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ENGINEERING, PRODUCTION, AND CHARACTERIZATION OF GLYCOSYLATED AND *in vivo* DEGLYCOSYLATED FORMS OF ANTI-PA83 IN *Nicotiana benthamiana*

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MASTER'S DEGREE THESIS

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Bu tez 14/07/2020 tarihinde jüri tarafından Oybirliği ile kabul edilmiştir.

Prof. Dr. Tarlan MAMEDOV (Danışman)

Prof. Dr. Nedim MUTLU

Doç. Dr. Muhammet TONGUÇ

ÖZET

GLİKOSİLE VE *in vivo* DEGLİKOSİLE EDİLMİŞ ANTİ-PA83 FORMLARININ *Nicotiana benthamiana* BİTKİSİNDE MÜHENDİSLİĞİ, ÜRETİMİ VE KARAKTERİZASYONU

Damla YÜKSEL

Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı

Danışman: Prof. Dr. Tarlan MAMEDOV

Haziran 2020 --- 42 sayfa

B. anthracis potansiyel bir ajan olarak kullanıldığından, güvenli, düşük maliyetli, yüksek etkili ve uzun süreli stabiliteye sahip bir şarbon aşısı geliştirebilmek çok önemlidir. Son yıllarda yapılan çalışmalar, bitki bazlı geçici ekspresyon sistemlerinin, yüksek ekspresyon kapasiteleri nedeniyle rekombinant proteinlerin güvenli, hızlı ve ucuz bir şekilde üretilmesini sağlayan umut verici bir teknoloji olduğunu göstermektedir. Monoklonal antikorlarla tedavi hastalığın etkilerini ve patojenin yayılmasını azaltmak için kullanılan kanıtlanmış bir yaklaşımdır. Tam uzunluktaki antikorların çoğu, rekombinant antikor üretme potansiyeline sahip olan memeli hücre kültürü, bakteri, maya, böcekler ve bitki ekspresyon sistemlerinde üretilir. Bitki ekspresyon sistemleri, patojenlere karşı pasif koruma sağlayabilen rekombinant terapötik mAb'ler üretmek için en etkili sistemlerden biridir. Bitki ekspresyon sistemleri, büyük ölçekli üretim, maliyet verimliliği, ölçeklenebilirlik ve güvenlik açısından faydalıdır. Şarbona karşı toksini nötralize etme kapasitesine sahip bitki monoklonal anti-PA83 antikoru, şarbona karşı güvenli, etkili ve düşük maliyetli aşı adayları geliştirmek önemlidir. Son çalışmalarda; bitki ekspresyon sistemi kullanılarak üretilen ve şarbon koruyucu antijene karşı etkili olan anti-PA83 monoklonal antikoru, mutasyon (Mett vd. 2011) ile glikosile ve deglikosile edilen bitkilerde üretildi. Bu tezde, anti-PA83 proteininin deglikosilasyonu için *in vivo* PNGase F (Mamedov vd. 2012) ve Endo H (Mamedov vd. 2017) deglikosilasyon stratejisi kullanılmıştır. Bu çalışmada, tam uzunlukta anti-PA83 proteinlerinin glikosile ve deglikosile formları, önceki çalışmalarda kodon optimize edilmiş ve Agrobacterium hücrelerine aktarılan HC ve LC genlerinin birlikte ekspres edilmesiyle üretilmiştir. Bu tez çalışmasında üretilen proteinler, amonyum sülfat ile çöktürülüp protein A kolon kromatografisi yardımıyla saflaştırılarak, antikor-antijen etkileşimini, protein stabilitesini ve antikorların toksin nötralize edebilme yeteneğini incelemek amacıyla kullanıldı. Tez sonuçlarına göre, Endo H ile deglikozile edilmiş anti-PA83, diğer anti-PA83 varyantlarından daha fazla stabilite ve aktivite göstermiş olup ve bu durum Endo H ile deglikozile edilmiş anti-PA83'nin iyi bir aşı adayı olarak kullanılabileceğini gösterir.

Anahtar Kelimeler: Bitkide Üretilmiş Monoklonal Antikor, Deglikosilasyon, Endo H, PNGase F, Geçici Ekspresyon, Şarbon Aşı Adayı

JÜRİ: Prof. Dr. Tarlan MAMEDOV

Prof. Dr. Nedim MUTLU

Doç. Dr. Muhammet TONGUÇ

ABSTRACT

ENGINEERING, PRODUCTION, AND CHARACTERIZATION OF GLYCOSYLATED AND *in vivo* DEGLYCOSYLATED FORMS OF ANTI-PA83 IN *Nicotiana benthamiana*

Damla YÜKSEL

Master's Degree Thesis, Department of Agricultural Biotechnology

Supervisor: Prof. Dr. Tarlan MAMEDOV

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Since *B. anthracis* has been used as a potential agent, it is crucial to be able to develop an anthrax vaccine that is safe, low-cost, high-effective, and long-term stability. Latest studies show that plant-based transient expression systems are a promising technology that allows safe, rapid and inexpensive production of recombinant proteins due to their high expression capacity. Monoclonal antibody treatment is used as one of the way for limiting the spread and effects of the pathogen. Most of the full-length antibodies are produced in mammalian cell culture, bacteria, yeast, insects, and plant expression systems that have potential to produce recombinant antibodies. Plant expression systems are one of the most effective systems to produce recombinant therapeutic mAbs that can provide passive protection against pathogens. Plant expression systems are beneficial in terms of large-scale production, cost-efficiency, scalability, and safety. The plant produced monoclonal anti-PA83 antibody, which is capable of neutralizing toxin against anthrax, is an important target to develop reliable, effective and cost-effective vaccine candidates against anthrax. In recent studies; anti-PA83 monoclonal antibody produced using plant expression system and effective against anthrax protective antigen was produced in plants glycosylated and deglycosylated by mutation (Mett et al. 2011). In this thesis, *in vivo* PNGase F (Mamedov et al. 2012) and Endo H (Mamedov et al. 2017) deglycosylation strategy was used for deglycolysis of anti-PA83 protein. In this study, glycosylated and deglycosylated forms of the full-length anti-PA83 proteins were produced by co-expressed with the HC and LC genes that were HC and LC genes which were codon optimized and transferred to Agrobacterium competent cells in previous studies. The produced proteins were used to examine the antibody-antigen interaction the protein stability and toxin neutralizing ability of antibodies. In results of thesis, Endo H deglycosylated anti-PA83 showed more stability and activity than other anti-PA83 variants and this indicates that Endo H deglysocylated anti-PA83 can be used as a good vaccine candidate.

Key Words: Anthrax Vaccine Candidate, Deglycosylation, Endo H, PNGase F, Plant-Produced Monoclonal Antibody, Transient Expression

JURY: Prof. Dr. Tarlan MAMEDOV

Prof. Dr. Nedim MUTLU

Doç. Dr. Muhammet TONGUÇ

PREFACE

In this thesis study, identification, production and stability studies of recombinant anti-PA83 protein that can be used as vaccine candidate for the treatment of anthrax have been performed.

Firstly; I would like to extend my thanks to my supervisor Prof. Dr. Tarlan MAMEDOV for their guidance and assistance from the determination of the thesis position.

In this project, I would like to thank Fatma AKÇAKALE for great help in my cell culture experiments. Also, I would like to extend my thanks to all the academic staff and graduate students of Akdeniz University Agricultural Biotechnology Department, which has made great contributions to my education life.

Throughout my experimental work, I would like to extend my thanks to Deniz SAY, İrem GÜRBÜZASLAN and all the other valuable students of our laboratory who support their technical assistance during my experimental studies, as well as providing spiritual support in all matters.

I offer my endless thanks and gratitude to my family and all my friends who never spared their support and love throughout my education and training life.

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BACKGROUND

ACADEMIC STATEMENT

I declare that this study titled “Engineering, Production and Characterization of Glycosylated and *in vivo* Deglikosile anti-PA83 Forms in *N. benthamiana*” 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.

……./……../20....

Damla YÜKSEL

SYMBOLS AND ABBREVIATIONS

Symbols

ºC : Degree Celsius

g : Gram

h : Hour

kDa : Kilodalton

kg : Kilogram

l : Liter

m : Month

µg : Microgram

µl : Microliter

mg : Milligram

ml : Milliliter

mM : Millimolar

min : Minute

M : Molar

w : Week

Abbreviations

anti-PA83 : Antibody for protective antigen (83 kDa)

dG-anti-PA83-E : Endo H deglycosylated anti-PA83

dG-anti-PA83-P : PNGase F deglycosylated anti-PA83

ddH_2O : Double distilled water

dpi : Day post infiltration

EF : Edema factor

Endo H : Endo-β-N-acetylglucosaminidase

ET : Edema toxin

Fab : Fragment for antigen binding

Fc : Fragment for constant region

FDA : Food and Drug Administration

HC : Heavy chain

IgG : Immunoglobulin G

LC : Light chain

LF : Lethal factor

LT : Lethal toxin

mAb : Monoclonal antibody

OD : Optical density

PA83 : Protective antigen (83 kDa)

PNGase F : Peptide-N-glycosidase F

PTM : Post-translational modification

SDS-PAGE : Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

TNA : Toxin neutralization assay

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1. INTRODUCTION

Anthrax disease is an animal-borne disease usually seen in herbivore animals. Anthrax disease caused by *Bacillus anthracis* is delivered to humans by contact with infected animals or by consuming food from these animals. This disease is quite likely to result in death. In the world, it is seen in Asia, Mediterranean countries, Africa and South America.

Anthrax disease was attracted attention with its use as a tool of bioterrorism in 2001 events in the USA. This disease is thought to occur as a result of laboratory studies and is used as a biological weapon.

The primary method used in the treatment of anthrax disease is antibiotic therapy. While antibiotics could destroy anthrax bacteria, the resistance of this bacterium to antibiotics has become insufficient to therapy. This situation encouraged the finding of new methods in the treatment of the disease. The most important way to treat this disease is immune gain through vaccination. Anthrax Protective Antigen, 83 kDa protein, is a preferred protein in immunization of anthrax. Proteolytic cleavage of this protein initiates pathogenicity by absorbing other anthrax endotoxin fragments into the cell. Although protein-based recombinant vaccines are generally concentrated on Anthrax Protective Antigen (PA), anthrax disease can also be treated with other action mechanisms of component that play a role in pathogenicity.

Monoclonal antibodies against antigen containing vaccines is one of alternative way in the anthrax treatment. While anthrax disease can be treated in protein-based vaccines containing toxins, it is an important method for pos-exposure treatment in recombinantly produced monoclonal antibodies.

In this study, the production of a recombinant monoclonal anti-PA83 form against the protective antigen (PA83) protein used in vaccine development against anthrax in *N. benthamiana* plant is aimed. The anti-PA83 protein is a member of an IgG family and is glycoprotein.

An important point in the development of recombinant proteins is to produce functional protein. Expression systems that are *E. coli*, yeast, plant, mammal and so on suitable to produce these functional recombinant proteins. In this study, plant expression system was used to generate recombinant anti-PA83 proteins. Plants are an important host system to produce vaccine antigens, antibodies, therapeutic proteins and others. Plant expression systems have advantages such as being reliable in terms of contamination of animal pathogens, high production capacity and relatively low cost.

Plant expression systems have an advantage in terms of post-translational modifications, but they have a disadvantage because they have a different N-glycosylation mechanism than mammals. Glycan differences in natural and plant produced forms can lead to conformational change in protein's molecular shape , leading to a alter in biological activity. These changes affect the function of the protein and the toxin neutralizing feature of anti-PA83 protein due to its glycoprotein property. Many studies have been conducted in the literature to prevent the effects of this aberrant glycosylation.

Mett et al. (2011) produced the deglycosylated form of the anti-PA83 protein by mutating the glycosylation site in the heavy chain (HC) of the anti-PA83 protein in order to prevent its aberrant glycolysis in the plant (Mett et al. 2011). He reported that this deglycosylated form produced by mutation provides complete protection against anthrax spores.

Another study took place in our laboratory. Mamedov et al developed PNGase F and Endo H *in vivo* deglycosylation strategies to prevent aberrant glycolysis (Mamedov et al. 2012 ; 2017). In these studies, deglycosylated forms of the recombinant PA83 protein, considered a promising vaccine candidate, were produced using in vivo deglycosylation technology (Mamedov et al. 2017).

Within the scope of this thesis; to produce glycosylated and deglycosylated forms of the antiPA83 protein, the heavy and light chain genes responsible for the expression of the anti-PA83 protein were produced by the Agroinfiltration in the *N. benthamiana* plant , resulting in a glycosylated form. In order to produce deglycosylated forms, target genes were produced co-expressed with Endo H and PNGase F genes and stability tests were performed to see the effect of glycolysis. As a result of these thesis studies, it was concluded that the glycosylated and deglycosylated forms of the anti-PA83 protein are found as functionally active and stable.

2. LITERATURE

2.1. Anthrax Disease and Clinical Forms

Anthrax is a infectious disease caused by *Bacillus anthracis* which directly or indirectly affects humans from animals and / or products of animal origin (Wang, J. Y. and Roehrl, M. H. 2005). The causative agent of anthrax is *Bacillus anthracis* that is a Gram-positive, rod-shaped, spore-forming bacterium (Hull A.K. et al. 2005). The natural hosts of anthrax spores are herbivores such as livestock. Humans are infected by the causative agent when they contact with infected herbivores or eat their products (Oncu et al. 2003).

Anthrax disease is happened naturally in both animals and humans and still exists in many parts of the world (Wang and Roehrl 2005). The most affected region in the world from anthrax is West Africa, while Central America, Central Asia, Greece, Turkey and the Middle East are also dramatically affected from anthrax (Oncu et al. 2003).

Anthrax spores naturally found in soil are highly resistant to heat, ultraviolet light, gamma irradiation and many disinfectants. Anthrax spores are about 1-2 millimeters in diameter, which allows them to inhale in the alveolar areas (Wang and Roehrl 2005). These small and durable spores can be easily dispersed and aerosolized into large populations by bombs, missiles, and planes and these resistant spores remain infectious for years. For this reason, *B. anthracis* is classified as Category A biological warfare agent by The Centers for Disease Control and Prevention of the USA (CDC) (Oncu et al. 2003).

The anthrax, which has existed as a biological warfare agent since the second world war, makes it a good biological weapon with high lethality, accessibility, low visibility, ease of production and dissemination (Kamal et al. 2011). Cases of anthrax spread by natural inhalation are not common before the attack in 2001. However, anthrax spores, which were accidentally released from a military lab in 1979, killed 64 people. In 2001, distribution of anthrax spores in the letter cause inhalational and cutaneous anthrax in the United States (Turnbull P.C.B. 2002).

Anthrax infection shows three common types, each with different signs and symptoms depending on the route of transmission: cutaneous, gastrointestinal and inhalation as seen Figure 2.1. Cutaneous anthrax constitutes 95% of human anthrax, while about 5% of disease cases are inhalational anthrax. This severe disease, transmitted by inhalation of aerosolized spores, has a mortality rate of about 86-89%. (Hull et al. 2005).

