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**MASTER THESIS**

Bu tez 16/02/2024 tarihinde jüri tarafından Oybirliği ile kabul edilmiştir.

Prof. Dr. Tarlan MAMEDOV (Danışman)

Prof. Dr. Nedim MUTLU

Doç. Dr. Hasan PINAR

# ÖZET

## 1 KG BİYOKÜTLE ÖLÇEĞİNDE ÜRETİLEN *NICOTIANA BENTHAMIANA* BİTKİSİNDE KUDUZ G PROTEİNİNİN SAFLAŞTIRILMASI

**Özlem BABACAN**

**Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı**

**Danışman: Prof. Dr. Tarlan MAMEDOV**

**Şubat 2024 --- 29 sayfa**

Kuduz hastalığı, *Lyssavirus* cinsi bir virüsün neden olduğu, tüm memelilerde ölümcül bir sonuçla seyreden zoonotik bir viral hastalıktır. Hastalık, merkezi sinir sistemini enfekte eder ve ensefalit ile karakterize edilir. Dünya Sağlık Örgütü (DSÖ), kuduz hastalığını, yılda 59.000'den fazla insan ölümüyle sonuçlanan en ölümcül bulaşıcı viral hastalıklardan biri olarak kabul etmektedir. Kuduz için mevcut aşılar memelilere uygulanabilmektedir. Ancak, bu aşılar maliyetli, üretimi karmaşık ve immünojeniteleri düşüktür. Bu nedenle, daha ucuz ve daha güvenli kuduz aşılarının geliştirilmesi ve büyük ölçekli üretimine ihtiyaç duyulmaktadır. Kuduz *Lyssavirus* (RABV) glikoproteini G, virüsün veya enfekte hücrelerin yüzeyinde homotrimerler oluşturur ve virüs-nötrleştirici antikorların hedefidir. Bu nedenle, virüsün birincil antijenik belirleyicilerini içerir. RABV'nin primer antijeni olarak belirlenen glikoprotein G'nin immünojenik indükleme yeteneği kanıtlanmıştır. Patogenezde önemli bir rol oynayan glikoprotein G hem reseptör bağlanmasını hem de membran füzyonunu katalize eder. Bir virüs organizmaya girdiğinde, aşılamadan sonra olduğu gibi, organizma virüse bağlanan ve onu etkisiz hale getiren virüs-nötrleştirici antikorlar oluşturur. G proteininin antijenik alanlarının virüs-nötrleştirici antikorları ortaya çıkarmada oldukça etkin olduğu bulunmuştur. Bitki geçici ekspresyon sistemi, aşılar ve antikorlarında rol oynadığı çeşitli rekombinant proteinlerin ekspresyonu için umut verici bir yöntemdir. Daha önce laboratuvarımızda, kuduz virüsünün G proteininin kesilmiş (yapay GCN4 tabanlı trimerizasyon alanı ile) formu olan RG2 ve RG3, geçici ekspresyon sistemi kullanılarak *Nicotiana benthamiana* (*N. benthamiana*) bitkisinde tasarlanmış ve üretilmiştir. Hem RG2 hem de RG3 proteinlerinin farelerde önemli ölçüde daha yüksek antikor titrasyonları ortaya çıkardığı gösterilmiştir. Bu tez, *Nicotiana benthamiana* bitkilerinden 1 kg ölçekte RG3 proteininin üretimi ve saflaştırılmasına odaklanmaktadır. RG3 proteini Ni-NTA kolonu kullanılarak *N. benthamiana* bitkisinden saflaştırılmış ve %53,69 saflıkta 55,87 mg RG3 proteini elde edilmiştir. Bu nedenle, sonuçlarımız bitki tarafından üretilen RG3 proteininin kuduz virüsüne karşı umut verici, uygun maliyetli ve güvenli bir aşı adayı olabileceğini göstermektedir.



**ANAHTAR KELİMELER:** Bitki Geçici Ekspresyon Sistemi, Kuduz, Kuduz G proteini, *Nicotiana Benthamiana*, Protein Saflaştırma

**JÜRİ:** Prof. Dr. Tarlan MAMEDOV

Prof. Dr. Nedim MUTLU

Doç. Dr. Hasan PINAR

## ABSTRACT

## PURIFICATION OF PLANT PRODUCED G PROTEIN OF RABIES AT THE 1KG BIOMASS SCALE FROM *NICOTIANA BENTHAMIANA* PLANT

**Özlem BABACAN**

**Master Thesis, Department of Agricultural Biotechnology**

**Supervisor: Prof. Dr. Tarlan MAMEDOV**

**February 2024; 29 pages**

Rabies is a zoonotic viral disease caused by a *Lyssavirus* species virus that is fatal in all mammals. The disease infects the central nervous system and is characterized by encephalitis. The World Health Organization (WHO) considers rabies to be one of the deadliest infectious viral diseases in the world, resulting in over 59.000 human deaths per year. Current rabies vaccines are available for mammals. However, these vaccines are expensive, complex to manufacture, and have low immunogenicity. Therefore, there is a need for the development and large-scale production of more affordable and safer rabies vaccines. The rabies *Lyssavirus* (RABV) glycoprotein G forms homotrimers on the surface of the virus or infected cells and is the target of virus-neutralizing antibodies. Therefore, it contains the virus's primary antigenic determinants. It has been demonstrated that the RABV's primary antigen, glycoprotein G, can induce protective immunity. Glycoprotein G, which plays an important role in pathogenesis, catalyzes both receptor binding and membrane fusion. The plant transient expression system is a promising method for expressing a variety of recombinant proteins, including vaccines and antibodies. Recently, in our laboratory truncated form of G protein, namely RG2 and RG3 (with artificial GCN4-based trimerization domain) of rabies virus was engineered and produced in *Nicotiana benthamiana (N. benthamiana)* plant, using transient expression system. It was shown that both RG2 and RG3 proteins elicit significantly higher antibody titers in mice. This thesis focuses on the production and purification of RG3 protein at 1 kg scale from *Nicotiana benthamiana* plants. RG3 protein was purified from *N. benthamiana* plant using Ni-NTA column, and a 55,87 mg of RG3 protein with 53,69% purity was obtained. Thus, our results demonstrate that plant produced RG3 protein could be a promising cost effective and safe vaccine candidate against the rabies virus.



**KEYWORDS:** *Nicotiana Benthamiana***,** Protein Purification, Plant Transient Expression System, Rabies, Rabies G protein

**COMMITTEE:** Prof. Dr. Tarlan MAMEDOV

Prof. Dr. Nedim MUTLU

Doç. Dr. Hasan PINAR

## PREFACE

Despite developments in the vaccine and pharmaceutical sectors, there is still no global effectiveness in the fight against rabies, which is a major threat to public health. As a result of our study, we hope to develop a domestic, reliable, cost-effective and highly immunogenic vaccine against rabies, especially in our country.

I would like to thank my valuable advisor Prof. Dr. Tarlan MAMEDOV for his knowledge and interest during my graduate education.

I thank all the professors of our Department of Agricultural Biotechnology, especially Prof. Dr. Nedim MUTLU.

I would like to thank my dear friend Damla YÜKSEL, the best of our laboratory, who has been with me throughout the process with her knowledge and experience in every field and who has never withheld her work and support.

Despite everything that has happened to me up to this age, I can never thank my loved ones, my mother Adile BABACAN and my father Adem BABACAN, who have never withheld their support, who have overcome every difficulty together, who have taught me to love people just because they are human, without abandoning honesty and kindness, and who have created my existence.

The most beautiful and precious thing that has happened to me in the world is having a brother. I would like to thank my best friend, my confidant, my guide, my sweetheart, PhD. Ümit BABACAN for everything he has added to my life. I wish you continued success in your academic life. I believe you will achieve everything you want... I love you so much...

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## ACADEMIC DECLARATION

I declare that this study titled “Purification of Plant Produced G Protein of Rabies At The 1kg Biomass Scale From *Nicotiana Benthamiana* Plant” that I submitted as a Master's Thesis is written in accordance with academic rules and ethical values, and I have shown the source of all information that does not belong to me.

16/02/2024

Özlem BABACAN

## SYMBOLS AND ABBREVIATIONS

**Symbols**

ºC : Degree Celsius

g : Gram

h : Hour

kb : Kilobase

kDa : Kilodalton

kg : Kilogram

L/l : Liter

m : Month

nm : Nanometer

µg : Microgram

µl : Microliter

mg : Milligram

ml : Milliliter

mM : Millimolar

min : Minute

M : Molar

**Abbreviations**

| | |
|---|---|
| $ddH_2O$ | : Double distilled water |
| dpi | : Day post infiltration |
| FDA | : Food and Drug Administration |
| CDC | : Centers for Disease Control and Prevention |
| IgG | : Immunoglobulin G |
| OD | : Optical density |
| Ni-NTA | : Nickel-Nitrilotriacetic acid |
| PTM | : Post-translational modification |
| PEP | : Post-exposure prophylaxis |
| PrEP | : Pre-exposure prophylaxis |
| RABV | : Rabies virus |
| RBD | : Receptor binding domains |
| RCF | : Relative Centrifugal Force (g force) |
| RIG | : Rabies Immunoglobulins |
| RG | : Rabies glycoprotein G |
| RNP | : Ribonucleoprotein |
| RPM | : Revolutions Per Minute |
| SDS-PAGE | : Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |

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# 1. INTRODUCTION

Rabies is a fatal disease that infects the central nervous system of mammals, usually transmitted by the bite of an animal with the virus that causes the disease. Progressing with encephalitis a serious health risk to both humans and animals. Given its large annual worldwide death toll and 100% fatality rate, rabies is regarded as one of the most significant zoonotic illnesses (Rupprecht et al., 2002).

