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AKDENIZ UNIVERSITY

PRODUCTION AND CHARACTERIZATION OF HEPTAMERIZED FORM OF PA83 OF *Bacillus anthracis* IN *Nicotiana benthamiana* PLANTS AS VACCINE CANDIDATE AGAINST ANTHRAX

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This thesis was accepted by the jury on 30/11/2021 with unanimity / ~~majority~~ vote.

Prof. Dr. Tarlan MAMEDOV (Advisor)

Prof. Dr. Nedim MUTLU

Prof. Dr. Muhammet ŞAKİROĞLU

Prof. Dr. Yaşar KARAKURT

Assoc. Prof. Dr. Sevil AKSU

ABSTRACT

PRODUCTION AND CHARACTERIZATION OF HEPTAMERIZED FORM OF PA83 OF *Bacillus anthracis* IN *Nicotiana benthamiana* PLANTS AS VACCINE CANDIDATE AGAINST ANTHRAX

Nilufer GUN

PhD Dissertation, Department of Biotechnology

Supervisor: Prof. Dr. Tarlan MAMEDOV

November 2021; 65 pages

Bacillus anthracis (B. anthracis) is the causative agent of anthrax, a gram-positive bacterium that is considered a weapon of biological terrorism in the world. It can be fatal for both animals and humans. Anthrax toxin comprises of three non-toxic proteins; Protective antigen (PA); Lethal factor (LF) and Edema factor (EF). PA is the central component of anthrax toxin and is responsible for the transition of EF and LF into the host cell. After proteolytic activation of PA83 (83 kDa), the PA20 (20 kDa) fragment is released; the remaining PA63 (63kDa) is rapidly self-assembled to form ring-shaped heptamers. The proteolytic cleavage is important for formation of heptamer structure, which allows binding with other two factors of LF /or EF.

In the native host, *B. anthracis* PA83 protein is not a glycoprotein but has some potential glycosylation sites. These glycosylation sites can be abnormally glycosylated in eukaryotic expression systems as well as in plant expression systems. Abnormal glycosylation can mask important epitopes of proteins, leading to improper folding. Recently, Mamedov et al., showed the *in vivo* deglycosylation strategy in plant expression systems. Plant-based expression systems are an alternative platform for the production of recombinant proteins which enables an economical, scalable and safe product. In addition, plant-based expression systems have eukaryotic post-translational modifications (PTMs) that affect the biological properties of proteins.

In studies, it has been shown that the *in vivo* glycosylated monomer form of PA83 produced in the plant is a highly active and stable protein for a vaccine candidate against anthrax. In this thesis study, we aim to demonstrate, the production of *in vivo* in heptamerized form of the PA83 protein using *Nicotiana benthamiana* (*N. benthamiana)* plant's transient expression system. Therefore, to produce the heptamerized form of PA63, the *B. anthracis* PA83 gene was first co-expressed with the deglycosylation enzymes Endo H and PNGase F and then *in vivo* cleaved with human furin.

Our plan was in this thesis; i) to confirm the heptamer form of PA63 with gel filtration system, ii) to optimize high level heptamerized form production, to purify, characterize and iii) examine proteins for immunogenicity and animal testing. Thus, using all these deglycosylation and furin processing strategies, the *in vivo*-deglycosylated, heptamerized form of the PA83 protein could be a new potential immunogenic vaccine candidate for mass vaccination against anthrax. Also the heptamer form of deglycosylated PA83 can be more stable because of its heptameric

structure. In addition, the study confirms the use of plant expression systems to get target proteins is a useful tool to produce safer and cheaper products in a short period, which is crucial for mass vaccination during the pandemic.

KEYWORDS: Anthrax vaccine, *Bacillus anthracis*, deglycosylation strategy, furin, heptamerization, PA83, post-translational modifications**,** recombinant protein transient expression system

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ÖZET

PRODUCTION AND CHARACTERIZATION OF HEPTAMERIZED FORM OF PA83 OF *Bacillus anthracis* IN *Nicotiana benthamiana* PLANTS AS VACCINE CANDIDATE AGAINST ANTHRAX

Nilüfer GÜN

Doktora tezi, Biyoteknoloji Anabilim Dalı

Danışman: Prof. Dr. Tarlan MAMEDOV

Kasım 2021; 65 sayfa

Bacillus anthracis (B. anthracis), dünyada biyolojik terör silahı olarak kabul edilen gram pozitif bakteri olan şarbonun etken maddesidir. Hem hayvanlar, hem de insanlar için ölümcül olabilmektedir. Şarbon toksini, toksik olmayan üç proteinden oluşmaktadır; Koruyucu antijen (PA); Ödem faktörü (EF) ve Ölümcül faktör (LF). Koruyucu antijen (PA), şarbon toksininin merkezi bileşeni olup, diğer iki proteinin konakçı hücreye taşınmasından sorumludur. PA83'ün (83kDa) proteolitik aktivasyonundan sonra, PA20 (20kDa) fragmanı serbest bırakılır; kalan PA63 (63kDa), halka şeklinde heptamerler oluşturmak için hızlı bir şekilde kendi kendine birleşmektedirler. Proteolitik bölünme, diğer iki faktör LF/veya EF ile bağlanmaya izin veren heptamer yapısının oluşumu için önemlidir.

Doğal konakçıda, *B. anthracis* PA83 proteini bir glikoprotein değildir, ancak ökaryotik ekspresyon sistemlerinde olduğu kadar bitki ekspresyon sistemlerinde de anormal şekilde glikozillenebilen bazı potansiyel glikosilasyon bölgelerine sahiptir. Anormal glikosilasyon, proteinlerin önemli epitoplarını maskeleyerek uygunsuz katlanmaya yol açabilmektedir. Son zamanlarda, Mamedov vd., bitki bazlı ekspresyon sistemlerinde *in vivo* deglikosilasyon stratejisini göstermiştir. Bitki bazlı ekspresyon sistemleri, rekombinant proteinlerin üretimi için alternatif bir platformdur. Bu sistemler ölçeklenebilir, ekonomik ve güvenli ürün sağlamaktadırlar. Ek olarak, bitki bazlı ekspresyon sistemleri, proteinlerin biyolojik özelliklerini etkileyen ökaryotik post-translasyonel modifikasyonlara (PTM) sahiptirler.

Daha önce yapılan çalışmalarda bildirildiği gibi, bitkide üretilen PA83'ün *in vivo* deglikosillenmiş monomer formu, şarbona karşı aşı adayı olarak aktif ve istikrarlı bir proteindir. Bu çalışmada, *Nicotiana Benthamiana* (*N. benthamiana)* bitkilerinde geçici ekspresyon sistemi kullanılarak, PA83 proteininin *in vivo* heptamerize formunun üretimini göstermeyi amaçlanmaktadır. Bu nedenle, PA63'ün heptamerize formunu üretmek için, *B. anthracis* PA83 geni önce deglikosilasyon enzimleri EndoH veya PNGaseF ile birlikte eksprese edildi ve daha sonra in vivo insan furini ile kesilmiştir.

Tezle ilgili yapılacak olan çalışma planımız; i) PA63'ün heptamer formunu jel filtrasyon sistemi ile doğrulamak, ii) yüksek seviyeli heptamerize form üretimini optimize etmek, saflaştırmak, karakterize etmek ve iii) proteinleri immünojenisite ve hayvan testleri için incelemektir. Bu nedenle, tüm bu deglikosilasyon ve furin işleme stratejilerini kullanarak, PA83 proteininin *in vivo*, deglikosile edilmiş, heptamerize

formunun, şarbona karşı toplu aşılama için yeni bir potansiyel immünojenik aşı adayı olabileceğini ummaktayız. Ayrıca, deglikosillenmiş PA83'ün heptamer formu, heptamerik yapısı nedeniyle daha stabil olabilmektedir. Çalışma, pandemi döneminde toplu aşılama için önemli olan daha kısa sürede daha güvenli ve ucuz ürünler sağlamak, hedef proteinlerin üretimi için bitki bazlı ekspresyon sistemlerinin umut verici kullanımını da doğrulamaktadır.

ANAHTAR KELİMELER: Şarbon aşısı, *Bacillus anthracis*, de-glikozilasyon, stratejisi, furin, heptamerizasyon, PA83, post-translatiyonal modifikasyonlar, rekombinant protein, geçici ifade sistemi.

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PREFACE

In this thesis research, my aim is to present a new vaccine candidate against anthrax, one of the most dangerous diseases in the world. This vaccine candidate will be economically important to the medical and agricultural fields around the world. I owe a debt of gratitude to my esteemed mentor Prof. Dr. Tarlan MAMEDOV, who helped me set out on this path and helped me move to an advanced level with his valuable ideas.

I want to thank Prof. Dr. Nedim MUTLU, who has guided me with his valuable experiences on this challenging journey and my valuable teacher on my thesis committee, Assoc. Prof. Dr. Sevil AKSU, for her valuable ideas, helps and assistance.

I want to thank my dear husband Yasin GUN, who always supported me and my family; my mom Gulshan NAGHIYEVA, father Gadir NAGHIYEV for raising me as a strong woman and making me stand on my own two feet. I dedicate this thesis to my beautiful little daughter Elif GUN.

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ACADEMIC DECLARATION

I would like to state that this study named "Production and Characterization of Heptamerized form of PA83 of *Bacillus anthracis* in *Nicotiana benthamiana* plants as vaccine candidate against anthrax", which I submitted as a doctoral thesis, was written under academic rules and ethical values, and the source of all the information that does not belong to me in this thesis was indicated.

30/11/2021

Nilufer GUN

SYMBOLS AND ABBREVIATIONS

Symbols

G : gram

µg : micro gram

mg : milligram

µg : microgram

ng : nanogram

^{o}C : degree of temperature

L : Liter

µl : microliter

ml : milliliter

M : molar

mM : mill mole

kDa : kilo Dalton

Abbreviations

PA : Protective antigen of *Bacillus anthracis*.

rPA : Recombinant PA

LF : Lethal factor

EF : Edema factor

PTM : Post-translational Modification

CV : Column Volume

OD : Optical Density

Comma (.) is used as decimal separator in the thesis (e.g. “21.01”).

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1. INTRODUCTION

Anthrax is caused by the aerobic and spore-forming, gram-positive, rod-shaped bacterium called *Bacillus anthracis (B. anthracis)* (Brachman et al. 1960; Meyerhoff et al. 2004; Goel et al. 2015). This lethal disease has a very high environmental resistance, and spores can become contagious after decades and years. The disease has a variety of exposure routes: cutaneous, gastrointestinal and inhalation (Inglesby et al. 2002). The inhalation form is the most dangerous form of the disease, which can act as a biological warfare agent and weapon.

B. anthracis categorized as A agent in the list of bioterrorism agents (Rotz et al. 2002) by CDC (Centers for Disease Control and Prevention of the USA). Additionally, *B. anthracis* spores can easily form transportable endospores. It is thought that these spores are likely to be used as a weapon of bioterrorism (Inglesby et al. 2002, 2002; Webb et al. 2003). The accidental spread of anthrax spores from a military laboratory in the former Soviet city of Sverdlovsk in 1979 (Abramova et al. 1993; Meselson et al. 1994; Guillemin et al. 1999) and the spore-containing letter attacks that occurred in the United States in 2001(Jernigan et al. 2001; Jernigan et al. 2002) support this notion.

B. anthracis virulence factors are encoded by two different genes, one of which is responsible for the synthesis of the poly-γ-D-glutamic acid of the capsule (Makino et al. 1989; Mock et al. 2001) that enhance phagocytosis of vegetative forms. The second type of the genes is liable for the synthesis of the three proteins exotoxin-protective antigen (PA), edema factor (EF) and lethal factor (LF) (Dixon et al. 1999; Okinaka et al. 1999). All of these proteins are non-toxic on their own (Leppla and Gordon 1994; Young et al. 2003).

Protective antigen (PA) is a B (binding) protein responsible for the entry of toxins into the host cell which is important for the host cell intoxication to form the lethal toxin (LeTx; PA + LF) and edema toxin (ETx; PA + EF) (Ascenzi et al. 2002; Moayeri et al. 2009). Edema factor (EF) and lethal factor (LF) are proteins that contain exotoxin (Tang et al. 2009; Friedleander et al. 2000; Swartz et al. 2001; Bromberg-White et al. 2010).

Moreover, Domain 4 of the protective antigen (PA) involves the host cell receptor binding site that can bind to the cell surface receptor when cell poisoning occurs. The furin protease cleavage site located in Domain I, Domain II and III are included in the formation of heptameric structure on the cell surface.

Lethal toxin (LeTx) induces the release of tumor necrosis factor-interleukin-1b (Swartz et al. 2001; Diane et al. 2009), which causes pathological effects, by macrophages, that can end up in vital results (Young et al. 2007; Hutt et al. 2014). The intoxication process begins with the binding of PA to mammalian host cell receptors (Young et al. 2007). After proteolytic cleavage by the human furin protease enzyme, the 20 kDa fragment attached to the N-terminal end is released (Leppla et al. 1988; Singh et al. 1989; Klimpel et al.1992), and the 63 kDa fragment at the carboxyl end attached to the cell receptors forms the heptameric complex (Beauregard et al. 2000; Elliott et al. 2000; Mogridge et al. 2002). The heptamers combine with LF and EF (Mogridge et al. 2002) then enter the host cell by endocytosis (Abrami et al. 2003).

Under the influence of low acidic pH, EF and LF are released into the cytosol to achieve their toxic effects (Koehler et al. 1989; Milne and Collier 1993; Bann et al. 2012; Jiang et al 2015) (Fig. 1.1.).

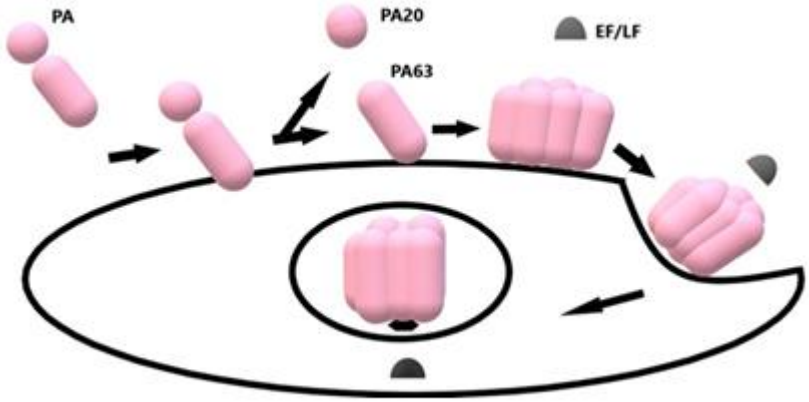


Figure 1.1. The steps of anthrax toxins entry to the cell

Vaccination is recognized as the most effective and critical way of protection from infectious diseases. Therefore, developing safe and long-term stable vaccines for mass vaccination remains a key concern for researchers. A vaccination human against anthrax with live spores has been limited to the former USSR and China (Shlyakhov et al. 1994). The first developed version of the live attenuated anthrax vaccine for humans was in 1940 Russia, in which live dry spores containing two different encapsulated *B. anthracis* variants were used. The currently used version of the vaccine is one of a non-capsulated *B. anthracis* strains in combination with PA adsorbed on aluminum hydroxide (Feodorova et al. 2001). The UK and USA use non-live subunit vaccines based on PA, due to concerns about the possibility of bacterial residue (Turnbull et al. 1991).