Cutaneous anthrax is the most common form, making up 95% of naturally occurring anthrax cases. Microbes found in infected animals and their products enter the body through itching, scratches and cuts in the skin. Arm, hand, head or neck are the most commonly affected anthrax lesions. After exposure, infection usually develops within 1 to 7 days. Nowadays, cutaneous anthrax can be treated with dressing and

antibiotics. Mortality rate of cutaneous anthrax is approximately 20% in untreated cases (Kamal et al. 2011).

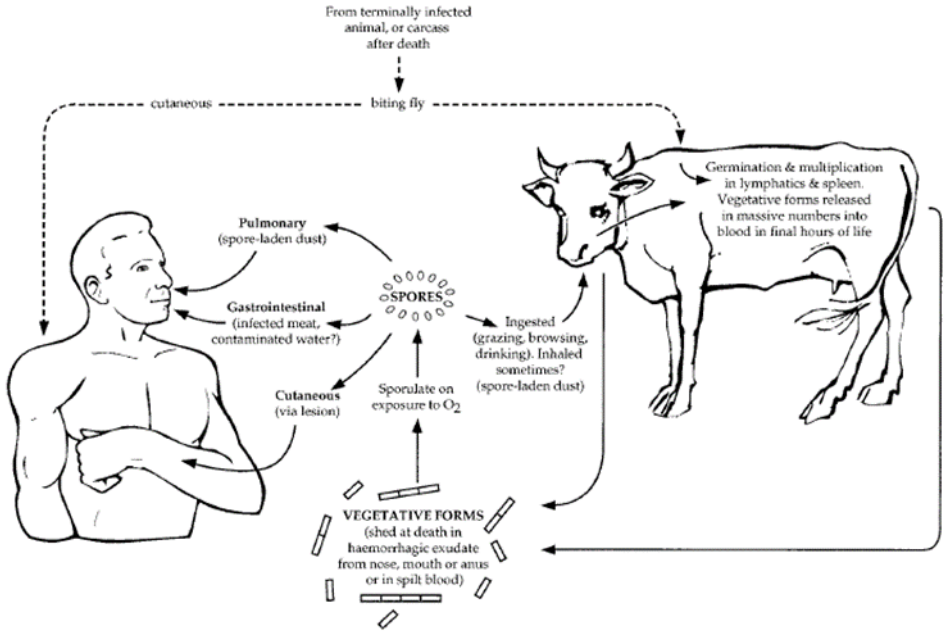


Figure 2.1. Infection cycle of the anthrax disease. In the infected host, spores produce vegetative forms that multiply and eventually kill the host. Some of these vegetative forms that are killed or released into the environment by the dead animal become ready to be taken by another animal and cause severe illness. (Collier and Young 2003)

Gastrointestinal anthrax, usually seen in developing countries, results from ingestion of contaminated meat that is infected with many bacilli or spores. Infection usually develops from 1 to 7 days after exposure. The points of entry to the submucous occur especially in the oropharynx and ileocecal region. It usually causes digestive system bleeding. In intestinal anthrax, mortality rate is 25% to 60% and may even reach 100% (WHO 2008).

Inhalational anthrax caused by inhalation of pathogenic endospores is the deadliest anthrax form. The US Department of Defense considers that the lethal dose for humans is about 8,000-10,000 spores. Anthrax spores are accumulated in the alveolar areas and ingested by macrophages. Spores transported to lymph nodes with infected macrophages then sprout into vegetative bacilli. Bacilli escaping from macrophages spread into the bloodstream and release a high level of exotoxin, which poison the host. After inhaling many spores, patients often complain of a disease with signs of upper respiratory tract infection. The infection, which usually develops within a week after exposure, may gradually worsen its symptoms and may last up to 2 months. The symptoms of this disease are rapidly progressing fever, cough, shortness of breath and so on. The patient usually dies of toxemia and respiratory failure within 24 hours of the onset of this second stage. Despite supportive treatment, including appropriate antibiotics, the mortality is very high (Wang and Roehrl 2005).

2.2. Pathogenesis of Anthrax

B. anthracis, Gram-positive bacterium with rod-shape, is immobile and can also be aerobic or facultative anaerobic (Figure 2.2). Vegetative cells, usually found in long chains in vitro, can also be present as single or short chains. (Kamal et al. 2011).

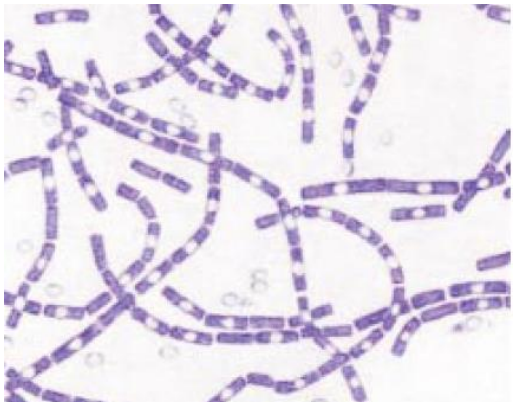


Figure 2.2. Microscopic image of *B. anthracis*. Causative agent of anthrax is a rod-shaped gram-positive bacterium (Kamal et al. 2011)

In inhalational anthrax, anthrax spores are phagocytosed by macrophages into the host and wherein the spores germinate into vegetative bacteria. Anthrax toxin, triple protein toxin, and the poly-D-glutamic acid capsule that protects the bacteria from phagocytosis by host neutrophils, are correlated to the lethality of anthrax (Hull et al. 2005). The expression of these virulence factors is regulated by host-specific factors, and the optimal conditions for their expression should be at 37ºC and a concentration of CO_2 of more than 5% (Wang and Roehrl 2005).

Two extrachromosomal plasmids as pXO1 and pXO2 exist in virulent strains of B. anthracis. pXO2 encodes poly-D-glutamic acid (PGA) capsule that is the anionic and poorly immunogenic polypeptide. Its negative charges provide the inhibition of bactericidal activity of the host so that it inhibits the phagocytosis of vegetative bacterium and cause the highly lethal infectious disease (Jamie 2002). pXO1 encodes the three partitate exotoxin complex. This exotoxin consists of protective antigen (PA) and two enzymatically active subunits: lethal factor and edema factor. PA is the non-toxic cell-binding component and functions as a cell-binding receptor for LF and EF to form lethal toxin (LeTx= PA+LF) and edema toxin (ET = PA+EF) (Peterson et al. 2006). After binding to the cell surface, PA is proteotically cleaved by furin as a cell protease into two fragments as 63-kDa protein fragment (PA63) and 20-kDa protein fragment (PA20). PA63 formed by proteolytic cleavage of PA83 is heptamerized for formation of pre-pore structure. Heptamerization creates the binding sites for LF and EF so that heptamerized PA mediates the cellular uptake of LF and EF. The membrane-bound exotoxin is transported into the host cell by receptor-mediated endocytosis. Acidic environment into the endosome cause the release of the LF and EF from the heptamer. EF and LF show their respective biological effects by entry into the cytosol (Chen et al. 2011).

Once LF and EF inside the cell, the immune responses of the cell are disturbed because of their effects on signal transduction pathways as shown Figure 2.3. EF is a calmodulin-dependent adenylate cyclase that converts ATP to the cyclic AMP. Edema toxin, the combination of EF with PA, cause increasing cellular amount of cyclic AMP and leads to the accumulation of fluids between cells in surrounding tissue called edema (Wang and Roehrl 2005). LF,a zinc metalloprotease, primarily acts on macrophages. LF causes inactivation of cell signalling by causing inactivation of members of the MAPKK (Mitogen Activated Protein Kinase Kinase) family. When LF and PA form a complex, lethal toxin increase the production of cytokines and this overproduction leads to the macrophage lysis (Kamal et al. 2011). Lethal factor rapidly blocks the signals and it causes hypotension, and shock. Side effects of edema toxin and lethal toxin trigger cells to apoptosis, causing cell death. (WHO 2008).

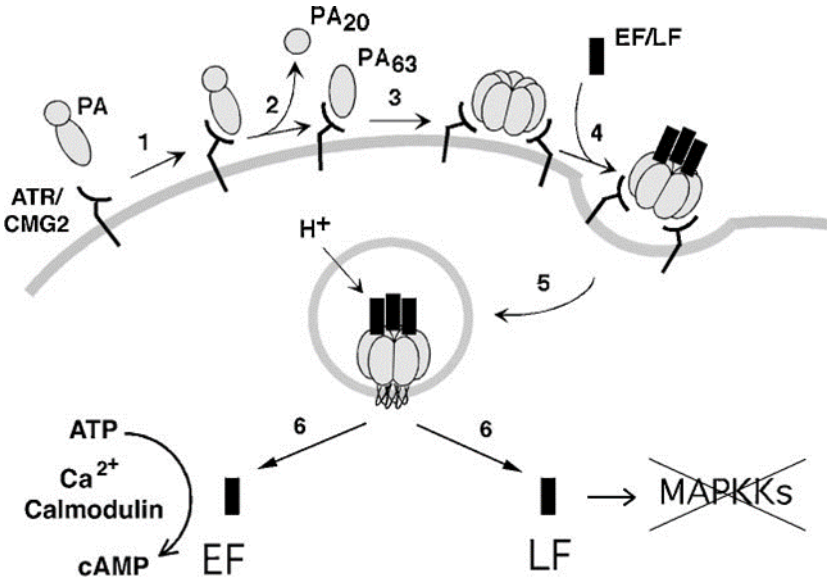


Figure 2.3 The virulence mechanism of anthrax. The protective antigen (PA) secreted by Bacillus anthracis binds to the cellular receptor for anthrax toxin. Proteolytic cleavage of PA causes the formation of a pore for EF and LF to enter the cell, leading to edema and cell death (Collier and Young 2003)

2.3. Anthrax Treatment

B. anthracis spores infect wild and domestic animals and humans by skin wounds, ingestion or inhalation, as well as affecting (Chen et al. 2011).There are several ways to protect from anthrax as vaccination, antibiotics, anti-toxin therapy and so on. For post-exposure prophylaxis, continuous protection is provided with a combination of antibiotics and vaccination (Wang and Roehrl 2005). Currently, the three-dose vaccination with antibiotic administration is defined as post-exposure therapy by the Food and Drug Administration. Additionally, passive immunization using a polyclonal or monoclonal antibody product can provide significant clinical benefit (Chen et al. 2011).

Antibiotic treatment used in anthrax is usually carried out by using antibiotic types that B. anthracis is sensitive to penicillin, ampicillin, doxycycline, ciproflaxin, streptomycin, clindamycin and other many antibiotics. Ciprofloxacin was the first identified antibiotic for use after anthrax exposure by the FDA (Jamie W. E. 2002). Cutaneous anthrax, the most moderate type of anthrax, can be treated with common antibiotics as penicillin, a tetracycline, ciproflaxin or another antibiotic. For post-exposure treatment of inhalation anthrax, which can be treated with high-dose antibiotic therapy, four antibiotics with doxycycline, ciprofloxacin, levofloxacin and penicillin G have been proposed by the FDA (Wang and Roehrl 2005).

In anthrax disease, vaccine protection is one of the most appropriate forms of treatment. While first animal vaccine is found in 1881, human vaccines began to be developed in the mid-twentieth century. Vaccines for anthrax treatment , which emerged with the use of anthrax spores as biological agents, began to be developed for human use in the 1940s. In 1970, a cell-free subunit vaccine called "adsorbed" (AVA) or Biothrax, prepared from microaerophilic cultures of B. anthracis's weakened, unencapsulated strain, V770-NP1-R, was the only FDA-licensed single human anthrax vaccine in the United States. This vaccine is recommended for use by people at risk of occupational anthrax, such as farm workers, veterinarians and so on. (WHO 2008).

One of the biggest obstacles to using AVA vaccine is that although its main preservative is PA, it also contains other anthrax proteins, EF and LF. In this case, the purity of the vaccine can cause allergic conditions and death. In addition, existing PA-based vaccines require at least 4 weeks for repeated application and development of anti-PA protective titers. Short incubation time and rapid advancement of anthrax disease reduce the possibility of vaccination to protect. For these reasons, other improved methods are needed in the treatment inhalation anthrax (Friedlander et al. 1999).

Another approach used in anthrax treatment is human monoclonal antibodies (mAb) that are specific to PA or LF toxin and can neutralize the toxin. Passive immunization with antibodies represents an attractive option for post-exposure treatment, with comprehensive protection. (Yusibov et al. 2016). Polyclonal or monoclonal PA IgG antibodies provide protection to mice, rabbits, rats, guinea pigs, and monkeys challenged with B. anthracis by intracutaneously or aerosolization (Leppla et al. 2002).

Passive immunization with polyclonal antibodies against PA is one of way for anthrax treatment (Yusibov et al. 2016). Many animal studies have shown that vaccines prepared with anthrax toxin-specific antibodies provide passive protection in animals. One alternative approach is production of toxin-neutralizing human monoclonal antibodies (mAbs) with unique to either PA or LF, or both (Figure 2.4) (Hull et al. 2005).

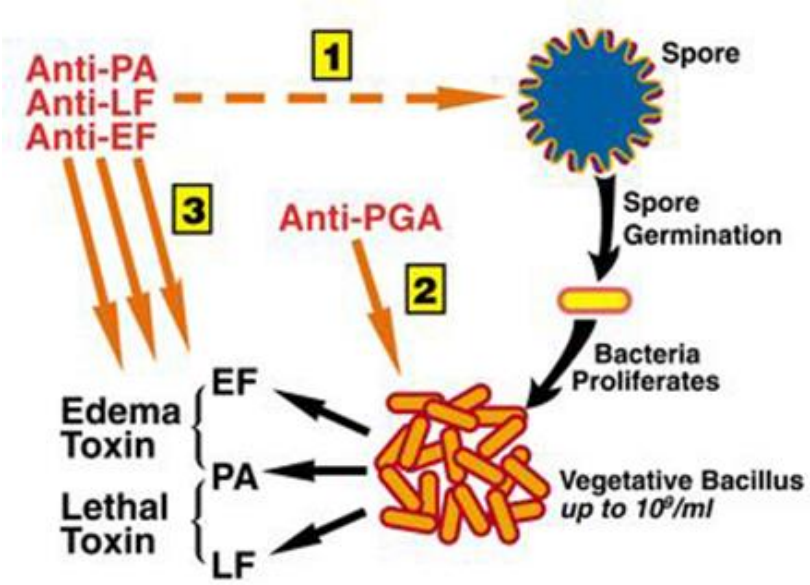


Figure 2.4. Inhibition scheme of anthrax toxins by monoclonal antibodies. Protection against the disease can be provided by a combination of mAbs specific to each of the virulence factors directly affecting the infection (Chen et al. 2011) (Chen et al. 2011)

Monoclonal antibodies can reduce the effects of the disease by various mechanisms, such asb inhibition of the toxin activity, restriction of infection spread etc. (Yusibov et al. 2016). These antibodies neutralize the PA by blocking proteolytic cleavage PA by furin, by inhibition of PA heptamerization, by interfering with association of the active subunits with PA, by inhibition of receptor binding and so on (Chen et al. 2011). The PA-based anthrax vaccine causes a protective immune response and provide immediate, specific and low toxicity protection. (Wang and Roehrl 2005).