According to the World Health Organization's (WHO, 2018a) study, most human rabies cases occur in developing nations because of a lack of access to healthcare and insufficient vaccination programs. According to a 2019 report, nearly 60.000 people in the world die from the virus every year, and estimates that the annual economic cost of rabies is around USD 8,6 billion (Centers for Disease Control and Prevention, 2019). The incidence of rabies in domestic animals has been greatly reduced by regular vaccination, however, rabies is still spread by wild animals (Fooks et al., 2014). While timely vaccination offers a powerful shield against rabies, once clinical symptoms emerge, the disease unfortunately presents most patients die from the infection (Yousaf et al., 2012). In general, there are two primary methods for preventing rabies. The initial method is immunization of humans, which can be administered either before or after exposure. Vaccinating dogs is the second method which, given that dogs are responsible for 99% of human cases, can stop the virus from spreading to people (Mamedov et al., 2020).

Rabies virus, which has a characteristic "bullet" form, belongs to the *Rhabdoviridae* family of negative-stranded RNA genomes and viruses with non-segmented. Lyssavirus is a member of the Rhabdoviridae family, which contains 15 different virus species. While all *Lyssavirus* species are capable of causing rabies in humans, the vast majority of rabies cases can be attributed specifically to the rabies virus (Wunner, 2003).

Nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) are proteins encoded by the rabies virus genome. The main structural elements of all Rhabdoviruses are core of the helical ribonucleoprotein (RNP) is surrounded by an envelope. The genomic RNA is tightly wrapped by the nucleoprotein of RNP, which is also associated with two viral proteins: phosphoprotein and polymerase (L-protein). On the surface of the virus, glycoprotein G forms characteristic homotrimers; it contains information about the primary antigenic determinants of the virus, which are targeted by infected cells and virus-neutralizing antibodies. Research has shown that rabies virus glycoprotein G is capable of inducing protective immunity (Lafon, 2005), (Brunker & Mollentze, 2018), (Albertini et al., 2011).

Since the first application of the rabies vaccine, significant progress has been made in the control of rabies. New alternatives have emerged, especially with a better understanding of the biology of the virus and advances in technology. Given the annual worldwide mortality rates of the rabies virus, there is a need for cheaper, effective, and stable vaccines. Studies have shown that the plant-based transient expression system has particularly favorable implications for recombinant protein production. Rapid production, simplicity, low cost, high production capacity, and the absence of

mammalian pathogens make the plant-based expression system more important (Mamedov et al., 2020).

Vaccines against rabies are already available for humans and animals. However, they are costly, difficult to produce, and have poor immunogenicity. It has become even more important to prevent rabies, which has a high annual mortality rate, as the world's and our country's populations grow. However, there is a need for more reliable, highly immunogenic, and cost-effective vaccines for rabies that can be manufactured in high capacity. According to the data of the Ministry of Health of the Republic of Turkey for the year 2022, it was reported that there were 250.375 rabies risk contact cases in our country. Rabies vaccine has not been produced in our country since 1996. The control of this disease, which is extremely harmful to human health, depends on the international marketplace. It has become too dependent on foreign sources. This thesis aims to contribute to the development of safe, low-cost, highly immunogenic, new-generation rabies vaccines for domestic and national production in our country.

# 2. LITERATURE

## 2.1. Rabies Disease

Rabies known to be viral zoonotic disease which is transmitted by the bite or scratch of an infected animal although can be preventable various way. The virus can also be transmitted through contact of infected animal saliva with the mouth, nose, or eyes (WHO, 2018b). Mammal's central nervous systems are infected by the rabies virus, which results in meningeal and brain inflammation. It culminates in deathly encephalitis and brain damage. Symptoms may include localized and mild paralysis, disorientation, irritability, anomalous behaving, anxiety, insomnia, and delusions (Giesen et al., 2015). For much of its history, rabies has also been known by the term hydrophobia, or "fear of water" (Graham & Dunlap, 1937). Death generally occurs two to ten days following the beginning of symptoms. Even in intensive care, survival after symptoms appear is virtually unknown. The CDC number of deaths per year following rabies virus infection is approximately 59.000, but this is likely an underestimate. Approximately 40% of these deaths are in children under 15 years of age (WHO, 2013). Dogs are the animals most associated with this illness worldwide. More than 99 percent of rabies cases in nations where dogs are often infected with the illness are directly related to dog bites (Fooks et al., 2017). Pre-exposure vaccination is already available for pets and humans; vaccination of stray animals is also required. The prevention of rabies in humans depends on a number of animal control strategies, such as vaccine campaigns and dog control (Hicks et al., 2012).

## 2.2. Transmission of Rabies Virus

*Saliva contact with a mucous membrane or a break in the skin: The virus is transmitted through direct contact with the saliva or brain/nervous system tissue of an infected animal (Wounded skin or mucous tissue of the mouth, eyes, and nose).

*Animal biting with virus infection: An animal biting humans or other animals typically spreads rabies. There are very few alternative ways of transmission except bites and scrapes.

*Corneal transplant: Although reports of rabies transmission during solid organ and corneal transplants exist, these cases are likewise extremely uncommon.

One possible non-bite mode of exposure is inhalation of aerosolized rabies virus; however, it is highly unlikely for individuals other than laboratory personnel to be exposed to rabies virus aerosol (Centers for Disease Control and Prevention, 2019; Fooks et al., 2014).

## 2.3. History of Rabies Vaccine

The most successful method of preventing infection from this deadly viral zoonosis is vaccination against rabies. This can be given as a preventative measure or as a treatment (Fooks et al., 2017). When given soon after rabies exposure, the current vaccines have more efficacy. The two main vaccination strategies recommended by WHO for rabies prevention are as follows:

1- **PEP (Post-Exposure Prophylaxis):** Wound cleaning at the site of virus exposure, using immunglobulins (rabies immunoglobulins: RIG) when necessary, giving multiple doses of vaccination,

2- **PrEP (Pre-Exposure Prophylaxis):** Giving multiple doses of vaccination before exposure to the virus (Hicks et al., 2012).

The first scientist to conceive of the possibility of a vaccine against rabies infection was the French pharmacist Apollinaire Bouchardat in 1852. First experimentation anti-rabies vaccine was carried out by the French veterinarian Pierre-Victor Galtier in 1881 by inoculating sheep with the rabies virus intravenously (Nagarajan & Rupprecht, 2020). The first vaccine for pre- and post-exposure prophylaxis was developed by Louis Pasteur on June 6, 1885. This represented the beginning of the age of first-generation vaccines (Natesan et al., 2023).

Louis Pasteur created the first rabies vaccination by physically inactivating the virus from an infected rabbit's spinal cord by drying it in the sun. Nine-years-old Joseph Meister, who had been bitten by a rabid dog several times, was the first person to receive the experimental rabies vaccination from him. The young child was given 13 injections of progressively more virulent rabbit spinal cord suspensions infected with the rabies virus, which had been air-dried for 11 days, about two days after the bite. Pasteur's strategic immunization rescued Meister from the ravages of rabies. This vaccine has a significant disadvantage as it contains the highly lethal rabies virus. Additionally, the number of reported cases of individuals developing rabies after vaccination was low, and there were concerns about the consistency of the inactivation process. Furthermore, an additional challenge was the lack of capacity to produce enough vaccine to support the need for large-scale vaccine production. But it took more than 50 years for the rabies vaccine to be prepared using Pasteur's approach before any notable modifications were introduced (Sureau, 2005).

Cell-cultured, inactivated rabies vaccines are widely utilized in numerous countries throughout around the globe. However, to elicit a robust humoral immune response, multiple administrations of these vaccines are necessitated. This requirement translates to increased cost, posing a significant obstacle to their widespread implementation in human and animal vaccination programs, particularly in resource-constrained developing countries (El-Sayed, 2018). Adverse side effects such as nerve paralysis have led to the use of cheaper inactivated nerve tissue vaccines being phased out in many countries (Nandi & Kumar, 2010). New generation vaccinations have been developed in response to the growing need for vaccines that are less reactive, safer, and more immunogenic (Zhu & Guo, 2016).