The currently licensed human vaccines are the anthrax vaccine adsorbed (AVA, Biothrax) in the US and the anthrax vaccine precipitated (AVP) vaccines in the UK. Both vaccines were obtained from the culture filtrate of toxic strains of *B. anthracis* absorbed to aluminum adjuvant (Wang and Roehrl 2005; Chitlaru et al. 2011; Kaur et al. 2013). Several studies have demonstrated that both vaccines can provide protection in different animal models, requiring repeated doses (Fellows et al. 2001; Pitman et al. 2001). The negative features of both vaccines, such as the vaccination schedule and the length of the dose interval, and the instability of PA, have led to the development of new recombinant PA (rPA)-based vaccine candidates (Geier 2002).

In this study, we aim to produce an *in vivo* heptamerized form of PA83 as a new recombinant anthrax vaccine candidate in *Nicotina benthamiana (N. benthamiana)* plants using the plant's transient expression system. Until now, many organisms have been used as hosts to produce recombinant proteins. Plant expression systems are a promising platform for safe and cost-efficient production of valuable recombinant proteins including antibodies, vaccines, and enzymes for medical, agricultural and industrial applications. In addition, the existence of eukaryotic post-translational

modification machinery makes plants more attractive to produce target proteins in their natural form.

In this work, PA83 was successfully deglycosylated with the *in vivo* deglycosylation enzymes EndoH and PNGaseF and processed further with *in vivo* human furin. With metal affinity purification (IMAC) and size-exclusion chromatography, our objective was to purify the required proteins and separate the PA20 fragment from the heptamerized form of PA83 protein. Proteins were examined by transmission electron microscope to observe heptameric ring structures and to ensure the structural accuracy of heptamerized PA63. Mass spectrometry analysis was performed for peptide sequence analysis to detect matches with *B. anthracis* PA similarities.

After the confirmation of heptamerized proteins, the toxin neutralization assays and animal tests were performed in order to describe the immunogenicity of the obtained heptamerized proteins. We expected that heptamerized form of PA83 will be more immunogenic and stable (which is a major concern in the development of vaccines) from its other variants due to its structure.

2. LITERATURE REVIEW

2.1. Anthrax

Anthrax disease is a serious animal and human pathogen. This disease has a cutaneous, gastrointestinal and inhalation forms that associated with animal infections. As mentioned above *B. anthracis* spores are very resistant to adverse conditions such as radiation, drought and chemicals.

Cutaneous form occurs, when people consume contaminated meat or injured part of a body contact with sick animals. The anthrax spores can diffuse the body with injured body skin. The 95% of anthrax cases are cutaneous form of the disease. Incubation period is 0.15 to 12 days and the mortality rate is 20% if there is no usage of antibiotics (Turnbull 1991).

Gastrointestinal form of anthrax occurs when people consume contaminated meat products. The incubation period of this form is between 1-7 days and the mortality rate can form 25-60% (Turnbull 1991; Brachman and Friedlander 1999).

The inhalation form is quite dangerous; it begins with the inhalation of dormant endospores. This form has rapid development and a high mortality rate (Hugh-Jones et al. 1999; Mal'tseva et al. 1990).

In general, consuming antibiotics is a current treatment method for anthrax treatment. Many antibiotics are effective on *B.anthracis* bacteria. However, the fact that bacterias become resistant to antibiotics day by day initiates new and different methods to develop in the treatment. Vaccination is known to be the most effective methods in the treatment of diseases. Currently, researchers are developing next-generation recombinant subunit vaccine components using a variety of expression systems (Chichester et al. 2007, 2013). The binding of the protective antigen to two toxins by target cells and inhibiting the entry of this toxin complex into the host cell is an important issue to obtain a protective immune response against anthrax (Ezzell et al. 1992; Pedro et al. 2015).

2.2. Vaccines Against Anthrax

In 1881, Pasteur demonstrated protection against anthrax by injecting heat-attenuated *B. anthracis* strains into sheep. Vaccination with attenuated anthrax Sterne strains, which was widely used after the 1930s, has decreased anthrax cases in domestic animals. The only human vaccine licensed against anthrax in the USA is the AVA (BioThrax) vaccine, which consists of an aluminum-hydroxide-absorbed and toxic culture non- capsulated *B. anthracis* Sterne strain. In the early 1950s, the AVA was improved, which contained only the protective antigen (PA) protein of *B. anthracis* (Leppla et al. 2002). Some limitations, such as instability, traces of LF and EF, lack of optimal vaccination schedule and doses, are the negative features of AVA vaccines in the case of mass vaccination.

2.2.1. Live bacterial vector based vaccines against anthrax

Due to their mucosal administration and stimulation of numerous immune responses, live bacterial vectors based vaccines are possible candidates against anthrax. Live bacterial vaccines based on *B. anthracis* spore vectors (Aloni-Grinstein et al. 2005) have protection against anthrax infection. Recently, *Salmonella spp., S. typhi* in particular is a promising platform for stable expression of anthrax toxin protective antigen (Galen et al. 2009). Other *Salmonella* based live attenuated vector vaccines are now under investigation. The limitations of live attenuated bacterial strains are their virulence activity; however, bacteria's such as lactic acid bacteria can be another alternative for live attenuated bacterial strains (Ding et al. 2018). To improve immune responses, further studies must be conducted.

2.2.2. DNA-based vaccines against anthrax

DNA-based vectors are a promising platform for designing candidate vaccines against anthrax infection. Because of their easy construction, stability and efficacy make these candidates more useable. However, the level of immune response induced by DNA-based vectors is not enough for conferring the immunity against the infection. Gu et al. (2005), first put forward the ability to elicit the toxin neutralizing activity of DNA-based anthrax vaccines. Although the immune response of DNA-based anthrax vaccines was very low, it is considered as one of the promising vaccine candidates against anthrax due to its easy of construction and time efficiency (Livingston et al. 2010; Darrell and Les Baillie 2005). Currently, studies are mainly directed towards developing DNA-based vector vaccines for the future.

2.2.3. Subunit rPA based vaccines against anthrax

Until today *Escherichia coli (E.coli)* (Sharma et al. 1996), *Bacillus substilis (B.substilis)* (Baillie et al. 1998), *Bacillus anthracis (B. anthracis)* (Ramirez et al. 2002), *Bacillus brevis (B. brevis)* (Baillie et al. 1998; Rhie et al. 2005) bacteria were mostly used for rPA production in bacterial expression systems. Every bacterium has its own advantages and limitations (Kondakova et al. 2019) as in clinical trials of rPA-based vaccine candidates, NCT01624532, NCT04148118 and NCT02655549 (clinicaltrials.gov), produced in the bacterial expression system, have been completed but the results have not been published.

The plant expression system is an alternative system for the expression of subunit vaccines due to its safe, cost-effective, scalability and eukaryotic post-translational modification machinery. Studies have shown that potato, tomato and tobacco plants were the first plants to express recombinant PA. Aziz (2002, 2005), despite their low expression efficiency and toxin neutralizing antibodies in transgenic, tomato and tobacco plants expressed recombinant PA for the first time. Watson (2002, 2005), showed that the expression of PA in tobacco plant chloroplasts can be increased by 360 million doses. Studies have shown that oral immunization of mouse with plant chloroplast-derived rPA survives the challenge of deadly toxin (Koya et al. 2015).

The protective antigen (PA) of *Bacillus anthracis* does not carry N-linked glycans in its native form. However, it has some potential N-linked glycosylation sites that can

glycosylate abnormally in eukaryotic expression systems and as well as in plant expression systems. Adding glycan groups to the protein chain can alter the biological behavior of proteins (Yusibov and Mamedov 2010), mask their important epitopes and cause them to misfold (Mamedov et al. 2012; Yusibov and Mamedov 2013). Mamedov et al. (2016, 2017) demonstrated an *in vivo* deglycosylation strategy of proteins with co-expression of bacterial PNGaseF (Mamedov and Yusibov 2013; Mamedov et al. 2016) and EndoH (Mamedov et al. 2017) enzymes in *Nicotiana benthamiana* plants. This strategy enables to generate recombinant proteins in plants with their native glycosylated forms (Mamedov et al. 2012; Yusibov and Mamedov 2013; Mamedov et al. 2016).

Chichester et al. (2007), demonstrated the transient expression of subunit anthrax vaccine candidate using tobacco mosaic virus in *Nicotiana benthamiana* plants. Mouse and rabbits were immunized with the target proteins produced in this study, resulting in the formation of high titers of lethal toxin neutralizing antibodies in the animals. Furthermore, Chichester and Yusifov (2007) reviewed six potential plant-produced vaccine candidates that are in Phase-I human clinical trials. The plant produced rPA based candidate vaccine NCT02239172 (ClinicalTrials.gov) clinical trials are completed but results have not been published.

AV7909 (NuThraxTM) is one of the new generation anthrax vaccine candidates developed for post- exposure prophylaxis against anthrax (Clinicaltrials.gov). It contains a combination of licensed AVA (BioThrax) with adjuvant CPG7909 and agonist Toll-like receptor nine (TLR9). CPG7909 is an immunostimulatory oligodeoxynucleotide (Bode et al. 2011) compounds which induce the B-cell to bind with Toll-like receptors (Aebig et al. 2007). High protection against anthrax challenge and toxin neutralizing antibodies have been demonstrated in both animal (Rynkiewicz et al. 2011; Savransky et al. 2017) and clinical studies (Hopkins et al. 2013, 2016; Savransky et al. 2019) by ensuring the safety of vaccine candidates using CPG7909 as an adjuvant against anthrax in rat models (Savransky et al. 2019). Also, Shearer et al. (2021), a protection on the candidate with less immunization for the AV7909 vaccine candidate has been conducted.

2.2.4. Adenovirus expressed rPA vaccine candidates

Another potential next generation anthrax subunit vaccine candidate is human adenovirus expressed rPA based vaccines. There are two different adenovirus expressed rPA-based vaccine candidates which were developed by PaxVax, Inc. (ClinicalTrials.gov NCT01979406) in 2013 and Altimmune, Inc in 2017 (ClinicalTrials.gov Identifier NCT03352466). The clinical trial NCT01979406 is completed but the data have not been published.

Table 2.1. Clinical and preclinical trials of some anthrax vaccine candidates[1]

Candidate Vaccines	States	References
Live bacterial vector vaccine		
Salmonella Typhi Ty21a vectored vaccine expressing PA83 (integrating the PA gene into the Ty21a chromosome) Ty21a-PA-01	Preclinical	
Bacterial expression system-subunit		
rPA83 PA83 protein expressed in *B.anthracis* Sterne-1	Clinical, Phase II	NCT00170469
rPA83 PA83 protein expressed in *E. coli*	Clinical, Phase II	NCT00170456
Wild type rPA (wtrPA)	Preclinical	
rPA	Clinical, Phase I I(terminated)	NCT00133484,
peptidoglycan-linked poly-γ-D-glutamate (GluPG) combined to PA (GluPG + PA)	Preclinical	
Subunit vaccine -plant expression system		
Transgenic *Brassica juncea* (*B. juncea*) plants expressing PA83 protein-PA (BJ-PA)	Preclinical/ Oral (crushed leaf material)	
Transplastomic *Nicotiana tabacum* (*N. tabacum*) plants expressing PA83 protein (NT-PA)	Preclinical/Mice Oral (total soluble protein)	
PA83- Fraunhofer Center for molecular biotechnology (FhCMB)	Clinical, Phase I	NCT02239172
Plant produced (pp)-PA83 nonglycosylated PA83	Preclinical	

[1] Information get from ClinicalTrials.gov website. Continues on next page

Continuation of **Table 2.1**

PA-D4-DNA vaccines encoding the codon-optimized PA-D4 (domain IV) protein fused with signal peptides and containing the SV40 enhancer	Preclinical	
Adenovirus-vectored vaccine		
Adenovirus4 (Ad4) -PA	Clinical, Phase I	NCT01979406
Adenovirus5 (Ad5)	Clinical	NCT03352466

The current state of rPA based vaccine studies underway to improve safety, stability, purity and immunogenicity. The major concern for the development of anthrax vaccines is to obtain highly pure subunit recombinant vaccine candidate that remain stable during storage. Selection of the appropriate expression system is important to produce stable and safe recombinant proteins. Currently, bacterial, yeast, insects, mammalian and plants expression systems are widely used. These expression systems have advantages as much as limitations and drawbacks. The expression system mainly used for rPA is bacterial and plant- based expression systems.

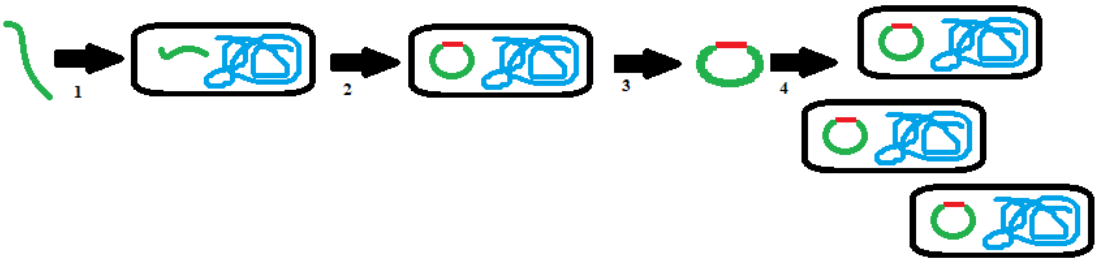
2.3. Expression Systems

Production of recombinant proteins has increased significantly due to the ever-increasing demand in research laboratories, medical fields, industry and agriculture. Selection of an appropriate expression system for the production of recombinant proteins should generally be chosen based on the biochemical and biological properties of the desired proteins. The most used expression system is bacterial expression systems; which provide cheap product and time efficient production.

