T.C.

AKDENİZ UNIVERSITY

ENGINEERING, PRODUCTION AND CHARACTERIZATION OF RBD AND NUCLEOCAPSID FUSION PROTEIN

Eylül Bilge SARI

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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MASTER'S DEGREE THESIS

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TARIMSAL BİYOTEKNOLOJİ

ANABİLİM DALI

YÜKSEK LİSANS TEZİ

Bu tez Prof. Dr. Tarlan MAMMEDOV yürütücülüğünde TÜSEB tarafından 8611 numaralı proje ile desteklenmiştir.

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Bu tez 23/01/2025 tarihinde jüri tarafından Oybirliği ile kabul edilmiştir.

Prof. Dr. Nedim MUTLU (Danışman)
Doç Dr. Münevver AKSOY
Prof. Dr. Ahmet KOÇ

ÖZET

ENGINEERING, PRODUCTION AND CHARACTERIZATION OF RBD AND NUCLEOCAPSID FUSION PROTEIN

Eylül Bilge SARI

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı

Danışman: Prof. Dr. Nedim MUTLU

Mart 2025; 56 sayfa

SARS-CoV-2, Şiddetli Akut Solunum Yolu Enfeksiyonu Coronavirüsü 2, insanda COVID-19 isimli enfeksiyona sebep olan tek zincirli RNA virüsüdür. SARS-CoV-2 dünya çapında büyük kayıplara sebep olan COVID-19 pandemisine yol açmıştır. COVID-19 pandemisinin ilk günlerinden itibaren enfeksiyonu önleyici ve iyileştirici tedavilerin geliştirilmesi önem kazanmıştır. SARS-CoV-2'nin hızlıca varyantlaşması ve virüsün tedavilere karşı tepkisinin düşük olması evrensel COVID-19 aşısı konusunu gündeme getirmiştir. SARS-CoV-2'nin spike proteininin RBD alt birimi (Reseptör baglama alanı) konağın ACE2 isimli reseptörünü tanır ve bağlanarak enfeksiyonu başlatır. RBD alt biriminin SARS-CoV-2 varyantları arasında korunması sebebiyle terapötik ajanların geliştirilmesinde öncül olmuştur. Nucleocapsid proteininin spesifik biyokimyasal yapısı ve enfeksiyon sürecindeki rolü ile terapötik protein olarak kullanımı diğer çalışmalardan farklı bir yorum getirmektedir. Rekombinant RBD ve nucleocapsid füzyon proteini, konakta enfeksiyon gelişmeden bağışıklığı tetikleyerek antijen görevi görür.

Bu tez çalışmasının amacı, *Nicotiana benthamiana* bitkilerinde RBD ve nükleokapsid proteinlerinden oluşan bir rekombinant füzyon proteininin üretilmesi ve daha önce laboratuvarımızda üretilen ve karakterize edilen rekombinant RBD ve nükleokapsid proteinlerinden oluşan antijen kokteyli ile fonksiyonel aktivitesinin karşılaştırılmasıdır. Bu amaçla, tasarlanan füzyon proteini bitki ekspresyon vektörü kullanılarak eksprese edilmiş ve üretilen füzyon proteini *N. benthamiana* bitkisinden saflaştırılmış ve karakterize edilmiştir. Bu yüksek lisans tezi kapsamındaki füzyon proteininin üretimi ve karakterizasyonu laboratuvarımızda halen devam etmektedir. Farelerde antikor titrelerini incelemek için immünojenisite çalışmaları devam etmektedir. İmmünojenisite çalışmaları tamamlandığında, füzyon proteininin kokteyl antijenleri gibi yüksek antikor titrelerini indüklemesi ve SARS-CoV-2'ye karşı aşı araştırmaları için umut verici bir yaklaşım olması beklenmektedir.

ANAHTAR KELİMELER: Antijyotensin dönüştürücü enzim 2 (ACE2), Rekombinant füzyon proteini, Reseptör baglama alanı (RBD), SARS-CoV-2

JÜRİ: Prof. Dr. Nedim MUTLU

Doç. Dr. Münevver AKSOY

Prof. Dr. Ahmet KOÇ

ABSTRACT

ENGINEERING, PRODUCTION AND CHARACTERIZATION OF RBD AND NUCLEOCAPSID FUSION PROTEIN

Eylül Bilge SARI

MSc Thesis in Agricultural Biotechnology

Supervisor: Prof. Dr. Nedim MUTLU

March 2025; 56 pages

SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2, is a single-stranded RNA virus that causes the infection known as COVID-19 in humans. SARS-CoV-2 has led to the COVID-19 pandemic, which has caused loss of life in millions deficit worldwide. Since the first days of the COVID-19 pandemic, the development of preventive and curative treatments has gained importance. The rapid variation of SARS-CoV-2 and the low response of the virus to treatments have brought the issue of a universal COVID-19 vaccine as topic. The RBD subunit of the spike protein of SARS-CoV-2 recognizes the host's ACE2 receptor and initiates the infection by binding. The conservation of the RBD subunit among SARS-CoV-2 variants has led to the development of therapeutic agents. The specific biochemical structure of the nucleocapsid protein, its role in the infection process brought about its use as a therapeutic protein bring a different interpretation from other studies.

The aim of this thesis was the production of a recombinant fusion protein comprising of RBD and nucleocapsid proteins in *Nicotiana benthamiana* plants and compare its functional activity with antigen cocktail, comprising of recombinant RBD and nucleocapsid, which was previously produced in Mammedov's laboratory and characterized. For this purpose, the engineered fusion protein was expressed using plant expression vector and produced fusion protein was purified from *N. benthamiana* plant and characterized. Immunogenicity studies will examine the antibody titers in mice. When the immunogenicity studies are completed, it is expected that the fusion protein may induce high antibody titers like the cocktail antigens and will be a promising approach for vaccine research against SARS-CoV-2.

KEYWORDS: Angiotensin converting enzyme 2 (ACE2), Receptor binding domain (RBD), Recombinant fusion protein, SARS-CoV-2

COMMITTEE:

Prof. Dr. Nedim MUTLU

Assoc. Prof. Dr. Münevver AKSOY

Prof. Dr. Ahmet KOÇ

PREFACE

First, I would like to express my deepest gratitude to my advisor Prof. Dr. Tarlan MAMMEDOV, who shared his knowledge and experience with me during my graduate education and experimental studies. His mentorship made me a scientist. Also, I would like to thank my co-advisor Prof. Dr. Nedim MUTLU for his support and patience.

I would like to thank my lab partner and friend Damla YÜKSEL, who has been with me since the first day I started working in the laboratory and shared everything she knew with me and guided me, Dr. Cansu ŞİMŞEK, who has been with me since the beginning of my biotechnology adventure, and my colleagues Doğuhan YALIM, Beyza YÜKSEL and Ahmet Turhan İKTEN, I am so grateful.

Lastly and most importantly, I would like to thank my mother Özgül AKBENİZ and my father Halil SARI, who have endlessly trusted and supported me.

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ACADEMIC STATEMENT

I declare that this study titled “ENGINEERING, PRODUCTION AND CHARACTERIZATION OF RBD AND NUCLEOCAPSID FUSION PROTEIN”, which I have submitted as my master’s thesis, has been written in accordance with academic rules and ethical values, and that I have shown the source of all information that does not belong to me in this thesis.

24/02/2025

Eylül Bilge SARI

SYMBOLS AND ABBREVIATIONS

Symbols

°C : Degree Celsius

g : Gram

kb : Kilobases

kDa : Kilodalton

µg : Microgram

µL : Microliter

µm : Micrometer

min : Minutes

mL : Milliliter

M : Molar

ng : Nanogram

nL : Nanoliter

V : Volt

% : Percent

Abbreviations

ACE2 : Angiotensin Converting Enzyme 2

bp : Basepair

CoV : Coronavirus

dpi : Days post infiltration

NTD : N-terminal domain

ORF : Open reading frame

RBD : Receptor binding domain

WHO : World Health Organization

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1. INTRODUCTION

The SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) is a virus from Coronaviridae family that causes COVID-19, which has started to spread in December 2019 and was declared a pandemic by the WHO on 11 March 2020. The disease progresses with severe respiratory infections, gastrointestinal problems, and neurological symptoms. The disease is believed to have first emerged in a seafood market in Wuhan, China, and spread rapidly around the world through travellers passing through Wuhan (Wu et al. 2020).

The SARS-CoV-2 is a single-stranded, positive-sense RNA virus. RNA viruses are prone to mutations due to the unstable structure of their RNA. SARS-CoV-2, like other RNA viruses, undergoes mutations and new variations emerge. New variants may be contagious and deadly than previous variants. From the first days of the pandemic, scientists began researching to understand the structure of the virus and develop a universal SARS-CoV-2 vaccine (Thakur et al. 2022).

The genome of SARS-CoV-2 is about 29.8 kb in size and contains 14 open reading frames (ORFs) encoding total 27 proteins, 4 of which are structural proteins (M, membrane; E, envelope; S, spike, and N, nucleocapsid). Structural proteins - especially the S protein - induce infection in the host and trigger the formation of antibodies (Pollet, Chen, and Strych 2021). For this reason, there are studies to understand the structural proteins and infection mechanisms to produce a universal SARS-CoV-2 vaccine by using recombinant protein technology.

SARS-CoV-2 shares homology with MERS-CoV (Middle Eastern Syndrome Respiratory Coronavirus) and SARS-CoV-1 viruses, which previously caused the disease in human. As a result of recent advancements in generation sequencing technologies, Lu et al. have found that SARS-CoV-2 is 50% identically like MERS-CoV and 70% like SARS-CoV-1 (Lu et al. 2020).

The number of people infected with COVID-19 have reached 776.000.000 by 2024 according to WHO. Several therapeutics against the disease have been tested in clinical trials. Treatment can involve chemical molecules that block the proliferation of the virus, such as protein inhibitors, and monoclonal antibodies that neutralize the infection (Drożdżal et al. 2021). Vaccines are mentioned in the literature as a preventive treatment, as they provide immunity before the infection begins. Vaccine production could take years, and it could be costly. Even so vaccination is the best way to prevent disease. Traditional and modern platforms are used in vaccine production. Examples of traditional vaccine development platforms include live viruses or attenuated virus vaccines. Modern vaccine development platforms include recombinant protein vaccines, nucleic acid-based vaccines and vector vaccines (Awadasseid et al. 2021).

Advances in biotechnology have resulted to the production of recombinant protein vaccines which offer enhanced safety. Recombinant proteins are proteins that can be produced by many living systems through recombinant DNA technology. Due to their low production costs and reliability, these proteins are widely used as therapeutic or food.

Living materials such as bacteria, animal cell lines or plants are used to produce recombinant proteins. Since therapeutic proteins targets is eukaryotic and the structure of eukaryotic proteins is complex, the production system must also be considered.

Due to the eukaryotic structure of plants, their rapid life cycles, low cost and minor risk of contamination, plant production systems are commonly used in the production of recombinant proteins. Plant transient expression systems are an approach that has been in development for over 30 years. The production of recombinant proteins in plants imitates *Agrobacterium* spp. or Tobacco Mosaic Virus (TMV), which naturally infect plants (Pogue et al., 2010).

Nicotiana benthamiana L. is an important plant from the Solanaceae family, that has 19 chromosomes and grows naturally in tropical climates. It is used in biotechnological studies due to its rapid life cycle, resistance to diseases and manageability of genetic manipulation (Kurotani et al. 2023).

Agrobacterium-mediated transient gene expression is a well-studied technique in plants such as *N. benthamiana* that purposing the production of therapeutic recombinant proteins rapid and accessible. Considering previous recombinant protein studies, recombinant protein production has been optimized by proper promoter selection, codon optimization, and development of experimental protocols. Production and engineering studies on recombinant RBD and nucleocapsid fusion protein as vaccine candidate for SARS-CoV-2 will be helpful in view of and fill the gap in the literature.

In the scope of this thesis study, a transient expression system was used to produce recombinant RBD and nucleocapsid fusion proteins in *N. benthamiana* plants. The RBD+N fusion gene was designed and cloned into an expression cassette. The relevant vectors were transformed into *N. benthamiana* plants by agroinfiltration via syringe. Protein purification was performed by collected infiltrated *N. benthamiana* leaves. Comparing the activity of the protein with other antigen cocktails produced in our laboratory, our data suggest that the produced fusion protein is active and may be suitable for future therapeutic purposes. To this end, future immunogenicity studies will continue.

2. LITERATURE

2.1. The Pandemic of COVID-19

In March 2020, the World Health Organization (WHO) declared COVID-19, caused by SARS-CoV-2, a global pandemic. The word "SARS" is given because it refers to "severe acute respiratory syndrome", the word "CoV" is given because the virus that causes the disease belongs to the *Coronaviridae* family, and the word "2" is given because it is genetically related to the SARS epidemic that occurred in 2003. The first reports of the disease were announced in Wuhan, China, in December 2019.

COVID-19 has been transmitted from person to person and has spread throughout the world. To date, according to the WHO, 775.522.404 cases have been registered and about 7 million people have died from this cause (World Health Organization, 2024).