### 2.4. Virus Structure and Life Cycle

The rabies virus (RABV), a bullet-shaped, negative-sense RNA virus of the *Lyssavirus* genus within the *Rhabdoviridae* family, measures approximately 180 nm in length and 75 nm in diameter. Its genome encodes five distinct proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) (Fooks et al., 2014). It has a non-segmented genome of approximately 12 kb in length and has linear, conserved 5 major genes arranged according to the sequence 3'-N-P-M-

G-L-5' (Guo et al., 2019). An envelope of lipids covers the outside of the virus. On the envelope are glycoproteins that help the virus bind to the host cell. The inside of the virus is encapsulated by a negative polarity, single-stranded RNA (ssRNA) genome, and a capsid that surrounds it. The capsid structure shows helical symmetry (Burrell et al., 2017). RABV consists of two functional units:

1-An inner nucleocapsid (NC) containing genomic RNA, nucleoprotein (N), and phosphoprotein (P) attached to viral polymerase. The capsid structure shows helical symmetry. The RNA genome is tightly encapsulated in nucleoprotein. The encapsulated RNA is called ribonucleoprotein (RNP).

2-External unit; It is represented by glycoprotein (G) with a bilayer lipid envelope with protruding spikes.

Between these two units is the matrix protein (M). The matrix protein condenses the NC, causing the G protein to interact with the endodomain (Walker et al., 2011). The functions of the virus genome-encoded structural proteins are as follows:

NC: Nucleocapsid protein. It has a phosphoprotein structure. It functions in viral replication.

N: Nucleoprotein. It is the major structural protein of the virus.

M: Matrix protein. Within the context of RABV virion architecture, the matrix protein serves as a crucial lynchpin, bridging the viral ribonucleoprotein complex (RNP) to the surrounding envelope. This protein interacts with the RNP, inducing its condensation and shaping it into the characteristic helical form observed within mature virions.

L: RNA-dependent RNA polymerase (large protein).

G: Glycoprotein on the envelope surface. It creates spikes on the outside of the envelope. The M protein demonstrably mediates RABV entry into host cells by directly recognizing and engaging cellular receptors. This interaction triggers fusion of the viral envelope with the cellular membrane, facilitating the delivery of the viral RNP core into the cytosol (Wiltzer et al., 2014).

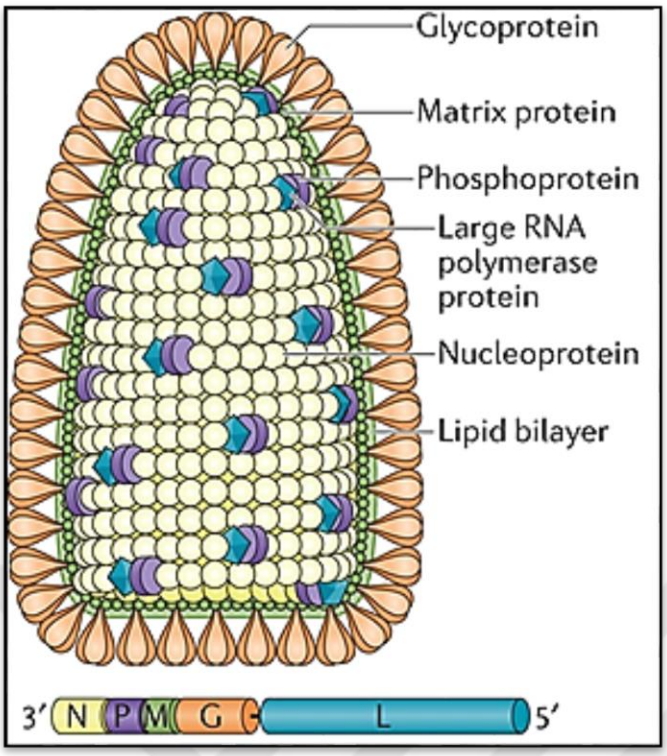


**Figure 2.1.** Rabies virus structure (Fooks et al., 2017).

The rabies virus life cycle can be divided into three distinct stages. First, it binds to the host cell through receptors. It enters the cell by endosomal transport system the plasma membrane. Meanwhile, RNP is released into the cytoplasm, decreased pH causes membrane fusion, allowing DNA to enter the cytosol. Next, the viral genome is transcribed and replicated through the P-L polymerase complex specifically targets viral ribonucleoprotein (RNP) as a template for new viral protein synthesis. Unbound RNA lacks the necessary structural context and cannot be recognized by the viral polymerase for replication. Cis-acting genomic regions and protein M regulate transcription. Finally, the virus leaves the infected cell (Finke & Conzelmann, 2005; Wunner, 2007). Most important role of glycoprotein G is to catalyze receptor binding and membrane fusion in pathogenesis. Additionally, mutant viruses without G proteins cannot propagate (Finke & Conzelmann, 2005).

As the infection progresses, the P-L polymerase undergoes a functional shift, transitioning from mRNA synthesis to the production of full-length positive-strand viral RNA genomes. These newly generated mRNAs act as templates for the subsequent synthesis of negative-strand genomes, which, upon complexation with N protein, form infectious ribonucleoprotein (RNP) cores capable of initiating new viral cycles (Albertini et al., 2008).

### 2.5. Glycoprotein G and Pathogenicity

The rabies virus glycoprotein (G) serves as the primary antigenic target, eliciting protective immunity against RABV infection. Following both natural exposure to the virus and vaccination, the host immune system generates virus-neutralizing antibodies

(NAbs) specifically directed against G protein epitopes. Notably, specific antigenic regions on G have been identified as particularly potent in inducing high titers of rabies specific NAbs. In particular, G homotrimers prominently displayed on the virion surface act as key immunogens, triggering robust humoral immune responses. Moreover, even a single vaccine dose in animal models has been shown to elicit significantly elevated levels of G-specific NAbs, highlighting the critical role of G in vaccine efficacy (Koraka et al., 2014; Yang et al., 2014). Recombinant G-proteins expressed using various protein expression systems are the basis of most vaccine candidate studies (Prehaud et al., 1989; Ramya et al., 2011). Consequently, as a protein-based subunit vaccine, the G protein has been stated to be a promising candidate (Mammadova et al., 2022).

### 2.6. Plant-Based Transient Expression Systems

The production of recombinant proteins has benefited greatly from diverse expression systems spanning bacteria, yeast, and mammalian cells. Initially, stable transformation of plant nuclear genomes served as the primary approach, whereby the gene of interest was integrated for long-term protein expression. While offering the advantage of stable production, this method often suffered from lengthy development times and limited yields. To address these constraints, transient gene expression systems in plants emerged as a complementary approach (Yusibov & Mammedov, 2010). Particularly for the synthesis of proteins that are challenging to produce, transient gene expression has emerged as a substitute expression mechanism (Klimyuk et al., 2014; Mamedov et al., 2021c). Compared to other expression platforms, this expression system has a number of benefits. For instance, plants have large production capacities and eukaryotic post-translational modification (PTM) processes. Within a week or less, for every kilogram of leaf biomass, hundreds of milligrams of recombinant protein are produced (Mammadova et al., 2022). Other benefits include simple scaling, reduced costs, and safety because there are no endogenous human infections. It has been shown that the method is highly helpful for expressing complex proteins. Certain medications made from plants, such enzymes, therapeutic human proteins, and vaccine antigens, are already in the clinical development phases (Mamedov et al., 2020; Mammadova et al., 2022). Using this system, many challenging to be expressed proteins like Factor IX human Furin (Mamedov et al., 2019b), Pfs48/45 protein (Mamedov et al., 2019a), human immunodeficiency virus (HIV); other complex proteins, including SARSCoV-2 spike protein receptor binding domains (RBDs) (Mamedov et al., 2021b) and functionally active monoclonal antibodies (de Melo et al., 2022), *Nicotiana benthamiana* plants have been used to successfully produce a variety of recombinant proteins.

The utilization of plant transient expression technology holds significant promise for the development of affordable, safe, durable, and highly immunogenic rabies vaccines. While attempts to produce the rabies virus G protein in transgenic plants yielded limited success due to low expression levels, a truncated version of the G protein (RG) emerged as a promising candidate when expressed transiently in *N. benthamiana* (Dietzschold et al., 2005). This expression system facilitates rapid production of high amounts of RG antigen, as demonstrated by studies in *N. benthamiana*. Moreover, immunogenicity studies revealed that plant-derived RG antigen effectively induces significantly higher antibody titers in mice compared to

other methods. These findings suggest that plant-produced RG antigen holds significant potential for the development of cost-effective and safe subunit vaccines against rabies virus (Mammadova et al., 2022).

## 2.7. Agroinfiltration

Agroinfiltration is a technique used, particularly lately in plant biotechnology, to produce a specific protein, genes are expressed transiently in a plant or even in cultures of plant cells. The procedure involves injecting the target gene into the Ti-plasmid (tumor-inducing plasmid) of *Agrobacterium tumefaciens* followed by activation of the pathogenicity mechanism (Chilton et al., 1980), directly injecting or suction infiltrating *A. tumefaciens* suspension into a plant leaf and followed by bacteria transferring the target gene into plant cells via T-DNA transfer (Fischer & Emans, 2000). A pathogenic bacteria called *A. tumefaciens* is responsible for causing crown gall disease, which is a plant tumor that affects many different types of plants (Lee et al., 2009).