Bacterial expression system; since post-translational modifications (PTMs) are not in bacterial expression systems, they are not a suitable system to produce of eukaryotic proteins. PTMs play a critical role in the biological and functional activity, solubility and stability of proteins (Table 2.2). Briefly, the target proteins are transformed into a competent E. coli cell to produce target proteins in the bacterial expression system. Positive clones are then selected and isolated from the plasmid or expression vector. Next, step is transformants amplification and isolation of the protein of interest. Last, the amplification and isolation of the protein transformant of interest (Figure 2.1.). Bacterial expression systems are mostly used for the production of proteins that do not need post-translational modifications, such as insulin and other uncomplicated human proteins (Mammedov and Yusibov 2010).

Table 2.2. The advantages and disadvantages of conventional protein expression systems

Expression system	Advantages	Disadvantages
Bacterial- *E. Coli*	Fast expression	No eukaryotic PTM
	Cost effective	Not properly folded proteins(some)
	Easy scale-up	Rare Secretion
	Suitable genetic	
Yeast-*Saccharomyces cerevisiae (S.cerevisiae)*	Relatively fast expression	Characteristic N-linked glycan structures of proteins
	Secrets intracellular proteins	Use of signal peptides requiring
	Cost effective	Safety precautions are required
	Able to most protein folding and post translational modifications (PTMs)	
Baculovirus/Insect cells	Relatively fast expression	Characteristic N-linked glycan structures of proteins
	Secreted, membrane and intracellular proteins expression	Expensive
	Able to most protein folding and PTMs	Difficult to scale-up
Mammalian cells (transient expression)	Relatively fast expression	Expensive
	Secreted and membrane proteins expression	Intracellular proteins yield are low
	Able to all protein folding and PTMs	Difficult to scale-up



1. Transformation of competent E.coli with gene of interest
2. Selection and expansion of clones
3. Isolation of plasmid/expression vector
4. Scale-up of transformants; isolation of protein of interest

Figure 2.1. Schematic illustration of the Protein expression system schedule in *E.coli* bacteria expression system

Yeast expression system; the other mostly used expression system is yeast expression system which is perfectly suitable for the production of complex eukaryotic recombinant proteins. This expression system is safe, cost-effective and scalable, and is free of mammalian viruses and toxins that are dangerous to humans. To exemplify;

i) The target proteins are transformed into a competent E. coli cell to produce gene of interest;

ii) Positive clones are then selected and isolated from the plasmid or expression vector;

iii) Isolated plasmid/expression vectors transformed into the yeast cells;

iv) Selection and scale-up of transformants and isolation of protein of interest (Figure 2.2.).

Recombinant proteins produced in yeast expression systems have hyper glycosylation that differs from mammalian ones (Rogan and Babiuk 2005).

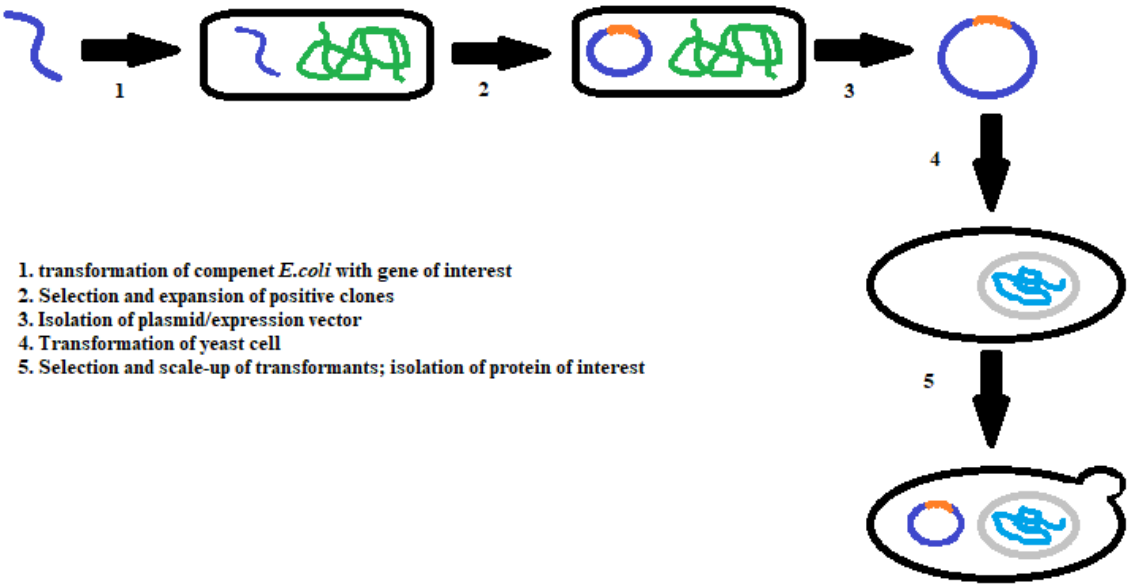


Figure 2.2. Schematic illustration of the protein expression system schedule in Yeast–*Saccharomyces cerevisiae* expression system

Insect expression system; baculovirus vector-inserted insect expression systems have advantages such as high-level target expression capacity and presence of post-translational protein modifications. Figure 2.3. shows, the transformation of the gene of interest into baculovirus vector-inserted insect expression systems. The disadvantage of these expression systems is the high mannose-type glycosylation and the absence of some complex oligosaccharides that differ from their mammalian counterparts (Rai and Padh 2001). In addition, gene cloning, lengthy engineering procedures, and difficult culture conditions make target proteins more difficult to express in these systems than their prokaryotic counterparts.

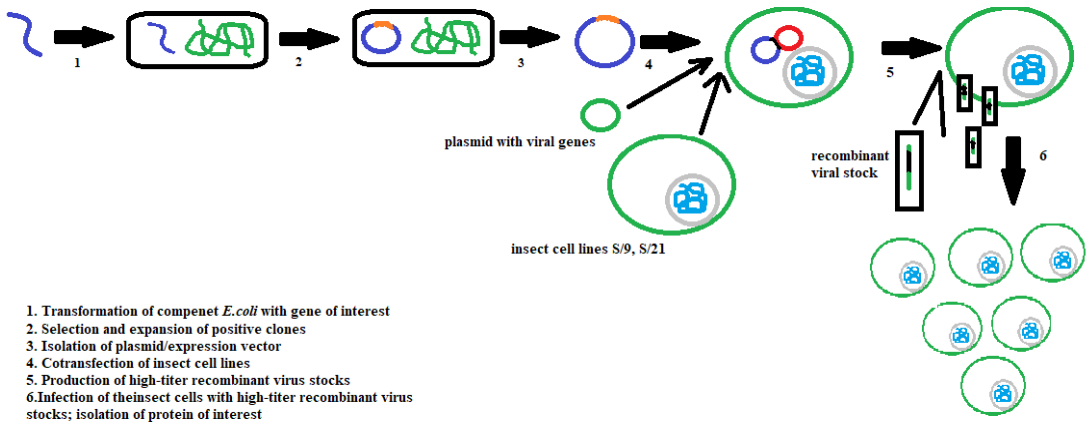


Figure 2.3. Illustration of the Protein expression system schedule in insects *Spodoptera frugiperda* (*S. frugiperda* Sf9 and *S. frugiperda* Sf21) expression system example

Mammalian expression system; the ideal expression system to produce complex proteins is regarded as the mammalian expression system. The transfer of target genes into the mammalian expression system is shown in Figure 2.4. These systems are capable of proper folding of proteins and post- translational modifications. Chinese Hamster Ovarian (CHO) cells and Human Embryonic Kidney (HEK 293) cells are the only mammalian expression systems that can achieve high protein yield (Rosano et al. 2014). Also, Table 2.2 shows the disadvantages and advantages of this expression system.

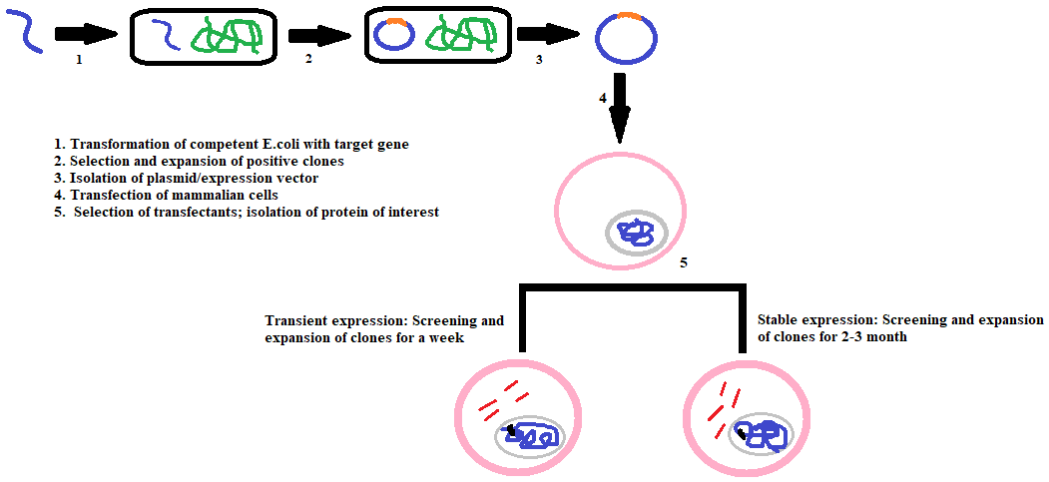


Figure 2.4. Illustration of protein expression system schedule in Mammalian cell expression systems–HEK293 and CHO example

2.3.1. Plant expression systems

Plant-based production systems are an alternative expression system that is potentially safe, scalable, and reduces the production cost of the targeted recombinant proteins (Nandi et al. 2016). The *Nicotiana* (*N. benthamiana* and *N. tabacum*) genus (*Solanaceous* species) is considered as the most suitable host due to its easy breeding, high biomass, easy genetic manipulation and lack of contamination of pathogens which enabling these plants to be used in molecular plant engineering (Bally et al. 2018). The important feature of *N. benthamiana* is that it contains a natural addition to the RNA-dependent RNA polymerase 1 gene (Bally et al. 2015). This feature results in reduced levels of gene silencing, ease of insertion, transformation, and development of expression vectors for transient expression systems (Bally et al., 2018). Also, *N. benthamiana* plants are preferred as hosts for the expression of recombinant proteins with the transient system, due to their susceptibility to plant viruses (Figure 2.5.). The non-food crop specificity of *N. benthamiana* plants provides advantages for minimizing the risk of recombinant proteins produced to involve the food chain (Rymerson et al. 2002; Twyman et al. 2003).

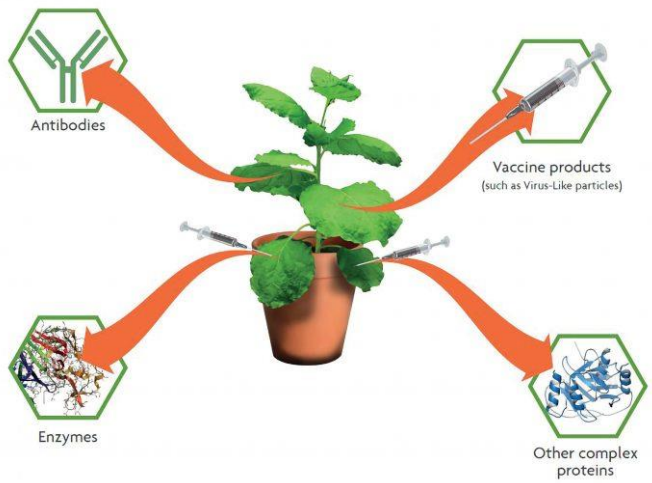


Figure 2.5. *N. benthamiana* plant-based production systems can produce antibodies, enzymes, vaccine products and other complex proteins for research applications (edited and quoted from Albor, 2019)

There are two ways to produce proteins in plants; transgenic (target gene is involved in the plant's nuclear or chloroplast genome) and transient expression system using *Agrobacterium* infiltration technology. Transgenic or stable transformed plants take a long time to produce the targeted gene or protein. This makes transient expression systems preferable to transgenic expression systems (Kapila et al. 1997). Transient expression in plant leaves allows time efficient production of recombinant proteins.

Generally, transient expression of the recombinant target proteins in plants are mediated by tissue infiltration using *Agrobacterium tumefaciens* or vector based plant viruses (Kapila et al. 1997). *A.tumefaciens* is able to pass its own DNA to the host plant genome with two separate regions called T-DNA and vir region (Gelvin et al. 2003) (Figure 2.6.). These two regions are essential for tumor induction and contain the genes that are encoded for proteins involved in biosynthesis of plant-type hormones and opine (Zupan et al. 2000; Chumakov et al. 2013). Opine is the source of nitrogen and energy for *Agrobacterium*, which induced by the expression of T-DNA in transformed plants. Vir genes contain seven loci and are not transferred to the host cell genome, but are required for T-DNA to transit into the host genome (Zupan and Zambryski 1995).

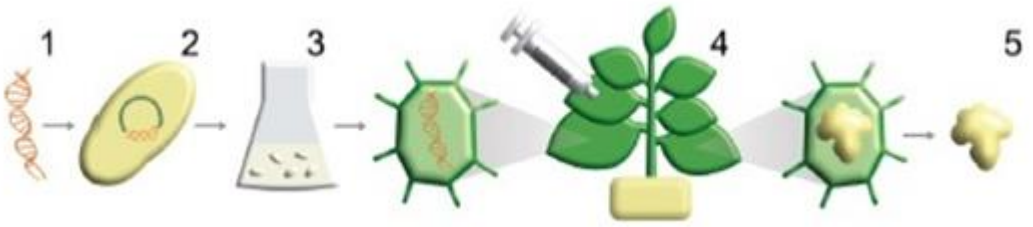


Figure 2.6. The general principle of transient expression in plants mediated by *A. tumefaciens* (edited and quoted from Helger, 2019)

Vector systems for expressing target protein genes in plant expression systems are often constructed by using sequences from self-replicating virus systems. Until now, several different plant viruses such as Tobacco mosaic virus (TMV), Potato X virus (PVX), Cowpea mosaic virus (CPMV), Tobacco etch virus (TEV) have been used for self-replicating viral vectors. The expression efficiency, promoter activity, vector size and gene silencing are the several factors affecting efficiency of proteins produced in plants expression system using viral expression systems. Peyret and Lomonossoff (2015) and Mardanova et al. (2017), showed that the P19 protein from the Tomato bushy stunt virus (TBSV) is one of the repressors to reduce post-transcriptional gene silencing effect.