2.1.1. SARS-CoV-2 and COVID-19 disease

The characteristics of COVID-19 disease are comprehensive. Symptoms can range from mild to severe. Frequent symptoms are fever (83%), cough (82%) and dyspnoea (31%) and gastrointestinal symptoms other than pneumonia diseases reported in 10% of patients (Ciotti et al. 2020).

SARS-CoV-2 causes pneumonia in humans, as well as birds and other mammals. Initial transmission to humans is thought to occur through direct contact of viral particles with the body of an animal. The genome of SARS-CoV-2, which causes COVID-19, is 96% like a *Betacoronavirus* (RaTG13) isolated from bats. The receptor binding motif (RBM) of SARS-CoV-2, which is associated with infection, is 98.7% like the RBM motif of pangolin-origin coronaviruses (pCoV) (Salian et al. 2021).

2.2. Viral Structure, Characteristics and Infection Mechanism of SARS-CoV-2

Coronaviruses are enveloped RNA viruses that can cause a variety of infections in humans and other animals. Following the infection by SARS-CoV-2, these viruses' precipitate complications in host the respiratory, digestive, or central nervous systems. Six coronaviruses have been identified that can infect humans. These are SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1 (Maier, Bickerton, and Britton 2015).

SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus) and MERS-CoV (Middle East Respiratory Syndrome Coronavirus) caused two major pneumonia epidemics before SARS-CoV-2. Given the SARS-CoV-2 pandemic, studying the molecular biology of infection mechanisms is important for the development of vaccines and treatments.

SARS-CoV-2 binds to a receptor on the surface of the host cell before entering the host cell. The S1 subunit of the spike protein on the surface of SARS-CoV-2 has a

receptor binding domain (RBD) that recognizes the host receptor, Angiotensin-Converting Enzyme 2 (ACE2).

For the fusion of SARS-CoV-2 with the host cell, proteolytic activation of the S1/S2 interface is achieved by a structural change in a cell surface protease called TMPRSS2 (Shang et al., 2020). Studies have shown that the presence of ACE2 is important for the occurrence of SARS-CoV-2 infection (He et al. 2020).

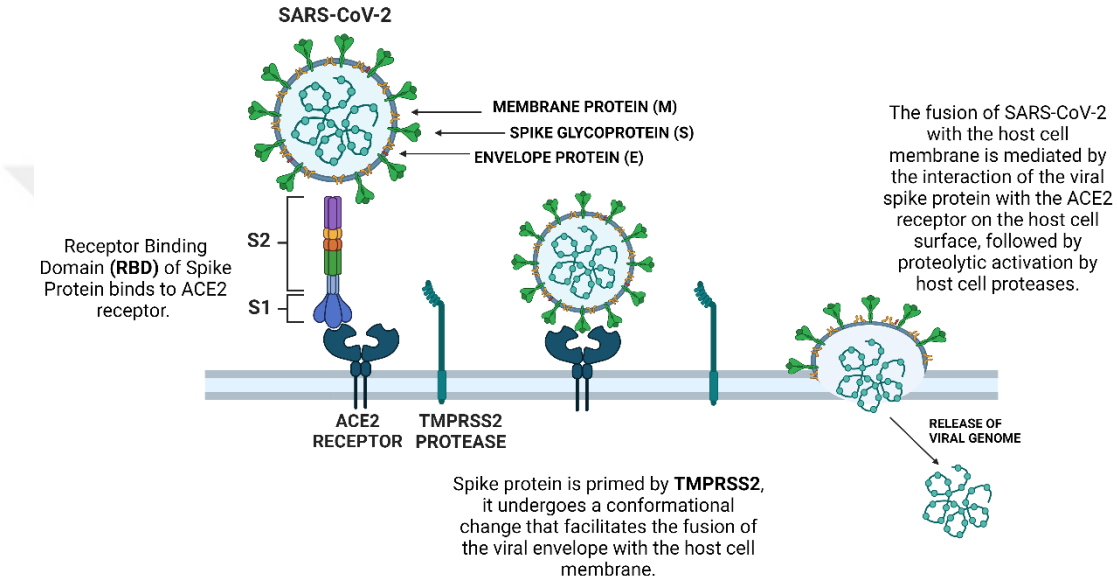


Figure 2. 1. Infectious mechanism of SARS-CoV-2. Viral infection and entry of host cell relies on the host cell receptor angiotensin-converting enzyme 2 (ACE2) to recognition and on the host transmembrane serine protease TMPRSS2 to prepare the SARS-CoV-2 spike (S) protein

2.3. Origin of SARS-CoV-2

According to phylogenetic analysis, SARS-CoV-2 forms a distinct lineage with Bat-SARS-like coronaviruses that belong to the order *Nidovirales*, family Coronaviridae, genus Betacoronavirus, and subgenus Sarbecovirus (Zhou et al. 2020).

The order *Nidovirales* includes the families Arteriviridae, Mesoniviridae, and Roniviridae, as well as the family Coronaviridae. Viruses of the order *Nidovirales* are enveloped positive-sense RNA viruses characterized by spikes on their surface. Their genomes are highly conserved and are the largest RNA viruses by genome size, reaching 33.5 kb (Fehr and Perlman 2015).

They can infect many birds and fish and mammals including humans. Their genomes encode at least three structural proteins, but their number and properties may differ. These proteins are the major surface glycoprotein or spike protein (S), the transmembrane (M) or matrix protein and the nucleocapsid protein (N) (Enjuanes et al. 2008).

According to phylogenetic analysis, SARS-CoV-2, together with Bat-SARS-like coronaviruses, forms a separate lineage belonging to the order *Nidovirales*, family Coronaviridae, genus Betacoronavirus and subgenus Sarbecovirus (Helmy et al. 2020).

2.4. Structure of SARS-CoV-2

The SARS-CoV-2 genome is approximately 29.9 kb in size, it encodes four structural proteins (spike, nucleocapsid, membrane, and envelope) and sixteen non-structural proteins (nsp1-16). The non-structural proteins are involved in pathways related to RNA processing, replication, infection and survive (Wang et al. 2020a).

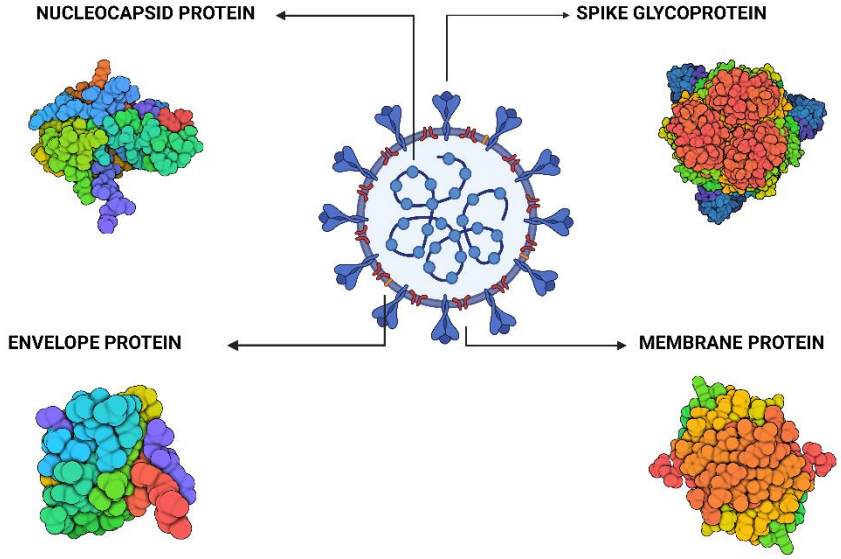


Figure 2.2.SARS-CoV-2's Structural Proteins; envelope protein has determined by X-Ray diffraction, PDB ID: 7QCT (Zhu et al. 2022). Spike protein has determined by electron microscopy, PDB ID: 5XLR(Gui et al. 2017). Nucleocapsid protein has determined by solution NMR, PDB ID: 7ACS(Dinesh et al. 2020). Membrane protein has determined by electron microscopy, PDB ID: EQJ(Miller et al. 2023)

2.4.1. Spike protein (S)

Spike glycoproteins are type I transmembrane proteins found on the surface of SARS-CoV-2. It has a trimeric structure consisting of subunits S1 and S2. Subunit S1 consists of a receptor binding domain (RBD) and an N-terminal domain (NTD). The RBD domain is responsible for receptor binding, and it binds to the host cell receptor angiotensin-converting enzyme 2 (ACE2). This is an essential requirement for infection by facilitating binding. The S2 subunit consists of a fusion peptide (FP), heptad repeat 1 (HR1), central helix (CH), connecting domain (CD), heptad repeat 2 (HR2), transmembrane domain (TM) and cytoplasmic tail (CT). The S2 subunit is responsible for fusion of the virus with the host and conformational changes. The boundary between the S1 and S2 subunits is called the S1/S2 protease cleavage site(Wang et al. 2020b).

2.4.1.1. The receptor binding domain (RBD)

RBD is located the S1 subunit of the spike protein of SARS-CoV-2 and specifically recognizes ACE2. Therefore, RBD is a suitable target for vaccine development, antiviral therapeutics and antibody production. The RBD conformation consists of two structural domains. These are the core and outer regions. The RBD core consists of five antiparallel β-strands connected by loops and short helices, as well as a disulfide bond between two β-strands. Between the β4 and β7 strands in the nucleus is the RBM (receptor binding motif), which contains the α4 and α5 helices. The RBM contains the binding site to the N-terminal domain of ACE2 and has a concave conformation, allowing the RBD to bind to ACE2 (Lan et al. 2020).

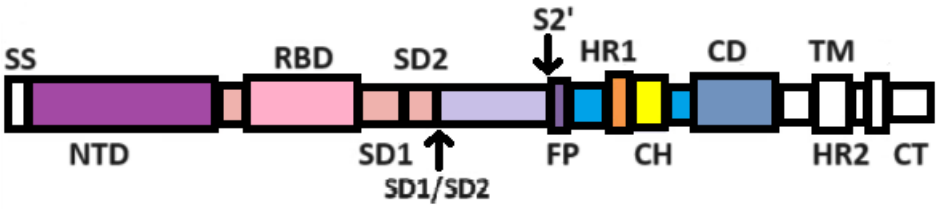


Figure 2.3. SARS-CoV-2's spike protein domains are single sequence (SS), N-terminal domain (NTD), Receptor Binding Domain (RBD), SD1 subdomain 1 (ssSD1), SD2 subdomain 2 (ssSD2), S1/S2 boundary, S1/S2 protease cleavage site, S2' and S2' protease cleavage site, Heptad repeat 1 (HR1), central helix (CH), connector domain (CD), heptad repeat 2 (HR2), transmembrane domain (TM) and cytoplasmic tail (CT). Meaning of arrow is the protease cleavage site

The human ACE2 gene is a 40 bp gene containing 18 exons located on chromosome Xp22 and encodes an endothelium-dependent carboxypeptidase called ACE2, which can be cleaved by different metalloproteases found on the cell surface and released in a soluble form. The SARS-CoV-2 spike (S) protein uses the human ACE2 receptor to enter target cells. Therefore, the interaction between SARS-CoV-2-S and ACE2 is decisive for the fusion, transmission and pathogenicity of the virus.

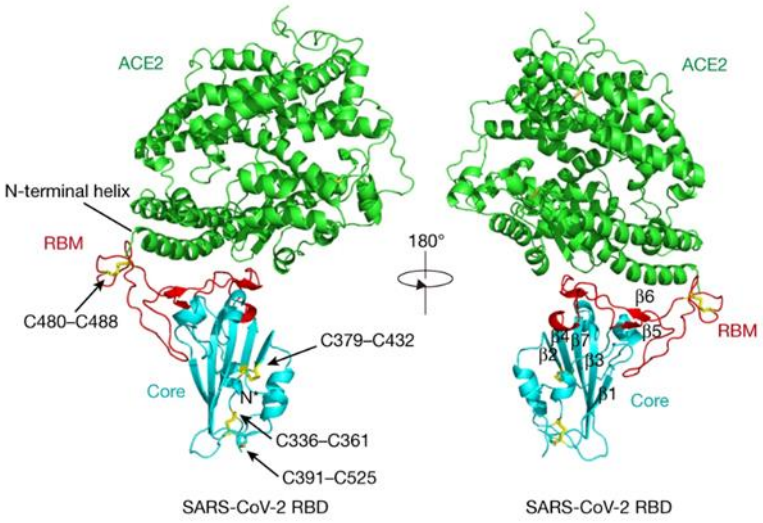


Figure 2.4. Total structure of the SARS-CoV-2 RBD mediated by ACE2; ACE2 is presented in green, the SARS-CoV-2 RBD core is presented in blue and the RBM is presented in red. Disulfide bonds in the SARS-CoV-2 RBD are displayed as sticks and indicated by arrows (Lan et al. 2020)

ACE2 has a homodimer structure and consists of two domains: PD (catalytic protease domain) as the substrate binding region and CLD (collectrin-like domain), which contains the transmembrane sequence and the cytoplasmic sequence (Gross et al. 2020).