*Agrobacterium* determines phenolic compounds generated from actively developing cells in a plant wound, T-DNA transfer begins. Numerous virulence (vir) genes are expressed in response to these phenolics, encodes proteins that break down T-DNA and move it through the bacterial membrane system and enters the plant cell, then integrates in DNA at a basically random site (McCullen & Binns, 2006). T-DNA encodes genes that are expressed, changing the level of plant hormones, and causing uncontrolled cell division and tumor development (Lee et al., 2009).

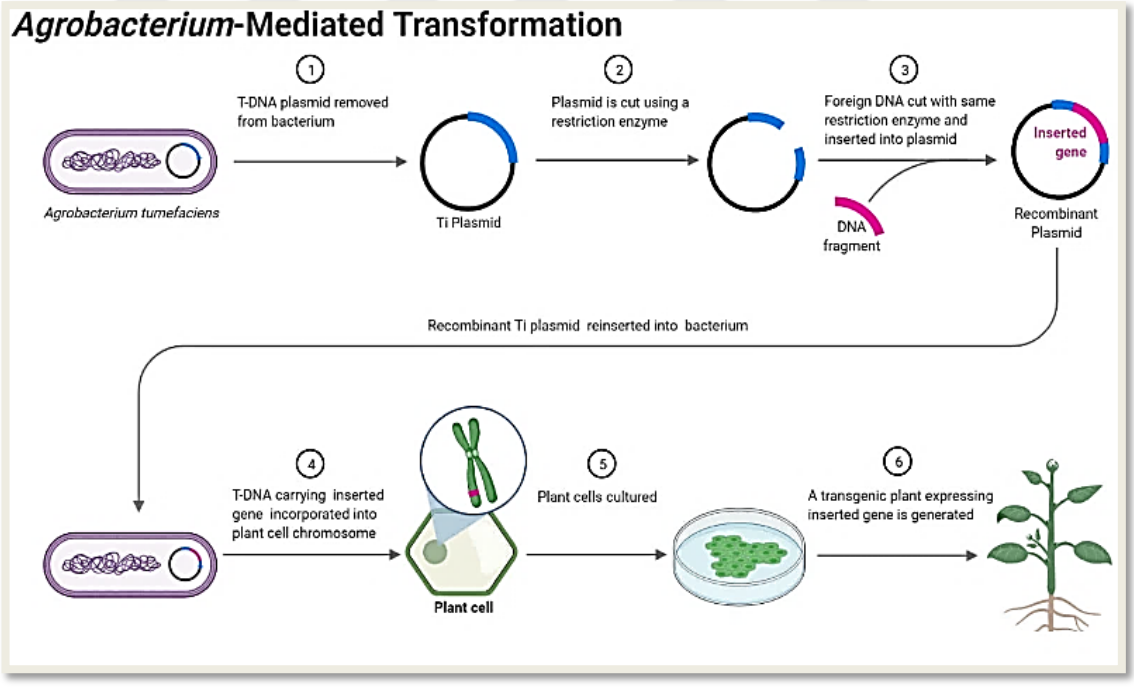


**Figure 2.2.** Agrobacterium-Mediated Gene Transfer (Transformation) in Plants (Sapkota, 2022).

When comparing agroinfiltration to the more conventional plant transformation method, the primary advantages are expediency and ease of use. In addition, the yield of

recombinant protein is generally higher and more consistent. It is a particularly good technique for observing the phenotypes of unstable and low-efficiency proteins, as these proteins have been reported to be undetectable even in transgenic plants (McGarvey et al., 1995). This technique may be used to process several plant species, although the most prevalent are *Nicotiana benthamiana* and, less frequently, *Nicotiana tabacum* (Kurotani et al., 2023).

Agroinfiltration is scalable enough to yield tens of milligrams of recombinant protein and can potentially be acceptable for preclinical studies without the requirement for stable transformed plants (Fischer & Emans, 2000). Proteins obtained through the agroinfiltration method in the plant transient gene expression system have been shown to produce up to twenty times more than stably transformed plants, and as a result, this method has become popular in both commercial and academic protein production (Sainsbury & Lomonossoff, 2008).

### 2.8. Protein Purification

Protein purification is the separation of a protein from other proteins and components using various techniques in order to research a specific biological property, structure, or function. Various techniques are applied to isolate the target protein from the cell extract. This is followed by cell lysis using methods such as mechanical, ultrasonication or homogenization. Chemical methods may also be utilized during this stage. After cell lysis, a cell extract is prepared, which facilitates the isolation of the target protein from the many different molecules and components it contains.

Several techniques are used to isolate the target protein from the cell extract. Protein isolation techniques include precipitation, chromatography (such as affinity and ion exchange), electrophoresis, and filtration. The isolated protein is usually concentrated and cleared of other contaminants through purification steps. Various techniques like Western Blot, SDS-PAGE, Mass Spectrometry, and NMR (Nuclear Magnetic Resonance), are used to assess the accuracy and purity of the protein.

The successful application of recombinant proteins across diverse fields, including molecular research, hinges critically on their efficient purification. This process often involves the incorporation of an affinity tag at the N- or C-terminus, facilitating precise and efficient isolation of the target protein from the complex cellular matrix (Spriestersbach et al., 2015). Among the diverse repertoire of protein fusion tags, the poly histidine (His) tag ranks as one of the most ubiquitous choices. Comprising six or more consecutive histidine residues, this compact tag exhibits minimal impact on the folding and functionality of its tethered fusion protein. This favorable behavior stems from the histidine residues' negligible size and neutral charge at physiological pH, effectively minimizing structural perturbations and preserving the native properties of the target protein (Carson et al., 2007). The unique characteristic of the His-tag, where its affinity for Ni-NTA solely relies on its primary conformation, enables the purification of His-tagged proteins under both native and denaturing conditions. This exceptional flexibility stems from the minimal influence of tertiary or quaternary structures on the His-tag's interaction with the metal ion, allowing for successful isolation regardless of the protein's folding state (Hochuli & Dobeli, 1987). Under denaturing conditions, the enhanced accessibility of the His-tag facilitates its optimal

interaction with the Ni-NTA matrix. This strengthened binding promotes efficient capture of the His-tagged protein, minimizing co-purification with untagged molecules due to steric hindrance and reduced nonspecific interactions (Spriestersbach et al., 2015).

# 3. MATERIAL AND METHOD

## 3.1. Mediums, Buffers, and Other Materials in Experiments

→ ***Nicotiana benthamiana*:** From seed planting for infiltration, plants were cultivated in a 3:1 mixture of peat and perlite at a heat was 24°C and humidity of 60% in climate chamber. The light cycle consisted of 18 hours of light followed by 6 hours of without light. For the experiments, plants were used that were 6-7 weeks old.

→ **BBL (SYS) Medium:** Prepare a growth medium by dissolving 10g soy hydrolysate, 5g yeast extract, and 5g NaCl in 800ml of autoclaved double-distilled water using a magnetic stirrer. Adjust the pH to 7,0 with 1M KOH or NaOH solution. To achieve a final volume of 1L, supplement with autoclaved double-distilled water. Sterilize the medium by autoclaving at 1 atmosphere pressure and 121°C for 30 minutes. Store the prepared medium at 4°C.

→ **MMA Medium:** Prepare a 50mM 2-(morpholinoethanesulfonic acid) (MES) buffer with 10mM $MgCl_2$ containing 800ml of autoclaved double-distilled water. Employ a magnetic stirrer to dissolve 1,952g MES and 2,03g $MgCl_2 \cdot 6H_2O$. Adjust the pH to 5,8 using 1M KOH or NaOH solution. To achieve a final volume of 1L, supplement with autoclaved double-distilled water. Sterilize the buffer by autoclaving at 1 atmosphere pressure and 121°C for 30 minutes. Store the prepared buffer at 4°C.

→ **Acetosyringone (AS) Stock Solution (100mM):** A stock solution of 100mM acetosyringone was prepared by dissolving 0,3924g in a 20ml solvent mixture containing 8ml autoclaved double-distilled water and 12ml 95% ethanol. The solution was then filter-sterilized using a 0,45µm membrane filter. Aliquots of 100µl were dispensed and stored at -20°C for future use.

→ **Kanamycin:** A 50mg/ml kanamycin stock solution was prepared. To achieve this, 0,5g of kanamycin monosulphate was dissolved in 10ml of sterile double-distilled water ($ddH_2O$) using sterile technique. The solution was subsequently filter-sterilized through a 0,45µm membrane filter. For long-term storage, aliquots of 500µl were dispensed and frozen at -20°C.