2.4. Antibodies and Their Properties

Anthrax antibodies that are immunoglobulins are glycoprotein molecules capable of specific combinations with the causative antigens for anthrax (Peterson et al. 2006). Antibodies for anthrax are usually in the structure of immunoglobulin G (IgG) (Peterson et al. 2006).

IgG for anthrax are Y-shaped molecules that consists of four polypeptide chains as two identical Heavy chain (HC) and two Light chain (LC) as shown in Figure 2.5. Molecular weight of HC is 50 kDa and molecular weight of the LC is 25 kDa so that total molecular weight of one complete immunoglobulin is 150 kDa (ThermoFisher Scientific).

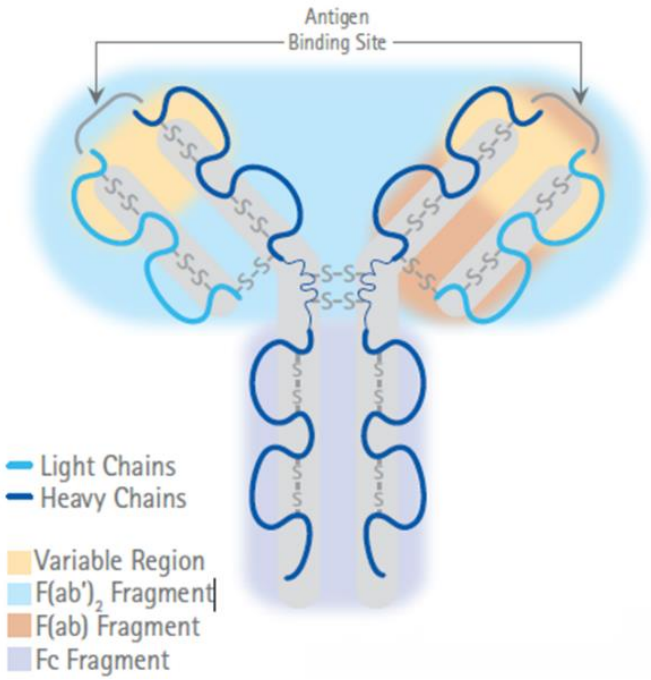


Figure 2.5. Illustration of Immunoglobulin G (IgG) structure (EMD Millipore 2014)

In complete antibody molecule, HC and LC are hold together by disulfide bonds. Two of interchain disulfide bonds are located between two HC at hinge region that is the flexible domain of antibody binding Fab to Fc portion. These disulfide bonds are responsible for the stabilization of domains on the chains (Wang et al. 2007). The Fab fragment of the antibody, formed with the variable regions of HC and LC, is responsible for antigen recognition and binding so that each antibody monomer contains two antigen binding sites. The Fc fragment representing the constant region of HC is responsible for the biological function of antibodies (Elgert 2009).

Glycosylation is important for antibody antigenicity and function. Glycosylation site located in the constant region of HC provides binding of glycans to antibodies. N-linked oligosaccharide chain attach on conserved asparagine residues at position 297 in CH2 domain. This oligosaccharide is generally found as fucosylated form in antibodies produced in hybridoma cells or CHO cell lines (Wang et al. 2007).

Antibodies show variety between classes in terms of charge, size, function and antigenicity (Elgert 2009). Production and structural differences are main criteria for antibodies classification such as their production as monoclonal and polyclonal and their structural differences as immunoglobulins. (ThermoFisher Scientific).

Some B-lymphocytes secrete only one specific antibody molecule when the immunological response is homogenous to antigen. This antibody type is named as monoclonal (mAb) antibody (EMD Millipore 2014). Different B-lymphocyte cell lines produce heterogeneous antibodies for same antigen during immune response. All these cells can recognize the same antigen epitope. Antibodies named as polyclonal

antibodies are purified from the heterogeneous sample of antigen-binding immunoglobulin (EMD Millipore 2014).

Monoclonal antibodies have great specificity for antigen binding. Polyclonal antibodies recognize multiple epitopes of the antigen so they can tolerate even minor changes in the nature of the antigen (EMD Millipore 2014).

2.5. Recombinant Antibody Production

A new generation of vaccines based on highly purified recombinant PA and anti-PA83 currently being developed and evaluated. Numerous attempts have been made to establish high-level PA expression systems based on viruses, E. coli, insects, mammalian cell lines as human embryonic kidney cells, hybridoma cells and plant expression systems with the potential to produce recombinant antibodies (Fischer et al. 2003). Mammalian expression system has variety of post-translational modifications so that they have capacity for protein production with all required PTMs. These PTMs, proper folding, and secretion provides the antibody production with less immunogenicity. However, mammalian expression system has risk for safety. Host-cell derived pathogens and oncogenic DNA sequences cause the contamination of antibodies (Mett et al. 2008).

2.5.1. Plant-based transient expression system

Plants are the preferred systems to manufacture of recombinant monoclonal antibodies and antibody fragments in terms of large scale production, cost efficiency and safety. Plant expression systems are effective systems to produce functional recombinant therapeutic mAbs that can provide passive protection against pathogens in animals (Peeters et al. 2001) These recombinant antibodies are produced as properly folded with all necessary PTMs. But they have risk for safety. Antibodies can be contaminated with human pathogens during their synthesis in mammalian expression systems, but plant expression system does not have any risk for human pathogens (Mett et al. 2008).

Recombinant antibodies are produced in plants either transient or permanent expression. Transiently produced genes show maximum level at 2-4 days after infection of plant tissues, and then a decrease in expression level occurs. Stable expression is carried out by the integration of T-DNA carrying the gene to be expressed in the host genome, and the production genes increases 10-14 days after infection of plant tissues (Krenek et al. 2015). Transient gene expression has a number of advantages compared to stable transformation, such as time efficiency, high expression level in a short time, scalability and so on. Transcriptionally active target gene including vector for target antibody is introduced into plant tissues by using particle bombardment, viral vector infection or Agroinfiltration that is the way of the introducing target gene carrying Agrobacterium vectors into tobacco leaf tissues (Mett et al. 2008). Tobacco and N. benthamiana leaf are widely used for simplicity of agroinfiltration and high conversion efficiency (Krenek et al. 2015).

Indirect transformation in plants is the method of transporting the desired gene to the target cell via bacteria that contain plasmids such as Agrobacterium tumefaciens

and Agrobacterium rhizogenes. The pathogenicity of Agrobacterium is used in genetic plant transformation, and the plasmid allows the foreign gene to be transferred to the plant cell. A. tumefaciens mediated gene transfer consists of several steps, including activation of the infection-causing system in bacteria, T-DNA transfer and the integration of T-DNA into the plant genome (Tzfira and Citovsky 2006).

Currently, temporary transformation using Agrobacterium tumefaciens is the preferred method for protein production due to its safe, high level and rapid expression. Generally, agrobacterium containing the gene that is transmitted through electroporation are introduced into extracellular leaf spaces by physical or vacuum infiltration, a process known as agroinfiltration (Norkunas et al. 2018). Agrobacterium tumefaciens Mediated transient expression utilizes infiltration of plant spaces with bacterial suspension (Rybicki 2010). Agroinfiltration is based on the infiltration of the recombinant Agrobacterium clone, which carries the target gene to the leaf area by syringe. The method uses the gene carried by the Agrobacterium strain AGL1 (Du et al. 2014). The infiltration preparation involves the growth of Agrobacterium in nutrient-rich media and subsequent resuspension of the pellet cells in the MMA medium for infiltration of target genes. Agrobacterium-mediated transient transformation may elicit necrotic responses in tissues as a result of plant defense responses (Krenek et al. 2015).

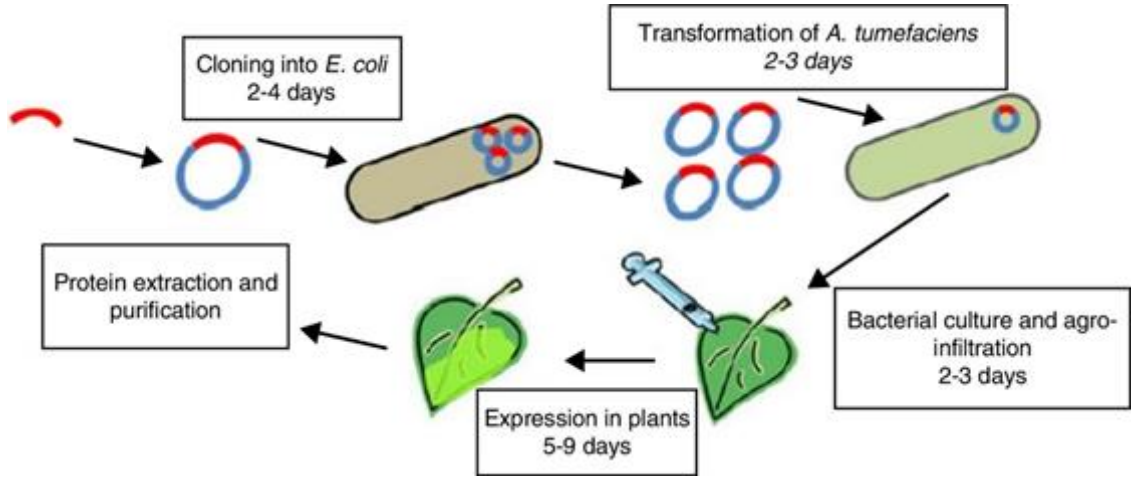


Figure 2.6. Overview of agroinfiltration

Two binary expression vectors are required as one for HC and one for LC to produce full-length antibodies by agroinfiltration in plants. These two vectors are co-infiltrated into plant leaves by using Agroinfiltration. Co-expression of HC and LC genes into plant leaves is resulted as proper assembly of full-length functional antibody with correct glycosylation (Sarrion-Perdigones et al. 2011). Full length antibodies undergo glycosylation to become active and they must be targeted to secretory pathway for glycosylation. For this purpose, protein synthesis can be optimized for high yield and secretion by adding signal sequences. N-terminal signal sequence is added for targeting to secretory pathway whereas C-terminal peptide signal (KDEL) for retrieving to Golgi apparatus (Fischer et al. 2003).

2.5.2. Glycosylation

Glycosylation is covalent binding of an oligosaccharide to protein (Blom et al. 2004). Glycosylation strongly affects the physicochemical properties of a protein such as specific activity, ligand-receptor interaction, and immunogenicity (Yusibov et al. 2016).

N-linked and O-linked glycosylation are most common glycosylation types (Blom et al. 2004). O-linked glycosylation usually occurs in Golgi apparatus by adding carbohydrates to the hydroxyl group (OH) of serine and threonine amino acid (Blom et al. 2004). N-linked glycosylation occurs between a specific carbohydrate group and the amino (NH2) group of asparagine (Asn) amino acid located on the consensus sequence as Asn-X-Ser-The motif and (Schwarz and Aebi 2011). N-linked glycosylation is performed in ER by enzymes called oligosaccharyl transferase (Yusibov, et al. 2016).

N-linked glycosylation is essential for many biological processes as correct folding, protein-protein interaction, stability and physiological activity of many proteins including recombinant proteins (Sarrion-Perdigones et al. 2011). N-glycosylation of proteins in plant cells begins by transferring the oligosaccharide precursor to the specific Asn residue (Asn-X-Ser / Thr) to form the immature protein. This immature glycoprotein is then transported along the secretion pathway that include ER and Golgi apparatus and subjected to some maturation processes involving steps such as removing glucose and mannose residues to produce high mannose containing N-glycans (Gomord et al. 2010). N-linked glycosylation mechanism of plants and animals share similarity, but they are still different. The core glycan structure of glycoproteins is conserved between plants and animals whereas terminal groups are different from to each other. Plant-derived glycoproteins include α-1,3-fucose and β-1,2-xylose residue but humans include β-1,4-galactose and sialic acid residue (Yusibov et al. 2016).

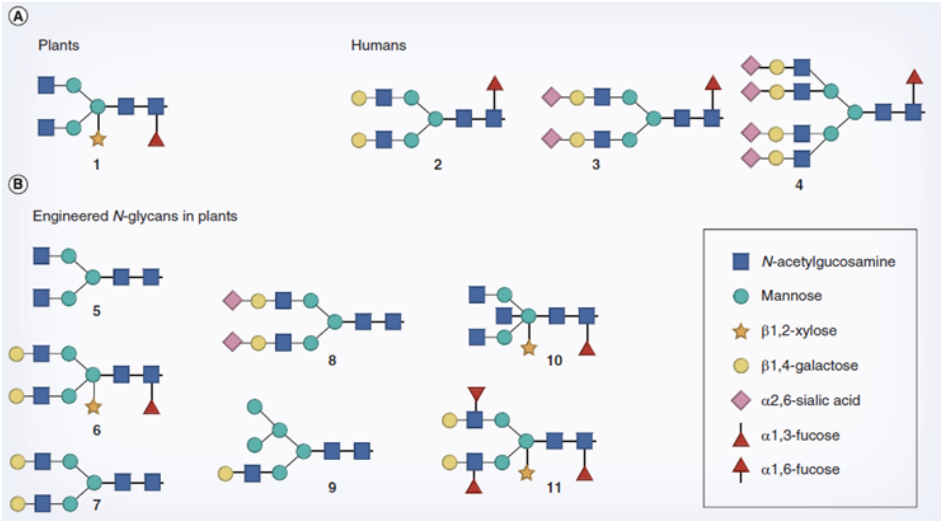


Figure 2.7. Schematic representation of N-glycan structures in plants and humans. a) Some typical types of N-glycans that can be found in plants and humans. Plant N-glycans contain β1,2-xylose and α1,3 fucose residues, while human N-glycans β1,4-galactose and sialic acid. b) Some N-glycan structures produced in plants by glycoengineering (Bosch and Schots 2010)

Bacterial proteins and some eukaryotic proteins may not contain N-glycans in the natural host, but because these proteins may contain more than one potential glycosylation site, they may be exposed to abnormal glycosylation when expressed in eukaryotic expression systems. For example, Plasmodium falciparum's Pfs48/45 protein, and Bacillus anthracis protective protein (protective antigen) does not carry N-linked glycans, and so they are not a glycoprotein in the nature (Mamedov et al. 2017). However, these proteins contain potential N-linked glycosylation sites, and these regions may undergo unwanted abnormal glycosylation during their expression in yeast, mammalian or plant systems. Differences in the glycan structure relative to the natural systems of proteins expressed in a heterologous system can potentially alter the molecular integrity of the protein, leading to a decrease in biological activity (Mett et al. 2011).

Various strategies avoid plant-specific N-glycosylation in order to overcome its potential effects on the protein. Mamedov et al. (2016; 2017) developed a strategy in the plant to produce target proteins without glycosylation. This strategy is based on transient gene expression through co-expression of the respective target gene and bacterial deglycosylation enzymes peptide-N-glycosidase F (PNGase F) or Endo-β-N-acetylglucosaminidase (Endo H) enzymes (Mamedov et al. 2016; 2017).