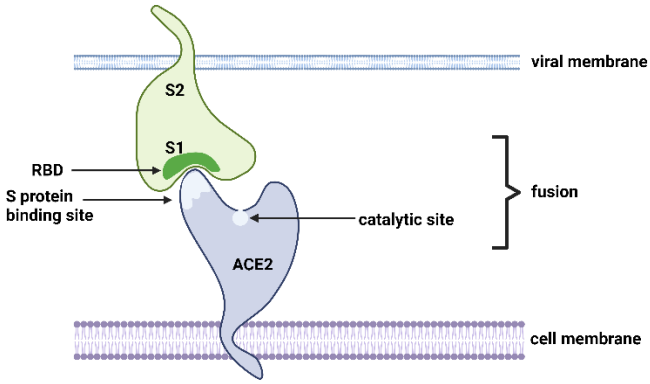


Figure 2.5. Binding and fusion interaction between spike protein of SARS-CoV-2 and ACE2; Cavities of both ACE2 and S protein shows compatibility, and it results in binding activity

2.4.2. Nucleocapsid protein (N)

The nucleocapsid protein is a 46 kDa protein that has an N-terminus (NTD) and a C-terminus (CTD) and is conserved across coronaviruses (Carlson et al. 2020).

The 5 antiparallel β-sheets in the NTD region are rich in the amino acids serine and arginine and therefore have basic properties. The β-sheets enter a stable state and

form a hairpin. The hairpin structure serves as a binding site for positively charged RNA (Chang et al. 2014).

This serine and arginine-rich protein is phosphorylated early in infection, leading to overexpression of proinflammatory factors. In studies in mice, overexpression of these factors has been shown to increase lung injury in the host (Pan et al. 2021). The nucleocapsid protein has two functions: transcription regulation and RNA compaction. The negatively charged nucleocapsid proteins tightly bind and package the positively charged genome. Due to its biochemical properties, it can be a precursor protein in vaccine development and antibody production research (Di et al. 2021).

2.4.3. Envelope protein (E)

Envelope protein is structurally the smallest protein in SARS-CVv-2's structural proteins. Its size is between 8.4 and 12 kDa. When the virus enters the host, producing of envelope protein increases. Its main function is to interfere with the C-terminal domain of protein E, the endoplasmic reticulum, the golgi intermediate region, and the ER-Golgi by creating a channel with a transmembrane domain that directly affects viral replication and thus affects the host's intracellular environment activity (Mandala et al. 2020).

2.4.4. Membrane protein (M)

The membrane protein is glycoprotein and 25.06 kDa in a size. It is also called glycoprotein E1 or matrix protein. The high GC content (40%) determines the content of hydrophilic amino acids, which distinguishes it from other structural proteins (Hu et al. 2003).

During SARS-CoV-2 infection, the membrane and envelope proteins localize to a region called ERGIC, which is situated between the endoplasmic reticulum and the golgi apparatus of the host cell. This localization allows for the expression of viral proteins in the host cell (Zhang et al. 2022).

The membrane protein can bind to all other structural proteins and helps them in glycation and functionalization (Thomas 2020).

2.5. Vaccines Against SARS-CoV-2

SARS-CoV-2 has spread rapidly since the first case was reported. Due to its rapid spread and high pathogenicity, many scientists focused on developing vaccines and treatments. Vaccination efforts accelerated after the WHO declared a global pandemic and the first vaccines were produced.

Traditional and reliable vaccines take many years to develop; however, due to exceptional circumstances, the first vaccines were not released until three months after the pandemic was declared. Vaccines can be produced on different platforms. Although

their reliability and effectiveness vary, it is essential that they are also effective against new variants.

2.5.1. Vaccine technologies

The human immune system has several lines of defence against infections. These can be chemical and physical such as body fluids, or biological in, such as immune cells. The immune system includes sensors that detect the presence of pathogens, cells that neutralize pathogens, and adaptive immune cells that protect against subsequent infections.

Vaccines activate the immune system by exposing the pathogen itself or part of it. Conventional vaccines contain weakened or inactivated pathogens. Next-generation vaccine technologies include nucleic acid vaccines, recombinant protein vaccines, and nanoparticle vaccines.

Table 2.1. Vaccine efficacy rates of SARS-CoV-2 vaccines (Ciotti et al. 2022)

Platform	Company	Efficacy (%)
Protein subunit	Novavax	60–89
	GSK-Medicago	Unknown
	Anhui Zhifeilongkoma Biopharmaceutical/Chinese Academy of Sciences	?
Viral vector	AstraZeneca	62–90a
	Gameleya	91.6
	Johnson & Johnson	66–85.4
	CanSinoBio	?
DNA	Anges	?
	Inovio	?
Inactivated viruses	Sinopharm	79
		73 (WIV04)
		78 (HB02)
	Chinese Academy Medical Science	?
	Sinovac	50
	Bharat Biotech	
mRNA	Pfizer/BioNTech	95
	Moderna	94
	CureVac	47

2.6. Recombinant Protein Production

Recombinant therapeutic proteins are exogenous proteins produced by various organisms and used in the diagnosis and treatment of various diseases in humans and animals. These proteins can be obtained from mammalian cell lines, insect cell lines, plants, yeast or bacterial cultures. The presence of post-translational modifications leads to difficulties in the production of therapeutic agents. Therefore, the use of organisms capable of carrying out PTM in production provides advantages in this area (Andersen and Krummen 2002).

2.6.1. Plant expression systems

Plant expression systems are widely used in recombinant protein production due to their advantages such as cost, lower risk of contamination, easy production of complex proteins and fast life cycle. Plant expression systems are divided into two: transient and stable.

Persistent plant expression systems involve manipulating the host's genome and continuously producing desired proteins using genetic engineering tools. Transient plant expression systems, on the other hand, enable gene transfer by manipulating natural pathogens such as viruses or Agrobacterium and involve transient expression of desired proteins. Transient expression systems are more often preferred due to their speed and cost-effectiveness in producing therapeutic agents.

Agrobacterium spp. from the Rhizobiaceae family are the bacteria that cause plant infections and various diseases, especially crown tumours. After molecular biology studies, it was shown that these bacteria are natural genetic engineers. Various species, especially *Agrobacterium tumefaciens*, have been used in genetic studies for over 20 years. The *Agrobacterium* Ti plasmid is transmitted during infection. The T-DNA is transferred part of the Ti plasmid to the host. Beside the T-DNA, it also contains parts such as the virulence region and the opine catabolism region. The virulence region is the region required for T-DNA transmission, and oncogenes for the synthesis of tumour-forming metabolites such as opines are also found outside the T-DNA. Manipulation of T-DNA has paved the way for foreign gene expression systems in plants. If genetically modified Ti plasmid, which does not contain the oncogene, is transferred to the plant, transformation occurs, but no carcinogenesis will observe. Genetic transformation studies are carried out on many plants, especially model organisms such as *Arabidopsis thaliana* (Hellens et al. 2000).

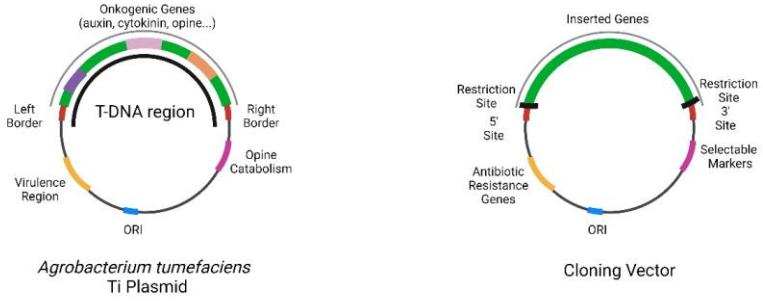


Figure 2.6. Natural Ti plasmid and cloning vector

In transient expression systems, the delivered DNA cassette is transiently expressed and integrated into the DNA of a very small number of cells (Nester 2015).

Agrobacterium tumefaciens is a soil-borne, gram-negative bacteria. A. tumefaciens' genes that expressing eukaryotic signals are found in the T-DNA and it leads to high specificity between host and pathogen (Tzfira, Hohn, and Gelvin 2017).

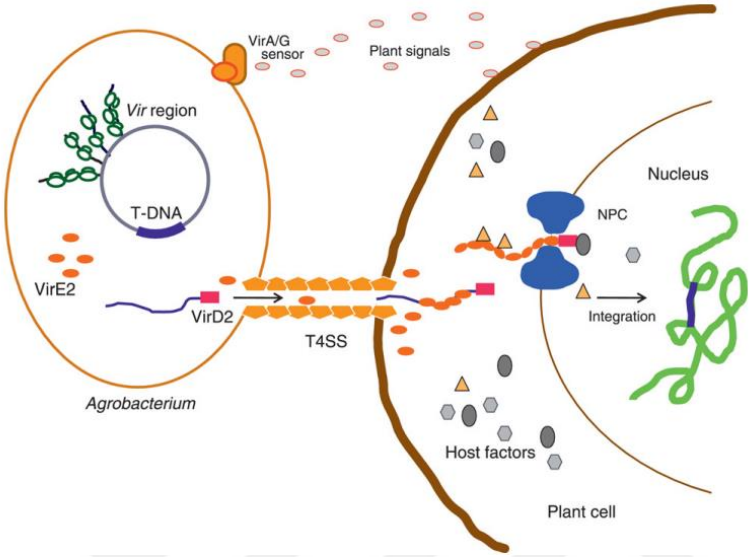


Figure 2.7. *Agrobacterium* infection mechanism (Tzfira et al. 2017)

Agrobacterium-mediated transformation can be performed by chemical or physical methods. While chemical methods such as electroporation method works successfully in animal cells, it can only be successful in protoplast cultures in plant systems. The ability of the protoplast to develop into a healthy plant is varies from species to species and it is the limiting factor of transformation (Yuan et al. 2012).

Physical transformation methods are biolistics (particle bombardment), vacuum infiltration (or syringe infiltration), ultrasound-mediated transformation, microinjection, and other methods. It may vary depending on the purpose of the experiment and the material type (Rivera et al. 2012).

2.6.2. Agroinfiltration

The agroinfiltration method is a rapid, convenient, inexpensive and effective plant transformation method. This method has been and is used in many experiments, such as understanding host-pathogen interactions, protein production and localization. It has been successfully applied to many plants such as *Nicotiana* spp. (*N. benthamiana* and *N. tabacum*) tomato, lettuce, *Arabidopsis thaliana*, pea and so on.

The short life cycle of *Nicotiana* spp. and its broad leaves make them suitable plants for agricultural infiltration (Ma et al. 2012).

Agroinfiltration is widely used in recombinant therapeutic protein production, especially because it is important to produce proteins with high purity and high efficiency in a short period of time. Since contamination with human pathogens can occur in animal systems and fermentation systems, the use of higher plants for therapeutic protein production, especially vaccine production, is advantageous (Circelli et al. 2010).

The eukaryotic structure of plants offers advantages to produce recombinant therapeutic proteins, including features such as folding, production of large proteins as one piece, and application of post-translational mechanisms. It is possible to produce the protein in high quantity and purity if the cassette to be delivered contains a suitable promoter for propagation in the host plant, a protein designed with a tag suitable for purification and signals suitable for production in the host (Ma et al. 2003).

