 500 mM NaCl (pH 7.4). Elution was performed with elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 200 mM imidazole (pH 7.4). The eluted fractions were dialyzed against phosphate-buffered saline (PBS) buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4) for the removal of imidazole. After being dialyzed, samples were concentrated using an Amicon® Ultra-15 centrifugal concentration filter. Samples were analyzed on SDS-PAGE.

The ImageJ 1.53t tool (National Institutes of Health, USA, Version 1.53t) was used to perform densitometric analysis of total protein. A Bradford assay was performed to determine the concentration of Gla-NTD and Gla-RBD proteins at 595 nm using bovine serum albumin as a protein standard.

Size exclusion chromatographic analysis of recombinant Gla-NTD and Gla-RBD proteins

The concentrated recombinant Gla-NTD and Gla-RBD proteins were applied to a Superdex 75 Increase 10/300 GL size exclusion chromatography column coupled to an HPLC system (AKTA Pure Chromatography System) to check the purity (Cytiva, USA). PBS buffer was prepared according to the manufacturer's instructions and used as equilibration, wash, and elution buffers. The elution was performed at a linear flow rate of 0.5 mL/min.

In vitro functionality assay by indirect ELISA

Indirect ELISA was used to evaluate the binding ability of recombinant Gla-NTD and Gla-RBD proteins to anti-IgG antibodies in human serum samples obtained from COVID-19 patients. Flat-bottomed 96-well ELISA plates were coated with Gla-NTD and Gla-RBD, separately. Final concentrations of recombinant proteins were adjusted to 10 µg/mL in a coating buffer containing 50 mM carbonate (pH 9.6). Blank wells were coated with only coating buffer. Non-fusion glucoamylase expressed in *A. oryzae* and an irrelevant recombinant protein expressed in *A. oryzae* were coated on wells as negative controls. For positive control, recombinant RBD protein expressed in *Pichia pastoris* (*P. pastoris*) was coated in positive wells. Plates were incubated overnight at 4 °C. After incubation, plates were washed with 200 µL of PBS-T (PBS buffer supplemented with 0.05% Tween-20). The plates were blocked with 200 µL of PBS-T containing 5% skim milk for 2 h at room temperature. Following that, 100 µL of human serum samples were diluted in PBS-T containing 5% skim milk in a 1:500 ratio and applied to the plates.

After being incubated at 37 °C for 1 h, the plates were washed with 200 µL of PBS-T three times. HRP-conjugated anti-human IgG antibody was diluted by 1:10.000 in PBS-T containing 5% skim milk and added to the wells. After 1 h of incubation at 37 °C, the plates were washed with 200 µL of PBS-T three times. For detection, 100 µL of TMB ELISA substrate was added and incubated at room temperature for 30 min in the dark. The reaction was terminated with 500 µL of 1 M H_2SO_4. Absorption was read at 450 nm using a Biotek microplate reader. Furthermore, different concentrations (20, 10, and 2.5 ng/L) of expressed Gla-NTD and Gla-RBD proteins were evaluated in an ELISA against human serum samples to investigate their binding capacity. All the acquired data for these recombinant Gla-NTD and Gla-RBD proteins was statistically examined using the Kruskal-Wallis H test.

Results and Discussion

Expression of Gla-NTD and Gla-RBD in *A. oryzae*

The *pyrG* gene locus was introduced into the *pyrG* auxotrophic *A. oryzae* genome by gene replacement using expression vectors containing Gla-NTD and Gla-RBD. Following transformation, multiple transformed colonies were selected from transformant plates and subjected to an expression test in a small flask volume.