→ **Tris-HCl Solution (1,5M, pH:8,8):** A 1M Tris-HCl (tris (hydroxymethyl) aminomethane) buffer pH:8,8, was prepared by dissolving 185g Tris in 800ml of autoclaved double-distilled water. The pH was then adjusted to 8,8 using concentrated hydrochloric acid (HCl). To achieve a final volume of 1L, the solution was supplemented with additional autoclaved double-distilled water.

→ **Tris-HCl Solution (0,5M, pH:6,8):** A 0,5M Tris-HCl buffer pH:6,8 was prepared by dissolving 60g Tris in 800 ml of autoclaved double-distilled water. The pH was then adjusted to 6,8 using concentrated hydrochloric acid (HCl). Finally, the solution was supplemented with autoclaved double-distilled water to achieve a final volume of 1L.

→ **Running Buffer (1X):** Using a magnetic stirrer, dissolved 3,03 grams of Tris and 14,3 grams of glycine in 800ml autoclaved dd$H_2O$. 10ml 10% SDS solution was added to this solution, making the final volume 1L.

→ **Transfer Buffer (1X):** 5,8g Tris and 2,93g glycine were added to 800ml autoclaved dd$H_2O$ and dissolved with a magnetic stirrer. 370µl of 10% SDS solution was added to the mixture, and volume completed to 1L.

→ **DIECA (Sodium diethyldithiocarbamate):** 1M DIECA was calculated as mw:225,31g/mol and prepared with dd$H_2O$.

→ **90% Glycerol:** Prepared in a 9:1 ratio with 100% glycerol and autoclaved dd$H_2O$. The prepared solution autoclaved at 1 atm, 121ºC for 30 minutes.

→ **1X PBS (Phosphate-Buffered Saline):** A 1X phosphate-buffered saline (PBS) solution was prepared by dissolving one commercially available PBS tablet in 200ml of autoclaved double-distilled water, following the manufacturer's instructions for reconstitution. The prepared solution was subsequently stored at +4°C for future use.

→ **Acrylamide / Bis-acrylamide Solution (40%):** A stock solution of 40% acrylamide and 1% bis-acrylamide was prepared by dissolving 194,8g acrylamide and 5,2g bis-acrylamide in 150ml of autoclaved double-distilled water (dd$H_2O$) using a magnetic stirrer. The mixture was stirred overnight to ensure complete dissolution. The final volume was adjusted to 500ml with additional autoclaved dd$H_2O$. The acrylamide/bis-acrylamide solution was then stored in a light-tight Erlenmeyer flask at +4°C.

→ **10% APS (Ammonium Persulfate):** 10µg of APS powder was dissolved in 90µl of autoclaved dd$H_2O$. The solution was always prepared fresh and used immediately.

→ **10% SDS Buffer (Sodium Dodecyl Sulfate):** Dissolved 10 grams of SDS in 90ml of autoclaved dd$H_2O$. It was left on a magnetic stirrer throughout the day. The solution was stored at room temperature.

→ **5X TBS (Tris-Buffered Saline):** A buffer solution containing 20mM Tris-HCl and 150mM NaCl (pH:7,5) was prepared as follows. Tris (12,115 g) and sodium chloride (NaCl) (43,88g) were dissolved in 800ml of autoclaved double-distilled water (dd$H_2O$) using sterile technique. The pH was then adjusted to 7,5 using concentrated hydrochloric acid (HCl). Finally, the solution was supplemented with additional autoclaved dd$H_2O$ to achieve a final volume of 1L.

→ **1X TBS:** 200ml of 5X TBS was taken and 800ml of distilled water was added.

→ **Sample Loading Solution for Western/SDS (5X, Laemmli Buffer):** Tris-HCl (1 M) 9,375ml; Glycerol 11,9ml; SDS 3,33 g; β-mercaptoethanol (25%) 7,5ml and Bromophenol blue stock suspension (0,01%) 660µl were mixed. The solution was adjusted to pH 6,8 by adding HCl. Protein samples were boiled with 1/4 volume (5X) of Laemmli Buffer before loading onto the gel. Laemmli buffer denatures proteins due to its mercaptoethanol content.

→ **SDS gel staining solution (Coomassie Blue):** A staining solution was prepared by dissolving 500ml of methanol, 100ml of glacial acetic acid, and 400ml of deionized water ($ddH_2O$) in a magnetic stirrer. Subsequently, 1g of Coomassie Brilliant Blue R-250 was added and stirred until completely dissolved.

→ **SDS Gel De-staining Solution:** A mixture containing 700ml $ddH_2O$, 200ml methanol and 100ml of acetic acid was prepared.

→ **Blotting Suspension (I-Block):** Dissolved 1,5g of blotting grade powder (I-block) in 150ml of 1X TBS using a magnetic stirrer.

→ **Preparation of gels (for 10% gel):** The preparation of the gel is shown in Table 1.1.

**Table 1.1.** 10% Polyacrylamide gel preparation compounds

| Gel ingredients | Resolving Gel | Steaking Gel |
|---|---|---|
| Autoclaved Distilled Water | 2,425 ml | 1,98 ml |
| Tris-HCl | 1,25 ml<br>(pH 8,8 using a 1,5 M Tris-HCl buffer) | 782 µl<br>(pH 6,8 using a 0,5 M Tris-HCl buffer) |
| Acrylamide/Bis-acrylamide, 40% solution | 1,25 ml | 312 µl |
| SDS 10% | 50 µl | 31,25 µl |
| TMEDA | 2,5 µl | 3,125 µl |
| APS 10% | 25 µl | 15,62 µl |

→ **Sodium Phosphate Buffer (20mM):**

- **1M $NaH_2PO_4$:** A solution of 13,8g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) was prepared in 100ml of distilled water.
- **1M $Na_2HPO_4$:** A solution of 28,38g of disodium hydrogen phosphate ($Na_2HPO_4$) was prepared in 100ml of distilled water.
- A phosphate buffer solution was prepared by adding 15,48ml of a solution containing 17,53g of disodium hydrogen phosphate ($Na_2HPO_4$) and 4,52ml of $NaH_2PO_4$ to 100ml of deionized water ($ddH_2O$). The pH of the resulting solution was adjusted to 7,0 and the final volume was brought to 1L.

→ **Stock Solution of Imidazole (100 mM):** 0,34 grams of imidazole were dissolved in 50 milliliters of 20mM sodium phosphate buffer.

The purification buffers contain specific amounts of imidazole. To achieve this, a stock solution of imidazole was initially prepared.

→ An equilibration buffer containing 10mM imidazole was prepared by combining 20mM phosphate, 500mM NaCl, and 10 mM imidazole.

→ The wash buffer contained 25mM imidazole, prepared by combining 20mM phosphate, 500mM NaCl, and 25mM imidazole.

→ An elution buffer containing 250mM imidazole was prepared by combining 20mM phosphate, 500mM NaCl, and 250mM imidazole.

→ The extraction buffer contained 100mM imidazole, prepared by combining 20mM phosphate, 500mM NaCl, and 100mM imidazole.

→ To remove dissolved gases and potential contaminants, all buffers employed in the purification process were subjected to degassing using a Millipore Durapore 0,22µm sterilizing filter unit.

### 3.2. Gene Cloning

Previous studies in our laboratory investigated the functions of two truncated G protein forms, RG2 and RG3. RG2 (GenBank accession number: AAA47204.1, 20-455 amino acids) is native, while RG3 incorporates an artificial GCN4-based trimerization domain and LIGGGGI linker at the N-terminus. Both genes were codon-optimized, synthesized de novo with the PR-1a signal peptide, His6 tag, and ER retention peptide (KDEL) at the C-terminus. Subsequently, they were cloned into the plant expression vector pEAQ to generate pEAQ-RG2-His6-KDEL and pEAQ-RG3-His6-KDEL. These plasmids were then transformed into *Agrobacterium tumefaciens* strain AGL1 for

infiltration into *Nicotiana benthamiana* leaves, enabling the production of RG2 and RG3 proteins (Mammadova et al., 2022).

### 3.3. Agrobacterium Culture Preparation for *Nicotiana Benthamiana* Infiltration

For large-scale (1kg) RG3 protein purification, *Agrobacterium tumefaciens* harboring the pEAQ-RG3-His6-KDEL plasmid was cultured in BBL media supplemented with kanamycin (concentration dependent on our selection marker) at 225 rpm and 28°C for 16-18 hours in an orbital shaker. Following growth, a portion (aliquot) of the *A. tumefaciens* culture containing the RG3 plasmid was cryopreserved for future use. This aliquot was mixed with 90% glycerol to prevent ice crystal formation during freezing and stored at -80°C in a deep freezer.

*Agrobacterium tumefaciens* harboring the pEAQ-RG3-His6-KDEL plasmid was transformed into the AGL1 strain and incubated overnight in BBL medium at 225 rpm and 28°C. The culture's optical density (OD) was measured at the end of incubation. Following OD measurement, the culture was centrifuged at 5,000 × g for 5 minutes, and the supernatant discarded. The pellet was then resuspended in MMA medium supplemented with acetosyringone (15 µl per 100 ml MMA, based on the measured OD). Six- to seven-week-old *Nicotiana benthamiana* plants were chosen for infiltration. After a 2-hour incubation in the MMA medium, the bacterial suspension was manually injected into the leaves of the selected plants. A visual representation of the process is presented in Figure 3.1.