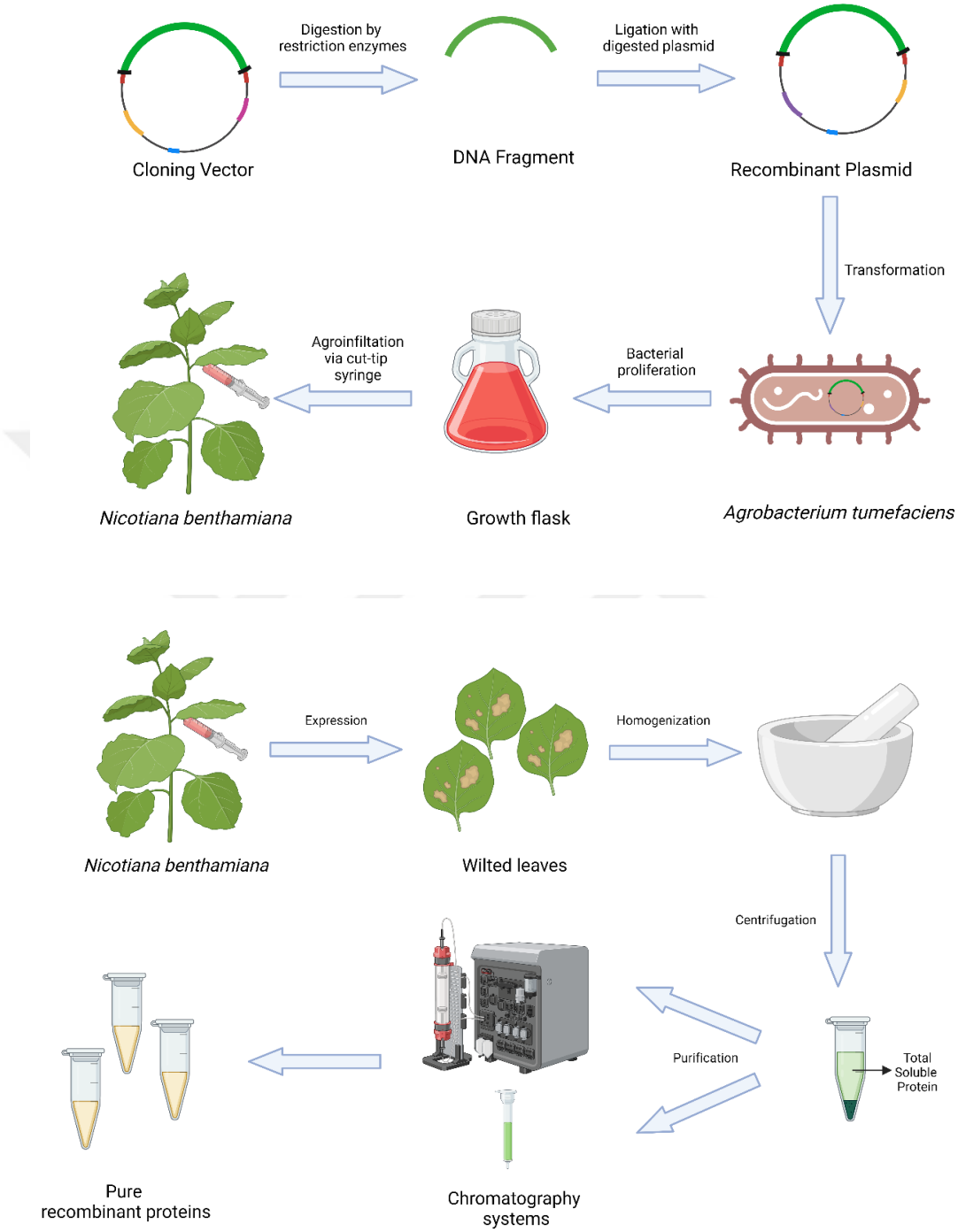


Figure 2.8. Recombinant protein production via agroinfiltration

3. MATERIAL AND METHOD

3.1. Plant Material

Nicotiana benthamiana is used as plant material in the transient expression system. *N. benthamiana* is cultivated in a controlled growth room under the following conditions: 25°C temperature, 16/8 hour light/dark photoperiod, and 50% relative humidity. After the seeds are planted, they are kept covered with aluminium foil for three days. Three days later, fertilizer and water are provided daily.

Figure 3. 1. *N. benthamiana* plants in controlled growth room

Figure 3.2. Suitable *N. benthamiana* plant size for agroinfiltration

3.2. Vectors

Escherichia coli strain DH5α and *Agrobacterium tumefaciens* strains AGL1 and EHA105 are used in the experiments.

3.3. Preparation of Solutions Used in Experiments

3.3.1. Antibiotics and phenolics

- Kanamycin

Kanamycin is prepared as 50 mg/ml. 0.5 g of Kanamycin (Sigma-Aldrich, Cat. No. BP861) is weighed and dissolved in 10 ml ddH_2O. It is filtered through Millex-HV syringe 0.45 µm filter system (Millipore, Cat. No. SLHV004SL) and aliquoted into 250 µl tubes. It is stored at -20°C.

- Acetosyringone

A 100 mM solution of Acetosyringone is prepared 100 mM as 5 ml. 0.098 g of 3′,5′-Dimethoxy-4′-hydroxyacetophenone (97%, Acros Organics, Cat. No. 115540050) is weighed and dissolved in 3 ml ethanol and 2 ml ddH_2O. The solution is filtered through a Millex-HV syringe 0.45 µm filter system (Millipore, Cat. No. SLHV004SL) and aliquoted into 250 µl tubes. The solution is stored at -20°C.

3.3.2. Buffers and solutions

- 10x TAE Buffer

14.61 g EDTA (Merck, Cat. No. 1.00944.1000) and 7 g NaOH are weighed, to prepare 0.5 M EDTA (pH: 8). The mixture dissolved in 100 ml ddH_2O and adjusted to pH: 8.00.

48.5 g Tris (RPI, Cat. No. TB0040) is weighed, 20 ml 0.5 M EDTA (pH: 8) and 11.4 ml acetic acid are added, and the volume is completed to 1 L. The pH is adjusted to 8.00 with NaOH.

- 10% SDS

20 g of SDS (Merck, Cat. No. 8.17034.1000) is weighed and dissolved in 180ml ddH_2O.

- Extraction Buffer

3 ml 1x PBS and 6 µl DIECA are mixed for each gram of infiltrated leaf.

- Running Buffer for Western Blot

3.03 g Tris (RPI, Cat. No. TB0040) and 14.3 g Glycine (Sigma Aldrich, G8898-1KG) are weighed and mixed with 10 ml of 10% SDS. The volume is completed to 1 L with ddH_2O.

- Transfer Buffer for Western Blot

5.8 g Tris (RPI, Cat. No. TB0040) and 2.93 g Glycine (Sigma Aldrich, G8898-1KG) are weighed, mixed with 370 µl 10% SDS and the volume is completed to 1 L with ddH_2O.

- 5x Laemmli Buffer

3.333 g of SDS is weighed. 9.375 ml of 1M Tris, 11.9 ml of glycerol, 660 µl of Bromophenol blue (0.01%), and 7.5 ml of β-mercaptoethanol are mixed with the SDS. The pH is adjusted to 6.8 with HCl. The Buffer is aliquoted and stored at -20 ºC.

- 1x PBS

1x PBS tablet (Fisher Scientific, Cat. No. BP2944100) is dissolved into 200 ml of ddH_2O.

- 5x TBS

12.115 g of Tris and 43.88 g of NaCl (Fisher Scientific, Cat. No. BP358-1) are weighed and dissolved in 800 ml of ddH_2O. The pH is adjusted to the 7.5 with HCl. The volume is completed to 1 L with ddH_2O.

- 1.5 M Tris-HCl (pH: 8.8)

181.65 g of Tris (RPI, Cat. No. TB0040) is weighed, and the volume is completed to 1 L with ddH_2O. The pH was adjusted to 8.8 with HCl.

- 0.5 M Tris-HCl (pH:6.8)

60.5 g of Tris (RPI, Cat. No. TB0040) is weighed, and the volume is completed to 1 L with ddH_2O. The pH is adjusted to 6.8 with HCl.

- Column Equilibration Buffer for Affinity Chromatography

2.922 g of NaCl (Fisher Scientific, Cat. No. BP358-1) is weighed and dissolved in 100 ml of 1x PBS. The pH is adjusted to 7.5.

- High Salt Wash Buffer for Affinity Chromatography

0.6057 g of Tris (RPI, Cat. No. TB0040) (50 mM in solution) and 11.688 g of NaCl (Fisher Scientific, Cat. No. BP358-1) (2M in solution) are weighed. 80 ml of ddH_2O is added. The pH is adjusted to 8.0 and the volume is completed to 100 ml with ddH_2O.

- Elution Buffer for Affinity Chromatography

0.75066 g of Glycine (Sigma Aldrich, Cat. No. G8898-1-KG) (200mM in solution) and 0.4388 g of NaCl (Fisher Scientific, Cat. No. BP358-1) (150mM in solution) are weighed, and 40 ml of ddH_2O is added. The pH is adjusted to 2.2 and the volume is made up to 50 ml.

- Neutralization Buffer for Affinity Chromatography

12.114 g of Tris (RPI, Cat. No. TB0040) (2M in solution) is weighed. 40 ml of ddH_2O is added and the pH is adjusted to 8.0. The volume is made up to 50 ml.

- 40% Acrylamide/Bis Solution

38.96 g of acrylamide is weighed and dissolved in 30 ml of autoclaved ddH_2O. 1.04 g of bis-acrylamide is weighed and mixed with the solution. The volume is completed to 100 ml with ddH_2O after completely dissolved. The acrylamide/bis-acrylamide solution is kept in the dark at +4ºC.

- 10% APS (Ammonium Per Sulphate)

APS (Sigma Aldrich, Cat. No. 248614-500G) is weighed that the ratio of solute weight to total volume is 10%. The volume is made up with ddH_2O.

- DIECA (Sodium Diethyldithiocarbamate)

DIECA (Sigma Aldrich, Cat. No. 228680-500G) is weighed to 225.31 g and dissolved in 1L of ddH_2O.

- Blotting Solution (I-Block)

1 g of blotting grade powder (Bio-Rad, Cat. No. 1706404) is dissolved in 100 ml of 1X TBS, including 100 µl of Tween-20.

- Coating Buffer for ELISA

0.15897 g of Na_2CO_3 and 0.29402 g of $NaHCO_3$ are weighed and dissolved in 100 ml of ddH_2O.

- Washing Buffer for ELISA

200 ml of 1X PBS and 0.2 ml of Tween-20 are mixed.

- OPD-Substrate Solution for ELISA

2.8392 g of Na_2HPO_4 is weighed and dissolved in 100 ml of ddH_2O to make a 0.2 M dibasic sodium phosphate solution.

2.1014 g of citric acid is weighed and dissolved in 100 ml of ddH_2O to make 0.1 M citric acid solution.

25.5 ml of 0.2 M dibasic sodium phosphate solution and 24.3 ml of 0.1 M citric acid solution are mixed, and volume is completed to 100 ml with ddH_2O. The pH is adjusted to 5.0.

12 ml of H_2O_2 and 28 ml of ddH_2O mixed to prepare 30% hydrogen peroxide solution.

Before use, 40 µl of 30% hydrogen peroxide solution is added to each 100 ml of 0.05 M phosphate-citrate solution.

OPD free base tablet (Sigma Chemical Company, Cat. No. SL3P6518V) is dissolved in 25 ml of ddH_2O, and mixed with 10 ml of 30% hydrogen peroxide solution to prepare substrate solution.

- Staining Buffer

1 g Coomassie Brillant Blue is weighed and mixed with 100 ml of acetic acid, 500 ml of methanol and 400 ml of ddH_2O.

- Destaining Buffer

200 ml of methanol, 100 ml of acetic acid and 700 ml of ddH_2O are mixed.

3.3.3. Mediums

- LB Broth

25 g of LB Broth is weighed, and total volume is completed to 1 L with ddH_2O. The Medium is autoclaved at 121 ºC for 30 minutes.

- LB Agar (Miller)

37 g of LB Agar (Thermo Fisher Scientific, Cat. No. BP9724-500) is weighed and total volume is completed to 1 L with ddH_2O. The medium is autoclaved at 121 ºC for 30 minutes. After autoclaving, medium is cooled to 50 ºC in water bath. When temperature is drops below 50 ºC, 1 ml of Kanamycin (50 mg/ml) is added to medium. 25 ml of medium is poured into per petri dishes.

- SYS (BBL)

10 g of soy hydrolysate, 5 g of yeast extract (Sigma Aldrich, Cat. No. Y1625-1KG) and 5 of NaCl (Thermo Fisher Scientific, Cat. No. BP358-1) are weighed. The volume of SYS medium is completed to 1 L with ddH_2O. The pH of the medium is adjusted to 7.00 with 1 M KOH solution. The medium is autoclaved at 121 ºC for 30 minutes.

- MMA

1.952 g of MES (Sigma Aldrich, Cat. No. M3671-250G) and 0.95 g of $MgCl_2.6H_2O$ (Tekkim, Cat. No. TK.120290.01002) are weighed. pH of the medium is adjusted to 5.8 with 1 M NaOH solution. The medium is autoclaved at 121 ºC for 30 minutes.

- SOC

1 g of tryptone, 0.25 g of yeast extract (Sigma Aldrich, Cat. No. Y1625-1KG), 0.0029 NaCl (Thermo Fisher Scientific, Cat. No. BP358-1), 0.009 g of KCl, 0.101 g $MgCl_2.6H_2O$ (Tekkim, Cat. No. TK.120290.01002) and 0.123 g of $MgSO_4$ are weighed. The total volume is completed to 50 ml with ddH_2O. The medium is autoclaved at 121 ºC for 30 min. 0.180 g of glucose is added to medium after autoclaving. The medium is sterilised with Millex-HV syringe 0.45 μm filter system (Millipore, Cat. No. SLHV004SL).

3.3.4. Gels

- Polyacrylamide Gel

Polyacrylamide gel is made up of two gels: resolving gel and stacking gel. Gels with density of 12% is used in experiments. Resolving gel is prepared and poured between glass plates and completed with propanol. After the gel is solidified, the propanol is evaporated, and the stacking gel is poured. The comb is placed between the glass plates.

Table 3.1. Ingredients of 12% resolving and stacking gels for 10 well-comb (1.5 mm, 66 µl)

INGREDIENTS	12% GEL
Autoclaved ddH_2O	2.175 ml
40%Acrylamide-Bisacrylamide Solution	1.5 ml
1,5 M Tris-HCl (pH: 8.8)	1.25 ml
10% SDS Solution	50 µl
TEMED	2.5 µl
10% APS	25 µl

INGREDIENTS	12% GEL
Autoclaved ddH_2O	1.917 ml
40% Acrylamide-Bisacrylamide Solution	374.4 µl
1,5 M Tris-HCl (pH: 6.8)	787 µl
10% SDS Solution	31.25 µl
TEMED	3.125 µl
10% APS	15.62 µl

- Agarose Gel (1% m/V)

1 g of agarose (Sigma Aldrich, Cat. No. A9539-100G) is weighed and the total volume is completed to 100 ml with TAE buffer. The solution is boiled in microwave oven for minute. After the temperature is dropped below 50 °C, 1.2 µl of EtBr is added. The solution is poured into gel tray and comb is placed.