PNGase F is a 34.8 kDa enzyme that breaks the bond between the N-Acetyl-D-glucosamine (GlcNAc) and asparagine residue, secreted by the gram-negative bacteria, *Flavobacterium meningosepticum*. (Mamedov et al. 2012). PNGase F is a glycoamidase. When it cuts the link between GlcNAc and Asn and releases the entire sugar chain, an amino acid change occurs in the glycosylation site (N-X-S / T) of the deglycosylated protein due to the deamidation of asparagine to aspartate (Mamedov et al. 2017). Another enzyme, Endo-β-N-acetylglucosaminidase H (Endo H), cuts the bond between two GlcNAc residues in the inner region of the high mannose non-complex N-linked glycan chain (Wang et al. 2015). When EndoH removes the glycan, single N-Acetyl-D-glucosamine residue is still attached to the protein without the concomitant deamidation of asparagine (Gomord et al. 2010).

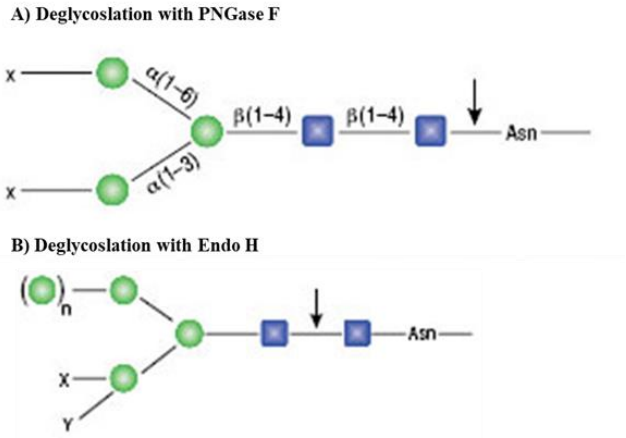


Figure 2.8. Illustration of deglycosylation of N-linked glycans. a) PNGase F cleavage occurs between N-Acetyl-D-glucosamine (GlcNAc) and asparagine residue. b) Endo H cuts the bond between two GlcNAc residues (Anonymous 1,2)

The *in vivo* enzymatic deglycosylation strategy has the potential to become a powerful strategy to produce glycan-free target proteins. In the literature, Mamedov et al. shows that PNGase F deglycosylated proteins showed more instability than Endo H deglycosylated proteins due to deamidation of the amino acids (Mamedov et al. 2016). Previous studies showed that the properties (activity, immunogenicity, etc.) of proteins produced by *in vivo* deglycosylation are different from their in vitro deglycosylated partners as more stable and provide a strong antibody activation (Mamedov et al. 2017).

2.6. Current studies about anti-PA83

Today, while many anthrax antibodies are produced with mammalian cell types, the production of these antibodies in plants has recently begun to spread. The functionality of the antibody is affected from differences between the plant and mammalian glycosylation mechanism. In addition, the natural form of anthrax proteins are not glycoproteins, but when they are produced in plants, they become glycoproteins. To eliminate this effect occurring in the plant expression system, proteins deglycosylated forms must be produced. In literature, deglycosylation of anti-PA83 was provided by mutation on the glycosylation site of the HC (Mett et al. 2011). They showed that nonglycosylated mAb anti-PA83 shows full protection to the anthrax spores and improved half-life (Mett et al. 2011).

In this study, we will do *in vivo* deglycosylation that is performed by co-expressed domains of the anti-PA83 with deglycosylating enzymes as Endo H (Endo-β-N-acetylglucosaminidase) and PNGase F (peptide-N-glycosidase F). When PNGase F removes the glycan, it causes the amino acid change in the N-X-S/T site, but Endo H does not (Mamedov et al. 2016). PNGase F cause the instability of the proteins due to this change. Therefore, in this thesis, anti-PA83 variants were be produced both glycosylated and deglysocylated forms with Endo H and PNGase F compared the anti-PA83 variants in terms of the activity and stability.

3. MATERIAL AND METHOD

3.1. Preparation of Solutions Used in Experiments

- **LB agar plate**

  - LB-Broth Miller with agar
  - Kanamycin
  - ddH_2O

  40 g LB-Broth Miller with agar is weighed and dissolved in 1 L autoclaved ddH_2O. Solution is autoclaved at 121ºC for 30 min. Just after autoclave, LB agar solution is cooled to 50ºC in water bath. After that, 1 ml 50 mg/ml Kanamycin is added into 1 L LB agar solution. LB agar solution is poured into petri dishes as 25 ml LB-agar per petri.

- **Kanamycin**

  - Kanamycin
  - ddH_2O

  Kanamycin stock is prepared as 50mg/ml. 0.5g kanamycin is dissolved into 10 ml autoclaved ddH_2O. It is filtered on 0.45 µm filter and aliquoted as 250 µl to keep -20ºC.

- **BBL Medium (SYS)**

  - Soyhydrolysate
  - NaCl
  - Yeast extract
  - ddH_2O

  10 g soyhydrolysate, 5 g yeast extract and 5 g NaCl are weighed and dissolved into 800 ml autoclaved ddH_2O. pH is adjusted to 7.0 with KOH. Volume is completed to 1 L with autoclaved ddH_2O. Finally, BBL medium is autoclaved at 121ºC for 30 min and it is kept at +4ºC.

- **90% glycerol**

  To prepare 90% glycerol, 90 ml 100% glycerol is mixed with 10 ml autoclaved ddH_2O It is autoclaved at 121ºC for 30 min.

- **MES Medium (MMA)**

  - MES (2-(N-Morpholino)ethanesulfonic acid)
  - MgCl2
  - ddH_2O

  MES medium is prepared as 10 mM MES, 10 mM $MgCl_2$. 1.952 g MES and 2.03 g $MgCl_2.6H_2O$ are dissolved into 800 ml autoclaved ddH_2O. pH is adjusted to 5.8 with NaOH and ddH_2O is added until volume is complete to 1 L. Medium is autoclaved and kept at +4 ºC.

- **Acetosyringone**

  - Acetosyringone
  - ddH2O
  - Ethanol (absolute)

  Acetosyringone is prepared as 100 mM stock. 0.3924 g acetosyringone is dissolved into 12 ml 95% ethanol and 8 ml ddH_2O. Stock is filtered on 0.45 µm filter and aliquoted as 250 µl and kept at -20ºC.

- **1x PBS**

  1X PBS buffer is prepared according to tablet instruction. 1X PBS tablet is dissolved into autoclaved 200 ml ddH_2O. It is kept at +4ºC.

- **DIECA (sodium diethyldithiocarbamate)**

  2 mM DIECA and 10mM DIECA are used in this thesis. 2mM DIECA is used to expression control of infiltrated leaves whereas 10 mM DIECA is used to extraction of infiltrated leaves during purification.

- **5X Laemmli Buffer**

  - Tris
  - Bromophenol Blue
  - SDS
  - B-mercaptoethanol
  - Glycerol

  5X Laemmli buffer is the buffer sample buffer for SDS-PAGE. Ingredient of Laemmli buffer is different from in terms of reducing or non-reducing samples. For reducing samples; 3.333 g SDS, 9.375 ml 1M Tris, 11.9 ml glycerol, 660 µl Bromophenol blue (0.01%), and 7.5 ml β-mercaptoethanol are mixed until SDS is completely dissolved. pH is adjusted to 6.8 with HCl. Buffer is aliquoted and kept at -20ºC. If non-reducing samples are used for SDS-PAGE, Laemmli buffer must include no β-mercaptoethanol. When performing polyacrylamide gel electrophoresis, four volume of protein sample is mixed with one volume 5X Laemmli Buffer.

- **40% Acrylamide/Bis-acrylamide Solution**

  - Acrylamide
  - ddH_2O
  - Bis-acrylamide

  38.96 g acrylamide and 1.04 g bis-acrylamide are weighed. Firstly, 38.96 g acrylamide is dissolved in 30 ml autoclaved ddH_2O. Then, bis-acrylamide mixed with solution. After completely dissolving, volume is completed to 100 ml with ddH_2O. Acrylamide/bis-acrylamide solution is kept into the aluminum capped bottle at +4ºC.

- **1.5 M Tris-HCl (pH:8.8)**

  - Tris
  - ddH_2O
  - HCl

  To prepare 1.5 M Tris-HCl, 92.5 g Tris is dissolved into 400 ml autoclaved ddH_2O. pH is adjusted to 8.8 with HCl. Volume is completed to 500 ml with ddH_2O.

- **0.5 M Tris-HCl (pH:6.8)**

  - Tris
  - ddH_2O
  - HCl for pH adjustment

  For preparing 0.5 M Tris-HCl, 30 g Tris is dissolved into 400 ml autoclaved ddH_2O. pH is adjusted to 6.8 with HCl and volume is completed up to 500 ml with autoclaved ddH_2O.

- **10% SDS**

  - SDS
  - ddH_2O

  To prepare 10% SDS solution, 10 g SDS powder is weighed and dissolved into 90 ml autoclaved ddH_2O.

- **10% Ammonium per sulphate (APS)**

  - Ammonium per sulphate
  - ddH_2O

  60 mg APS is dissolved into autoclaved ddH_2O to a final volume of 600 µl. APS is freshly prepared and used.

- **5X TBS and 1X TBS**

  - Tris
  - HCl for pH adjustment
  - NaCl
  - ddH_2O

  5X TBS includes 20 mM Tris (pH:7.5), and 150 mM NaCl. 12.115g Tris and 43.88 g NaCl are dissolved into 800 ml autoclaved ddH_2O. pH is adjusted to the 7.5 with HCl. Volume is completed to 1 L with autoclaved ddH_2O.

  1X TBS is prepared as dilution of 5X TBS. 200 ml 5X TBS is mixed with 800 ml autoclaved ddH_2O.

- **Polyacrylamide gel preparation for SDS-PAGE**

Polyacrylamide gels consists of two gel part as resolving and steaking gels. They are prepared as seen below table.

Table 3.1. Ingredients of 10% and 12% polyacrylamide gels.

a. **A. RESOLVING GEL**		
Ingredients	**10% Gel**	**12% Gel**
Autoclaved ddH_2O	2,425 ml	2,175 ml
40% Acrylamide- Bisacrylamide Solution	1,25 ml	1,5 ml
1,5M Tris-HCl (pH: 8.8)	1,25 ml	1,25 ml
10% SDS Solution	50 µl	50 µl
TEMED	2,5 µl	2,5 µl
10% APS	25 µl	25 µl

B. STEAKING GEL		
Ingredients	**10% Gel**	**12% Gel**
Autoclaved ddH_2O	1,98 ml	1,917 ml
40% Acrylamide- Bisacrylamide Solution	312 µl	374.4 µl
0,5 M Tris HCl (pH: 6.8)	787 µl	787 µl
10%SDS Solution	31,25 µl	31,25 µl
TEMED	3,125 µl	3,125 µl
10% APS	15,62 µl	15,62 µl

- **Running Buffer**

  - Tris
  - SDS
  - Glycine
  - ddH_2O

3.03 g Tris, 14.3 g Glycine, and 10 ml 10% SDS are dissolved into the 800 ml autoclaved ddH_2O and volume is completed to 1 L.

- **Transfer Buffer**

  - Tris
  - SDS
  - Glycine
  - ddH_2O

  5.8 g Tris, 2.93 g Glycine, and 370 µl 10% SDS are dissolved into the 800 ml autoclaved ddH_2O and volume is completed to 1 L.

- **Blotting solution (I-block)**

  - Blotting grade powder
  - 1X TBS

  1 g blotting grade powder is dissolved into 100 µl Tween-20 including 100 ml 1X TBS (1 µl Tween 20- 1000 µl 1X TBS).

- **Gel staining solution (Coomassie Blue)**

  - Glacial acetic acid
  - Coomassie Brilliant blue
  - Methanol
  - ddH_2O

  100 ml glacial acetic acid, 500 ml methanol, and 400 ml ddH_2O are mixed. 1 g Coomassie blue is dissolved into the mixture.

- **Destaining solution**

  - Methanol
  - ddH_2O
  - Acetic acid

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

- **Extraction, Binding, and Dialysis Buffer (50 mM Tris-HCl; pH:7.5, 10 mM EDTA, 1 mM PMSF)**

  - Tris
  - DIECA
  - EDTA
  - ddH_2O
  - PMSF
  - HCl for pH adjustment

  To prepare 50 mM Tris-HCl; 1 M Tris is prepared and 25 ml 1 M Tris is taken for 50 mM Tris in 1 L. To prepare 10 mM EDTA; 0.5 M EDTA is prepared and 10 ml 0.5 M EDTA is taken for 10 mM EDTA in 1L.

  25 ml 1M Tris, and 10 ml 0.5 M EDTA are mixed and volume is completed to the 1 L with ddH_2O. PMSF is added into the buffer as 1 mM. pH is adjusted to the 7.5 with HCl. This buffer is used as extraction buffer, binding buffer, and dialysis

buffer. If it is used as extraction buffer, 10 mM sodium diethyldithiocarbamate (DIECA) must be added.

- **Wash Buffer (1X tablet PBS)**

  - 1X PBS tablet
  - ddH_2O

  1X tablet PBS is dissolved into autoclaved 200 ml ddH_2O.

- **Elution Buffer (100 mM Glycine pH:2.5)**

  - Glycine
  - ddH_2O
  - HCl for pH adjustment

  To prepare 100 mM glycine, 1.5014 g Glycine is dissolved into 150 ml ddH_2O.pH is adjusted to 2.5 with HCl. Volume is completed to 200 ml with autoclaved ddH_2O.

- **Cell Culture Medium (10% FBS containing culture medium)**

  To prepare the cell culture medium 44 ml High Glucose DMEM, 5 ml Fetal Bovine Serum (FBS), 500 ul Pen -Strep (100x) (antibiotic- antimycotic), and 500 ul Sodium Pyruvate are mixed. This medium is kept at +4ºC

3.2. Transient expression of anti-PA83 variants in *N. benthamiana* by infiltration

In the previous studies within the scope of the project, heavy chain (HC) and light chain (LC) genes were engineered and optimized for expression in *N. benthamiana* plants and they were transformed to the *Agrobacterium tumefaciens*. Both HC and LC were expressed in the *N. benthamiana* plants and their expression was confirmed. Following that, next step will be produced and characterized full-length glycosylated and deglycosylated anti-PA83.

To produced glycosylated anti-PA83 (G-anti-PA83); pGR-HC and pGR-LC carrying Agrobacterium strains were grown in the BBL medium that consists of soy hydrolysate, yeast extract, NaCl, and kanamaycin overnight at 28ºC and then their absorbance values were measured at OD600. Agrobacterium cultures were centrifuged at 5000g for 5 min and their pellets were resuspended Acetosyringone (150 µM) including MES buffer (10mM MES, 10 mM $MgCl_2$) for 2 hours for induction. To co-express pGR-HC and pGR-LC constructs, they were introduced into 6-7-week-old *N. benthamiana* plants by manual infiltration as a mixture with a silencing suppressor gene at a ratio of 5:4:1 (HC:LC:TL).

For production of deglycosylated anti-PA83; the HC, LC and Endo H or PNGase F constructs were co-expressed. Each construct was grown in the BBL medium overnight at 28ºC. Then, they were prepared as mentioned above. To produce dG-anti-PA83 with EndoH; pGR-HC, pGR-LC and pGR-Endo H including bacterial suspensions and a silencing suppressor were mixed at a ratio of 4:4:1:1 (HC: LC: EndoH: TL). To produce dG-anti-PA83 with PNGase F; pGR-HC, pGR-LC and pGR-

PNGase F including bacterial suspensions and *A. tumefaciens* strain AGL1 carrying a silencing suppressor were mixed at a ratio of 4:4:1:1 (HC: LC: PNGase F: TL).

