Affinity purification of SARS-CoV-2 spike protein receptor binding domain produced in a C1 fungal expression system

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Introduction

The Receptor Binding Domain (RBD) of the spike protein of SARS-CoV-2 has shown promise for diagnosis, treatment, and development of vaccines for COVID-19. However, two problems persist with large scale production of RBD: 1) lack of high productivity upstream cell culture, 2) absence of a commercial, highly selective affinity resin. In an effort to overcome these limitations, we evaluated two novel technologies for the production and purification of RBD.

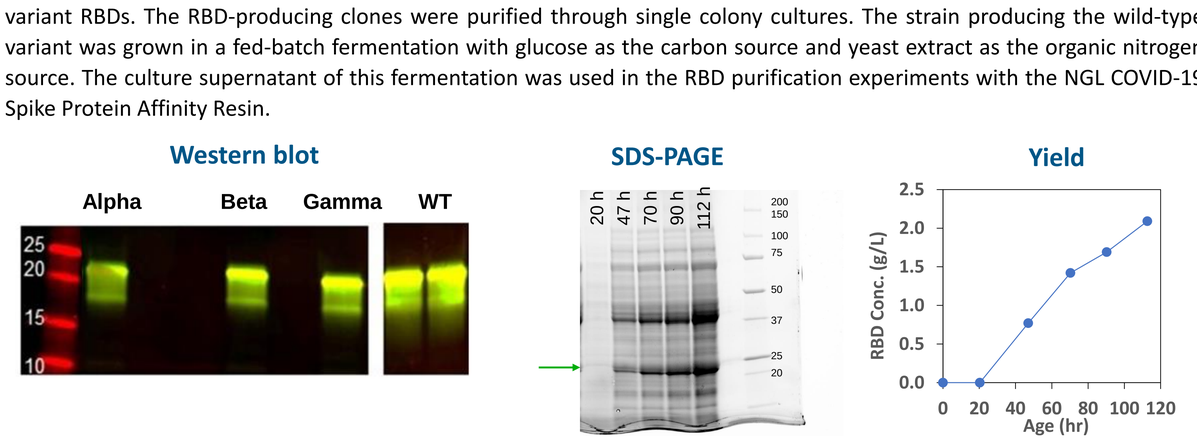
Briefly, RBD was expressed using C1, an engineered fungal strain of *Thermothelomyces heterothallica (Dyadic International[1]).* The C1 platform expresses glycosylated antigens with high productivity, stability, and purity. RBD was purified using a novel affinity resin[2] known to produce yields of 90% to 95% purity in one chromatography step.

Affinity purification did not affect protein quality, as demonstrated by ACE-2 binding of RBD. The novel affinity resin showed excellent base stability, consistent product quality, and similar ACE-2 binding activity over 40 cycles.

RBD produced in C1, in conjunction with affinity purification using a novel affinity resin, provides a breakthrough in the large-scale production of affordable COVID-19 protein-based vaccines.