3.4. Transformation

Prof. Dr. Tarlan MAMMEDOV has designed the vector for the co-expression of the SARS-CoV-2 spike protein receptor binding domain (RBD) gene and nucleocapsid

protein (N) gene with codon optimization and the addition of the FLAG epitope. The vector is commercially purchased for use in *N. benthamiana* plant.

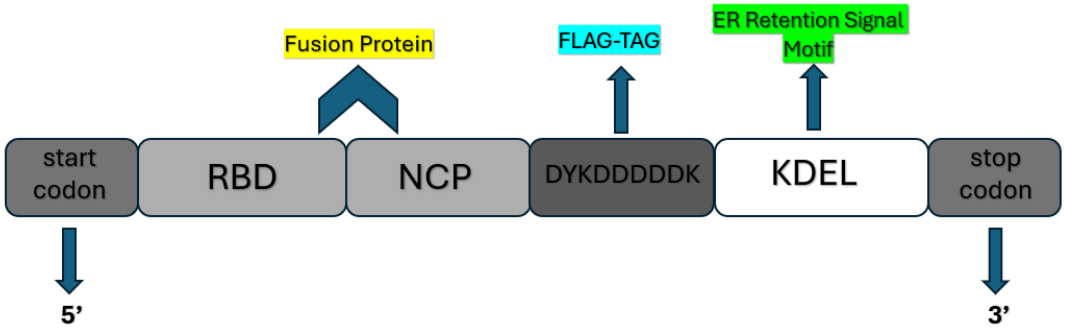


Figure 3.3. Transformation cassette designed by Prof. Dr. Tarlan MAMMEDOV

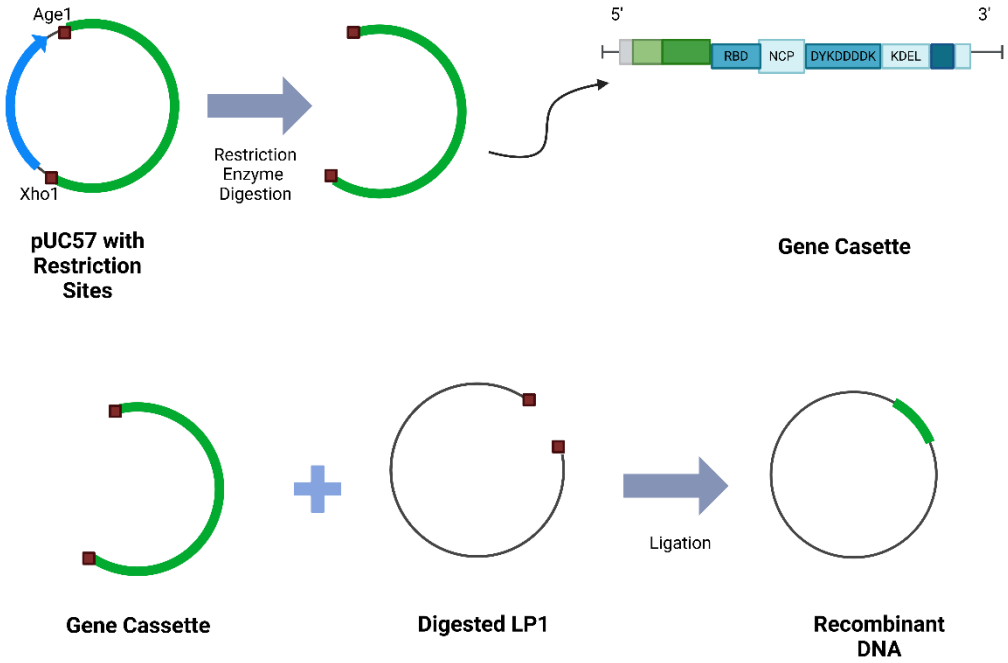


Figure 3.4. Molecular cloning workflow

The vector is provided in commercial pUC57 plasmid. The plasmid is restriction digested using Age1 (New England Biolabs, Cat. No. R0552L) and Xho1 (New England Biolabs, Cat. No. R0146S). A mixture of 30 µl plasmid, 3 µl Age1 and 3 µl Xho1, 6 µl

1:1 buffer and 8 µl nuclease free ddH_2O are prepared in tube. The tube is incubated in 37 °C for 1 hour.

Agarose gel electrophoresis is performed to confirm the digestion reaction. After confirmation, another agarose gel electrophoresis is performed for gel recovery assay. Gel recovery assay is carried out using the Monarch® DNA Gel Extraction Kit (New England Biolabs) according to kits manual. Agarose gel electrophoresis is performed to confirm gene recovery.

Ligation reaction is set up with using 3 µl of LP1 plasmid, 6 µl of gene insert, 2 µl of nuclease free ddH_2O , 10 µl of 2x Ligation Buffer and 1µl of Ligase. The reaction tube is incubated at the room temperature for 20 minutes.

Plasmid transformation into *Escherichia coli* is performed using heat-shock method, where cells are exposed to sudden temperature changes. Competent *E. coli* cells are incubated on ice for 30 minutes, then mixed with ligated DNA containing the gene. Later than, mixture is incubated 42 °C for 50 seconds and after that incubated in ice for 5 min. After the incubation, 400 µl of LB Broth is added and incubated at 37 °C for 1 hour with shaking at 225 rpm in orbital shaking incubator. Mixture is concentrated by centrifuge in 16000xg, 2 min. Pellet is dissolved with 100µl supernatant and spread LB Agar + Kan50 plate. Plate is incubated 37 °C for overnight. On the following day, colonies are selected and sub-cultured in LB Broth. Culture is incubated 37 °C for overnight in the orbital shaking incubator. The next day concentration is measured by UV/Vis Spectrophotometer. Plasmid purification is performed using Monarch® Plasmid Miniprep Kit. Digestion reaction is set up after concentration is enough with using 5µl plasmid, 5µl master mix (4.5 µl Agel, 4.5 µl Xhol, 9 µl 1:1 Buffer and 27 µl ddH_2O) and incubated 37 °C for 1 hour. Electrophoresis is performed to confirm the reaction.

Transformation of plasmid into *Agrobacterium tumefaciens* is carried out using the electroporation method. All procedures are performed on ice. 100 µl of competent *A. Tumefaciens* cells are mixed with 3 µl plasmid and placed into an electroporation cuvette. The cuvettes are subjected to high and pulsating electric fields by using the Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories). 1 ml of SOC medium is added each cuvette and pipetted into Eppendorf tubes. The tubes are incubated in 28 °C for 2 hours while shaking. After incubation, the samples are concentrated by centrifugation at 5000 x g for 25 minutes. The pellet is resuspended in 100 µl of supernatant and spread onto LB Agar + Kan50 plate. The plate is incubated 28 °C for 3 days. After 3 days, colonies are selected and sub-cultured in BBL + Kan50 medium. The culture is incubated at 28 °C for overnight with shaking at 225 rpm.

3.5. Transient Expression of RBD+N Protein in *N. benthamiana* by Infiltration

The optical density is measured using spectrophotometer, after incubation. *A. tumefaciens* cells are precipitated by centrifugation at 5000 x g for 5 minutes in 4 °C. The pellets are resuspended in MMA medium. The MMA medium is prepared according to OD value* amount of precipitated cells. 15 µl of AS is added per 100 ml of MES medium. The dissolved cells are incubated at room temperature for 2 hours with shaking. After incubation, the medium is infiltrated into mature *N. benthamiana* plants via syringe with cut-tip.

Figure 3.5. Agroinfiltration to *N. benthamiana* plants

3.6. Expression Analysis with Western Blot

Infiltrated leaves are collected 5 dpi. Expression analysis is performed with western blotting. The leaves are measured, then ground with extraction buffer in sterile marble mortar. The ground leaves are centrifuged at 20000 x g for 5 minutes in 4 °C. The supernatant of sample, which is referred as total soluble protein, is collected into clean a tube. 5x Laemmli buffer is added in 1:4 ratio to TSP and boiled at 100 °C for 5 minutes.

A Polyacrylamide gel is placed in electrophoresis tank, and running buffer is added. Samples are loaded into the wells of polyacrylamide gel. The gel is run at 100 V for 15 minutes and then at 200 V for 45 minutes. The transfer sandwich is assembled with polyacrylamide gel and polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA) between methanol-activated filter papers in a transfer cassette. The transfer cassette is placed into the tank, and transfer buffer is added. The transfer cassette is run at 100 V for 1 hour. The transferred membrane is blocked with blocking solution while shaking in room temperature for 1 hour. 10 of µl of primer antibody (Anti-DYKDDDDK Tag, Biolegend, Cat. No. 637301) and 10 ml of blocking solution is mixed. The membrane is blocked in solution with shaking in room temperature for 1 hour. The membrane is washed with blocking solution 3 times for 5 minutes each. 2,5 µl of seconder antibody (Anti-Rat, Biolegend, Cat. No. 405405) is mixed with 10 ml of blocking solution The membrane is incubated in solution with shaking in room temperature for 1 hour. Membrane is washed with blocking solution 2 times for 5 minutes and 1 time with 1x TBS for 5 minutes. Membrane is incubated with 2.5 ml of Enhancer and 2.5 ml of

peroxidase mixture (SuperSignal™ West Pico PLUS Chemiluminescent Substrates, Thermo-Fisher Scientific, Cat. No. 34578). Western Blot images is scanned by GeneSys Software (Syngene Bioimaging, UK) and band intensities were measured by GeneTools Software (Syngene Bioimaging, UK).

Figure 3.6. Extraction of proteins from infiltrated leaves

3.7. Purification of RBD+N fusion proteins from *N. benthamiana* Leaves by Affinity Chromatography

Plant-produced recombinant proteins are precipitated and purified by using affinity chromatography with anti-DYKDDDDK resin (Anti-DYKDDDDK Tag (L5) Affinity Gel, BioLegend, Cat. No. 651503). The column is set up, and filter is placed into column. The column is washed three times with ddH_2O. 1 ml of anti-DYKDDDDK resin is added to column, followed by 15 ml of binding buffer which is flowed through the column. The total soluble protein (TSP) is passed through the column again and flow-through is collected. Wash buffer is flowed from column and concentration is measured at the same time. Once the concentration is decreased below 0.1 µg/µl, elution buffer is flowed through the column and elute is collected to the clean collection tube. Elutes are mixed and concentrated with concentrators (Pierce™ Protein Concentrators, Thermo-Fisher Scientific, Cat. No. 29924) in centrifuge at 14000 x g. Final concentration is measured.

Figure 3.7. Affinity chromatography system for purification of FLAG tagged proteins

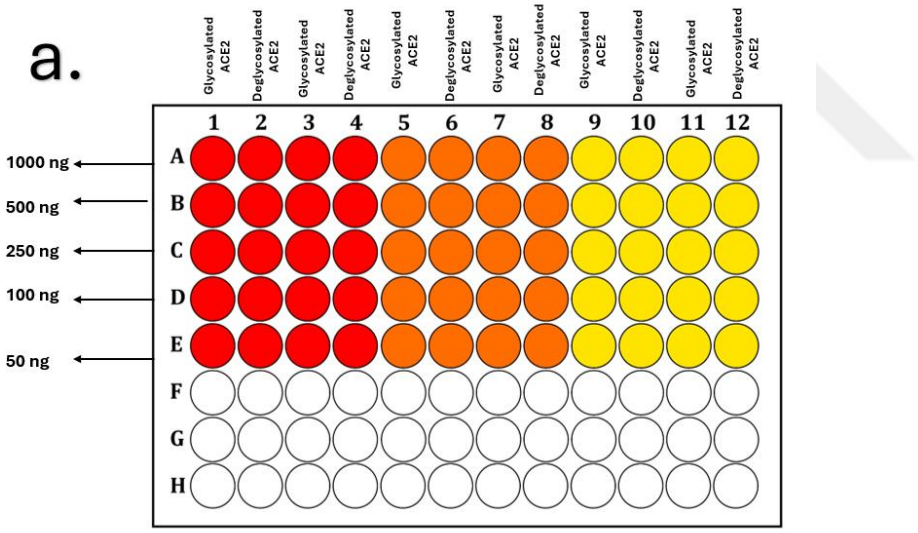
3.8. Stability Analysis

Stability analysis is performed to assess the protein stability of recombinant proteins at body temperature. Samples are prepared in five tubes with 150 ng protein in 15 µl of solution. Tubes are incubated in 37 °C. One tube is collected and placed in a -80 °C refrigerator every day for five days. Western blot analysis is performed to assess stability.