Table 3.2. Combination of constructs for infiltration

	G-anti-PA83 (5:4:1)	**dG-anti-PA83-E (4:4:1:1)**	**dG-anti-PA83-P (4:4:1:1)**
pGR-HC	0,5	0,4	0,4
pGR-LC	0,4	0,4	0,4
TL	0,1	0,1	0,1
pGR-Endo H	-	0,1	-
pGR-PNGase F	-	-	0,1

The leaves were taken at 6 days post-infiltration (dpi). Leaf samples were homogenized in the three volumes of extraction buffer (1X PBS, 2 mM sodium diethyldithiocarbamate (DIECA)). Homogenates were centrifuged at 20000g for 5 min and then supernatant was apart from the pellet. Supernatants were mixed with 5X Laemmli Buffer at the ratio of 4:1 for reducing conditions.

Expression level of G-anti-PA83, dG-anti-PA83-Endo H and dG-anti-PA83-PNGase F were detected with Western blot analysis. Samples that were prepared with Laemmli buffer were boiled at 100ºC for 5 min. After that, they were loaded to the 10% SDS-PAGE that is prepared with refences to Table 1 and run at 100 V 15 min-200V 45 min into running buffer. Then, proteins were transferred onto the polyvinylidene fluoride membrane with transfer buffer during 1 hour at 100V. Membrane was blocked with 1% blotting grade solution. Anti-PA83 proteins were detected with HRP-conjugated anti-human secondary antibody. Signal was generated by chemiluminescent substrate as peroxidase and enhancer.

3.3. Purification of anti-PA83 variants from N. benthamiana leaves by protein A column chromatography

G-anti-PA83, dG-anti-PA83-Endo H and dG-anti-PA83-PNGase F proteins were precipitated and purified with protein A column chromatography.

3.3.1. Ammonium sulphate precipitation and dialysis

Plant tissues for target proteins were extracted with three volumes of extraction buffer (50 mM Tris-HCl, pH:7.5; 10 mM EDTA;1 mM phenylmethylsµlfonyl fluoride (PMSF)) containing 10 mM sodium diethyldithiocarbamate (DIECA). Extract was centrifuged 13000g for 15min at 4ºC. After centrifuge, supernatant was taken into the beaker for ammonium sulphate $(NH_4)_2SO_4$ precipitation. Ammonium sulphate was used

according to Figure 3.1 that shows ammonium sulfate quantities required to reach given degrees of saturation. Firstly, anti-PA83 proteins were precipitated with 25% ammonium sulphate saturation. Enough $(NH_4)_2SO_4$ for 25% saturation was added slowly on the supernatant by mixing with magnetic stirrer. After mixing, it was incubated for 30 min at +4ºC. After incubation, it was centrifuged at 13000g for 10 min at 4ºC. Then, supernatant was taken into the new beaker for 60% $(NH_4)_2SO_4$ saturation. Supernatant was precipitated slowly adding of $(NH_4)_2SO_4$ as 60% $(NH_4)_2SO_4$ saturation. After mixing, it was incubated for 30 min at 4ºC. After incubation, it was centrifuged at 13000g for 10 min at 4ºC. Pellet was re-suspended into 1.5 ml extraction buffer and it was kept in the extraction buffer and dialyzed at +4ºC. After that, pellet solution was taken from dialysis cassette. Solution was centrifuged 13000g for 10 min at +4ºC. Pellets were re-suspended into extraction buffer for further purification.

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent	Amount of ammonium sulfate to add (grams) per liter of solution at 20°C saturation																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Figure 3.1. Scheme for amount of ammonium sulphate required to reach given degrees of saturation

3.3.2. Protein A column chromatography

20 grams of infiltrated leaves for G-anti-PA83, dG-anti-PA83-Endo H and dG-anti-PA83-PNGase F proteins were extracted and proteins were precipitated as mentioned above. Precipitated proteins were purified with Protein A Agarose Fast Flow resin. Resin was put on the column and liquid phase was drained. Resin was equilibrated with 10 resin-bed volumes of binding buffer (50 mM Tris-HCl, pH:7.5; 10 mM EDTA;1 mM phenylmethylsulphonyl fluoride (PMSF)). Re-suspended pellets that were obtained as a result of dialysis was passes through the column. Flow through was collected into new tube. This step was re-applied as 4-5 times. Then, resin was washed with 10-15 resin-bed volumes of 1X PBS as wash buffer. Before elution; enough 5M Tris was added into each eppendorf tube for neutralization of elutes. Antibodies were

eluted with 10 resin-bed volume of elution buffer (100 mM Glycine pH:2.5). Concentration of elutes were measured by BioDrop. Elution fragments with high concentrated were combined. The combined fragments were concentrated at 4°C at 8000g with Millipore concentrator (10K MWCO) and buffer interchanged against PBS. Purified proteins were filtrated and aliquoted as 1 µg/µl. Purified plant produced anti-PA83 variants were analyzed by SDS-PAGE at both reducing and non-reducing conditions. The reduced samples contained 2-mercaptoethanol, while the non-reducing samples were prepared with 5X Laemmli buffer that did not contain 2-mercaptoethanol. Coomassie Blue staining is used to visualize SDS-PAGE gels.

3.4. Examination of the interaction between antigen-antibody by Western blot analysis

While anti-PA83 variants were used as primary antibody to analyze the interaction of plant-produced anti-PA83 proteins with plant-produced PA83 variants (gPA83, dPA83 (E) and dPA83 (P)), PA83 variants were used as antigens. PA83 variants were loaded as 100 ng and run on the 10% polyacrylamide gel and transferred to polyvinylidene fluoride membrane. Proteins transferred to the membrane were labeled with anti-PA83 variants at a concentration of 5 µg/10 ml. The marked proteins were labelled with HRP-conjugated anti-human secondary antibody and they were visualized with the chemiluminescent substrate (SuperSignal West Pico, Thermo Fisher Scientific, Grand Island, NY).

3.5. Study of stability analysis of anti-PA83 variants using SDS-PAGE

pp-antiPA83 variants as glycosylated anti-PA83, deglycosylated anti-PA83 with EndoH, and deglycosylated anti-PA83 with PNGase F were analyzed for stability assessment. The concentration of proteins were adjusted to 1 mg/ml into eppendorf tube. They were incubated at 37ºC, +4ºC, and room temperature for a longer period as weekly and monthly. These periods were continued for 5 weeks and 6 months. For 5 weeks, samples were taken immediately prior to and after incubation at one-week intervals and mix with SDS loading dye. For 6 months, samples were taken just before and after incubation at one-month intervals and mixed with SDS loading dye. They were kept at -80 till to use and analyzed by 12% SDS-PAGE.

3.6.Cell culture preparation and toxin neutralization assay (TNA)

3.6.1. Cell culture

The J77A.1 (mouse macrophage cell line, ATCC TIB-67, Manassas, VA) cells was removed from liquid nitrogen. The tube containing the cells was kept until dissolved in a 37°C water bath. After defrosting, DMEM medium, a pre-heated nutrient medium, was gradually added to the cells to reduce the effect of DMSO, which is present in the freezing medium. Cells were centrifuged at 37°C at 300 * g for 5 minutes. The pellet was dissolved by slowly pipetting in the culture medium. Then, the cells were placed in flasks and left for incubation in 37°C 5% CO_2 environment. The next day, cells were visualized under a microscope. If they were not enough, growth mediums were changed and left at 37°C 5% CO_2 incubation. When the cells covered every part of the flask, they were removed from the medium flasks. Cells attached to the

flask were washed 3 times with PBS buffer. Trypsin was added to the cells to separate the cells from flask and incubated in the incubator for 3 minutes. If all the cells were removed as a result of observation under the microscope, DMEM medium was slowly added on them and the cells were transferred from flask to falcon tube. Shield cells were centrifuged at 300 * g for 5 minutes. The supernatant was removed, and the pellet was slowly pipetted and dissolved in 6 ml medium. Then, cells were counted under a microscope using the Bright-line Hemocytometer coverslip. Cells were plated in a 96-well plate as $2.5x10^4$ cells/well in 50 µl and were incubated in 37°C 5% CO_2 medium overnight incubation. Cells left for incubation were used for Toxin neutralization (TNA) assay 1 day later.

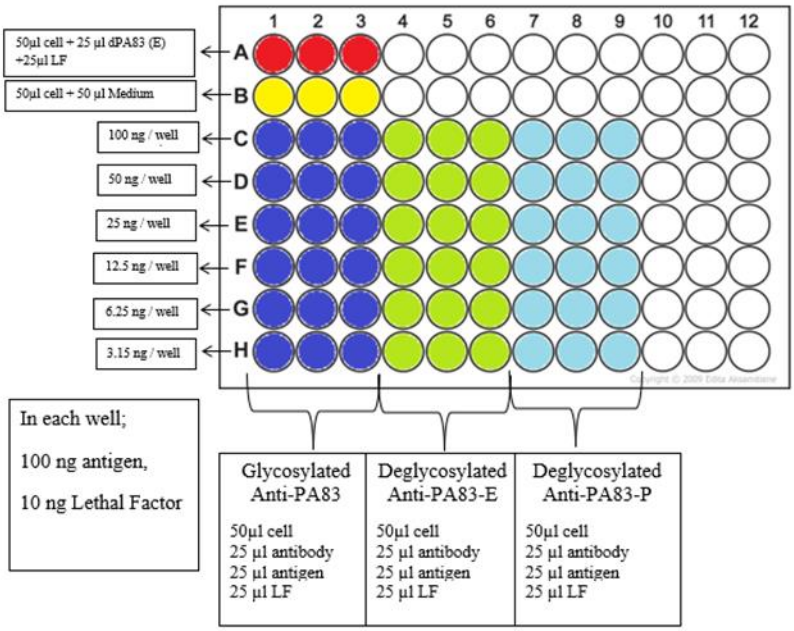


Figure 3.2. The loading scheme of proteins on the 96-well plate. Plant produced antibody variants (G-anti-PA83, dG-anti-PA83-E, and dG-anti-PA83-P), antigen as plant produced dPA83 (E) and lethal factor were used.

3.6.2. Toxin neutralization assay (TNA)

The mouse macrophage cell line (J774A.1 cells, ATCC TIB-67, Manassas, VA) was plated in a 96-well plate as $2.5x10^4$ cells/well in 50 µl. To determine the protein purity and their real concentration, concentrations calculations of plant-produced anti-PA83 variants, glycosylated antiPA83 (G-anti-PA83), Endo H deglycosylated anti-PA83 (dG-anti-PA83- E) and PNGase F deglycosylated anti-PA83 (dG-anti-PA83-P), were made based on SDS-PAGE analysis of proteins. These proteins were prepared by serial dilution to be 4000 ng/ml, 2000 ng/ml, 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml. As an antigen, plant-produced Endo H deglycosylated PA83 (dPA83 (E)) protein was used and prepared to be 4000 ng/ml. Lethal factor (List Biological Laboratories, Campbell, CA) was prepared to be 400 ng/ml. As shown in the Figure 3.6.2, it was added onto the cells plated in the 96-well plate in one row only cell, and in the other row only cell + antigen + LF. First, the antibodies were loaded in three replicates at 100ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng and 3.15 ng in 25 µl of each well. Immediately

after adding the antibodies, dPA83 (E) toxin was added to each well at 10 ng. Finally, LF was added on the antigens and antibodies, with 100 ng in each well. The plate with antibody, antigen and LF was incubated for 12-15 hours at 37°C in 5% CO2 environment. After 12 hours, cell proliferation agent WST-1 (Roche Applied Sciences, Indianapolis, IN) was added and left for 2.5 hour incubation. Cell viability was measured by an ELISA reader (OD450) at 450 nm.

4. FINDINGS AND DISCUSSION

4.1. Expression determination of anti-PA83 proteins by western blot analysis

Within the scope of this project, anti-PA83 protein was produced in *N. benthamiana* plant using pGR-HC and pGR-LC genes, which were previously cloned and transferred to Agrobacterium plasmid. As described in the Material and Method, pGR-HC, pGR-LC, pGR-EndoH and pGR-PNGase F genes were co-infiltrated to produce the glycosylated and deglycosylated forms of anti-PA83 protein in the plant.

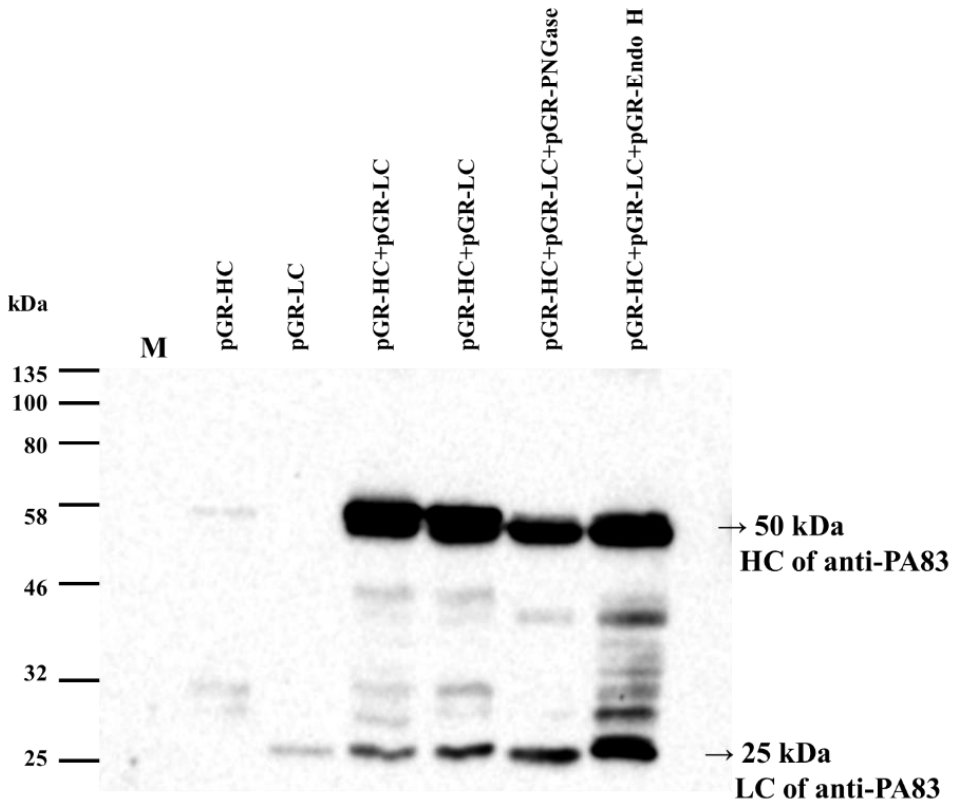


Figure 4.1. Western Blot analysis of anti-PA83 variants produced in *Nicotiana benthamiana* plants. *N. benthamiana* plants were infiltrated with co-expression of pGR- HC and pGR- LC constructs to produce G-anti-PA83; and also these constructs were co-infiltrated with pGR- Endo H and pGR- PNGase F to produce deglycosylated variants of anti-PA83.