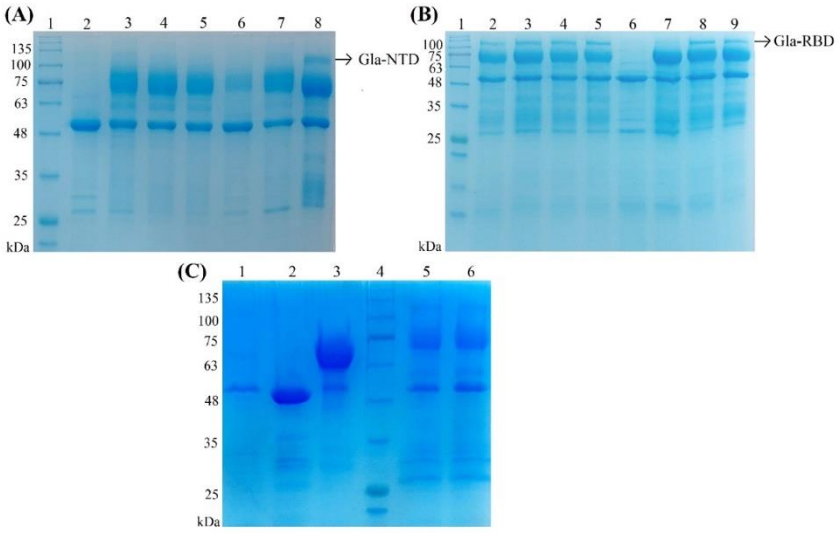

Figure 2. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis of expressed Gla-NTD and Gla-RBD proteins in *A. oryzae*. **(A)** Protein expression test of multiple transformed colonies on a small scale for Gla-NTD. Lane 1: Gangnam-Stain Protein Ladder. Lane 2–7: The colonies not expressing recombinant protein. Lane 8: The colony expressing Gla-NTD. **(B)** Protein expression test of multiple transformed colonies on a small scale for Gla-RBD. Lane 1: Gangnam-Stain Protein Ladder. Lanes 2–5, 8, and 9: colonies expressing Gla-RBD. Lanes 6 and 7: the colonies not expressing recombinant protein. **(C)** The comparison of the mock control and the colonies expressing glucoamylase, amylase, and fusion proteins. Lanes are as follows: mock transfection control (Lane 1), colony-expressing amylase (Lane 2), colony-expressing glucoamylase (Lane 3), colony-expressing Gla-RBD protein (Lane 5), and colony-expressing Gla-NTD protein (Lane 6). Gangnam-Stain Protein Ladder is located in Lane 4.

SDS-PAGE analysis of the culture samples after 7 days of incubation revealed that the expression of Gla-NTD fusion and Gla-RBD proteins was successfully maintained in *A. oryzae* (Figure 2). The recombinant colony expressing the Gla-NTD protein is shown in Figure 2A, and the recombinant colonies expressing the Gla-RBD protein are shown in Figure 2B. The recombinant Gla-NTD and Gla-RBD proteins were shown as bands measuring approximately 110 kDa in the SDS-PAGE investigation. Figure 2C compares differences in colonies expressing fusion proteins to the mock transfection control, the colonies expressing amylase and glucoamylase. Consequently, *A. oryzae* was able to express and secrete the recombinant Gla-NTD and Gla-RBD fusion proteins into the culture medium, as illustrated in Figure 2.

Bacterial expression systems in heterologous protein production offer advantages like simplicity, affordability, quick generation times, and scalability (He et al., 2021). Tantiwiwat et al. (2023) evaluated the potential of the *E. coli* expression system for RBD production to be used in neutralizing antibody detection kits, showing comparable results to the glycosylated RBD expressed in mammalian cells. Although the *E. coli* expression system is preferred for its advantages in recombinant SARS-CoV-2 protein production (Conzentino et al., 2021, 2022; Gao et al., 2022), the lack of post-translational modification makes it challenging, particularly for native RBDs, which are glycosylated proteins with eight cysteines that cause improper folding during production (Balasubramaniyam et al., 2022; He et al., 2021; Li et al., 2003; Tripathi et al., 2016).

In order to prevent the formation of inclusion bodies in *E. coli*, various strategies were used, including enzyme-assisted expression to produce RBD with comparable binding capacity to mammalian-produced RBD (Kim et al., 2022) and fusion protein in tandem with a carrier peptide (Brindha et al., 2022; McGuire et al., 2022). Fitzgerald et al. (2021) addressed the solubilization challenges of *E. coli*-expressed spike protein fragments, including RBD, through denaturation and refolding. Prahlad et al. (2021) employed a cytoplasmic disulfide bond formation system to overcome inclusion bodies and to avoid denaturing and refolding steps because of the reductive environment of the *E. coli* cytoplasm. However, mammalian-expressed RBD generally exhibits stronger immunogenicity and binding affinity than *E. coli*-expressed RBD (Maffei et al., 2021; Merkuleva et al., 2022).

A. oryzae possesses a robust secretion mechanism that allows for large-scale protein production. It has been utilized successfully as an expression system for expressing heterologous proteins derived from higher eukaryotes (He et al., 2019; Ntana et al., 2020). In our study, we employed the *pyrG* auxotrophic *A. oryzae* for the first time to produce recombinant glucoamylase fusion NTD and RBD proteins by utilizing its advantage in heterologous protein production, which includes complex protein production at the industrial level.