3.9. ELISA of RBD+N Fusion Protein Binding Affinity with ACE2

Enzyme-linked immunosorbent assay (ELISA) is used to quantitively measure the binding activity of ACE2 and recombinant proteins. The ELISA plate is coated with coating buffer and protein mixture (100µl per well). The coated plate is incubated in +4 °C, overnight. The coating buffer is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated at 37 °C for 2 hours. The blocking buffer is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated 5 minutes. The blocking buffer is removed. ACE2 is added as serial dilutions and incubated at 37 °C for 2 hours. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The Washing buffer is

removed. The blocking buffer is added (200 µl per well) and incubated at 5 minutes. The blocking buffer is removed. The primer antibody is prepared as 13 ml blocking buffer and 13 µl anti-ACE2 antibody. Primer antibody is added (100 µl per well) and plate is incubated in room temperature for one hour. The primer antibody is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated at 5 minutes. The blocking buffer is removed. The secondary antibody is prepared by mixing 15 ml blocking buffer, 3.5 µl anti-human antibody (BioLegend, Cat. No.:410902) and 3.5 µl anti-rat antibody (BioLegend, Cat. No.: 405405). The seconder antibody is added (100 µl per well) and plate is incubated in room temperature for one hour. The seconder antibody is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated for 5 minutes. The blocking buffer is removed. 25 ml OPD solution is prepared and added (100 µl per well) and incubated at room temperature in dark for 30 minutes. Chemiluminescence is measured by using UV/Vis Spectrophotometer.



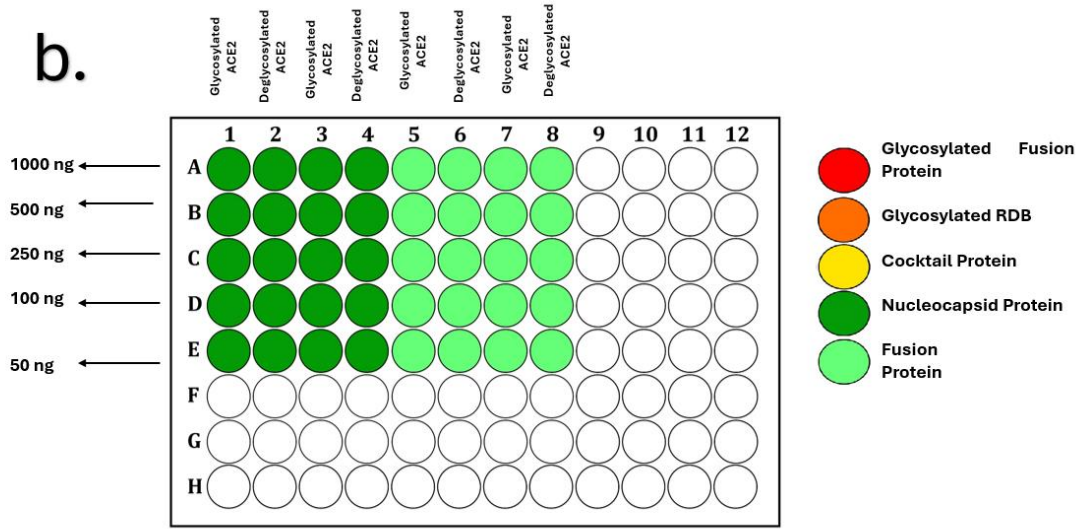


Figure 3.8. a); b) ELISA of protein binding affinity with ACE2

3.10. ELISA of RBD+N Fusion Protein Binding Affinity with Immunized Mice Serum

Enzyme-linked immunosorbent assay (ELISA) is used for immunized mice serums and recombinant proteins binding activity quantitatively. The ELISA plate is coated with coating buffer and protein mixture (100µl per well). The coated plate is incubated in +4 °C, overnight. The coating buffer is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated at 37 °C for 2 hours. The blocking buffer is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated 5 minutes. The blocking buffer is removed. Mice serum is added as serial dilutions and incubated at 37 °C for 2 hours. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated at 5 minutes. The blocking buffer is removed. The secondary antibody is prepared by mixing 10 ml blocking buffer with 3.5 µl anti-mice antibody. The secondary antibody is added (100 µl per well) and plate is incubated in room temperature for one hour. The secondary antibody is removed. The plate is washed three times with wash buffer (200 µl per well) for 5 minutes each time. The washing buffer is removed. The blocking buffer is added (200 µl per well) and incubated at 5 minutes. The blocking buffer is removed. A 25 ml OPD solution is prepared and added (100 µl per well) and incubated in room temperature in dark for 30 minutes. Chemiluminescence is measured by using UV/Vis Spectrophotometer.

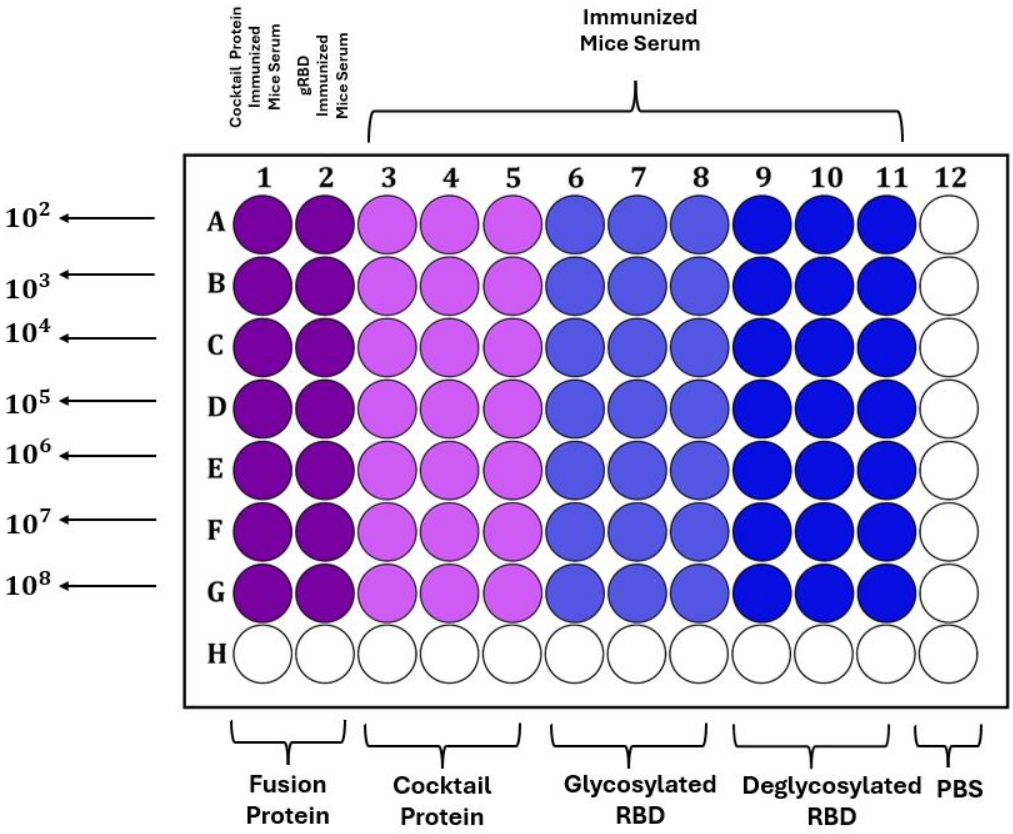


Figure 3.9. ELISA of protein binding affinity with immunized mice serum

3.11. Statistical Analysis

The data obtained in the thesis study were analysed using GraphPad Prism 9.0 software was used. One-way ANOVA was used in statistical evaluation of the findings. The results were evaluated at a significance level of $p<0.05$.

4. FINDINGS AND DISCUSSION

4.1. Bacterial Transformation

The commercial vector was transformed into *E. coli* strain DH5α by heat shock. *E. coli* cells were spread in LB agar medium and incubated (37 °C, overnight). The colonies were selected and incubated in LB broth medium (37 °C, 225 rpm). The cloned vector was isolated with Monarch® Plasmid Miniprep Kit (New England Biolabs, Cat. No. 0041607). The plasmid concentration was measured using a BioDrop µLite+ Microvolume Spectrophotometer. Enzymatic digestion was made to detect the presence of the desired gene, and the reaction products were run on a 1% agarose gel (30 min, 150 V). Image of the gel was taken using a gel imaging system. Digested genes were conformed on a 1% agarose gel and the gene of interest was cut on gel on to UV transilluminator. Recovery from the gel was performed using Monarch® DNA Gel Extraction Kit (New England Biolabs, Cat. No. 10029808). The recovered gene was run on a 1% agarose gel (30 min, 150 V). The gel image was captured and confirmed by using gel imaging system.

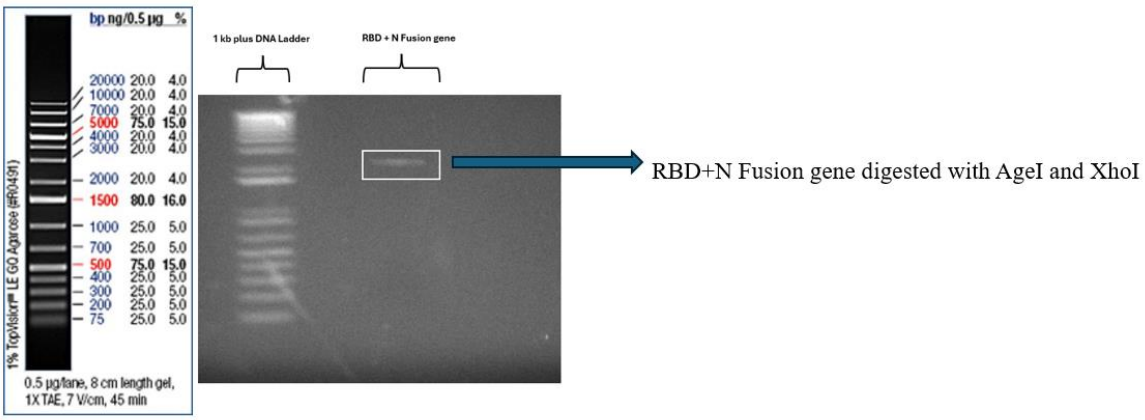


Figure 4.1. Agarose gel image after gene recovery assay

The resulting gene was combined with the vector in our laboratory using a ligation reaction. The new vector was transformed into *E. coli* strain DH5α by heat shock. Transformed *E. coli* cells were incubated in LB agar medium (37 °C, overnight). The colonies were selected and incubated in LB broth medium (37 °C, overnight, 225 rpm). Plasmid purification was performed using Monarch® Plasmid Miniprep Kit (New England Biolabs, Cat. No. 0041607). Plasmid concentration was measured using a BioDrop µLite+ Microvolume Spectrophotometer. Enzymatic digestion reaction was performed to confirm, and the reaction products were run on a 1% agarose gel (30 min, 150 V). Gel imaging system was used to capture an image of the gel.

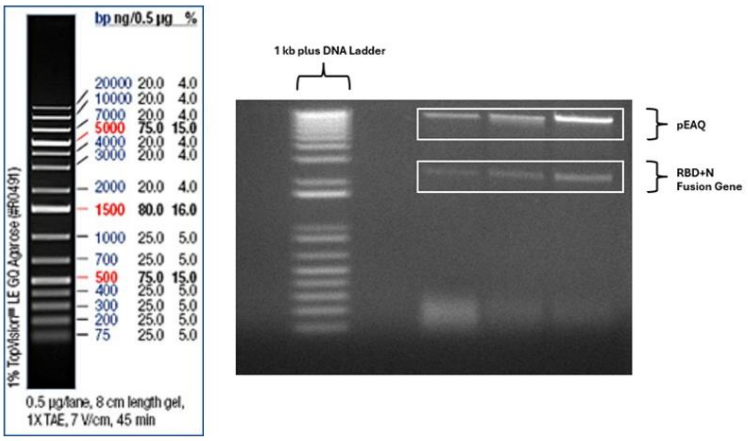


Figure 4.2. Confirmation of ligation reaction by performing enzyme digestion with AgeI and XhoI. To confirm the results, an enzymatic digestion reaction was carried out, and the reaction products were electrophoresed on a 1% agarose gel (30 min, 150 V)

The confirmed plasmid was transformed into *A. tumefaciens* strain AGL1 by electroporation (2500 V, 25 uF, 400Ω).

4.2. Agroinfiltration and Expression in *N. benthamiana*

Transformed cells were spread on LB agar with kanamycin plate and incubated (3 days, 28 °C). The colonies were selected and incubated in BBL medium (overnight, 28 °C, 225 rpm). The optical density (OD) of the medium was measured by using a UV/Vis spectrophotometer. The medium was centrifuged (5 min, 5000 x g). The pellet was dissolved in MES medium with acetosyringone and incubated (RT, 225 rpm, 2 h). The medium was infiltrated into mature *N. benhamiana* leaves via syringe with a cut tip.

4.3. Presence Determination of RBD+N Fusion Proteins by Using Western Blotting

The infiltrated plants were watered daily with fertilizer. To examine how many days it took for maximum expression to occur, leaves were collected on days 4, 5, and 6 after infiltration and grinded with extraction buffer in a mortar. The grinded leaves were centrifuged (20000 x g, 5 min). The supernatant was collected and labelled as total soluble protein. 5x Laemmli buffer was added to the tubes at a ratio of 4:1 and tubed were boiled (100 °C, 5 min).