**Figure 3.1.** Demonstration of infiltration to *Nicotiana benthamiana* leaves

### 3.4. Harvesting and Western Blot Analysis of Infiltrated Leaves

Five days post-infiltration (dpi), infiltrated leaves were harvested from *N. benthamiana* plants. The total collected leaf mass was recorded, and the leaves were subsequently stored at -80°C. This harvest-and-storage process was repeated until a

cumulative total of 1kg of infiltrated leaf tissue was obtained.

Frozen plant leaf tissue (1g) was thawed on ice and homogenized in a mortar and pestle with 3ml of ice-cold phosphate extraction buffer (1X PBS, 2mM DIECA). The homogenate was transferred to Eppendorf tubes and centrifuged at 20,000 × g for 5 minutes at 4°C. The supernatant was carefully collected and aliquoted into 20µl Eppendorf tubes.

Twenty microliters (µl) of each protein sample were combined with 5µl of 5X Laemmli buffer and thoroughly mixed. The resulting mixtures were then incubated in a 100°C water bath for 5 minutes to denature the proteins. Following protein denaturation, 10µl of each sample were carefully loaded into individual wells of a 10% polyacrylamide gel alongside pre-prepared western blot standards. The gel was then assembled in the Western blot tank with running buffer and electrophoresed at 100V for 15 minutes followed by 200V for 45 minutes. This separation step allowed for visualization of the proteins based on their size and charge.

Following gel electrophoresis, proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane using a transfer buffer at 100V for 1 hour. To prevent non-specific antibody binding, the membrane was subsequently blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 hour at room temperature with shaking.

Following primary antibody incubation, the membrane was washed three times for 5 minutes each with 1xTBS to remove unbound primary antibody. Subsequently, the cleaned membrane was incubated with 2,5µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (catalog number 405306, BioLegend, USA) diluted in 10ml of 1% BSA-TBS for 1 hour at room temperature with shaking. Finally, the membrane was washed again three times for 5 minutes each with TBS-T and once with 1X TBS to remove unbound secondary antibody.

Finally, the membrane was incubated with a chemiluminescent detection reagent containing 2,5ml each of hydrogen peroxide and luminol/enhancer solution (SuperSignal West Pico, Thermo Fisher Scientific, Grand Island, NY) for 5 minutes in the dark. Chemiluminescence was then visualized using the GeneGnome XRQ Chemiluminescence imaging system (Syngene Corp, USA) to detect protein bands bound by the secondary antibody.

### 3.5. Purification of Plant-Produced RG3 Protein from *N. Benthamiana*

The RG3 protein has been purified by affinity Ni column (HiScale™ 26/20 cat # 28-9645-14, Cytiva, Sweden) chromatography using Ni-NTA Resin (Ni Sepharose 6 Fast Flow, cat # 17-5318-02, Cytiva, Sweden) at the ÄKTA Start system. The instrument in mentioned is shown in Figure 3.2.


**Figure 3.2.** ÄKTA Start system

The purification buffers for the device were prepared following the previously described method. The resin was placed in the column according to the manufacturer's specifications. The first step was to pass a binding buffer through the column to remove the alcohol from the resin. The protocol was run on the instrument. After passing the equilibration buffer through the column, the supernatants of the plant sample extracts were passed through the resin. Wash buffer was added after the samples were passed through the device. Finally, the elution buffer was started for protein collection from the column. Concentrations of each fraction collected measured (BioDrop µlITE (cat # 80-3006-55) was used for this procedure. The image of the mentioned procedure is presented in Figure 3.3.

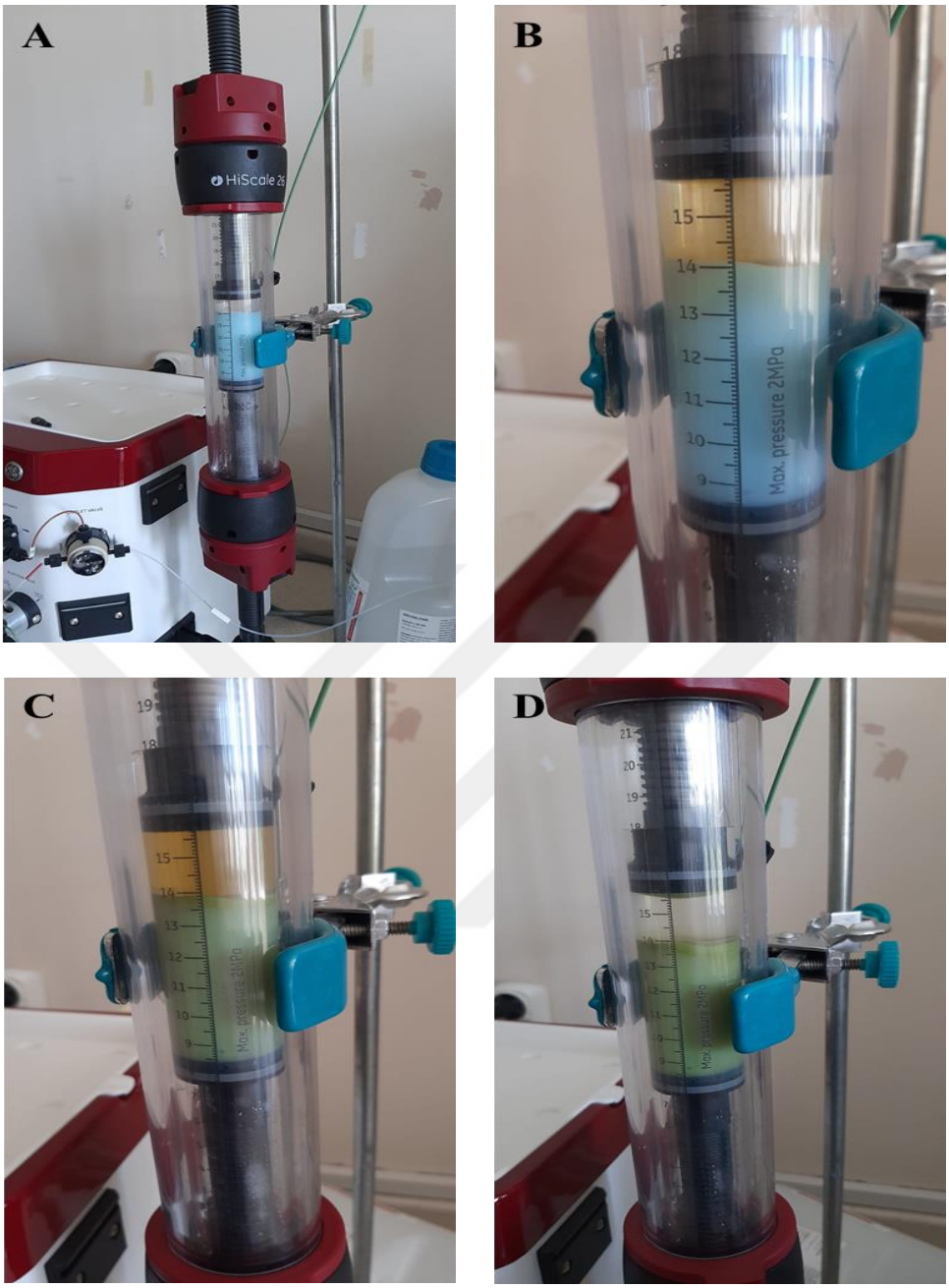


**Figure 3.3.** Ni column (HiScale™ 26/20) with Ni-NTA Resin (Ni Sepharose 6 Fast Flow) A) Conditioning of the column, B) First entry of the extracted material into the column, C) Transition of the extracted material during purification, D) Image of the column as a result of the purification process

Fractions that matched the elution profile and had high concentrations were combined. Protein concentration was required due to the high volume of protein present. The fractions were combined and passed through the 50K MWCO Millipore concentrator (Merck Millipore, Amicon®Pro, cat # ACS505024). Imidazole in the buffers used in the purification needs to be removed; the imidazole was washed with PBS (10ml of 1X PBS for every 5ml of protein), which allows for longer storage, as it will degrade proteins in the long term.

# 4. RESULTS AND DISCUSSION

## 4.1. Infiltration of *N. benthamiana* plant to produce 1 kg of plant material for further purification of RG3 protein.