The development of binary vector systems containing a disarmed Ti plasmid, (Bevan et al. 1984) and any type of DNA can be replaced if made with *Agrobacterium* infection of plant tissues. Moreover, the absence of tumor-inducing plasmids and modifying only non-productive tissues in Agro strains has been considered environmentally safe (Frank and Lomonossoff 2014; Krenek et al. 2015). Production efficiency is 5-20 times higher with agro infiltration. Temporary plant-based gene expression has become a potentially appealing option for the production of proteins in both academic and commercial interests (Sainsbury and Lomonossoff 2008).

2.4. Plant Post-Translational Modifications (PTMs)

Post-translational modifications are important for the biological activity of proteins. Phosphorylation, acetylation, glycosylation and others are post-translational modifications performed in eukaryotic expression systems (Figure 2.7.) (Dupas et al. 2007).

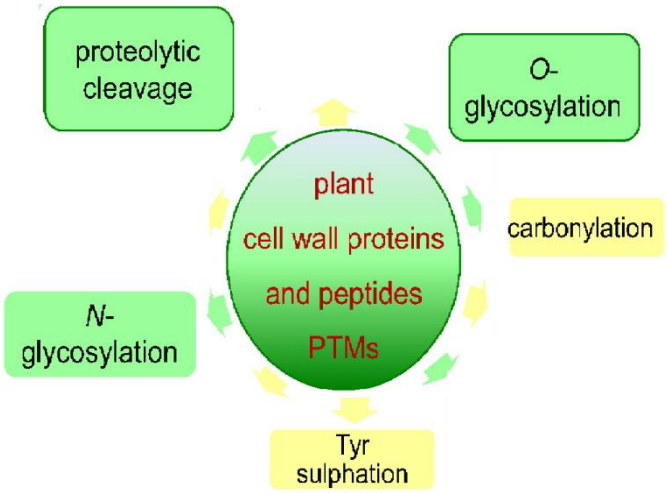


Figure 2.7. Some critical PTMs in plant secretory pathway

2.4.1. Glycosylation

Glycosylation is the reaction in which a carbohydrate group is attached to a hydroxyl or other functional group of molecule in order to form a glycans. N-linked glycans are glycans linked to the amide group of asparagine (Asn); O-linked glycans the

ones linked to the hydroxyl group of Serine (Ser), threonine (Thr), hydroxylysine or hydroxyproline (Hyp) residues of proteins. N-linked glycosylation is one of the most important types of glycosylation, can affect the proper folding, stability, transport of the proteins (Helenius and Aebi 2001, 2004). Glycosylation can affect the proper folding, stability, resistance to proteolysis, transport and classification of proteins (Gomord V et al. 2010). Studies show that there is a relationship between N-glycosylation status of cellular proteins, drug sensitivities and susceptibility to infections (Faye et al. 2005).

2.4.2. N- linked glycosylation in plants

N-linked or asparagine linked glycosylation of proteins are the main post-translational modification for the eukaryotic proteins. N-glycan maturation departs from plants and mammalians which results in the absence of α (1,6)-linked fucose and sialic acids and the presence of bisecting (1,2)-xylose and core α(1,3)-fucose in the N-glycans in late Golgi apparatus (Figure 2.8.) (Gomord et al. 2004).

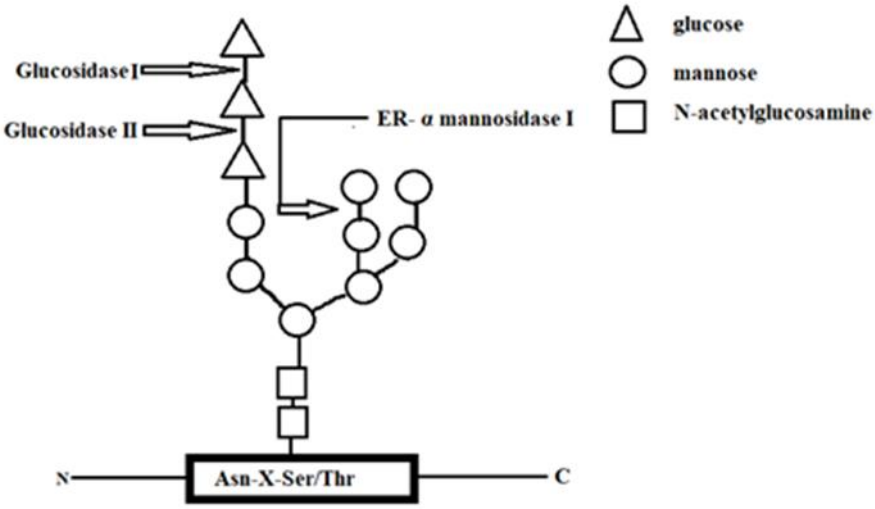


Figure 2.8. The N- linked core oligosaccharide

Understanding the importance of N-glycosylation has helped to understand the relationship between N-glycosylation and human diseases. The observations suggest a possible role of N-glycans as a potential cause of disease in cells. Therefore, information on the regulatory properties of N-glycosylation may be promising in designing of new therapeutic proteins (Kukuruzinska and Lenon 1998).

2.4.3. *In vivo* de-glycosylation strategy with deglycosylation enzymes EndoH and PNGaseF

N-glycosylation is required for cell viability in some eukaryotic proteins such as structural proteins, blood proteins, immunoglobulins and growth hormones. The addition of glycan groups to the protein chain can strongly alter the biological behavior of proteins, which is often undesirable (Mamedov and Yusibov 2013). Abnormal

glycosylation can mask important epitopes of proteins, leading to improper folding of the proteins (Mamedov and Yusibov 2012).

Recently, to avoid this unwanted aberrant glycosylation of plant-made recombinant proteins, Mamedov T et al., proposed *in vivo* deglycosylation of proteins by co- expressing with bacterial deglycosylation enzymes PNGase F (Mamedov et al. 2012, 2016) and EndoH (Endo-β-N-acetylglucosaminidase) (Mamedov et al. 2017) in plants using transient expression systems. Experiments have demonstrated that *in vivo* deglycosylation of recombinant proteins co-expressed with bacterial PNGase F in *N. benthamiana* plants are able to cleave N-linked glycans from all proteins tested. It has also been observed that these PNGase F deglycosylated proteins are more stable than their glycosylated forms (Mamedov et al. 2012, 2016).

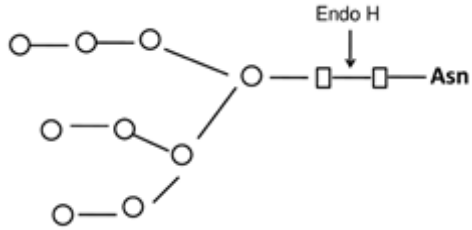


Figure 2.9. Cleavages of Endo H (quoted from Mamedov et al. 2017)

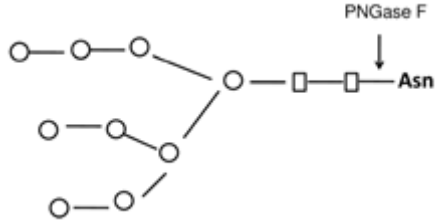


Figure 2.10. Cleavages of PNGase F (quoted from Mamedov et al. 2017)

Deglycosylation of target proteins with PNGaseF make deamidation of asparagine to aspartate in N-X-S/T sites, but deglycosylation of EndoH catalyze the cleavage between the two acetylglucosamine (GlcNAc) residues of N-linked oligosaccharides without any deamination contaminants of asparagine (Figure 2.9). Therefore, EndoH prevents target proteins to be in native conformation (Mamedov et al. 2017, 2019a, 2019b).

It has been reported that, the EndoH *in vivo* deglycosylated form of (PA) B. Anthracis is a potential vaccine candidate that is more stable and active which has a high immunogenicity against anthrax compared to its PNGaseF deglycosylated counterpart. EndoH can cleave the linkage between the two acetylglucosamine residues of highly active N- linked oligosaccharides while preserving the natural structure of the proteins (Figure 2.10.). Therefore, EndoH *in vivo* deglycosylated proteins give more stable results at different temperatures. This is a very important matter for the stability of the vaccine candidates in the production processes (Mamedov et al. 2017).

2.5. Furin Processing

The human furin is an endoprotease which is reliable for the post- translational cleavage for a number of pioneer proteins. The bond of maturation of gamma-carboxylation of glutamic acid and formation of disulfide is important for furin cleavage of the proteins.

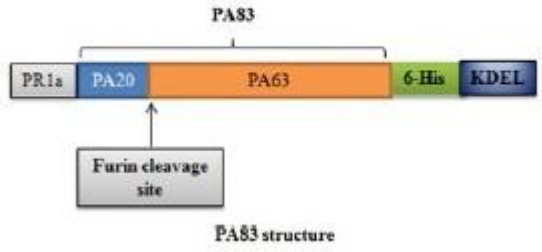


Figure 2.11. PA83 gene structure (edited and quoted from Mamedov et al. 2019a)

Furin also functions in the entry of pathogens such as viruses, bacteria into the host cells by activation of their proteins. Such examples are; Spike protein of the novel Coronavirus SARS-CoV-2, envelope proteins of HIV (Bour et al. 1995), dengue fever (Zybert et al. 2008), and influenza virus (Volchkov et al. 1981).

Furin also activates anthrax toxin (Gordon and Leppla 1994). PA of anthrax toxin must be cleaved by furin-like proteases to become functionally active (Figure 2.11.). The human furin gene was engineered and produced in *N. benthamiana* plants in a highly active form, which enabled the processing of target proteins (Factor IX, PA83) in plants (Mamedov et al.2019a).

Tyrosine sulfation is one of the mammalian cell PTMs. This PTM commonly describes neutralizing antibodies that target HIV envelope glycoproteins (Lee et al. (Lee et al 2017). There is only one reported engineering of tyrosine sulfation in a plant-based expression system (Loos et al. 2005).

In addition, γ-carboxylation is one of the important PTMs that affects the activity of coagulation factors. In theory, γ-carboxylase can be expressed in plant secretory pathways, requiring additional PTMs. Mamedov et al., (2019a), demonstrated the transiently co-expression of blood-clotting factor IX in *N. benthamiana* plants (Mamedov et al. 2019a).

3. MATERIALS AND METHODS

3.1. Buffers and Suspensions

3.1.1. Western blot and SDS –PAGE buffers

SDS-Polyacrylamide Gel Electrophoresis (PAGE):

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

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.

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

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 6NHCl and volume is completed up to 500 ml with autoclaved ddH_2O.

- **Running Buffer (1X):**

Tris-base 3.03 g; Glycine 14.3 g; SDS (%10) 10 ml were mixed and the final volume was arranged to 900 ml with autoclaved distilled water.

- **SDS / Western Gel sample loading solution (5X, Laemli Buffer):**

Tris-HCl (1 M) 9.38 ml; Glycerol 11.9 ml; SDS 3.3 g; 2- mercaptoethanol (%25) 7.5 ml; Bromophenol blue stock suspension (100 mg/20 ml**)** 660 µl add and mix. The pH was adjusted to 6.8 with HCl by the addition of 11.9 ml of glycerol, 9.4 ml of 1 M Tris solution and 3.3 ml of SDS. The final volume was arranged at 40 ml and aliquot into 1 ml tubes, stored at -20 °C for future use. The protein samples are boiled with 1/4 volume (5x) Laemli Buffer, before loading the gel. All acrylamide gels were prepared at 10%.

- **SDS gel staining solution (Coomassie staining):**

500 ml of methanol; 100 ml of glacial acetic acid and 1 g of Coomassie Blue R250 were dissolved in autoclaved distilled water and arranged the final volume to 1000 ml.

- **SDS gel destaining solution:**

700 ml distilled water; 200 ml of methanol and 100 ml of glacial acetic acid.

- **APS Solution (%10):**

60 mg APS solved in 600 µl distilled water. APS is freshly prepared and used.

- **SDS Solution (%10):**

10 g SDS dissolved in 90 ml distilled water.

- **Preparation of the Gels (%10-for two gels):**

Table 3.1. The %10 gel preparation compounds

Gels	Stacking	Resolving
Distilled water	2.45 µl	1.98 µl
40% Acrylamide-Bis solution	1.250 ml	0.368 µl
Tris-HCl	1.250 ml (p.H: 8.8)	0.780 (pH: 6.8)
%10 SDS	50 µl	50 µl
TEMED	2.5 µl	3.125 µl
%10 APS	25 µl	30 µl

After preparing and stacking gel, the 650 µl isopropanol was pouring to the surface of the gel, and then let for polymerizing for 20 minute at room temperature.

- **Transfer Buffer for Western Blot (1X):**

5.8 g Tris, 2.93 g Glycine, and 370 µl 10% SDS are dissolved into the 800 ml autoclaved ddH2O and volume is completed to 1 L.

- **5XTBS:**

5X TBS includes 20 mM Tris (pH: 7.5), and 150 mM NaCl. 12.12 g Tris and 43.9 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.

- **1XTBS:**

200 ml of 5 × TBS were taken, and the final volume was arranged to 1000 ml with distilled water.

- **I-Block;**

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). 0.5 g I-block was mixed completely 100 ml of 1XTBS.

- **Primary Antibody (Western Blot 1: 1000):**

10 µl antibodies were mixed in 10 ml I-block.

- **Secondary Antibody (Western Blot 1: 5000):**

2 µl of antibodies were mixed in 10 ml I-block.

3.2. Bacterial Grows and Mediums

- **SOC medium:**

20 g bactotriptone, 5 g bacto yeast extract, 2 ml of 5M NaCl, 2.5 ml of 1M KCl, 10 ml of 1M $MgCl_2$, 10 ml of 1M $MgSO_4$ were dissolved in autoclaved distilled water of 800 ml, autoclaved for 30 minutes at 121°C. After removing the solution from the autoclave, the temperature was reduced to 50 ° C. Then, 20 ml of 1M glucose was added under sterile cabinet and left at + 4 ° C.

- **SYS (BBL) medium:**

10 g of soyhydrolyzate, 5 g of yeast extract and 5 g of NaCl were solved in 800 ml of autoclaved distilled water. pH is adjusted to the 7.0 with 1M KOH or NaOH, the final volume was arranged to 1000 ml with autoclaved distilled water. It was autoclaved at 121°C for 30 minutes.

- **MMA medium:**

MES medium is prepared as 10 mM MES, 10 mM $MgCl_2$. 1.9 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.