The Western Blot analysis was made with samples extracted from leaves collected after infiltration to perform the expression analysis and its result is shown in Figure 4.1 . It was demonstrated by this Western Blot analysis that the plant produced anti-PA83 monoclonal antibody had 50 kDa heavy chain and 25 kDa light chain. While 2 bands were observed in the HC of glycosylated anti-PA83 protein, one band was observed in the HC of deglycosylated anti-PA83 variants. This indicates that deglycosylation is successfully carried out in the plant and full-size anti-PA83 proteins are successfully produced.

4.2. SDS-PAGE analysis of purified plant produced anti-PA83 proteins

Purification of recombinant anti-PA83 variants was performed as described in Material and method. Firstly, proteins were precipitated by ammonium sulphate and dialyzed and then purified by protein A affinity column chromatography. After purification, anti-PA83 proteins were analyzed by SDS-PAGE. As can be seen in the Figure 4.2 , the proteins were obtained in full size in a pure manner without any loss in structure. As a result of this analysis, a similar image was obtained with the previous Western Blot image.

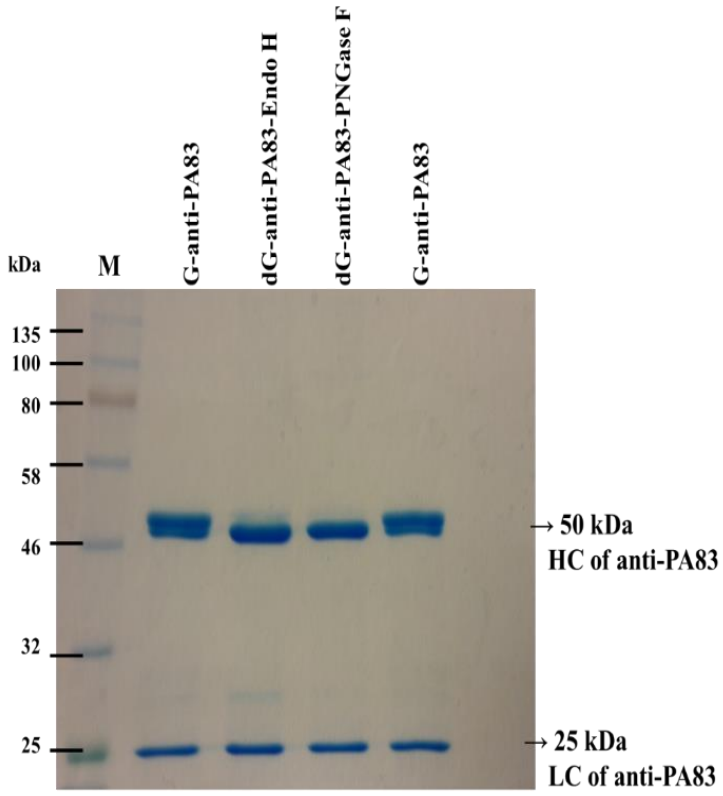


Figure 4.2. SDS-PAGE analysis of plant produced, purified anti-PA83 variants. SDS-PAGE analysis of purified plant produced anti-PA83 from *N. benthamiana* plant at reducing conditions. 5 µg samples were loaded in each line. M- color prestained protein standard (New England Biolabs).

The purified plant produced anti-PA83 variants were tested by SDS-PAGE analysis in both reducing and non-reducing conditions. Samples prepared in reducing conditions contain reducing agents such as β-mercaptoethanol, while samples prepared in non-reducing conditions do not contain reducing agents. The purpose of doing it in reducing conditions was to see protein subunits, while the purpose of doing it in nonreducing conditions was to analyze the full-size anti-PA83 protein. As shown in the Figure 4.3, two separate bands, HC and LC, were observed in reducing conditions, while single band was observed in non-reducing conditions.

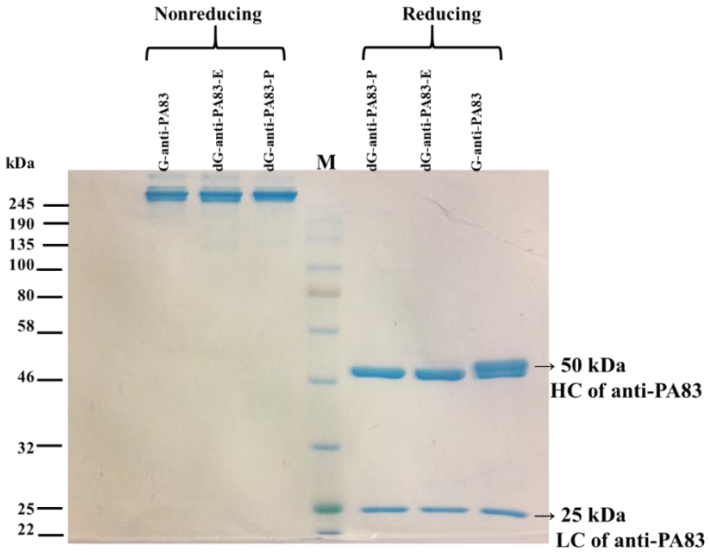


Figure 4.3. SDS-PAGE analysis of plant produced anti-PA83 at both reducing and non-reducing conditions. Anti-PA83 variants were prepared at both reducing and non-reducing conditions and run on the 10% SDS-PAGE gel. 5 µg samples were loaded in each line.

4.3. Examination of the interaction between antigen - antibody by Western blot analysis

The interaction of plant-produced anti-PA83 proteins with plant-produced PA83 antigens was analyzed by Western blot. While anti-PA83 proteins purified during this thesis period as G-antiPA83, dG-anti-PA83-E and dG-anti-PA83-P were used as antibody, PA83 variants produced in our laboratory – gPA83, dPA83(E) and dPA83(P)- were used as antigens. As shown in the Figure 4.4, all anti-PA83 variants used as primary antibodies showed the same cross-reactivity for all three antigens.

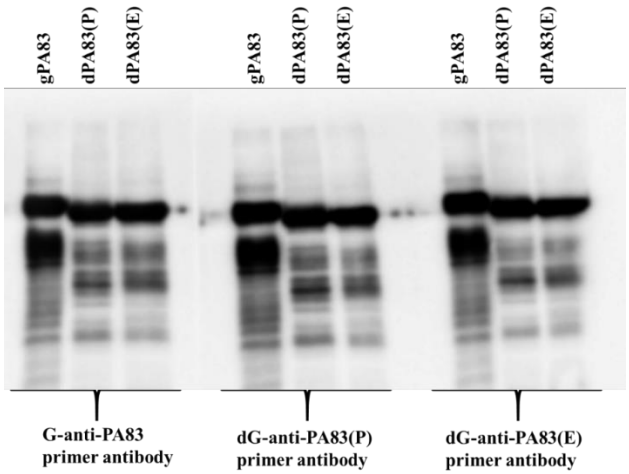


Figure 4.4. Interactions of anti-PA83 proteins with PA83 protein variants. PA83 proteins were loaded to 100 ng in each well and imaged by treating each membrane with a different primary antibody.

4.4. SDS-PAGE analysis for stability studies

Stability analyzes were performed as described in Material and method, and the results were analyzed by SDS-PAGE. Stability analyzes for all three proteins were done in three different environments, 37ºC, 4ºC and room temperature. While the first part of the stability analysis was carried out in daily period and 5-week period, the second part was carried out in a 6-month period.

4.4.1. SDS-PAGE for stability analysis of G-anti-PA83 for daily period

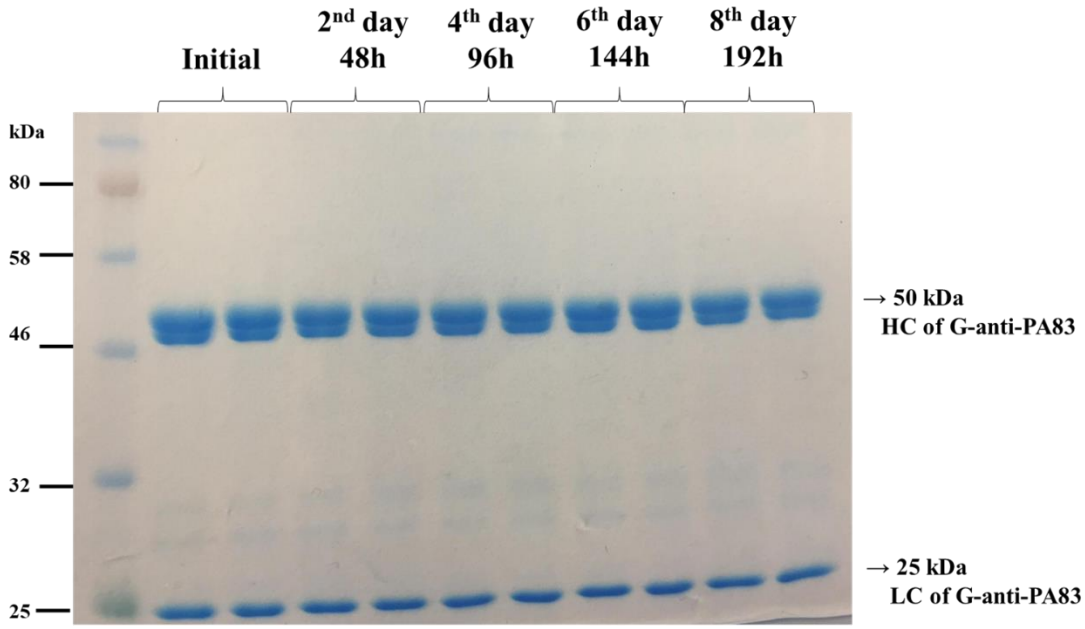


Figure 4.5. SDS-PAGE analysis for daily period at 37ºC. Plant produced G-anti-PA83 was stored at 37ºC during 192h. Samples were prepared at reduced condition. Each sample was loaded as 5 µg per lane. M- Color Prestained Protein Standard (New England Biolabs)

Stability analysis was initiated with daily period. Plant produced glycosylated anti-PA83 protein was incubated at 37ºC during 192h. As shown in Figure 4.5, G-anti-PA83 did not any change in stability. Therefore, period of stability analysis was done as weekly and monthly period.

4.4.2. SDS-PAGE for stability analysis of anti-PA83 variants for 5-week period

4.4.2.1. At 37ºC;

Samples for all three variants were kept at 37ºC for 5-week period and prepared as reducing conditions. As shown at figure, anti-PA83 variants do not show any stability loss.

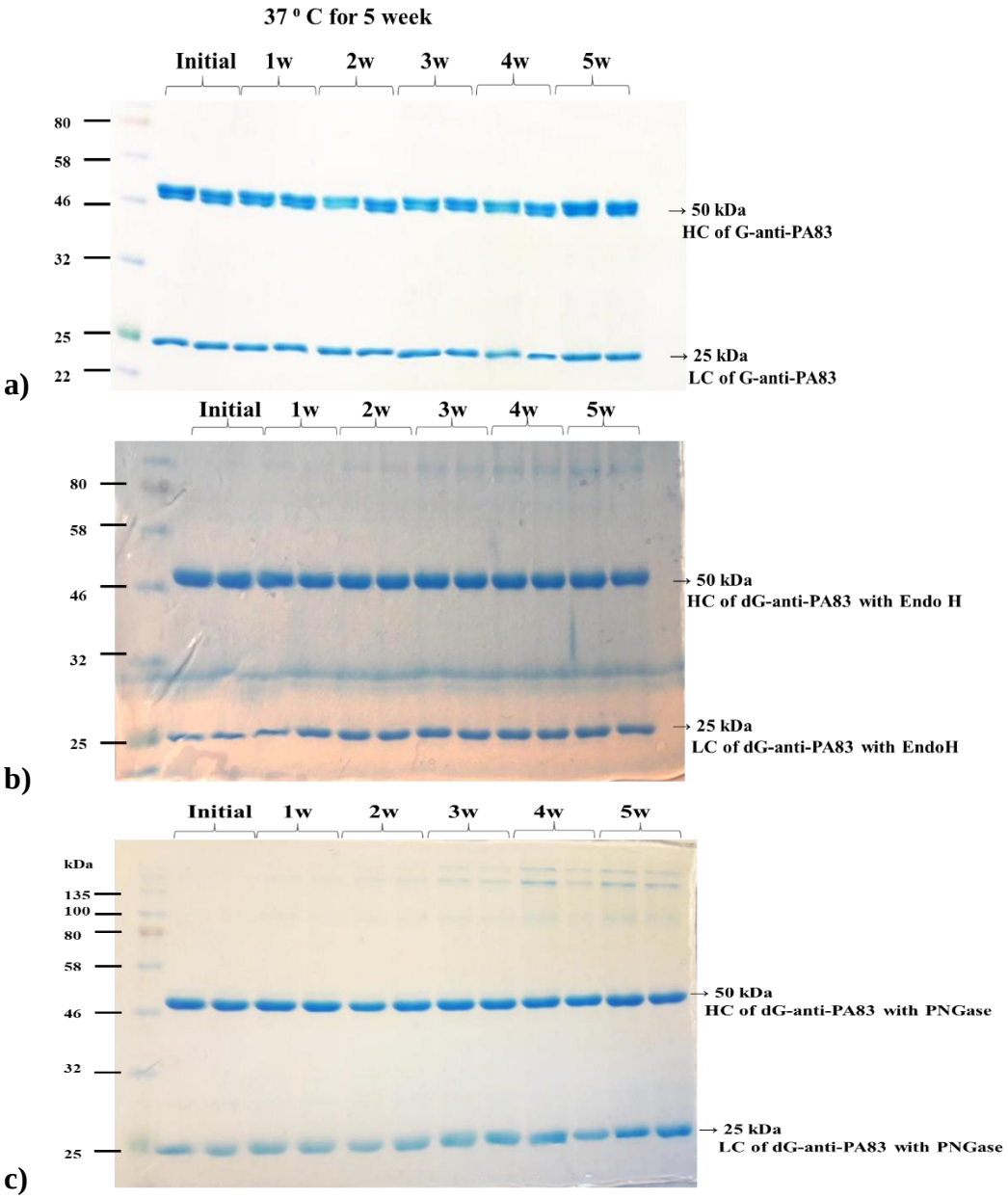


Figure 4.6. SDS-PAGE for stability analysis of anti-PA83 variants at 37ºC for 5 weeks. Plant produced G-anti-PA83, dG-anti-PA83-E and dG-anti-PA83-P were stored at 37ºC for 5 weeks. a) for G-anti-PA83; b) for dG-anti-PA83-Endo H; c) for dG-anti-PA83-PNGase F. Samples were prepared at reduced condition. Each sample was loaded as 5 µg per lane. M- Color Prestained Protein Standard (New England Biolabs)