For infiltration of RG3 gene into *N. benthamiana* plant, *A. tumefaciens* strain AGL1 harboring pEAQ-RG3-His6-KDEL were cultured as described in Material and Method, then infiltrated into *N. benthamiana* plant to produce a plant at 1kg scale. After 5 dpi plant leaves were harvested and stored at -80$^{o}$C until use. Western blot analysis was utilized on protein extracts from leaves harvested post-infiltration to confirm RG3 expression. Delivery of the RG3 protein to *N. benthamiana* plants was achieved through the infiltration method outlined in the Materials and Methods section. The experimental environment was a climate chamber, maintained at a constant temperature of 24°C with a relative humidity of 60%. Plants were subjected to a controlled light cycle of 18 hours light and 6 hours dark. At five days post-agroinfiltration, manual harvesting of plant material and subsequent processing were performed as established in prior methods. The resulting cell extracts were subjected to centrifugation, yielding a supernatant combined with Laemmli buffer for gel loading. Protein separation was achieved through polyacrylamide gel electrophoresis followed by blotting. Finally, a molecular marker facilitated precise identification of protein locations on the blot.

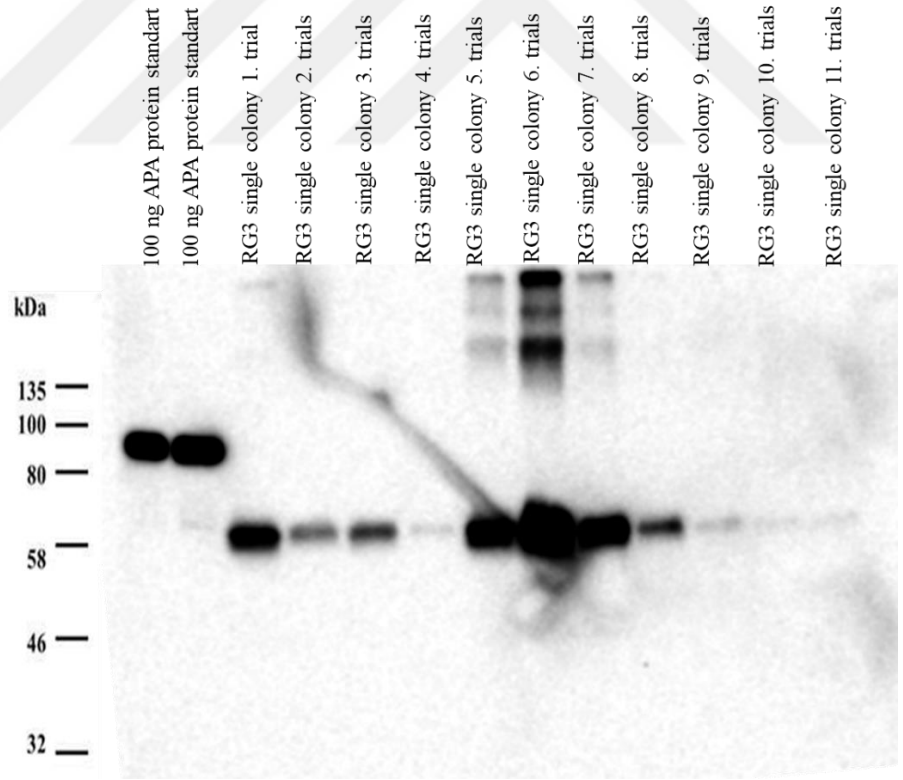


**Figure 4.1.** RG3 protein produced by *Nicotiana benthamiana* plants analyzed by Western blot.

The results were consistent with previous studies in our laboratory. Infiltration continued from plant groups with high expression.

### 4.2. Purification of Plant Produced RG3 Protein and Western Blot Analysis of Elution Fragments

Ni column affinity chromatography with Ni-NTA Resin was used to purify the RG3 protein produced in the plant. (Ni Sepharose 6 Fast Flow, cat#17-5318-02, Cytiva, Sweden). In the Western blot analysis that we performed; we combined the groups of plants that belonged to the bands where we thought the expression was high in 2 groups of 500 grams each. Due to the volume of the device and the column, we did all 1kg of plants in 2 times. Purification was performed using the device's appropriate protocol following the protocol detailed in the materials and methods section.

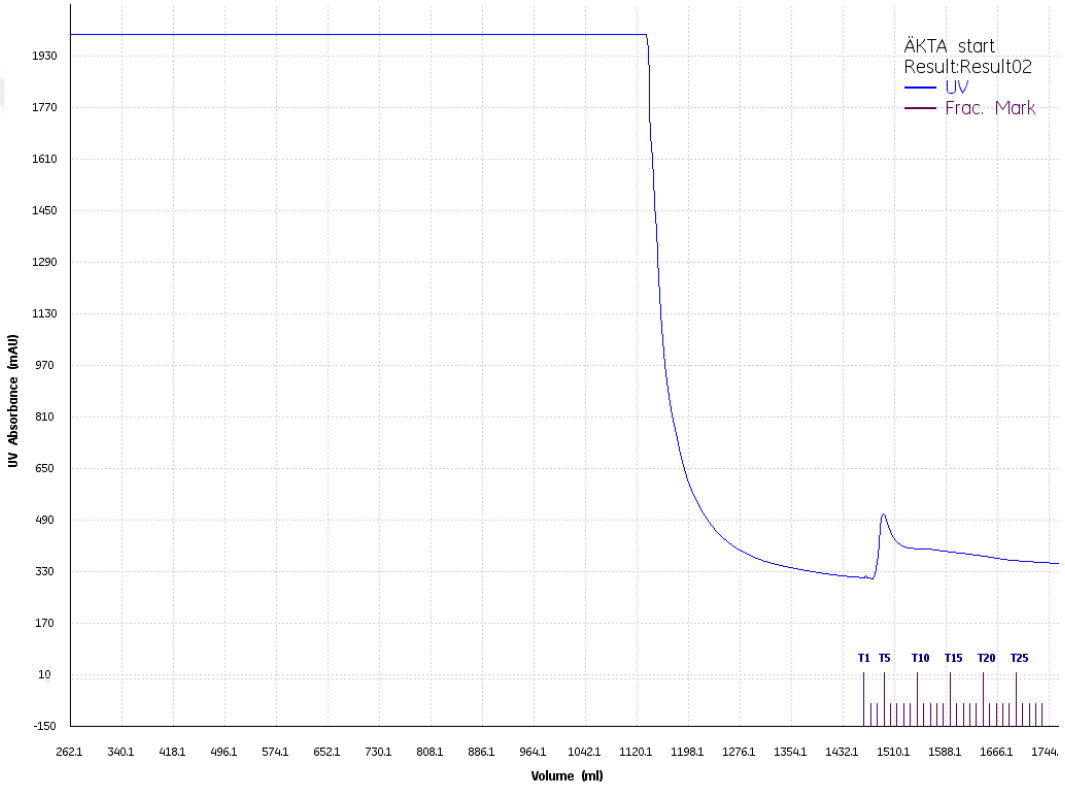


**Figure 4.2.** Chromatogram showing the purification profile of RG3 protein on ÄKTA start.

Guided by peak detection on the device, specific fractions corresponding to the elution peak were collected. The collected fractions were subjected to OD measurements employing the previously described protocol. Subsequently, fractions exhibiting high RG3 protein concentration and congruent elution profiles were pooled to generate a concentrated protein sample.

**Table 4.1.** Elution concentrations (µg/µl)

| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|
| 0,047 | 0,08 | 0,127 | 0,567 | 0,913 | 0,896 | 0,881 | 0,880 | 0,867 | 0,865 |
| **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
| 0,849 | 0,849 | 0,848 | 0,834 | 0,833 | 0,833 | 0,818 | 0,739 | 0,718 | 0,709 |
| **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** |
| 0,701 | 0,617 | 0,617 | 0,586 | 0,554 | 0,486 | 0,454 | 0,391 | 0,388 | 0,360 |

Utilized Western blot analysis to assess the expression levels of RG3 protein in the elution fractions that will be grouped according to their concentrations and results are shown in Figure 4.3.

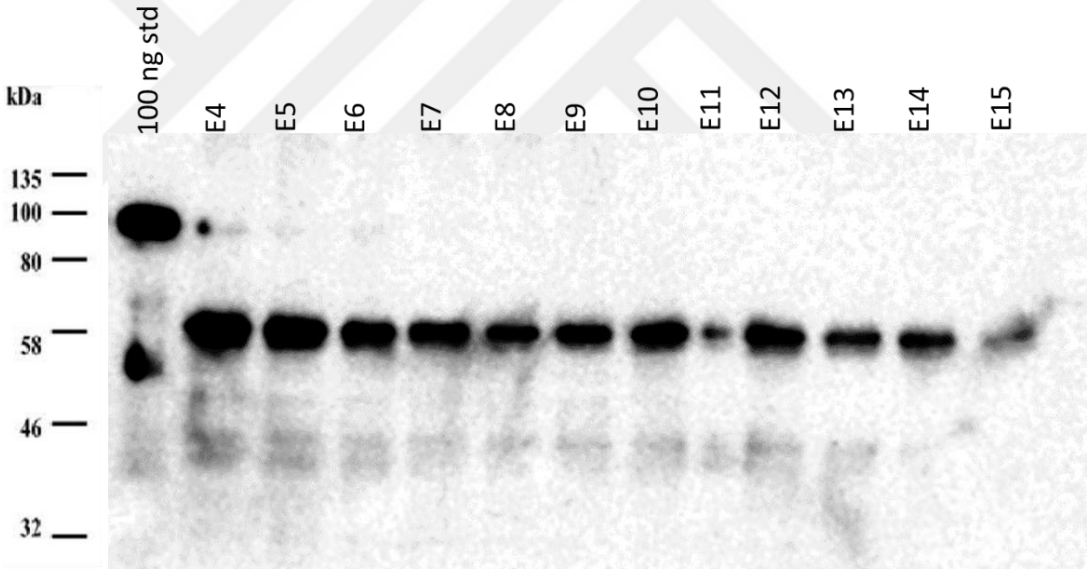


**Figure 4.3.** Confirmation of RG3 protein presence in high-concentration elution fragments using Western blot

The elution fractions were combined based on their concentrations and Western Blot results. The fractions were concentrated in 4 groups using a concentrator as previously described. In this way the imidazole was removed, and the volume reduced. The final concentrations obtained are the following.