- **100 mM stock solution of Acetosyringone (AS):**

0.4 g Acetosyringone was weighted and dissolved in 12 ml of 95% ethanol and 8 ml of distilled water and arranged to the final volume of 20 ml. For 1L MMA 150 µl Acetosyringone is required (15 mM).

3.3. Protein Purification (His-tag) Buffers

- **1 M NaH_2PO_4:**

To prepare 1 M NaH_2PO_4: 13.8 g NaH_2PO_4 (H_2O) dissolved into the 100 ml distilled water.

- **1M Na_2HPO_4:**

To prepare 1 M Na_2HPO_4: 28.38 g Na_2HPO_4 dissolved into the 100 ml distilled water.

- **Sodium phosphate solution (20mM):**

15.48 ml 1 M Na_2HPO_4; 4.52 ml NaH_2PO_4; 17.53 g NaCl are mixed in 100 ml distilled water. pH adjusted to the 7.0 with HCl and volume is completed to the 800 ml with distilled water.

- **100 mM imidazole stock solution:**

0.34 g Imidazole was mixed in 50 ml Sodium phosphate solution (20 mM).

- **Column Equilibration Buffer (10 mM):**

5 ml of imidazole stock solution was mixed with 45 ml Sodium phosphate solution (20 mM).

- **Extraction buffer(10 mM):**

1 mM Dieca weighted randomly for the targeted material and mixed with Equilibration Buffer (10 mM).

- **High Salt Wash Buffer (25 mM):**

12.5 ml of imidazole stock solution was mixed with 37.5 ml Sodium phosphate solution (20 mM).

- **Elution Buffer (250 mM):**

0.85 g imidazole dissolved into the 50 ml Sodium phosphate solution (20 mM).

- **1xPBS tablet buffer:**

1 tablet was dissolved in 200 ml of distilled water (The PBS tablet contains 137 mM NaCl, 2 mM KCl and 10 mM phosphate buffer).

3.4. Gel Filtration Buffers:

- **Cleaning buffer (%20 ethanol);**

40 ml of ethanol are mixed with 160 ml distilled water. The buffer used for cleaning of column.

- **Equilibration and Elution buffer:**

1.2 g Tris-base dissolved into the 500 ml double distilled water; pH adjusted to the 8.5 with HCl (20 mM Tris-base) ; 11.1 g CaCl dissolved into the 100 ml double distilled water (1 M).

3.5. Gel Preparation Buffers for Mass Spectrometry

0.4 g NH_4HCO_3 dissolved into the 50 ml distilled water (100 mM). Buffer prepared and used freshly.

- **Reducing solution:**

7.7 mg DTT dissolved into the 5 ml 50 mM NH_4HCO_3 buffer.

- **Alkylation solution:**

10 mg Iodoasetamide 1 ml dissolved into the 5 ml 50 mM NH_4HCO_3 buffer.

3.6. Elisa Buffers

- **Coating buffer:**

0.16 g Na_2HCO_3 (15 mM) and 0.3 g $NHCO_3$ (35 mM) dissolved into the 100 ml double distilled water. The buffer should be stored at room temperature.

- **Washing buffer:**

0.1% Tween-20 was mixed with 1xPBS.

- **Blocking buffer:**

0.5 % Blocking-grade was solved in 1xPBS containing 0.1% tween-20.

- **OPD-Substrate solution (0,05 M phosphate-citric acid buffer);**

2.84 g Na_2HPO_4 dissolved into the 100 ml double distilled water (0.2 M). 2.10 g citric acid dissolved into the 100 ml double distilled water (0.1 M). 25.7 ml from 0.2 M Na_2HPO_4 and 24.3 ml 0.1 M citric acid and 50 ml double distilled water are mixed (pH: 5.0).

- **30% Hydrogen peroxide:**

12 ml Hydrogen peroxide and 28 ml double distilled water are mixed. The solution should be stored at +4C.

3.7. Cell Culture Preparation

43.5 ml high glucose DMEM (Sigma, lot: RNBH7255). 5 ml fetal bovine-serum (gibco REF: 10270-106 LOT: 41G8349K). 500 µl Pen-strep (100X) (gibco REF: 15140-122 LOT: 16882254).

3.8. Transient Expression of PA83 of *B. anthracis* with Furin, EndoH and PNGaseF in *N. benthamiana* Plants by Infiltration

Constructs of PA83 of *B. anthracis*, EndoH, PNGase F and human furin genes were available in our laboratory and used for the production of heptamerized form of PA83 in *N. benthamiana* plants. The PA83 and EndoH; PNGaseF and human furin constructs 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 9:1 or 8:1:1 (PA83:EndoH:Furin) (Figure 3.1.). The leaf samples were picked on 5-6 days post-infiltration (dpi), homogenized in three volumes of extraction buffer (2 mM sodium diethyldithiocarbamate (DIECA) solved in 1xPBS) and centrifuged as described previously (Mamedov T et al. 2016).

Figure 3.1. The infiltration procedure in *Nicotiana benthamiana* plants leaves

3.9. Purification of Co-expressed Plant-produced gPA83, dPA83 –EndoH, dPA83 – PNGaseF and Co-expression with Human Furin Variants by Ni-NTA Column

Purification of deglycosylated PA83 variants and furin cleaved deglycosylated PA83 variants were performed with IMAC (Immobilized Metal Affinity Chromatography) using HisPur Ni-NTA resin (Thermo Fisher Scientific, Cat. No. 88221) as mentioned previously (Mamedov T et al. 2016, 2017, 2019a). To outline shortly, a 20 g leaf of each sample was homogenized (20 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole and 1mM DIECA, pH 7.4). The crude extract of plant the leaves were passed from gauze strip and centrifuged at 13000 g for 25 minutes. After centrifugation the plant cell supernatants were passed through 0.45 μm filter (Millipore), using HisPur Ni-NTA resin (Thermo Fisher Scientific, Cat. No. 88221) which was previously equilibrated with 2 column volume (CV) of equilibration buffer (20 mM sodium phosphate, 0.3 M NaCl, 10 mM imidazole, pH 7.4).

After passing all filtrated supernatant from the column, the column was washed with 8 CV of washing buffer (20 mM sodium phosphate, 0.3 M NaCl, 25 mM imidazole, pH 7.4). Elution buffer (20 mM sodium phosphate, 0.3 M NaCl, 250 mM imidazole, pH 7.4) was passed through the column to collect the proteins from the column resin. The purificated protein concentrations were combined and controlled with Biodrop which later measured the concentrated elutes. Elution fragments with high concentration were combined later fragments were concentrated at 4°C at 8000 g with Millipore concentrator (30K MWCO).

The deglycosylated samples with EndoH and PNGaseF (dPA83-E and dPA83-P) buffer interchanged against 1xPBS buffer, furin cleaved deglycosylated samples buffer interchanged against 20 mM Tris-HCl, pH 8.5, 1 mM $CaCl_2$, and 150 mM NaCl buffer. Purified proteins were filtrated and aliquoted as 1 μg/μl. Purified plant produced PA83

variants were analyzed by SDS-PAGE and Western-blot analysis at reducing conditions.

3.10. SDS-Page and Western Blot Analysis

The plant leaves after 6 days of post infiltration were picked up and homogenized in the three volumes of extraction buffer (2 mM sodium diethyldithiocarbamate (DIECA) solved in 1xPBS). Following the extraction the plant leaves crude extract was centrifuged at 13000g for 5 minutes and the supernatant was mixed with 5xLaemmli Buffer (100 mM Tris, 2% SDS, 20% glycerol, 4% β -mercaptoethanol, pH 6.8) at the ratio of 4:1 in reduced conditions and boiled at 100^{o}C for 5 min.

After that, they were loaded to the 10% SDS-PAGE that is prepared with references on Table 3.1 and run at 100 V 15 min-200V 45 min into running buffer. After running the gels the stacking part was removed and washed with distilled water for 5 minutes. After washing the gel, it was stained with Coomassie staining buffer for 1 hour, and distained with destaining buffer for 15-20 minute intervals. The destaining of the gels continued until the gel color cleared. In the final step the gel photos were taken.

There are two steps in Western Blot analysis: The first step is the same as the SDS-PAGE analysis. In the second step, proteins were transferred onto the polyvinylidene fluoride membrane (Millipore, Billerica, MA) with transfer buffer for 1 hour at 100V. Membrane was blocked with 1% blotting grade solution (0.5% I-Block (Applied Biosystems, Carlsbad, Calif.) in 1xTBS) (Figure 3.2).

Proteins were detected with HRP-conjugated anti-mouse secondary antibody (Cat. No., Abcam). Signal was generated by chemiluminescent substrate as peroxidase and enhancer (Stable Peroxide (2.5 ml) and Luminol / Enhancer (2.5 ml)). Protein amounts were calculated mathematically using Gene Tools Software. Gel photographs were obtained using a GeneGnome XRQ chemiluminescence imaging system (Syngene Corp., USA).

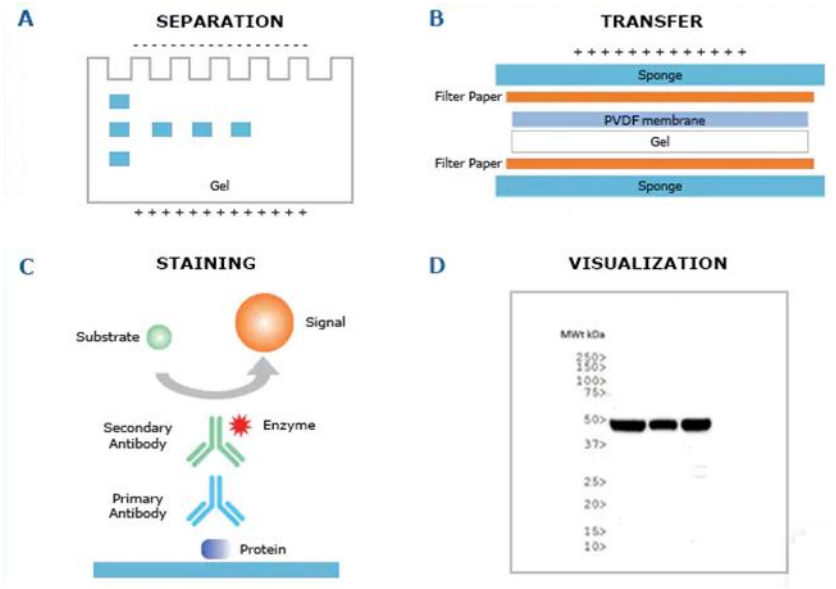


Figure 3.2. General representation of the conventional Western Blot. **A)** Seperation of proteins by PAGE; **B)** transfer of blotted proteins to a membrane (PVDF/nitrocellulose); **C)** Primary antibody specific to target proteins, followed by an enzyme conjugated secondary antibody. The enzyme (horseradish peroxidase, HRP) acts on a substrate (electro-chemiluminescence, ECL) to emit light; **D)** the signals were captured on chemiluminescence imaging system (edited and quoted from https://www.novusbio.com)

3.11. Gel Filtration Chromatography Analysis

The gel filtration chromatography analysis was performed using an AKTA Start Fast Purification Liquid gel filtration chromatography device using Hiprep 16/60 Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) (Figure 3.3.). Column resin was equilibrated with 1.2 (CV) of equilibration buffer (20 mM Tris-HCl pH 8.5, 1 mM CaCl2, 150 mM NaCl). After equilibration, protein samples were passed through the column. Before testing sample proteins, Protein standard Mix 15-600 kDa (Sigma, cat. No. 69385-30MG) was used as the standard protein in the experiments. The systems flow chart was shown in Table 3.2. The combined fractions were concentrated, aliquot and stored at -80 ^{o}C until future use.

Table 3.2. Akta Start Fast Chromatography Systems running flow chart

System Pressure Limit	0,15 MPa
System Flow rate	4 ml/min
Column Volume	120 ml
Column Equilibration Volume	1.2 CV
Column Elution Volume	1 CV
Sample Volume	1 ml
Fraction Volume	4 ml

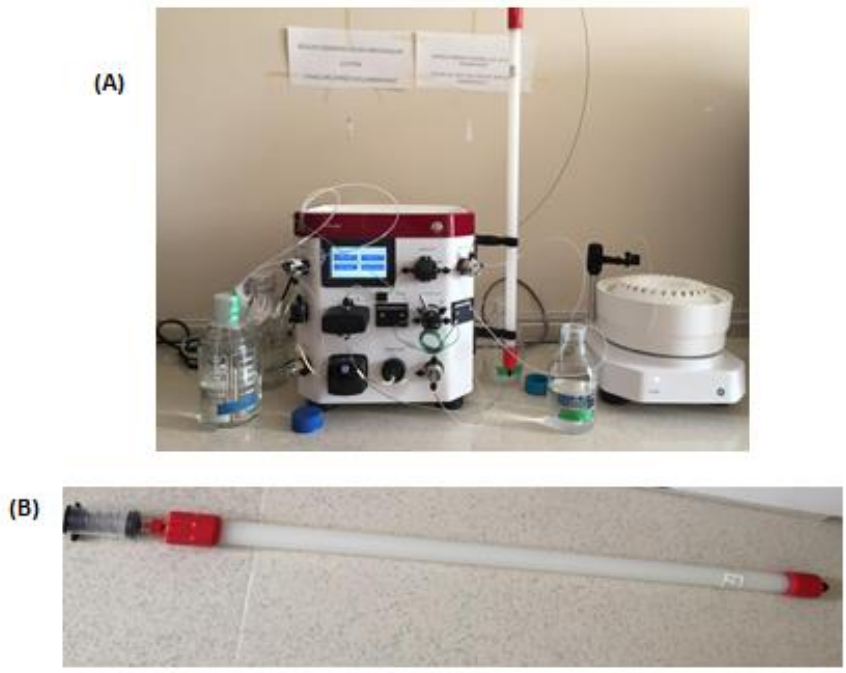


Figure 3.3. A) AKTA Start Fast Purification Liquid gel filtration chromatography System; **B)** Hiprep 16/60 Sephacryl S-200 High Resolution (HR) column

3.12. Transmission Electron Microscope (TEM)

Gel filtrated, negatively stained heptamer structure of furin cleaved protein samples were examined with Transmission Electron microscope (TEM) at Medical school in Akdeniz University. Plant produced gel filtration protein samples were applied to carbon film grids for 2 minutes, excess solvent was removed using blotting paper and stained with uranyl acetate (1%, w/v). After air dry, the grids were examined by a Zeiss Leo 906 E TEM (Germany) operated at 120 kV and then photographed.