Purification of recombinant Gla-NTD and Gla-RBD proteins

Following harvesting and filtering the expression culture medium, the recombinant proteins were purified under native conditions. The amount of

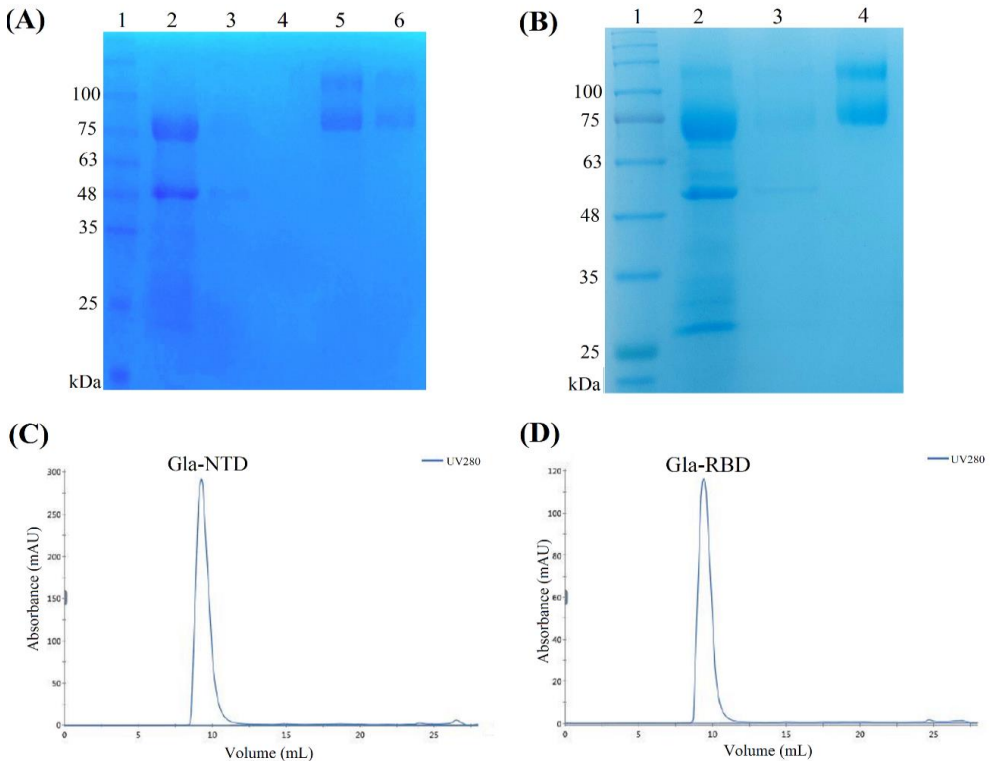

Figure 3. Purification of expressed Gla-NTD and Gla-RBD proteins. **(A)** Purification of His-tagged Gla-NTD by using the Ni-NTA column. Lane 1: Gangnam-Stain Protein Ladder. Lane 2: The culture medium of the colony expressing the Gla-NTD. Lanes 3–4: The wash solutions collected from the Ni-NTA column. Lanes 5–6: The eluted sample containing Gla-NTD. **(B)** Purification of His-tagged Gla-RBD by using the Ni-NTA column. Lane 1: Gangnam-Stain Protein Ladder. Lane 2: The culture medium of the colony expressing the Gla-RBD. Lane 3: The wash solution collected from the Ni-NTA column. Lane 4: The eluted sample containing Gla-RBD. **(C) (D)** Chromatograms of size exclusion chromatography purification using a Superdex 75 Increase 10/300 GL column. The peaks indicate the purified Gla-NTD **(C)** and Gla-RBD **(D)**.

recombinant His-tagged protein attached to the nickel resin was increased by co-incubating the sample and the resin for 1 h before purification. Following purification, the eluted samples were analyzed. SDS-PAGE analysis indicated the existence of protein bands with a molecular weight of approximately 110 kDa (Figures 3A and 3B). As expected, Gla-RBD has a slightly lower molecular weight than Gla-NTD (Figures A–B).