4.4.2.2. At 4ºC;

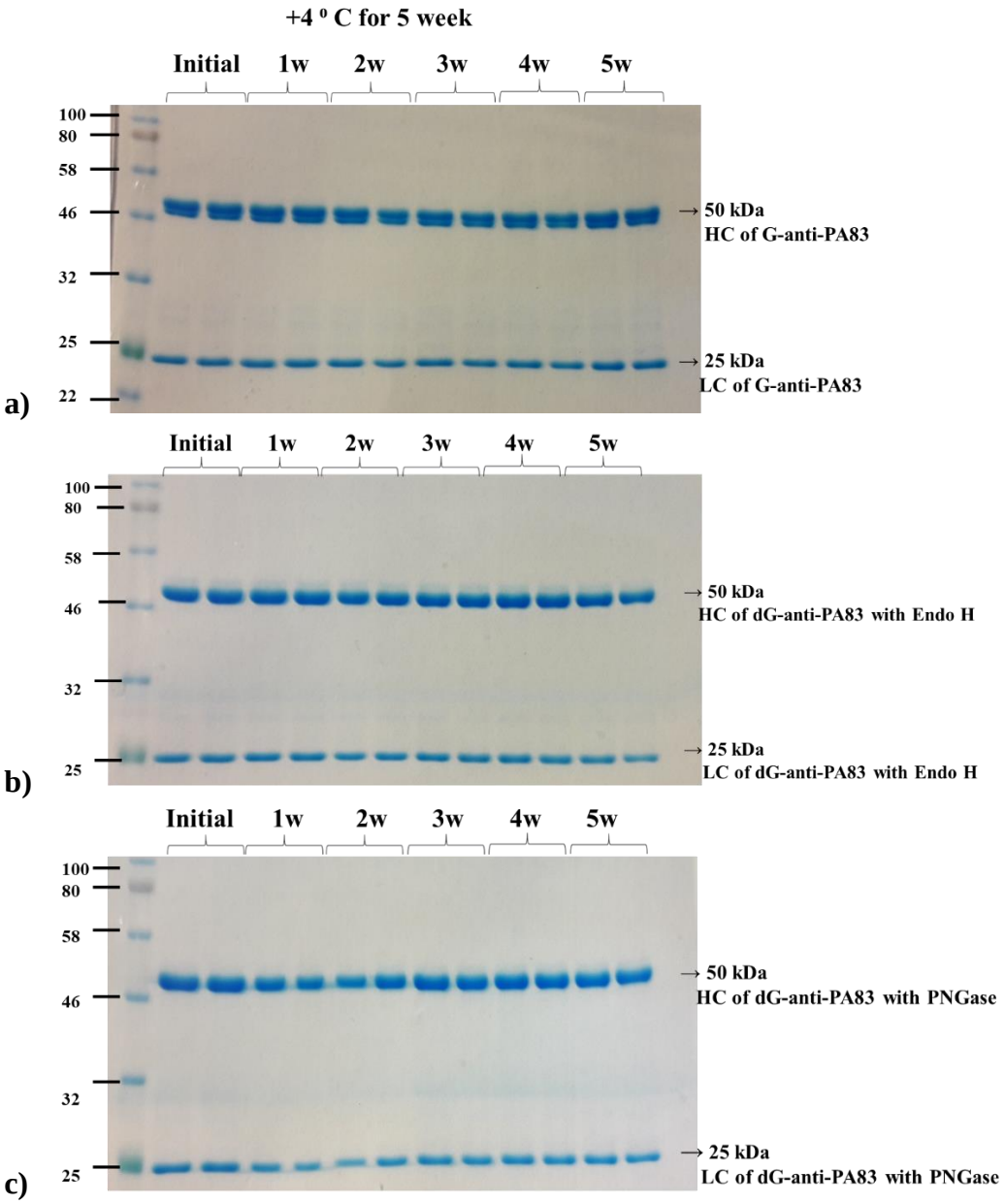


Figure 4.7. SDS-PAGE for stability analysis of anti-PA83 variants at 4ºC. Purified plant produced G-anti-PA83, dG-anti-PA83-Endo H and dG-anti-PA83-PNGase F were kept at 4ºC for 5 weeks. a) for G-anti-PA83 proteins; b) for dG-anti-PA83-Endo H proteins; c) for dG-anti-PA83-PNGase F proteins. Samples were prepared at reduced condition. Each sample was loaded as 5 µg per lane. M- Color Prestained Protein Standard (New England Biolabs)

Samples for all three variants were kept at +4ºC for 5-week period and prepared as reducing conditions. As shown at Figure 4.7, anti-PA83 variants do not show any stability loss.

4.4.2.3. At Room Temperature;

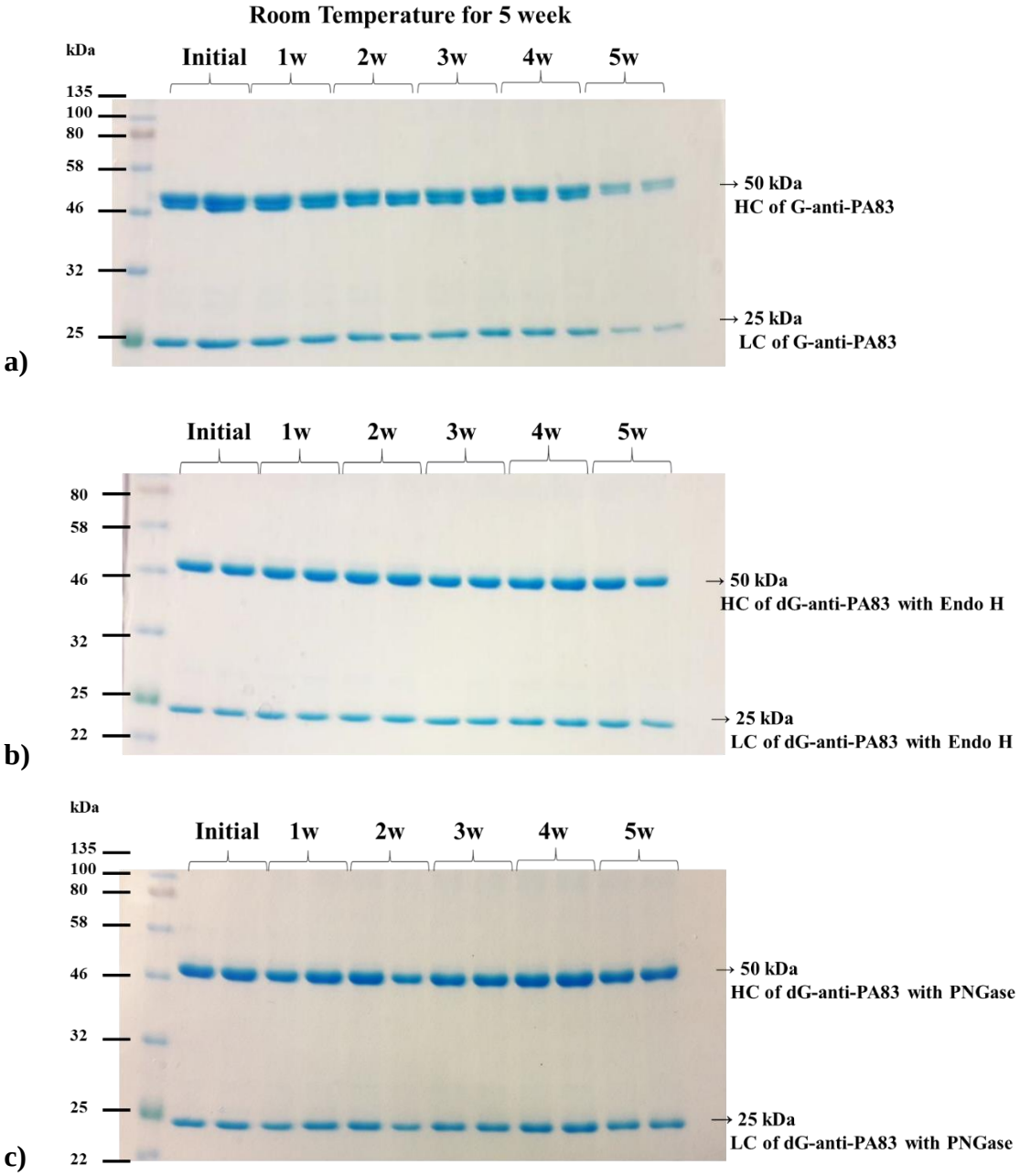


Figure 4.8. SDS-PAGE for stability analysis of glycosylated and deglycosylated anti-PA83 variants at room temperature. Purified plant produced G-anti-PA83, dG-anti-PA83-Endo H and dG-anti-PA83-PNGase F were kept at room temperature for 5 weeks. a) for G-anti-PA83 proteins, b) for dG-anti-PA83-Endo H proteins. c) for dG-anti-PA83-PNGase F proteins. Samples were prepared at reduced condition. Each sample was loaded as 5 µg per lane. M- Color Prestained Protein Standard (New England Biolabs).

Samples for all three variants were kept at room temperature for 5-week period and prepared as reducing conditions. As shown at figure, G-anti-PA83 showed a bit stability loss at 5th week and another anti-PA83 variants do not show any stability loss.

4.4.3. SDS-PAGE of stability of purified anti-PA83 variants for 6-month period

4.4.3.1. At 37ºC;

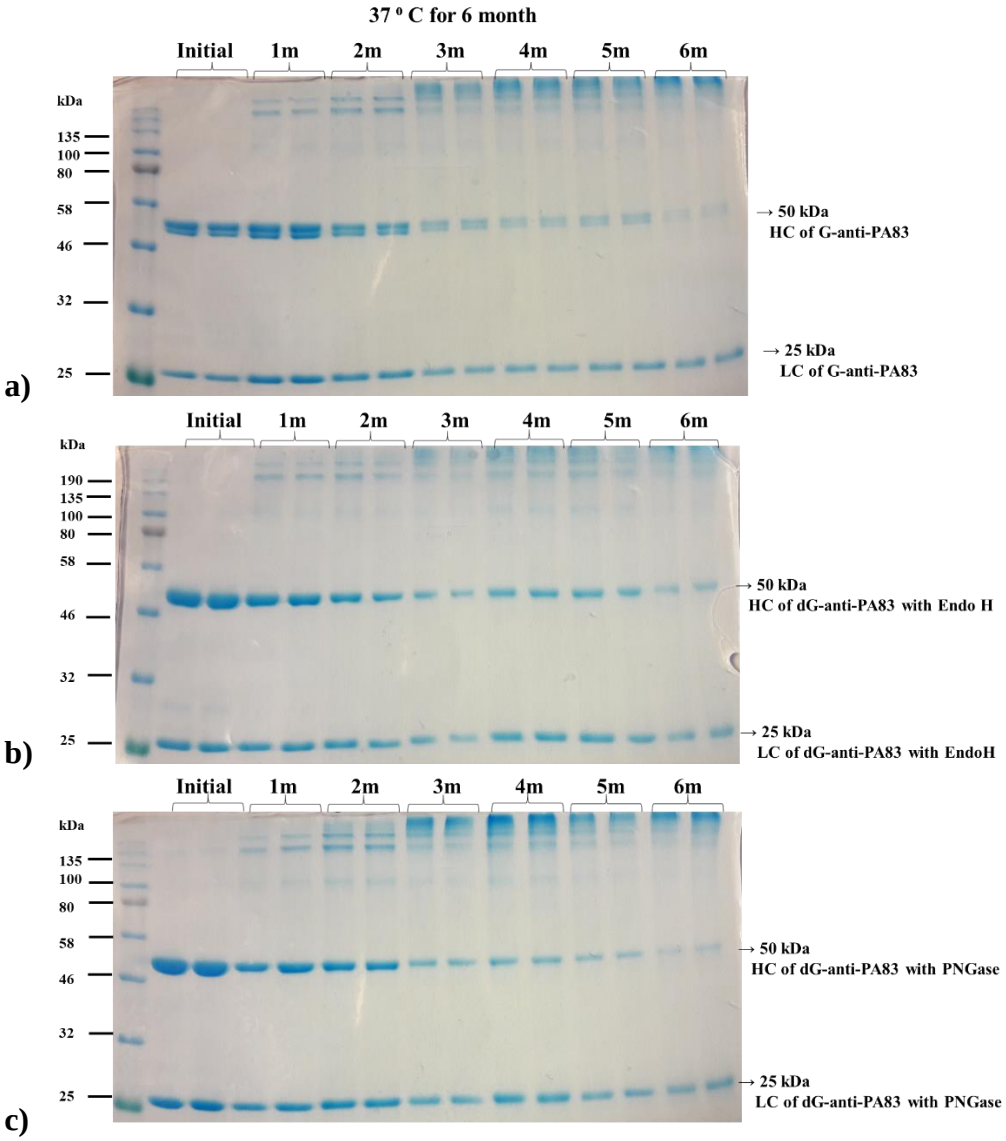


Figure 4.9. SDS-PAGE analysis for stability studies of glycosylated and deglycosylated anti-PA83 variants at 37°C for six months. Purified anti-PA83 variants were stored at 37°C for 6 months. a) G-anti-PA83 proteins, b) dG-anti-PA83 (E) proteins, c) dG-anti-PA83 (P) proteins. Samples were prepared as reduced. Each sample was loaded as 5 µg.