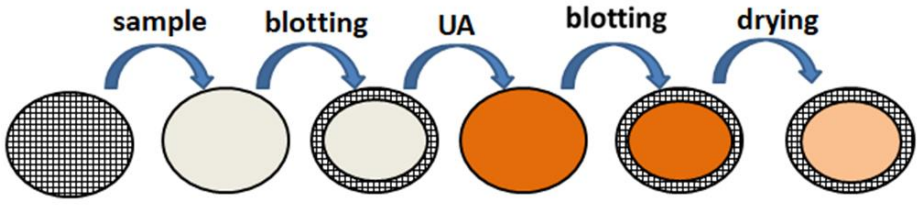


Figure 3.4. The sample preparation for the observation of transmission microscope

3.13. Mass Spectrometry Analysis

Respectively, the reduced pure protein samples were cut from %10 acrylamide-gels after running in SDS-PAGE. After washing with wash buffer (50 mM Ammonium Bicarbonate, 50% acetonitrile), the gel particles were the reducing with reduced buffer

(100 mM Ammonium Bicarbonate, 10 mM DTT). Later, the washed gel particles were alkylated and reduced with alkylation buffer (100 mM Ammonium Bicarbonate, 50 mM Iodoacetamide). After alkylation, suspended lyophilized trypsin (20 µg/vial) (prepared in 1 ml of 50 mM ammonium bicarbonate) was added to the gel pieces in minimal volume and incubated overnight at 37°C.

The next day, the centrifuged gel plugs were extracted with 50% acetonitrile and 0.1% TFA, and the prepared protein samples were lyophilized in Akdeniz University Faculty of Medicine. For Mass Spectrometry Analysis, MALDI-TOF MS Ultraflextreme MALDI-TOF, Bruker Daltonics instrument from Hacettepe University was used.

Saturated HCCA (alpha-Cyano-4-hydroxy cinnamic acid) was used as matrix for experiment with 30:70 (v/v) Acetonitrile: TFA 0.1% H2O. Lyophilized protein samples were mixed with 10 µl of HCCA matrix solution, 0.5 µl of the mixture was taken and left to dry by dripping onto the plate. Instrument calibration was performed at 700-3500 Da using Bruker Peptide Mix. Reflection positive mode, as a result, targets were ground steel (Maria et al. 2017; Apweiler et al. 1999).

3.14. Cell Culture Preparation and Toxin Neutralization Assay (TNA)

3.14.1. Cell culture

The J77A.1 (mouse macrophage cell line, ATCC TIB-67, Manassas, VA) (Figure 3.5.) cells were 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 and slowly pipetted 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 for incubation. When the cells covered every part of the flask, they were removed from the medium flasks. Cells attached to the the flask were washed 3 times with PBS buffer.

Trypsin was added to the cells to separate the cells from flask and incubate 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 the 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 2.5x 104 cells/ well in 50 µl and were incubated overnight at 37°C with 5% CO_2 medium. Cells left for incubation were used for Toxin neutralization (TNA) assay a day later.

3.14.2. Toxin neutralization assay (TNA)

Toxin-neutralizing activity was determined using a mouse macrophage (J774A.1 cells, ATCC TIB-67, Manassas, VA) cell line culture (Figure 3.5) and a WST-1-based

cell viability quantitation assay (Roche Molecular Biochemicals, Indianapolis, IN). Proteins were diluted as serial dilutions (4000 ng/ ml, 2000 ng/ ml, 1000 ng/ ml, 500 ng/ ml, 250 ng/ ml, 125 ng/ ml, 62.5 ng/ ml) in a fresh 96-well flat bottom whole DMEM (Sigma, D6429-500ML LOT: RNBH7255) supplemented in a fetal bovine serum (FBS, GİBCO ref: 10770 LOT: 41G8349K).

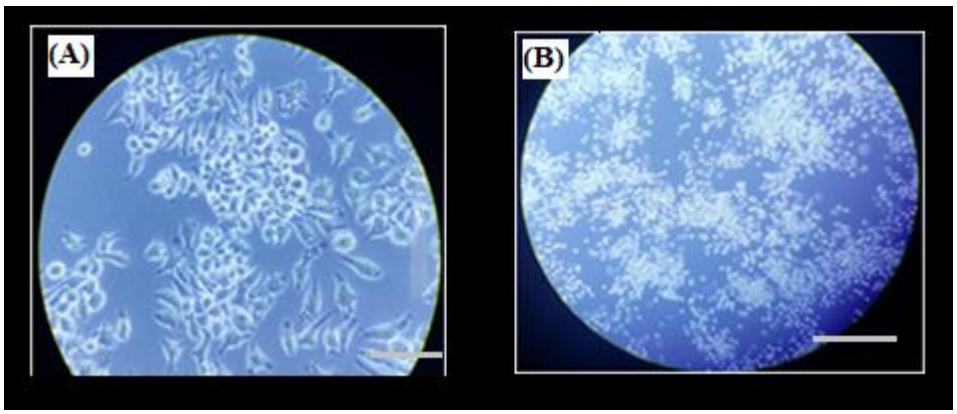


Figure 3.5. The mouse macrophage J774A.1 cells (ATCC TIB-67, Manassas, VA) culture view with electron microscope. **A)** Low density; **B)** High density

The mouse macrophage cell line (J774A.1 cells, ATCC TIB-67, Manassas, VA) was plated in a 96-well plate as 2.5 x 104 cells /well in 50 µl and incubated for 16–19 hours at 37°C with 5% CO2 in a microtiter plate in D-MEM for approximately 4 hours prior to the assay. Lethal factor (List Biological Laboratories, Campbell, CA) was prepared to be 400 ng/ ml.

As shown in Figure 3.6., proteins were added onto the cells in the 96-well plate in one and second row only cell + LF. First, the proteins were loaded in three replicates at 2000ng, 1000 ng, 500 ng, 250 ng, 125 ng and 62.5 ng in 25 µl to each well. After adding the proteins, LF was added with 100 ng in each well. The plate with cell, protein and LF was incubated for 4 hours at 37°C in 5% CO2 environment.

After a 4 hour incubation at 37°C with 5% CO_2, cell viability was accessed by adding WST-1 (Roche Applied Sciences, Indianapolis, IN), a proliferation reagent, followed by a spectrophotometric measurement at 450 nm (OD450) as described previously (Stephen et al. 1996; Miriam et al. 2010; Wycoff et al 2011; Hang et al. 2013).

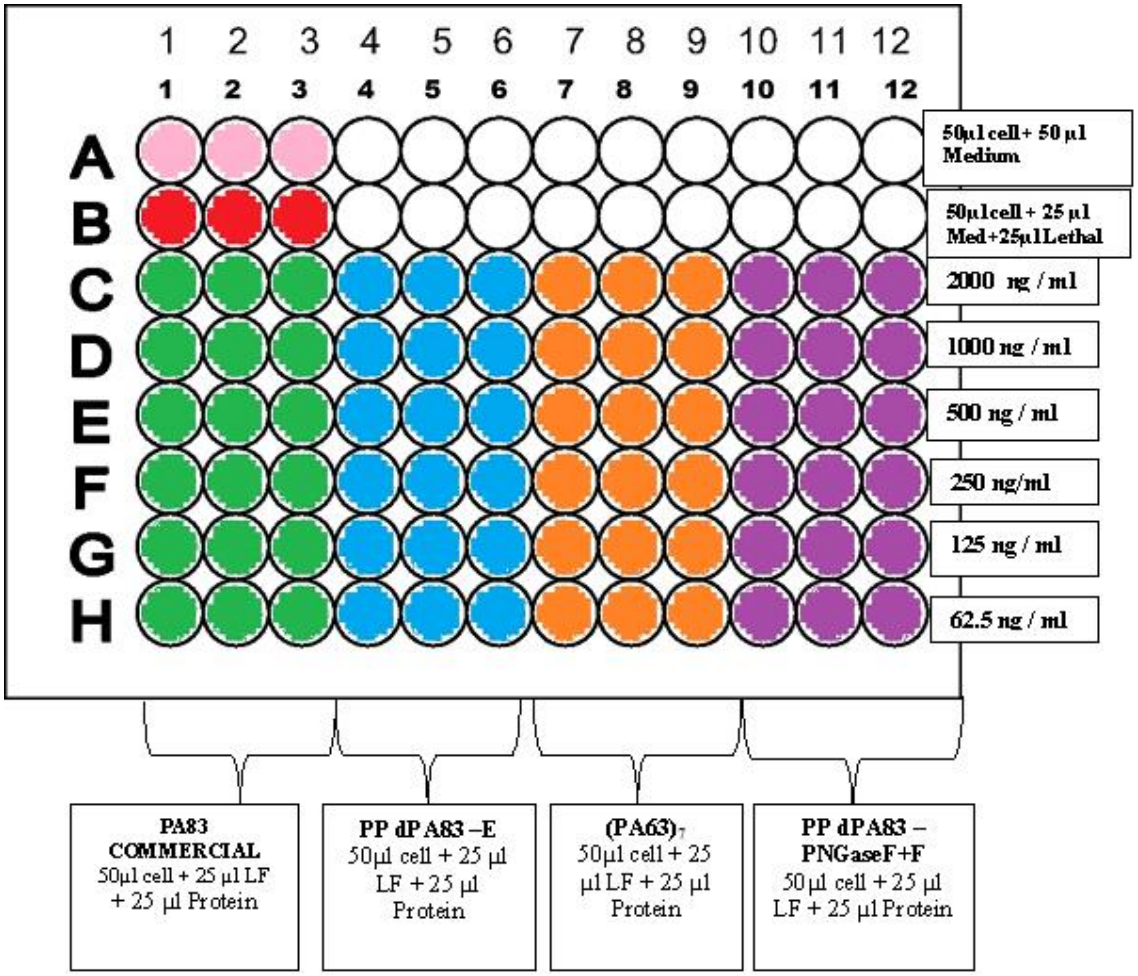


Figure 3.6. The loading scheme of the proteins on the 96-well plate

3.15. Immunization of Animals with Target Antigens

Immunogenicity assays were performed intraperitoneally in seven- to eight-week-old mice (6 animals/group) with 5 µg of plant-produced dPA83-E and $(dPA63E)_7$ proteins. Mice were injected with two doses of protein samples adsorbed in 0.3% Al hydrogel at three-week intervals (day 0, 21). On day 42, serum samples were taken from the abdominal aorta of the mouse under an anesthetic agent.

Recombinant PA (rPA) obtained from *B. anthracis* (purchased from List. Bio. Lab) was used as a positive control and 1xPBS was used as a negative control. Anti-PA IgG titers were determined by enzyme-linked immunosorbent assay (ELISA). Each point on the graph was derived from three replicas for each dilution. Dilutions of serum controls were titrated on plates containing dPA83-E protein (0.5 mg/well) and datas are expressed as geometric mean titer (GMT) ± standard error (SE) per group. Serum dilutions endpoint titers were determined in samples giving an OD value three times greater than pre-immune control samples.

3.16. Enzyme Linked Immunosorbent Assay (ELISA)

As mentioned before serum samples anti-PA IgG was detected by enzyme-linked immunosorbent assay (ELISA) as described previously (Kazutoyo et al. 2008). Data's were expressed as geometric means of titers (GMT) ± standard error (SE) per groups. End titers were defined as reciprocal serum a dilution that gives a mean to OD than the pre-immune control samples. Flat-bottom 96-well ELISA plates were coated with 0.5 μg/ml per well of test and the protein was diluted with coating buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate). Later, the plates were wrapped in plastic wrap, stored at 4 ◦C more than 12 hours.

To initiate the test, the plates were washed in 3 replicates at room temperature (RT) for 5 minutes with 200 μl/well of wash buffer (0.1% Tween-20 in PBS). Plates were then blocked with 200 μl/well of blocking buffer (5% (w/v) milk powder in 0.1% Tween-20 in PBS) for 2 hours at room temperature (RT).

Diluted serum was added to the coated wells (100 μl/ well) and incubated for 2 hours at room temperature (RT). After blocking, the plates were incubated with 100 μl/well of anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Cat. No. ab98790, Abcam) for 1 hour at room temperature (RT) (Suzanne et al. 2018).

After similar blocking and washing, substrate tablet(s) were dissolved in stop buffer (0.05 M phosphate-citrate buffer, pH 5.0, typically OPD concentration 0.4 mg/ml), plus 40 μl of fresh 30% hydrogen peroxide (per 100 ml should be used) added plate was stored in the dark for 30 minutes at room temperature. After incubation, spectrophotometric measurements of the plates were made at 450 nm.

4. RESULTS AND DISCUSSION

4.1. Expression, determination of PA83 of *B. anthracis*, Human Furin, EndoH and PNGaseF in *N. benthamiana* Plants

PA83 protein variants produced in *N. benthamiana* plant were previously cloned and transferred to *Agrobacterium* plasmid (Mamedov et al. 2016, 2017). As described in the Material and Method, PA83, EndoH and PNGase F genes were co-infiltrated to produce deglycosylated forms of PA83 protein in the plant (Figure 4.1.). As can be seen in Figure 4.1., the deglycosylation enzymes EndoH and PNGaseF were perfectly deglycosylated with the plant produced PA83 (pp-Pa83) (Figure 4.1. lane 3, 4).

In furin-cleaved samples, a high molecular band was observed with EndoH and PNGaseF deglycosylated PA83 variants (Figure 4.2. lane 4, 5), but not with glycosylated PA83 (Figure 4.2. lane 6). This suggests that the formation of the heptamerized form of glycosylated PA83 is likely to be blocked by the N-linked glycans plant.