Maffei et al. (2021) investigated the differences between native and recombinant RBD expressed in *E. coli*, insect cells, and human HEK-293 cells. According to Maffei et al. (2021), RBD was expressed in soluble form in insect cells (6.5 mg/L) and mammalian cells (1.8 mg/mL), while *E. coli*-expressed RBD (2.5 mg/L) was recovered from inclusion bodies using a denaturing/refolding strategy. Chen et al. (2022) purified a stable and monomeric recombinant RBD protein from *P. pastoris* with a yield of 493 mg/L via hydrophobic interaction and anion exchange chromatography. In our study, size exclusion chromatography experiments indicated that *A. oryzae*-expressed Gla-NTD and Gla-RBD fusion proteins are stable and correctly folded. Following size exclusion chromatography (Figures 3C and 3D), the fractions containing Gla-NTD and Gla-RBD were concentrated. Consequently, the purified recombinant Gla-NTD protein yield was determined to be 14 mg/L, while the purified recombinant Gla-RBD protein yield was determined to be 12 mg/L. Although the *E. coli* expression system offers cost-effective high-yield production than other expression systems, its use for complex proteins can be hindered by requiring refolding. On the other hand, despite the advantages of insect and mammalian expression systems in the production of complex proteins, their utilization in industrial production poses drawbacks due to their associated high costs and time-intensive production processes. *P. pastoris*, like *A. oryzae*, is commonly utilized in fermentation technology and has been demonstrated to produce RBD suitable for industrial production (Chen et al., 2022). In our study, we have demonstrated the feasibility of producing functional Gla-NTD and Gla-RBD proteins in *A. oryzae*, implying potential for industrial-scale production. This approach also allows us to circumvent the limitations associated with other expression systems.

The binding ability of Gla-NTD and Gla-RBD proteins

Indirect ELISA was performed using human sera obtained from patients suffering from COVID-19 to assess the binding ability of recombinant Gla-NTD and Gla-RBD proteins and to validate their in vitro functionality. Non-fusion glucoamylase-expressed *A. oryzae* culture mediums and irrelevant recombinant protein-expressed *A. oryzae* culture mediums were employed as negative control groups in order to

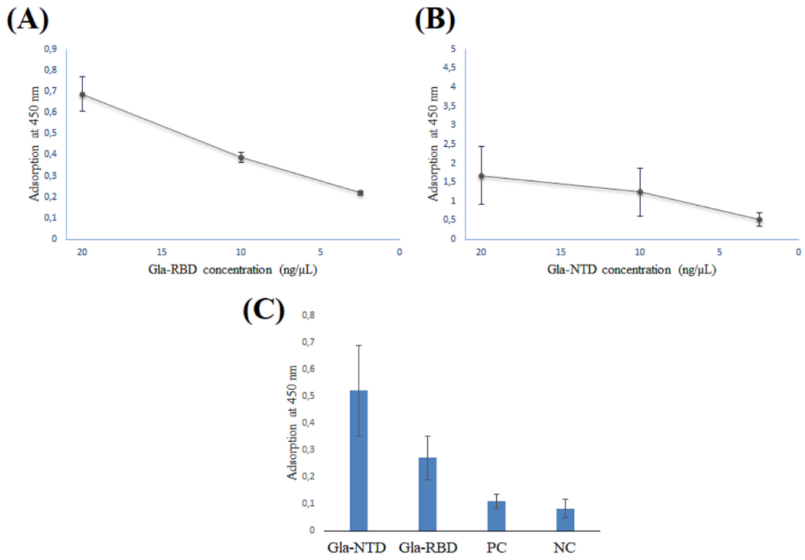

Figure 4. ELISA assays for purified recombinant Gla-RBD **(A)** and Gla-NTD **(B)** proteins compared to *P. pastoris*-expressed RBD and irrelevant recombinant protein **(C)**. The binding of the Gla-RBD (P = 0.011) and Gla-NTD (P = 0.006) proteins to human serum antibodies was detected using an HRP-conjugated anti-human IgG HRP antibody. Absorbance was measured at 450 nm, and bars indicate standard deviations. PC: positive control; recombinant RBD expressed in *P. pastoris*; NC: negative control; irrelevant protein.

investigate non-specific binding. Checkerboard titrations were used to evaluate the optimal concentration of recombinant Gla-NTD and Gla-RBD proteins as well as the ideal dilution ratio of human serum samples. For the ELISA, *P. pastoris*-expressed RBD, which was verified for its binding affinity of ACE2 homolog peptides (Azar et al., 2023), was utilized as a positive control group to confirm the binding ability of human anti-IgG antibodies to recombinant Gla-NTD and Gla-RBD.

ELISA was also used to qualitatively assess the IgG detection performance of recombinant fusion proteins in serum samples. The findings indicated that recombinant Gla-NTD (P = 0.006) and Gla-RBD (P = 0.011) proteins significantly bind to human anti-IgG antibodies in serum samples. As a result, the Gla-NTD and Gla-RBD proteins have correctly folded structures, allowing them to serve as antigen-antibody interactions. Furthermore, the data revealed a proportional relationship between recombinant fusion protein concentration and binding capacity (Figure 4).