RBD in C1 fungal expression system

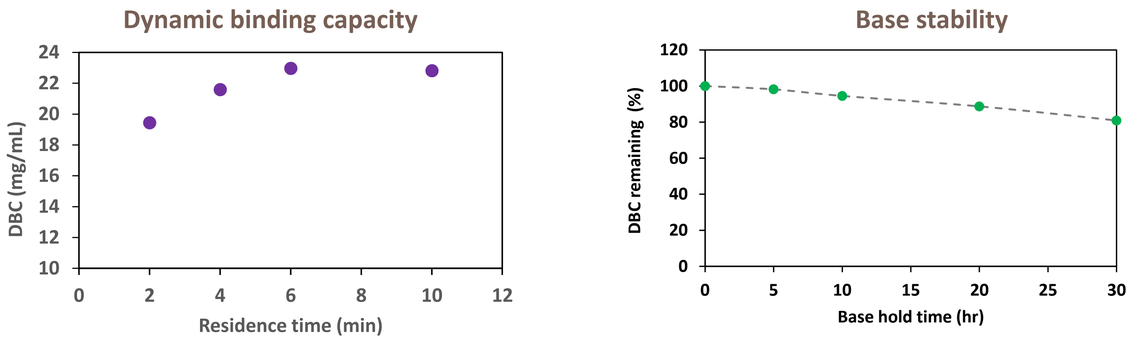
The receptor binding domains (RBD) of Covid-2 spike proteins were expressed in the fungal production platform C1 of Dyadic. Codon-optimized synthetic genes encoding the wild-type, alpha, beta, and gamma variants were made by GenScript®. Expression vectors with two target protein expression cassettes with the C1 *bgl8* promoter were constructed. C1 CBH1 signal sequence was used in the constructs, and a C-tag was added to the C-terminus of the RBD gene. The expression vectors were transformed into a C1 strain with 14 protease deletions, and transformants were screened from 24-well plate cultures by Western blotting with detection of the C-tag. Positive transformants were found for each of the variant RBDs. The RBD-producing clones were purified through single colony cultures. The strain producing the wild-type variant was grown in a fed-batch fermentation with glucose as the carbon source and yeast extract as the organic nitrogen source. The culture supernatant of this fermentation was used in the RBD purification experiments with the NGL COVID-19 Spike Protein Affinity Resin.



- Western blotting from 24-well plate culture supernatants of C1 transformants of the RBD variant expression constructs. The detection was done with Biotin-C-tag antibody conjugate and streptavidin-800CW fluorescent detection reagent.
- SDS-PAGE Coomassie stained from fermentation broth samples (wild-type RBD variant). Timepoints in hours (h).
- Graph showing the yield in g/L of wild-type RBD variant reached along fermentation.

NGL COVID-19 Spike Protein Affinity Resin

An affinity ligand for the SARS-CoV-2 spike protein, co-developed by Repligen and Navigo Proteins GmbH, was expressed in recombinant *E.coli* culture. This ligand was purified and immobilized on a Praesto® Epoxy 85 Resin (Purolite Corporation). The Dynamic Binding Capacity (DBC) at 10% breakthrough for this resin was estimated for SARS-CoV-2 spike protein RBD. The stability of this ligand was also tested by incubating it in 0.1 M sodium hydroxide.



- The DBC at 2 min residence time was ~19 g/L and increased to ~22 g/L at 6 min residence time.
- The affinity resin maintained ~80% of its DBC after 20 hr exposure to 0.1 M NaOH, equivalent to 120 cycles with 15 min exposure each cycle.

Objectives and experimental

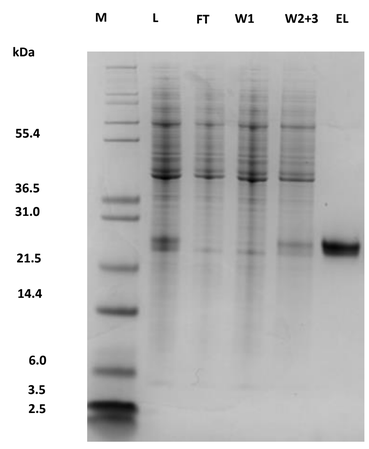
- Purify RBD produced in C1 fungal expression system using NGL COVID-19 Spike Protein Affinity Resin
- Estimate DBC and yield after every 10th cycle to show stability and purification performances of the resin
- Estimate monomer content of purified samples to show consistent product quality
- Conduct ACE-2 inhibition assay to measure activity of the purified RBD protein