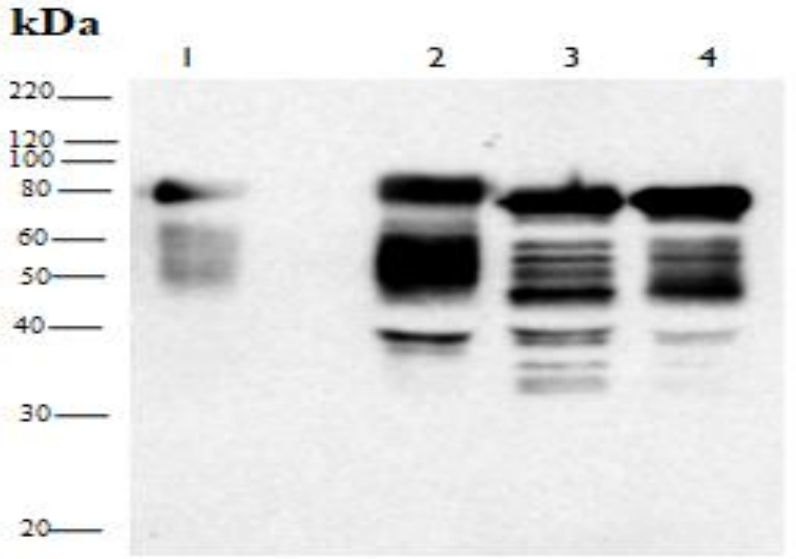


Figure 4.1. Western Blot analysis of *in vivo* deglycosylated PA83 with deglycosylation enzymes EndoH and PNGaseF. 1-glycosylated PA83 standard protein (100 ng/ml); 2-glycosylated PA83 protein (gPA83); 3- EndoH deglycosylated PA83 protein (dPA83-E); 4-PNGaseF deglycosylated PA83 (dPA83-P) protein[2]. 10 or 25 ng protein samples were loaded into gel

[2] Because the experiment was run under reducing conditions, the lines below the protein bands are denatured protein lines

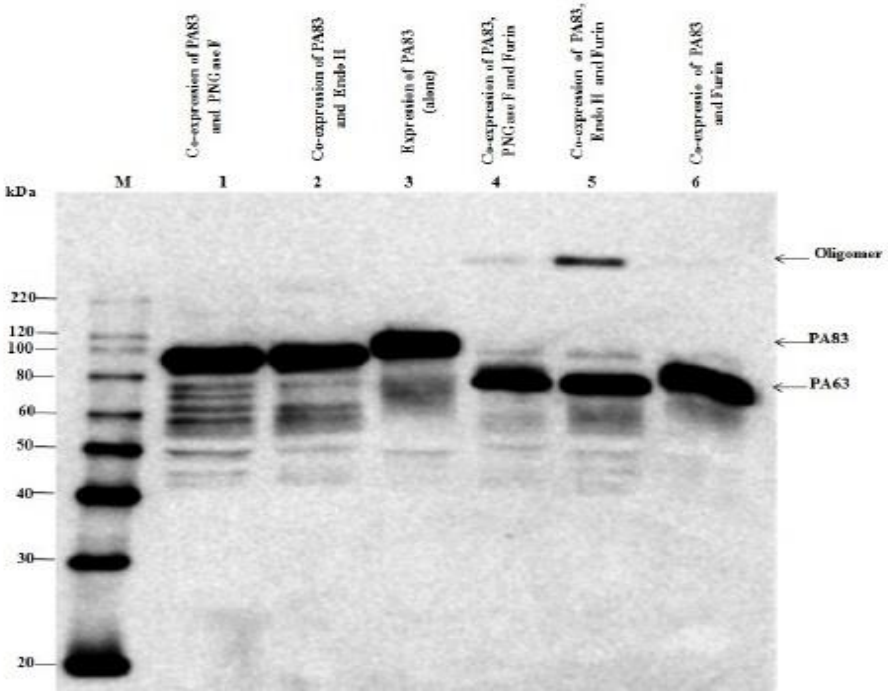


Figure 4.2. Western blot analysis of PA83 variants with furin, with/without deglycosylation enzymes EndoH and PNGaseF produced in *Nicotiana benthamiana* plants. Lanes: 1-PA83 co-expressed with PNGaseF (dPA83-P) (not furin cleavage); 2-PA83 co-expressed with EndoH (dPA83-E) (not furin cleavage); 3-glycosylated PA83 (gPA83) (not furin cleavage); 4-PA83 co-expressed with furin and PNGaseF (dPA83-P+F); 5-PA83 co-expressed with furin and EndoH (dPA83-E+F); 6-furin cleaved and glycosylated PA83 protein (gPA83+F). 10 or 25 ng protein samples were loaded into gel

4.2. Purification of Furin with De-glycosylated PA83 of *B. anthracis* (with EndoH and PNGaseF)

Plant-produced furin-cleaved, deglycosylated PA83 (dPA83-E, dPA83-P) protein variants were purified with an IMAC (Ni-NTA column) chromatography system as reported in Materials and Methods. Purified proteins treated with furin the buffer interchanged against 20mM Tris-HCl pH 8.5, 1mM $CaCl_2$, 150mM NaCl. Concentrated proteins were aliquot and placed at -80°C for future use (Figure 4.3 and Figure 4.4.).

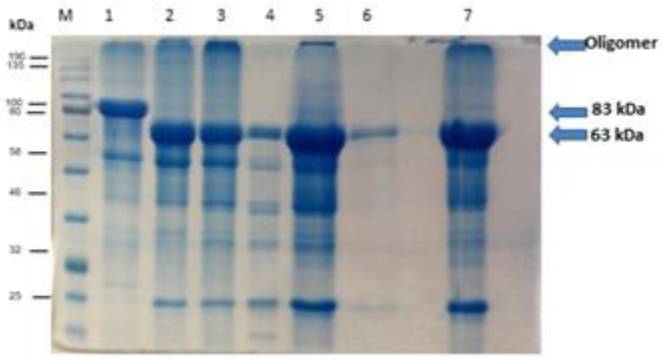


Figure 4.3. SDS-PAGE analysis of purified, co-expressed of PA83 with furin, with/without deglycosylation enzymes EndoH and PNGaseF from *N. benthamiana* plants. Lanes: 1-PA83 co-expressed with EndoH (dPA83-E); 2-Co-expression of PA83 with furin and PNGaseF deglycosylation enzyme; 3-Co-expression of PA83 with furin and EndoH deglycosylation enzyme; 4-Co-expression of PA83 with furin and PNGaseF deglycosylation enzyme; 5-Co-expression of PA83 with furin and EndoH deglycosylation enzyme; 6-Co-expression of glycosylated PA83 with furin; 7-deglycosylated PA83 with EndoH (dPA83-E) non-purificated form. 10 or 25 ng protein samples were loaded into gel. M: color prestained protein standard (NEB)

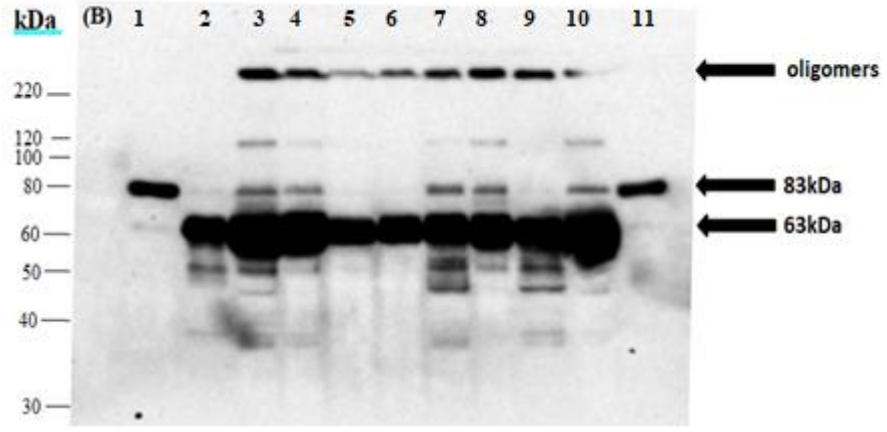


Figure 4.4. Western blot analysis of purified, co-expressed of PA83 variants with Furin, with/without deglycosylating enzymes EndoH and PNGaseF from *N. benthamiana* plants. Lanes: 1-Glycosylated PA83; 2-furin cleaved glycosylated PA83; 3-9-furin cleaved EndoH deglycosylated PA83 protein $(dPA63\text{-}E)_7$–purificated in different dates; 10-furin cleaved PNGaseF deglycosylated PA83 protein; 11-deglycosylated PA83 with EndoH (dPA83-E). 10 or 25 ng protein samples were loaded into gel

4.3. Gel Filtration Chromatography Analysis

After cleavage with furin PA83 release PA20 (20kDa) fragment and PA63 rapidly self-associates to form a ring-shaped heptamers. So, in our experiments, furin

cleaved *in vivo* deglycosylated PA83 variant (dPA83-E) protein solution contains, $(PA63)_7$ –heptamer form, PA63 (63 kDa) and the PA20 (20 kDa) molecule masses.

Gel filtration chromatography was used to isolate the heptamerized form of the PA63 molecules, $(PA63)_7$, from the mix of 63 kDa and 20 kDa molecules. After equilibration of the column, the protein standard Mix 15-600 kDa (Sigma, cat. No. 69385-30MG) was first passed through the column to obtain the protein elution profile (Figure 4.5.). The list of proteins containing the protein standard Mix 15-600 kDa (Sigma, cat. No. 69385-30MG) is shown in Table 4.1 below.

Table 4.1. Protein standard Mix 15-600 kDa containing protein

Protein standard Mix 15-600 kDa	**Molecular weights**
Thyroglobulin bovine	670 kDa
y-globulins	150 kDa
albumin chicken egg grade	44,3 kDa
ribonuclease A type	13,7 kDa

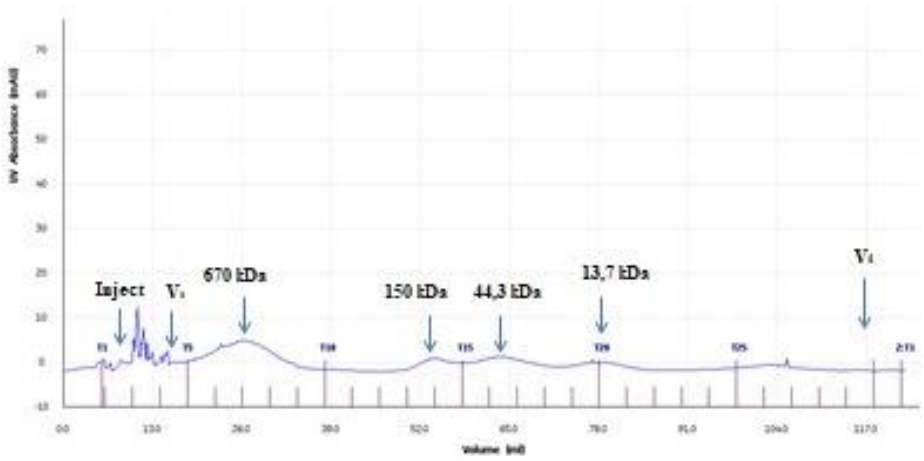


Figure 4.5. Gel filtration chromatography analysis of Protein standard Mix 15-600 kDa (Sigma, Cat. no. 69385- 30MG) on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA). Column equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$, 150 mM NaCl buffer. Molecular weight protein standards: thyroglobulin bovine (670 kDa) (0,5g/l) elution volume 26 ml; y-globulins (150 kDa) (1g/l) elution volume 52 ml; chicken egg grade (44,3 kDa) (1g/l) elution volume 65 ml and ribonuclease A

type (13,5 kDa) (1g/l) elution volume 74 ml. The void volume (V0)- was 15 ml, Vt – 117 ml[3]

Filtered gel elution results of purified, glycosylated (gPA83) and deglycosylated (dPA83-E; dPA83-P) protein samples are shown in Figure 4.6., Figure 4.7., and Figure 4.8. In the plant produced, purified glycosylated gPA83 protein elution volume was 52 ml (Figure 4.6.), EndoH *in vivo* deglycosylated dPA83-E protein elution volume was 54 ml (Figure 4.7.) and PNGaseF *in vivo* deglycosylated dPA83-P protein elution volume was 54 ml (Figure 4.8.).

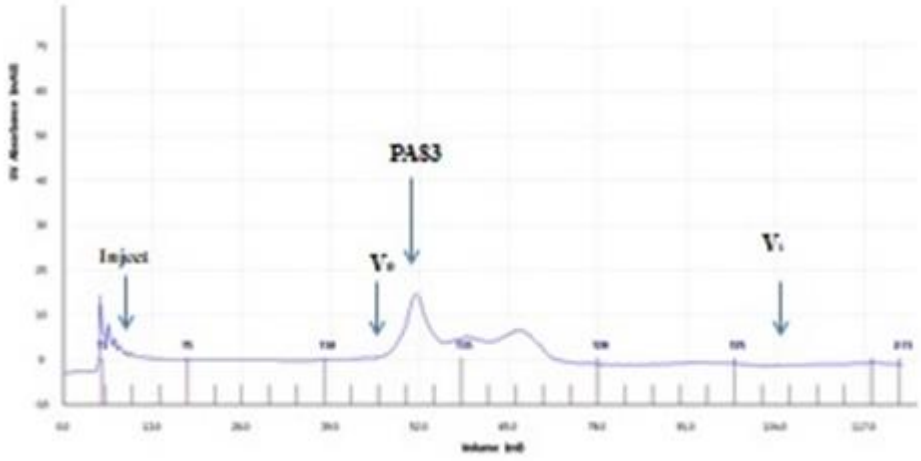


Figure 4.6. Gel filtration chromatography analysis of plant produced, Ni-NTA column purified gPA83 protein from *N. benthamiana* plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$, 150 mM NaCl buffer. Protein concentration was 0.7 µg /ml. Protein elution was 52 ml; the V_0- was 46 ml; Vt – 106 ml

[3] The void volume is that volume of buffer that passes through the column first and cannot contain any protein regardless of protein size. Vt –total physical volume of column

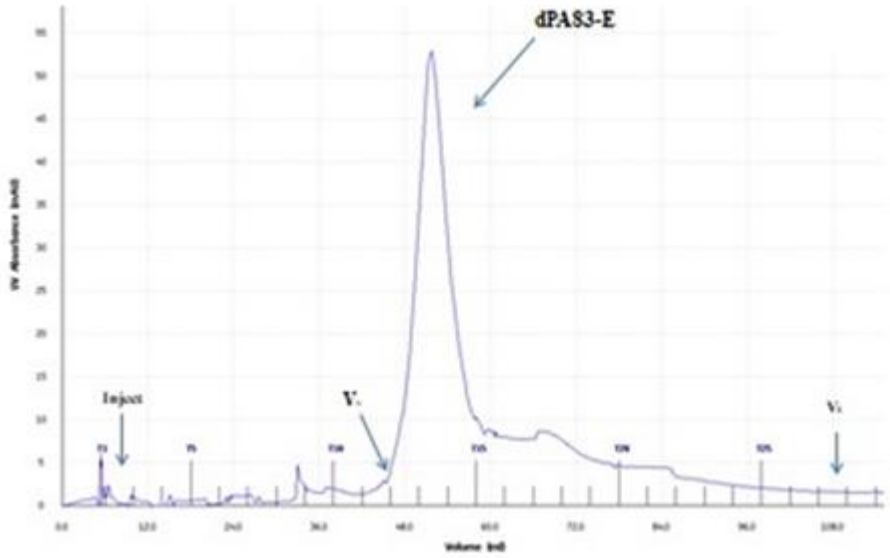


Figure 4.7. Gel filtration chromatography analysis of plant produced, Ni-NTA column purified dPA83-E (*in vivo* deglycosylated with EndoH) protein from *N. benthamiana* plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM CaCl2, 150 mM NaCl buffer. Protein concentration was 1 µg /ml. Elution was 54 ml; the V0- 48 ml; Vt – 108 ml