4.4.3.2. At +4ºC;

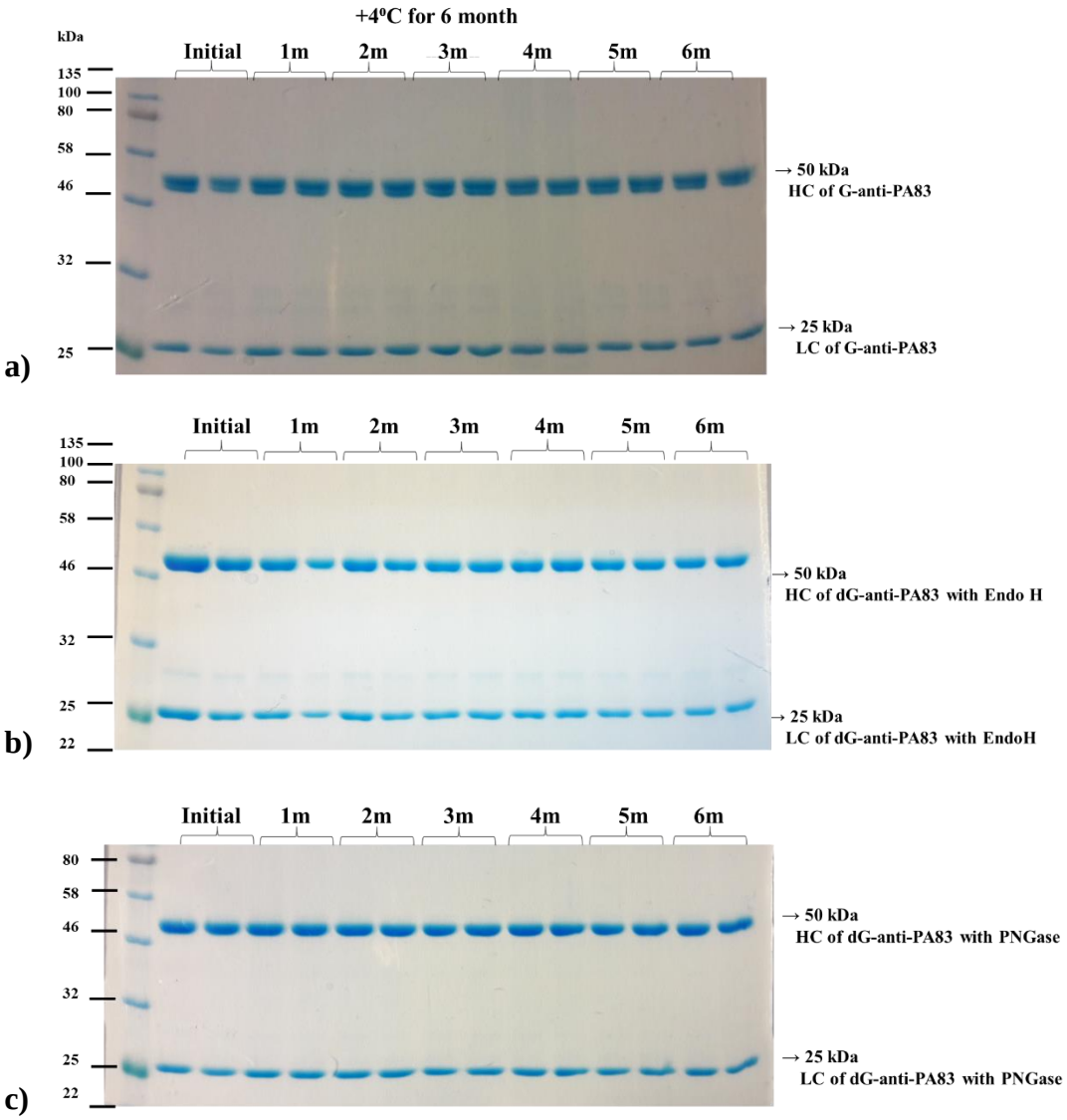


Figure 4.10. SDS-PAGE analysis for stability studies of glycosylated and deglycosylated anti-PA83 variants kept at +4°C for six months. Purified anti-PA83 variants were stored at +4°C for 6 months. a) For G-anti-PA83 proteins; b) For dG-anti-PA83 (E) proteins; c) For dG-anti-PA83 (P) proteins. Samples were prepared as reduced. Each sample was loaded as 5 µg.

4.4.3.3. At Room temperature;

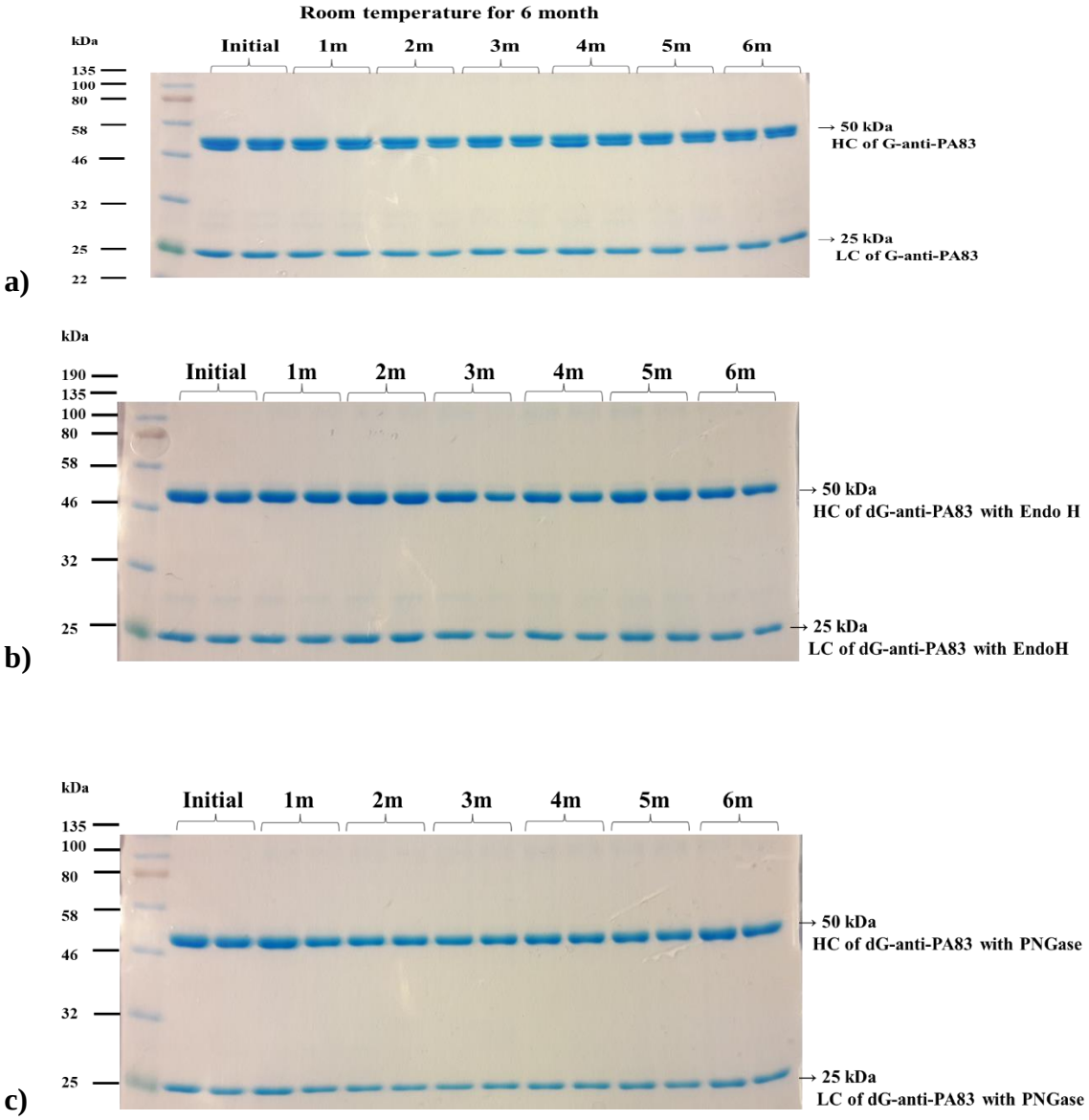
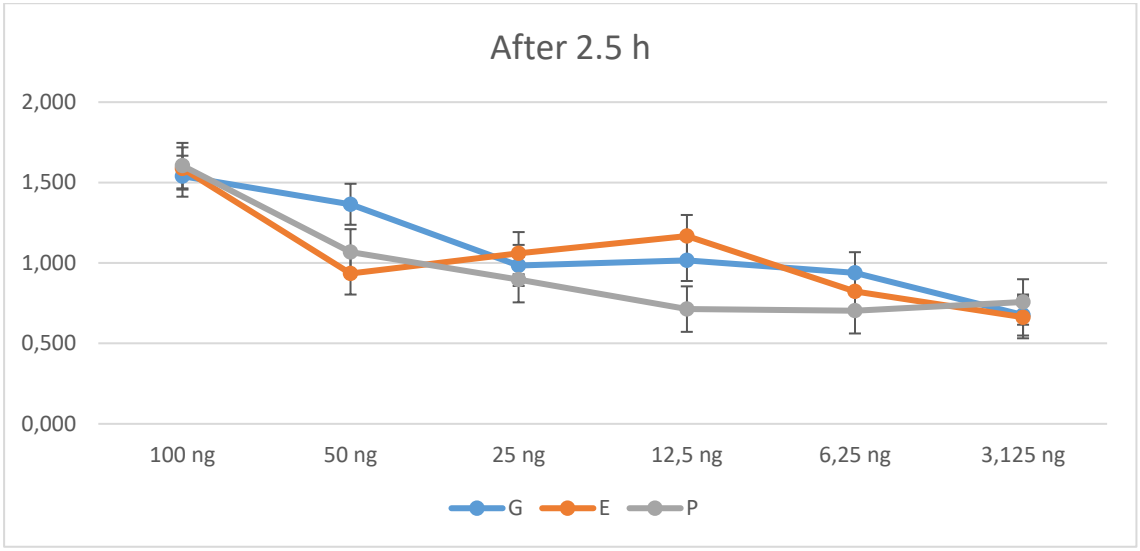


Figure 4.11. SDS-PAGE analysis for stability studies of glycosylated and deglycosylated anti-PA83 variants kept at room temperature for six months. Purified anti-PA83 variants were stored at room temperature for 6 months. a) For G-anti-PA83 proteins; b) For dG-anti-PA83 (E) proteins; c) For dG-anti-PA83 (P) proteins. Samples were prepared as reduced. Each sample was loaded as 5 µg.

4.5. Toxin neuralization assay

Toxin neutralization assay (TNA) is a method used to evaluate the ability of antibodies to protect cells from the action of specific toxins. The toxin neutralization ability of plant produced anti-PA83 antibodies was examined by the toxin neutralization assay on macrophage cell line. While the plate covered with macrophage cells was first treated with antibodies, then the plant produced antigen dPA83 (E) and lethal factor were added as toxins and the antibody's response to the antigen was observed. The results of the TNA experiment are as shown in the graphic in the Table 4.1.



a)

b)

Table 4.1. Graphs for TNA analysis of anti-PA83 variants. TNA results were obtained from ELISA reader and line chart and column chart were created by Excel. a) Line chart; b) Column chart for TNA analysis. No toxin is a control that represents cells grown without the addition of toxins. Toxin is another control that shows cells grown in the presence of toxins, PA and LF. G refers to G-anti-PA83, E refers to dG-anti-PA83-E and P refers to dG-anti-PA83-P. The graphics were created by averaging the OD values of triple replicas.

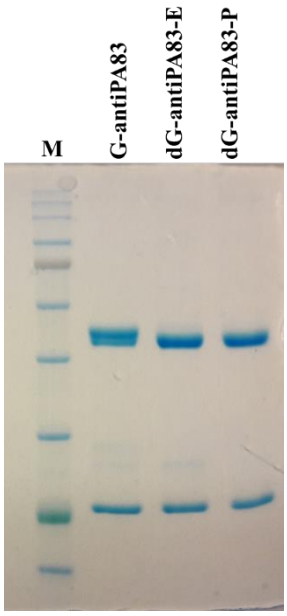


Figure 4.12. SDS-PAGE image for proteins that were used in TNA. Each protein was loaded as 5 µg/well. M- color prestained protein standard (New England Biolabs).

As seen in the Table 4.1, while each antibody shows close activity with each other, it has been observed that the antibodies administered at a dose of 3.125 ng / ml can be effectively neutralized toxins and provide high viability.

5. CONCLUSION

This thesis carried out within the scope of vaccine development against anthrax disease, was established as a strategy to produce the anti-PA83 monoclonal antibody in the plant transient expression system to be used against the PA83 protein, the most important toxin known as the disease agent. The HC and LC genes encoding the anti-PA83 protein were cloned and optimized in previous studies so that the engineering and production of glycosylated and *in vivo* deglycosylated anti-PA83 forms in *N. benthamiana* and characterization of them in terms of their affinity, stability and TNA is aimed in this thesis.

The use of anthrax, an infectious disease, as a bioterrorism tool, requires the development of medical measures against this biological agent. Treatment with monoclonal antibodies is a proven approach to reduce the burden of this disease and the impact and spread of the pathogen.

Recombinant antibodies and other proteins used as vaccine candidates are produced using expression systems such as primarily plant and mammalian and bacteria, insects and yeast expression systems. Recently, plant-based transient expression systems have been used as an alternative route to produce recombinant proteins. The plant-based expression system has eukaryotic post-translational modifications and the ability to produce a high amount of protein per minimum leaf biomass in a short time and many other advantages. Other advantages of plant expression systems are easy to scale, economical and short-term in production, and being safe in terms of free for human pathogens.

The production of recombinant protein by developing post-translational modifications appropriately and accurately is most important point to develop protein-based subunit vaccines. In plant-based expression systems, proteins produced by undergoing N-linked glycosylation have a different glycan structure in the origin. Plant-derived glycoproteins contain α-1,3-fucose and β-1,2-xylose residue, but humans contain β-1,4-galactose and sialic acid residue. Although the anti-PA83 protein is originally a glycoprotein, the addition of a different glycan structure than the original may affect the functionality of the anti-PA83 protein when produced in the plant expression system. In order to prevent this glycosylation problem, anti-PA83 protein was produced with bacterial PNGse F and Endo H enzymes, and deglycosylated forms of anti-PA83 protein were obtained by *in vivo* deglycosylation.

As part of this thesis study; the production of the full-length anti-PA83 protein was demonstrated by co-expression of the HC and LC genes by establishing a disulfide bond between them in the plant expression system.

Western blot analysis was performed to examine the affinities of anti-PA83 variants produced in the plant with the PA83 antigens produced in the plant. anti-PA83 variants produced in the plant and used as primary antibodies showed similar cross reaction to all three antigen types.

In order to test the effect of the N-linked glycosylation mechanism and the *in vivo* deglycosylation strategy on protein stability, stability studies were performed in

three different environments (+ 4°C, 25°C and 37°C) with anti-PA83 variants. These stability studies were carried out in two different periods, 6 weeks and 6 months. When the results of the weekly stability studies are analyzed, 3 different variants of the anti-PA83 protein did not show any loss of stability in these three different environments. In the stability studies carried out in monthly periods, there was no change in the stability of the samples that were kept at + 4°C and 25°C for six months, while the samples that were kept at 37°C lost stability. As seen in SDS-PAGE analysis of samples kept at 37°C, aggregation event increased at the same time as it started to lose its stability in three anti-PA83 variants from the 2nd month. While the heavy chain of anti-PA83 protein loses its stability at 37°C, no change in its light chain (LC) was observed. As a result, when all stability results were compared, no loss of stability was observed in three proteins at + 4°C and 25°C. While the glycosylated anti-PA83 protein at 37°C shows less stability; Endo H deglycosylated anti-PA83 protein showed better stability compared to other anti-PA83 variants.

Toxin neutralization activity of plant produced anti-PA83 antibodies was evaluated in the toxin neutralization experiment. The toxin-neutralizing activity of antibodies varies depending on the dose. When applying 100 ng, maximum cell viability is observed for each antibody, while Endo H deglycosylated anti-PA83 protein has been observed to be more effective. As seen in the graphic, even at a dose of 3.125 ng antibodies to able to neutralize toxins and this can be explained by maintaining cell viability.

As a result, the methods used in this study proved that the anti-PA83 protein was folded correctly and the deglycosylated anti-PA83 proteins produced by the *in vivo* glycosylation strategy are also functional. These results show that EndoH *in vivo* deglycosylated anti-PA83 protein is more stable in all applied conditions and can be used as a vaccine candidate for anthrax. In addition, the *in vivo* deglycosylation strategy implemented in this study offers a different approach not only to vaccine development against anthrax but also to many other vaccine antigens, antibodies, and therapeutic protein development studies.

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BACKGROUND

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MESLEKİ VE İDARİ GÖREVLER

Bursiyer (TUBİTAK projesi) 2018-halen	Şarbona Karşı Aşı Adayı Olarak Kullanılabilir Koruyucu Antijen (Pa)'nın Bitkide Üretilmiş Deglikozile Formunun Üretilmesi, Karakterizasyonu Ve Klinik Öncesi Değerlendirilmesi