The samples were loaded onto polyacrylamide gel. Running buffer was added to the system. The gel was run (100 V, 15 min and 200 V, 45 min). The gel was transferred to the membrane. Transfer buffer was added and run (100 V, 60 min). The membrane was blocked with I-block. The membrane was coated with primary antibody (anti-DYKDDDDDK). Membrane was washed with I-block. The membrane was coated with secondary antibody (anti-rat). The membrane was washed with TBS buffer. The membrane was incubated with chemiluminescent substrates in dark (5 min). The image was captured with gel imaging system.

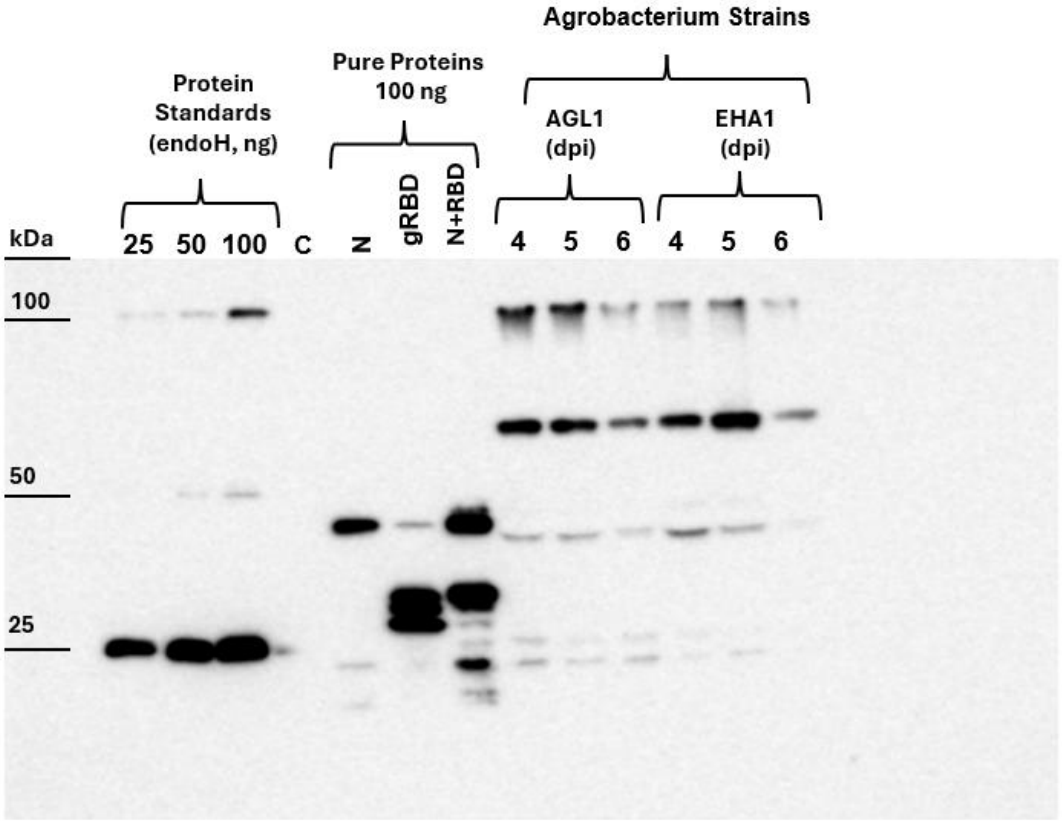


Figure 4.3. Extracted proteins were loaded and run on polyacrylamide gel to determine maximum expression; pure proteins were loaded to compare (N stands for nucleocapsid protein; gRBD stands for glycosylated RBD and N+RBD stands for fusion protein.) For comparison, the EndoH as protein standard was calculated and loaded as 25, 50, and 100 ng. Proteins extracted fr om leaves collected on days 4, 5, and 6 after infiltration (dpi) with *Agrobacterium tumefaciens* strains AGL1 and EHA105 were loaded.

4.4. Affinity Chromatography of Plant Produced RBD+N Fusion Proteins

The infiltrated leaves were weighed and three times their weight of extraction buffer was added. Leaves were homogenized. The homogenate was centrifuged (13000 x g, +4 °C, 15 min). The supernatant was reserved for affinity chromatography. Filter was inserted into the column to set up the affinity chromatography system and washed three times with ddH_2O. Resin was added to the column and waited until the resin and solvent separated from each other (Anti-DYKDDDDK affinity resin mixture, 1:1 resin and solvent). Binding buffer, ten times the volume of resin, was added and flowed through. Samples flowing through the column were collected and passed through the column again. The column was washed with wash buffer containing ten times the volume of resin. Elution buffer was passed through the column and fractions were collected. The concentrations of the fractions

were measured and concentrated. Western blot analysis was performed on samples collected at each stage.

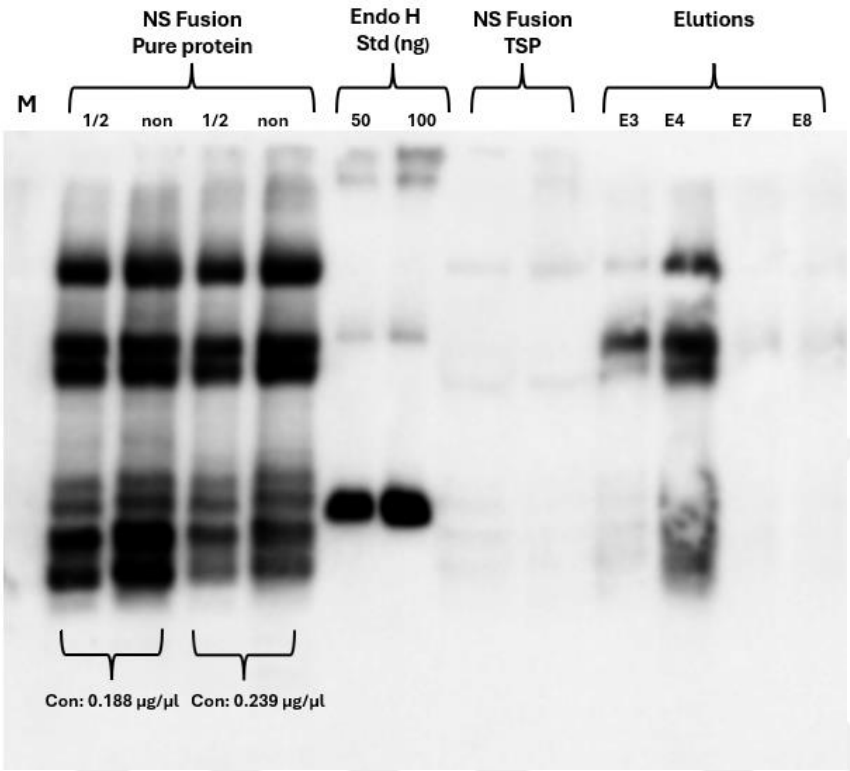


Figure 4.4. Western blotting image of before and after performing affinity chromatography. Pure proteins in dilutions (6.25 µg/µl and 12.5 µg/µl), EndoH protein as control, total soluble protein (TSP) containing the NS fusion protein, and elutions obtained during affinity chromatography were loaded onto a polyacrylamide gel, and a Western blot analysis was performed.

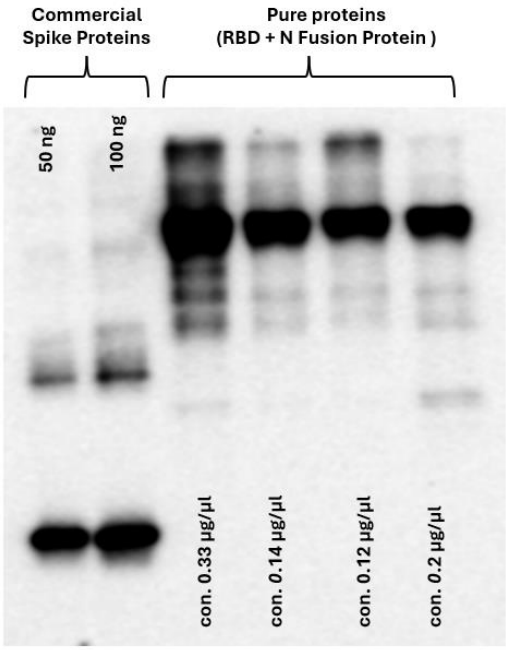


Figure 4.5. Immunoblot assay for detect spike protein; Anti-Spike antibody (SARS-CoV/SARS-CoV-2 Spike RBD Polyclonal antibody, BioLegend, Cat. No. MBS2563840) was used as primer antibody and anti-rabbit antibody was used as seconder antibody

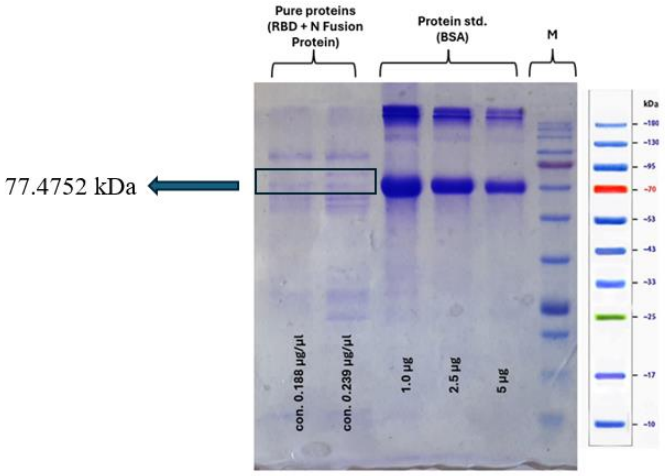


Figure 4.6. SDS-Page image of purified proteins; different concentrations of purified RBD+N fusion protein and BSA protein standards were calculated, loaded onto a polyacrylamide gel, and subjected to SDS-PAGE

4.5. Western Blot Analysis of Stability Assay

Temperature stability is important for the use of purified recombinant proteins as vaccine candidates. Thermostability test was performed to find out how long the protein maintains its stability at human body temperature. The proteins were incubated at 37 °C for 5 days. Every 24 hours, two tubes were placed in a -80 °C refrigerator. Western blot was performed to analyse.

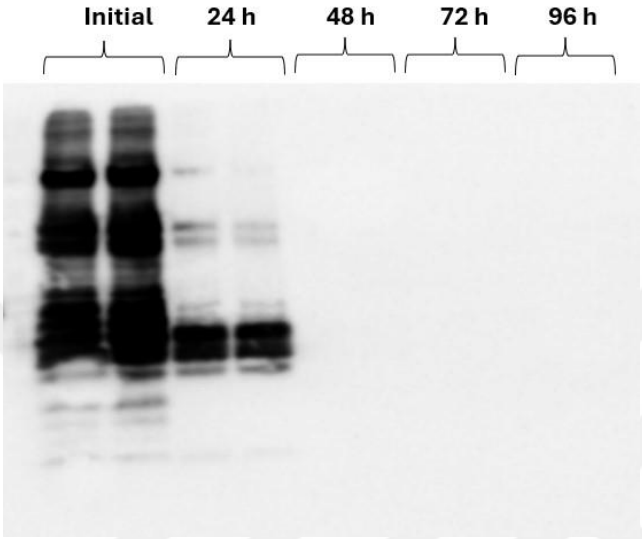


Figure 4.7. Gel image after western blot; stability analysis has been performed after determination of target proteins. Western Blot has been performed instead of SDS PAGE because collected NS fusion protein concentrations were low. Every well loaded as 19.5 µl (15 µl protein + 4.5 µl 5x Laemmli Buffer) and two repeats

4.6. Binding affinity of RBD+N proteins to ACE2

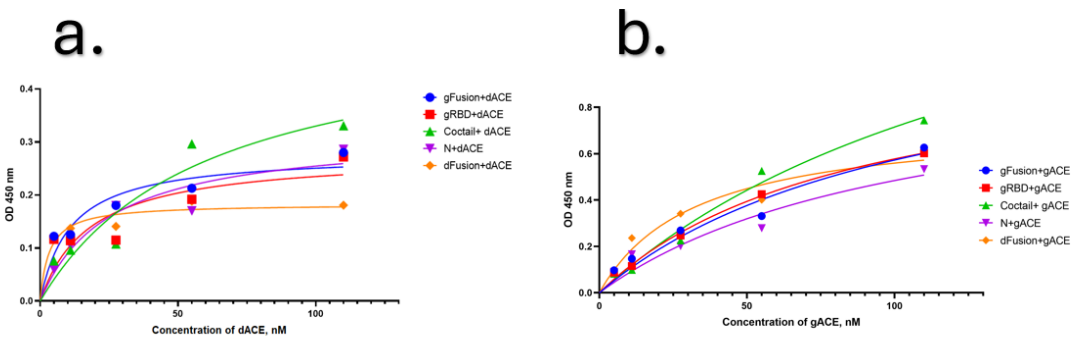


Figure 4.8. Regression analysis. **a)** Regression analysis based on binding affinity of pure proteins to dACE2; **b)** Regression analysis based on binding affinity of pure proteins to gACE2

Kd values of deglycosylated ACE2 Kd values were calculated with GraphPad Prism 9.0 software. Concentrations were place into X, and absorbance values were place into Y axis in XY data table. To analyse Kd value, one site specific binding option were chosen from the saturation binding equations section belong to the nonlinear regression. The higher value of Kd indicates the strong interaction between enzyme and substrate. Fusion protein (marked in green) showed strong binding affinity to both gACE2 and dACE2.