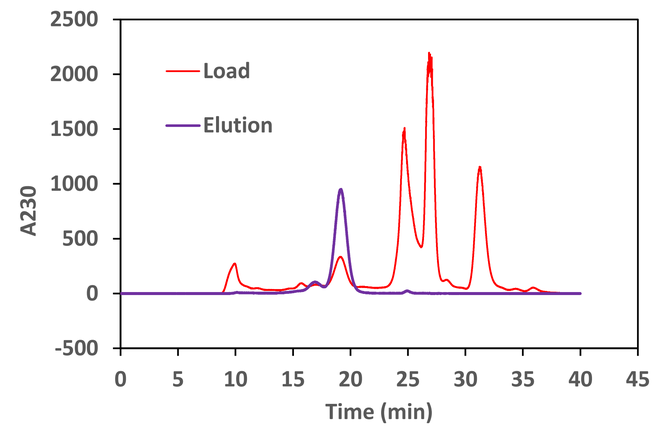
Step	Buffer	Volume (CV)	Residence time (min)
Equilibration	1x PBS	5	2
Wash 1	1x PBS	5	2
Wash 2	1x PBS + 1 M NaCl	5	2
Wash 3	1x PBS	5	2
Elution	0.1 M Acetic Acid, pH 3.5	3	2
Strip	0.2 M Acetic Acid	5	2
Sanitization	0.1 M NaOH	5	3
Re-Equilibration	1X PBS	7	2

- **Column volume:** 1 mL, **Loading:** ~80% of DBC, **Titer in feed:** 1.8 g/L, **Load residence time:** 4 min

Purification with NGL COVID-19 Spike Protein Affinity Resin

A column packed with 1 mL affinity resin was used for purification of C1 generated RBD. The load, flow through, wash, and elution streams were also analyzed using SDS-PAGE and size exclusion chromatography (SEC).

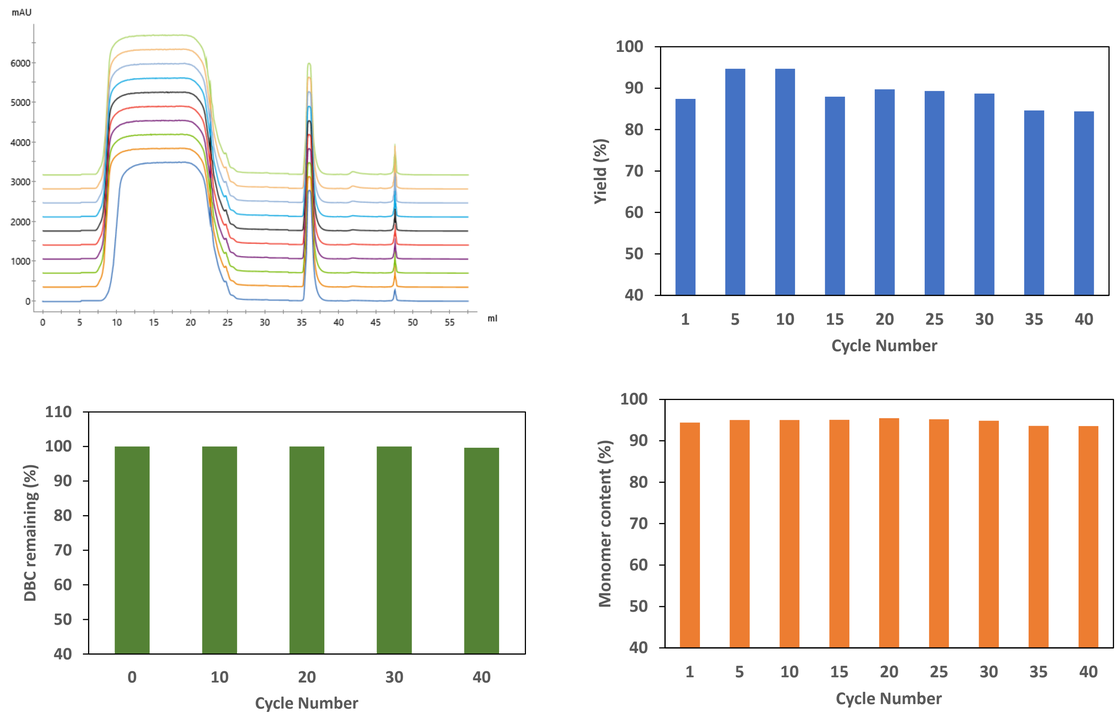




- SDS-PAGE showed a pure elution sample which indicates excellent selectivity of the resin.
- SEC elution profile indicates that the affinity resin binds RBD (elution peak, purple) and that the eluted product has low amount of impurity. This provides further evidence that the NGL COVID-19 Spike Protein Affinity Resin displays excellent selectivity for RBD. RBD monomer purity was estimated to be ~95%.