RG3 pure proteins (combined fractions):

- RG3-1 = conc:3,300 µg/µl vol:1,2 ml
- RG3-2 = conc:2,388 µg/µl vol:3,2 ml
- RG3-3 = conc:3,154 µg/µl vol:3,4 ml
- RG3-4 = conc:3,812 µg/µl vol:8,8 ml

Using the concentrations and volumes obtained, we calculated the amount of protein available.

***amount of protein=concentration X volume***

- RG3-1 = 3,96 mg
- RG3-2 = 7,6416 mg
- RG3-3 = 10,7236 mg
- RG3-4 = 33,5456 mg

} **55,87 mg** pure proteins

As a result of the calculations, followed purification protocol enabled the isolation of 55,87 mg of pure protein from 1 kg of *N. benthamiana* leaves.

### 4.3. Characterization of Purified Plant-Produced RG3 Protein by SDS-PAGE and Western Blot Analysis

SDS-PAGE analysis was performed on the four groups generated based on the data obtained in the previous step. As previously mentioned in Material and Method, to monitor protein migration and purity, samples were electrophoresed on a 10% polyacrylamide gel and subsequently stained with Coomassie Brilliant Blue.

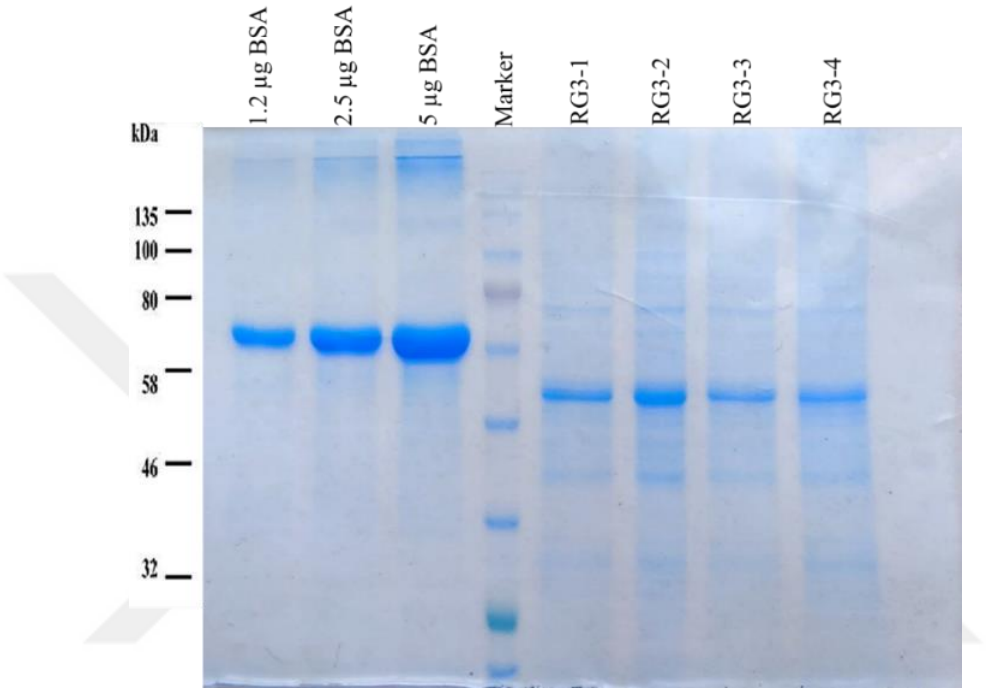


**Figure 4.4.** Confirming Purity and Expression Level of Plant-Derived RG3 Protein by SDS-PAGE

In the SDS-PAGE, Rubisco is observed at about 55kDa, while the RG3 protein is expected to be visualized at about 60kDa (Mamedov et al., 2019b). Rubisco contains half or more of all proteins in plant leaves and is the most plenty soluble protein in the chloroplast. The SDS-PAGE procedure we use contains β-mercaptoethanol, which separates proteins into subunits. β-mercaptoethanol is an effective agent for cleaving and reducing disulfide bonds, as well as denaturing the protein of interest.

According to the result of SDS-PAGE analysis, we could not distinguish this in this image, so we performed Western blot analysis again. Wash, flow through, TSP (total soluble protein) elutions collected during the purification phase; with the aim of verifying successful protein expression and evaluating purity, four purified protein fractions were analyzed by SDS-PAGE on a 10% polyacrylamide gel. As evident in Figure 4.4, distinct bands corresponding to the expected size confirm our successful protein isolation.

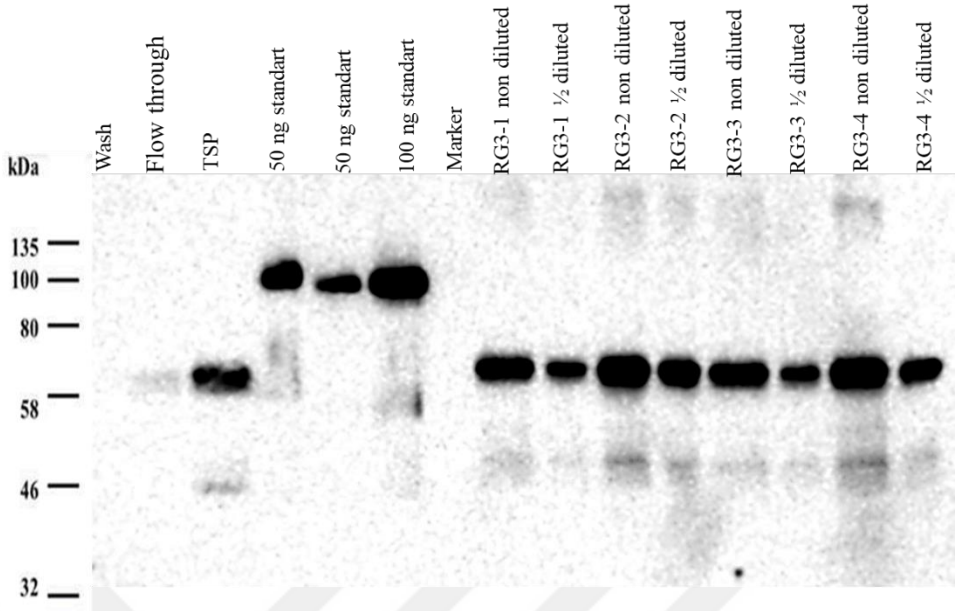


**Figure 4.5.** Analyzing Purity and Identity of Plant-Produced RG3 Protein using Western Blot

To ascertain the purity and specificity of RG3 protein purified from *Nicotiana benthamiana*, Western Blot analysis with a targeted antibody was employed. Estimations of band thickness then enabled protein quantifications.

*1µl extraction contains an estimated 10ng of proteins.

*1g leaf contains 3000µl extraction buffer = 30µg proteins

*1000 grams of leaves =3000ml= **30mg** proteins (estimated)

When the results of the concentration calculation were compared with the western blot bands, protein with a purity of **53,69%** was obtained (30mg / 55,87mg = 53,69%).

## 5. CONCLUSION

While existing rabies vaccines offer protection for both humans and animals, their widespread implementation is hindered by a combination of challenges: high cost, complex production processes, and suboptimal immunogenicity. Addressing these limitations through the development of novel rabies vaccines capable of large-scale, cost-effective production with enhanced safety and immunogenicity presents a critical unmet need in global public health.

The production of 55,87mg of RG3 protein with 53,69% purity from 1 kg of plant biomass in this study, although promising, highlights the need for optimization of purification procedures to realize the full potential of this protein as a cost-effective and efficient rabies vaccine candidate. Further research in this direction is warranted based on the immunogenic properties of RG3.

Research highlights the plant-based transient expression system as the most compelling option for recombinant protein production, particularly in the context of addressing the rabies pandemic. This system offers numerous advantages over traditional methods, including rapid production cycles, simplified processes, cost-efficiency, high yield capacity, and crucially, the absence of potential mammalian pathogens.

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# CURRICULUM VITAE (CV)

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| | |
|---|---|
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