4.7. Binding Affinity of RBD+N Fusion Protein to Immunized Mice Serum

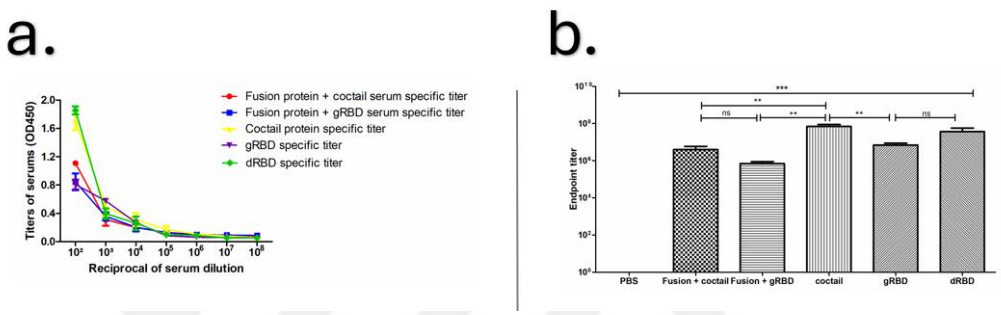


Figure 4.9. Binding affinity of RBD+N fusion protein to immunized mice serum**.** a**)** Regression analysis based on binding affinity of pure proteins to immunized mice serums; b) The endpoint titer of pure proteins and immunized mice serum concentrations from spectrophotometric measurement of ELISA (** $p<0.01$, *** $p<0.001$)

5. CONCLUSION

The SARS-CoV-2 virus is caused a pandemic in Wuhan, China, in March 2020 and it led to thousands of deaths worldwide. When the disease first emerged, hundreds of scientists began researching the virus, the disease it causes, and its treatment. The genetic structure of the virus and the proteins it expresses have studied, and genetic libraries began to create.

SARS-CoV-2 expresses four structural proteins like other SARS-CoVs and MERS-CoV: nucleocapsid (N), spike (S), envelope (E), and membrane (M). Studies have shown that the spike protein plays a role in recognizing the host's ACE2 receptor. The RBD subunit of the spike protein, which plays a role in recognition, has been identified and is known to have an immunogenic effect. It's known about the ACE2 that has the greatest binding affinity to the RBD subunit of SARS-CoV-2's S protein. In our laboratory, recombinant protein production studies from *Nicotiana benthamiana* plants are performed using Agrobacterium-mediated transient expression method performed the production and characterization of plant produced ACE2. The binding affinity and stability of the plant produced glycosylated and deglycosylated ACE2 were compared with commercial glycosylated and deglycosylated ACE2. Results promise rapid and efficient use in recombinant protein (Mamedov et al. 2021). Plant produced glycosylated ACE2 and deglycosylated ACE2 has triggered Barb/c mice immunity. Neutralization assay performed on SARS-CoV-2-infected Vero E6 cells using immunized mice serum. Glycosylated ACE2 and deglycosylated ACE2 has a neutralizing effect on cell lines (Mamedov et al. 2021a).

The human immune system has many mechanisms to fight viral infections, but in cases where the disease has not yet occurred or in cases where immunity is insufficient, illness and death can occur. Vaccination is the most effective and popular method of preventing viral infections. Vaccines have been produced for more than two centuries using traditional methods such as inactivated or killed viruses. New perspectives in vaccine production are started to use such as, mRNA, recombinant subunits and viral vectors.

Bacterial systems, mammalian cell lines or plants can be used to produce vaccines and recombinant therapeutic proteins. Alternatively, plants are also suitable as expression systems. Transgenic plants can express therapeutic proteins. However, due to their low expression levels, the fact that production is time-consuming and economically unsuitable (Mammedov 2010). Plant transient expression systems are a viable method to produce recombinant proteins because plants are eukaryotes, therefore post-translational mechanisms and protein folding occur naturally, which makes the overall process more applicable, rapid and inexpensive. Plant transient expression systems is well studied in Mammedov's laboratory.

Recent studies of successful recombinant therapeutic protein production examples in our laboratory can be given examples as the Pfs48/45 antigen that prevents malaria transmission (Mamedov et al. 2019), human Furin enzyme that have important functions in viral inhibition and its deglycosylated form by co-expression of human Endo H (Mamedov, et al. 2019), the Protective Antigen (PA83) that is highly immunogenic proteins from *Bacillus anthracis* and deglycosylated forms via co-expression with Endo

H (Mamedov et al. 2024).Although the advantages of N. benthamiana transient expression systems using plants eukaryotic nature for the therapeutic use o in humans and other animals, some differences in post-translational mechanisms cannot be ignored. The fact that the resulting protein carries N-glycan regions may cause problems in triggering the required level of immunogenicity in the target organism. Enzymatic deglycosylation of N-glycans occur naturally in bacteria. The ability to generate N-glycan-free recombinant proteins via co-expression with endo-β-N-acetylglucosaminidase H (Endo H) (Mamedov et al. 2017) and PNGase F (Mamedov 2013) is possible thanks to Tarlan MAMMEDOV's patented methods (Mammedov 2023)(Mammedov 2017).

The aim of this study is to produce and characterize recombinant RBD and N fusion proteins as potential vaccine candidates against all variants of SARS-CoV-2 virus caused infection. Since the early days of the COVID-19 pandemic, Tarlan MAMMEDOV's laboratory group has been producing, engineering and characterizing of recombinant SARS-CoV-2 proteins using an Agrobacterium-mediated transient expression system in N. benthamiana. Plant-produced recombinant RBD, S1, N and RBD+N proteins, as well as their glycosylated and deglycosylated variants, have been proved that is possible SARS-CoV-2 vaccine candidate (Mamedov et al. 2020).

For this study, Prof. Dr. Tarlan Mamedov designed the transformation cassette by combining the RBD subunit coding sequence, nucleocapsid protein coding sequence, FLAG purification tag and KDEL signal sequence and completed codon optimization as shown in Figure 3.3. The designed vector was commercially purchased. The vector was transformed into *E. coli* strain DH5α by the heat shock method to cloning. Transformed *E. coli* cells were first propagated in agar medium under appropriate conditions as described in the experimental part and then colonies were picked and propagated in appropriate medium. Plasmids were purified with the kit and cut with restriction enzymes. Each step of the experiment was controlled and performed. The DNA fragment was ligated to the pEQ plasmid that used in our laboratory with the ligation enzyme (Figure 4.2).

The obtained plasmids were transformed into the EHA105 and AGL1 strains of *Agrobacterium tumefaciens* used in our laboratory by the electroporation method as described in the experimental section. The transformed bacteria were first propagated in agar medium as described in the experimental section, and then colonies were picked and propagated in the appropriate medium.

Promoted *A. tumefaciens* bacteria were centrifuged and concentrated. The concentrated cells were incubated in medium containing phenolic acetosyringone for infiltration. Acetosyringone is added to the medium because it enhances gene transfer in Agrobacterium-mediated transformation. After incubation, the medium was agroinfiltrated from the under of mature *Nicotiana benthamiana* leaves via a syringe with a cut-off tip. The infiltrated plants were watered daily with fertilizer containing essential minerals. On the fourth, fifth and sixth days after infiltration, leaves were collected to understand the expression levels. The leaves were homogenized with extraction buffer. The homogenate was centrifuged, and the resulting supernatant was denatured. Denatured proteins were analysed by western blot. As shown in Figure 4.3, it was observed that the AGL1 strain showed maximum expression on the 5th day of infiltration.

Strain AGL1 was used to produce the optimized proteins. The experiment was repeated several times, and the leaves were collected. Infiltrated leaves were homogenized and purified by affinity chromatography using a FLAG tag. The purified protein was quantified and analysed. The presence of the protein was detected by Western blot analysis as in Figure 4.4 and SDS-PAGE analysis as in Figure 4.6. Protein concentrations were measured using GENE-SYS software. The results were consistent with spectrophotometric measurements.

After confirming the purity and presence of the protein with anti-flag, western blot was performed using anti-spike antibody to observe its activity. In the experiment where previously produced proteins in our laboratory were used as controls (Mamedov, et al. 2021), the fusion protein showed cross-reactivity and bound with the anti-spike antibody, as seen in Figure 4.5.

One of the most important elements in producing therapeutic proteins is that the protein remains stable under environmental conditions and is not easily denatured. For thermostability analysis, the purified protein was incubated at 37 °C for 5 days. The reason for choosing the temperature of 37 °C is that the target of the vaccine is humans. The proteins were collected every day for 5 days and analysed by Western blot. As can be seen in Figure 4.7, the recombinant RBD+N fusion protein remained stable for 24 hours.

In Mammedov's laboratory, glycosylated and deglycosylated variations of the RBD protein, glycosylated nucleocapsid protein, and RBD and N cocktail proteins were obtained by similar procedures before (Mamedov et al. 2021b). These recombinant proteins were used in animal experiments. The binding affinities of the recombinant fusion RBD+N protein and other recombinant proteins with serum of experimental animals were analysed by ELISA. The OPD is used in the ELISA experiment that has a white colour under normal conditions, but it easily oxidizes and turns lemon yellow. As can be seen in Figure 4.10, the protein acquired a lighter colour when diluted. The ELISA plate was measured at 450 nm in a spectrophotometer and one-way ANOVA analysis was performed using GraphPad 9.0 software with Tukey multiple comparison (** $P < 0.01$, *** $P < 0.001$). The recombinant protein is observed to interact with the obtained immunized mouse sera. The graphs in Figure 4.11 show that the data are significant.

Angiotensin-converting enzyme 2 (ACE2) is an enzyme present in many tissues in soluble form. It recognizes and interacts with the ACE2 enzyme, the spike protein of SARS-CoV-2. This interaction is important for virus fusion, infectivity and pathogenicity.

The glycosylated and deglycosylated states of the commercial ACE2 enzyme were analysed by ELISA to measure the binding affinity with the recombinant RBD+N fusion protein. In our laboratory, glycosylated and deglycosylated variations of RBD protein, glycosylated and deglycosylated variations of nucleocapsid protein and RBD and N cocktail proteins were compared with recombinant RBD+N fusion proteins by similar procedures (Mamedov et al. 2021b). As shown in Figure 4.8, it is observed that at high protein concentrations, it interacts with ACE2 giving a lemon-yellow colour. The ELISA plate was measured at 450 nm in a spectrophotometer and one-way ANOVA analysis was performed using GraphPad 9.0 software. The correlation between the absorbance values

of interaction of the purified protein with glycosylated ACE2 and deglycosylated ACE2 and the protein concentrations was performed using regression analysis using GraphPad Prism 9.0 software as shown in Figure 4.9. The KD value was calculated.

The high variability and rapid diversity of the SARS-CoV-2 virus reduces the effectiveness of the vaccines available. Many scientists are working on vaccine production and immunization. To prevent SARS-CoV-2 from becoming a global problem again, scientists continue to study emerging variants, their properties and their impact on public health. The antibody formation profile of SARS-CoV-2 in patients has been studied and it has been observed that antibodies against the spike and nucleocapsid protein were detected earlier and more frequently in the course of the disease (Brochot et al. 2020) The antigenic effects of the S1 and S2 subunits and at the same time RBD and NTD regions of the spike protein were studied separately and tested as vaccine candidates. RBD is less toxic therefore, it is better vaccine candidate than using the entire S1 subunit (Rezaei, et al. 2022) The use of the nucleocapsid protein in vaccination, which is as important as the spike protein during infection, is more highly conserved than the spike protein during new variant emerging. It can be important approach in the development of a universal COVID-19 vaccine because nucleocapsid protein has high immunoreactivity and can be easily detected in the serum of patients (Azimova et al., 2023).

In Mamedov laboratory, the RBD and nucleocapsid proteins were expressed in the *Nicotiana benthamiana* as mentioned in this thesis. Antigen cocktail was injected to mice group and their immunogenicity was tested. The use of RBD and nucleocapsid proteins as an antigen cocktail could be effective against the Omicron and Delta variants that has been previously reported (Mamedov et al, 2021).

Development of recombinant protein technology and therapeutic usage has become prevalent. Fusion antigens allow components to be obtained on a single platform rather than cocktail antigens. In this study, several properties of the fusion antigens were compared, including their binding affinities compared to antigen cocktail. We found that while the binding affinity of the antigen cocktail to the serum of immunized mice was high, therefore there was no significant difference in the binding of the fusion proteins shown in Figure 4.9. The structure of the fusion antigen provides targeting, half-life extension, advantage on purification (Marsh, et al, 2023). Producing two antigens as a fusion rather than producing them individually offers advantages in terms of speed and avoiding against experimental errors.

In conclusion, many studies have shown that nucleocapsid and RBD causes strong immunity against COVID-19. In addition, the fusion protein approach used in the design contributes new perspective to literature. The production of recombinant fusion nucleocapsid and RBD proteins in *N. benthamiana* and their therapeutic applications are promising.

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