Purification performance

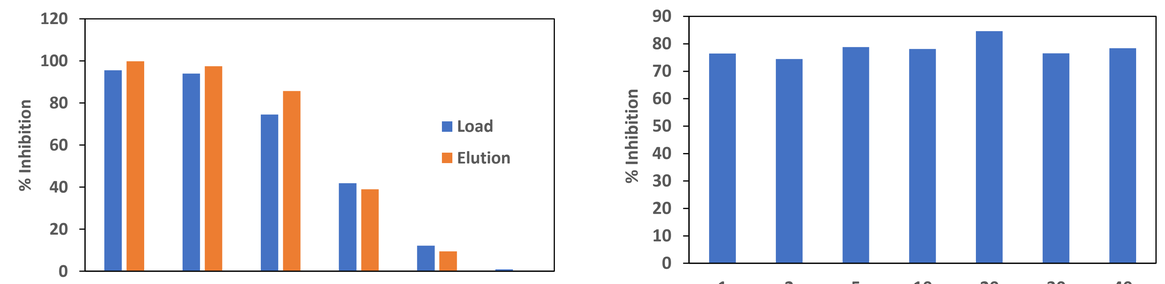
Repeatability of the affinity column was tested by conducting 40 purification cycles using C1 RBD cell culture fluid. 0.1 M NaOH was used for sanitization in every cycle. The DBC of the resin was estimated after every 5th cycle.



- The overlay of the AKTA™ chromatogram shows repeated reproducible purification cycles. Yield was ~90% for 40 cycles.
- Yield remained ~90% over 40 cycles.
- DBC of the resin remained ~100% of initial DBC after 40 cycles showing excellent base stability.
- Monomer content of the product is ~95% for 40 cycles indicating consistent product quality.

Activity of purified RBD

Binding of purified SARS-CoV-2 Spike Protein C1 RBD to ACE-2 was assessed using an ELISA based inhibition assay and compared with the pre-purified RBD contained in the load CCF. RBD was serially diluted and mixed with a fixed concentration of ACE-2 and then incubated in a well coated with RBD. The concentration of available ACE-2 was determined using an anti-ACE-2 secondary antibody colorimetric assay with detection at 450 nm. As the concentration of purified RBD increased, the amount of free ACE-2 available to bind to immobilized RBD decreased, demonstrating successful binding of RBD purified with the NGL COVID-19 Spike Protein Affinity Resin to its ACE-2 target. C1 RBD eluates were tested at a single concentration of 2 µg/ml to evaluate performance over many purification cycles.



- ACE-2 inhibition was nearly identical for load and elution samples (IC50 ~ 0.2 µg/ml), indicating that RBD activity was not affected by affinity purification.
- Percent inhibition from individual cycle eluates were all within 10% at a 2 ug/ml fixed concentration.
- Uniform product quality throughout the 40 affinity purification cycles was demonstrated.

Conclusions

- RBD produced in C1 fungal system was successfully purified using NGL COVID-19 Spike Protein Affinity Resin.
- Affinity purification with the novel resin produced ~95% pure RBD in a single step.
- The resin was stable for the 40 cycles when using 0.1 M NaOH for the sanitization steps.
- RBD quality and yield were consistent across 40 purification cycles.
- RBD activity was not impacted by affinity purification. ACE-2 binding of RBD was sustained over 40 cycles.
- To learn more about this resin: https://www.repligen.com/technologies/resins/ngl-COVID-19-spike-protein-affinity-resin

[1] C1 is an engineered Thermothelomyces heterothallica fungus developed for high productivity, increase stability and purity by Dyadic International, Inc. Dyadic International, Inc. , 140 Intracoastal Pointe Drive, Jupiter, Florida 33477 USA.

[2]The Novel affinity resin for the purification of the SARS CoV-2 Spike RBD Protein for vaccine development and manufacturing was co-developed by Repligen Corporation and Navigo Proteins GmbH.