The selection of the antigen exhibiting a stronger association with virus neutralization is critical to the efficacy of an immunoassay (McAndrews et al., 2020). The most dependable and commonly used technique for identifying antibodies produced against a particular antigen is ELISA (Bastos et al., 2020). While the specificity of antigen-based methods typically reaches around 100%, their sensitivity tends to fluctuate between 30% and 80% (Márquez-Ipiña et al., 2021). Studies on nucleoprotein-based ELISA assays for SARS-CoV-2 detection demonstrated sensitivity and cost-effectiveness in the detection of anti-IgG antibodies in serum samples (Tozetto-Mendoza et al., 2021). Additionally, RBD is an appropriate target antigen for developing serologic immunoassays. Márquez-Ipiña et al. (2021) investigated the binding ability of *E. coli*-expressed RBD in ELISA assays utilizing human sera exposed and non-exposed to SARS-CoV-2. In their study conducted for *E. coli*-expressed RBD, the binding affinity was determined to be approximately 75%. However, owing to its rapid applicability and cost-effectiveness, the immunoassay offers substantial advantages on a large scale compared to techniques reliant on reverse transcription and polymerase chain reaction. In our study, the binding abilities of *A. oryzae*-expressed Gla-NTD and Gla-RBD proteins were investigated in human serum using a low-cost ELISA as a straightforward and reliable immunoassay. The research has demonstrated that recombinant Gla-NTD and Gla-RBD proteins exhibit significant and concentration-dependent binding to human anti-IgG antibodies in serum samples.

RBD can be expressed in *P. pastoris* as a vaccine agent with comparable yields and immunization results to *E. coli* (Chuck et al., 2009; Liu et al., 2022; Mi et al., 2022; Pino et al., 2021; Xing et al., 2022; Zang et al., 2021). Kalyoncu et al. (2023) utilized a fermentation system to produce RBD in *P. pastoris*, achieving over 1 g/L yield with a high immune response. As a potential vaccine agent, Chen et al. (2022) assessed the immunogenicity of *P. pastoris*-expressed RBD in mice. Notwithstanding that the RBD expressed in *P. pastoris* possesses high mannose-glycan-type glycosylation, it is able to stimulate immunization in mice just as the RBD is expressed in mammalian cells (Argentinian Anti-Covid Consortium, 2020). On the other hand, the results of our investigation indicate that *A. oryzae* can be used as a biotechnological platform to produce NTD and RBD fusion proteins. Because of its low cost of handling, easily applicable purification process from the gene to the recombinant protein, and ease of scale-up to obtain high protein titers in fermentation, *A. oryzae* is an

attractive tool for the production of SARS-CoV-2 antigens to be used in protein-based subunit vaccines.

Conclusion

The mass manufacture of functional NTD and RBD proteins provides an improvement in the development in COVID-19 research, diagnostics, and treatment. The solubility and functionality of RBD are the main problems in the case of its expression in *E. coli* (Argentinian Anti-Covid Consortium, 2020; Chen et al., 2005). In the mammalian expression system, the low final yield of transient transfection is a limiting issue for serologic assays (Esposito et al., 2020). *A. oryzae* is a useful biotechnological platform for producing functional Gla-NTD and Gla-RBD proteins on a large scale without the requirement for cell lysis or laborious purification procedures. In this study, for the first time, *pyrG* auxotrophic *A. oryzae* was used as a robust platform to produce heterologous glucoamylase-fused NTD and RBD proteins of the S protein of SARS-CoV-2. The ELISA assay results, employed to investigate the binding capabilities of recombinant fusion proteins to anti-human IgG antibodies in serum, have validated the functions of *A. oryzae*-expressed Gla-NTD and Gla-RBD. This study could pave the way for the efficient, affordable, and high-level production of various viral antigens in *A. oryzae* in future investigations.

Ethical Statement

The institutional review board approval for the study was granted by the Bezmialem Vakif University Institutional Review Board and by the Turkish Ministry of Health, and all procedures were in accordance with the approval. The subject had given informed consent for their samples to be stored and used for serological testing in the future.

Author Contributions

EK: Project Administration, Investigation, Methodology, Visualization, Validation, Formal Analysis, Resources, Writing -original draft, Writing-review, and editing; SU: Conceptualization; Supervision, Methodology, Resources, Writing-review, and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank all staff from Department of Medical Microbiology, Medical School and Beykoz Institute of Life Sciences and Biotechnology, Bezmialem Vakif University, for human serum collection.

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