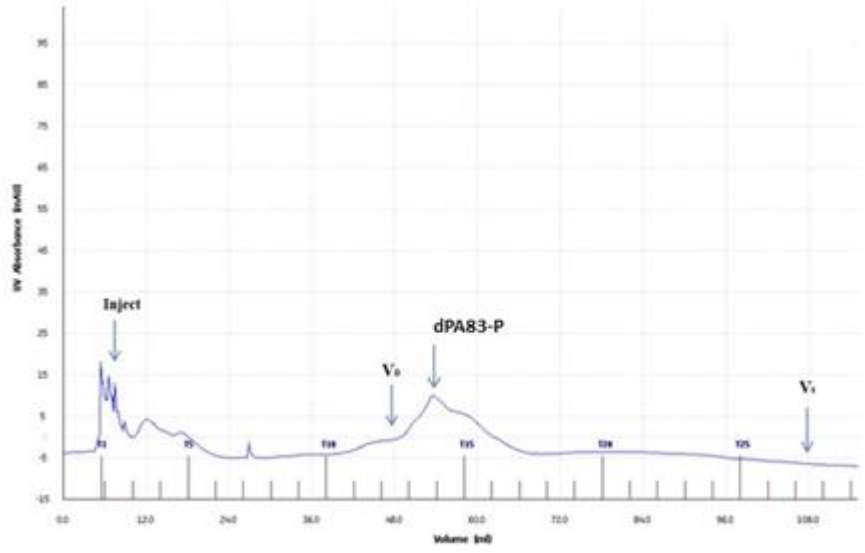


Figure 4.8. Gel filtration chromatography analysis of plant produced, Ni-NTA column purified dPA83-P (*in vivo* deglycosylated with PNGaseF) protein from *N. benthamiana* plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM CaCl2, 150 mM NaCl buffer. Protein concentration was 1 µg /ml. Elution was approximately 54 ml; the V0-48 ml; Vt –108 ml

The molecular mass of the heptamer form is approximately 441 kDa, so, when we pass through the column, deglycosylated, the heptamerized form of the furin cleaved dPA83-E (83kDa) must pass between 670 and 150 kDa, in other words, it is the 26-40 ml elutions observed from the protein mix elution profile on Figure 4.5. and Table 4.1.

The glycosylated furin cleaved gPA83, passed through the column at 55 ml (Figure 4.9.). As can be seen from the Figure 4.2., furin successfully cleaved gPA83, but heptamer formation was not observed.

In Figure 4.10., furin cleaved dPA83-E protein shows the first elution at 26-40 ml, most probably in heptamer form $(PA63)_7$, the second elution PA63 (63kDa) at 60 ml and the third elution PA20 (20kDa) at 104 ml. Furin cleaved dPA83-P protein was cleaved by furin, but probably heptamer formation was too small in amounts to observe Figure 4.11.

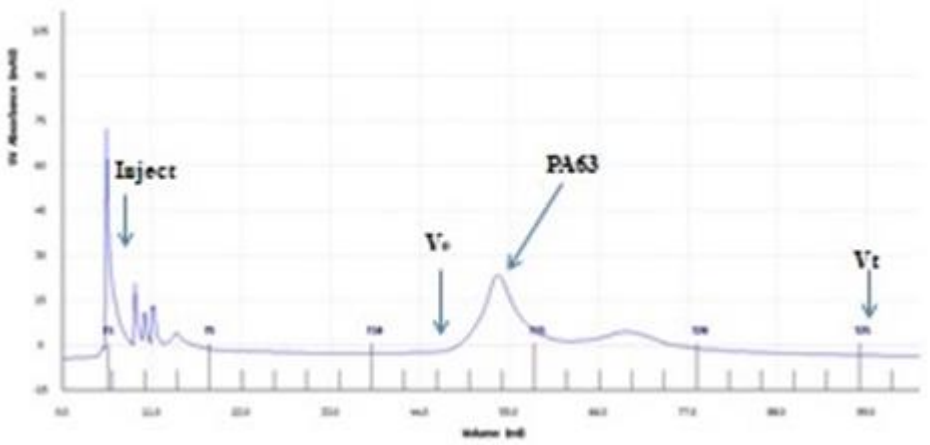


Figure 4.9. Gel filtration chromatography analysis of plant produced, glycosylated furin processed PA83 (gPA83) protein from *N. benthamiana* plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM CaCl2, 150 mM NaCl buffer. The protein eluted 56 ml; V0-50 ml; Vt –102 ml, high molecular mass protein volume not observed

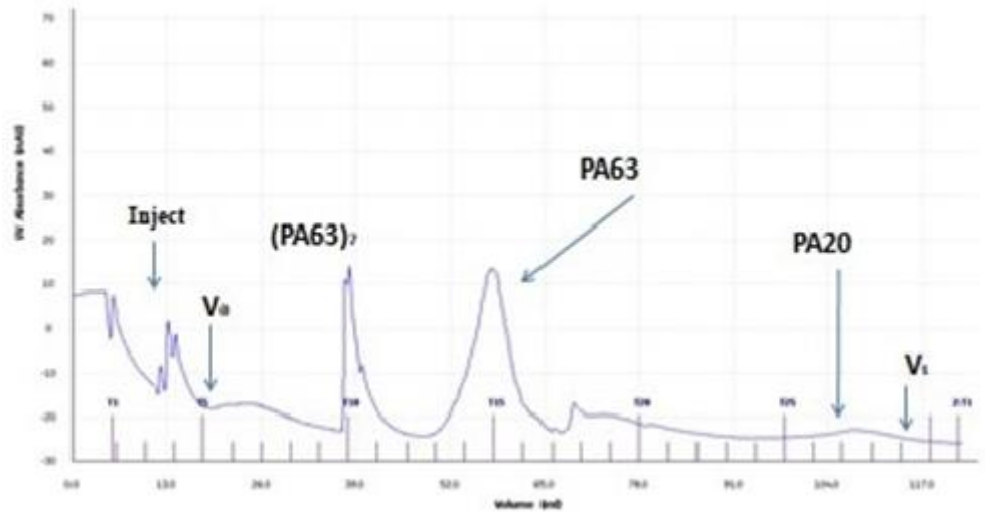


Figure 4.10. Gel filtration chromatography analysis of plant produced, purificated, *in vivo* furin cleaved PA83 deglycosylated with EndoH (dPA83-E) protein from *N. benthamiana* plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$, 150 mM NaCl buffer. The heptamer eluted 26 ml-40 ml; PA63 elution volume was 60 ml; PA20 elution volume was 104 ml; V_0- was 20 ml;Vt – 113 ml

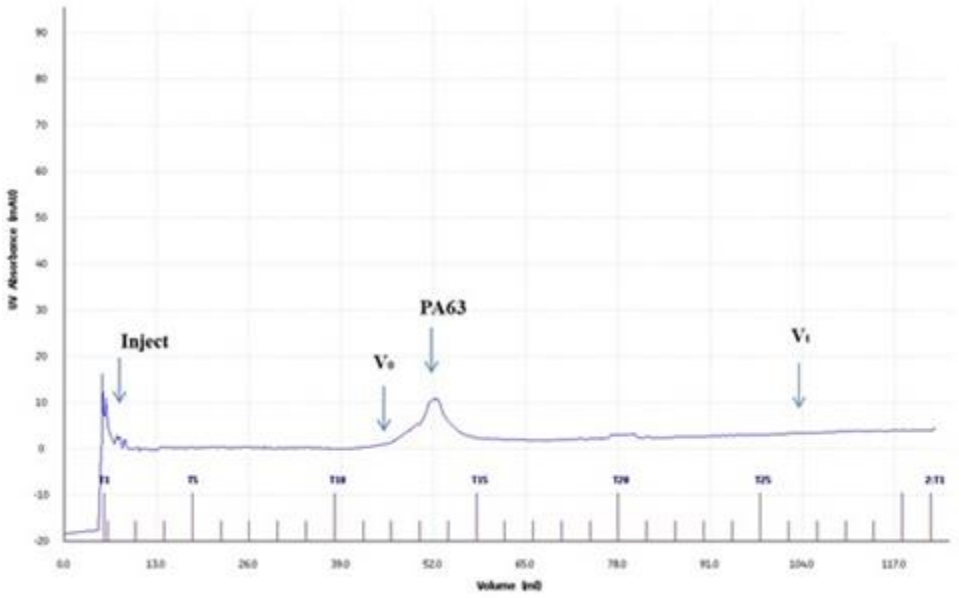


Figure 4.11. Gel filtration chromatography analysis of plant produced, Ni-NTA column purified, *in vivo* furin cleaved and deglycosylated PA83 with PNGaseF (dPA83-P) protein from N. benthamiana plants. Analytical gel filtration chromatography on a Sephacryl S-200 High Resolution (HR) column (GE Healthcare, WI, USA) equilibrated with 20 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$, 150 mM NaCl buffer. The protein eluted 52-54 ml; V_0- 42 ml; Vt –104 ml. High molecular mass protein volume not observed

After collection, elution fractions were concentrated and analyzed at SDS-PAGE and Western Blot. As shown in Figure 4.12. and Figure 4.13., 20 kDa fraction of PA was completely separated from the heptamer form.

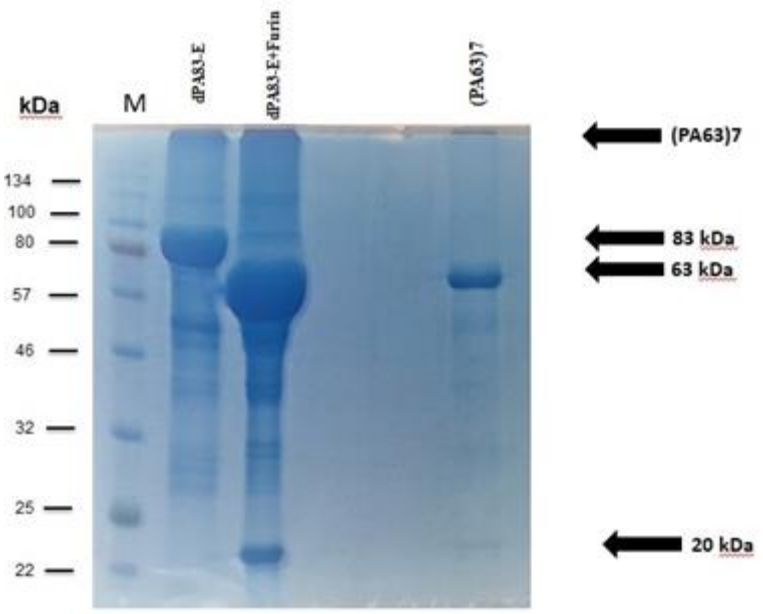


Figure 4.12. SDS-PAGE analysis of plant produced (Ni-NTA column) purified PA83 protein variants gel filtration, concentrated fractions results. $(PA63)_7$ - heptamer form; dPA83-E, *in vivo* deglycosylated PA83 with EndoH; dPA83-E, furin cleaved *in vivo* deglycosylated PA63 with EndoH; M- color prestained protein standard (cat. no. P7712S New England Bio labs)

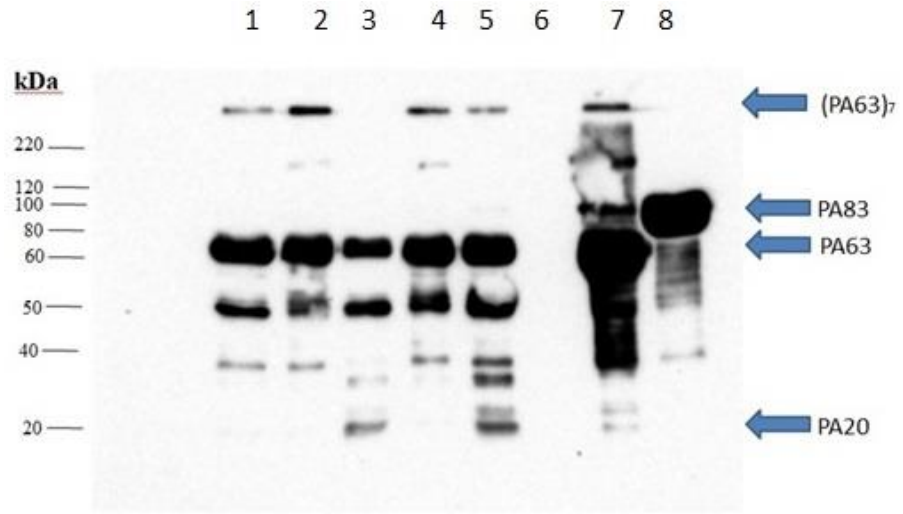


Figure 4.13. Western Blot analysis of plant produced, Ni-NTA column purified PA83 protein variants gel filtration results. 1-2-4; different fractions of $(PA63)_7$ –heptamer form- isolated from *in vivo* furin cleaved, deglycosylated dPA83-E with gel filtration; 3-

fraction of gel filtrated, furin cleaved gPA83; 5- Plant produced, Ni-NTA column purified, *in vivo* furin cleaved, PNGaseF deglycosylated PA83 (dPA83-P) protein; 6-null; 7-Plant produced, Ni-NTA column purified, furin cleaved deglycosylated dPA83-E protein (non-gel filtrated); 8-Plant produced, Ni-NTA column purified, EndoH deglycosylated dPA83-E (non-gel filtrated). Lane 5; 7; 8 were used as controls

4.4. Transmission Electron Microscope (TEM)

To observe the heptameric structure of $(PA63)_7$ the proteins, all control groups and protein samples were observed at TEM. All concentrated protein samples were negatively stained with 1% uranyl acetate, pH 4.5 and analyzed by TEM. EndoH deglycosylated form of PA83 (dPA83-E) was used as control protein. Concentrations of protein samples were set at 0.4 mg/ ml.

Figure 4.14 showes the examined furin cleaved gPA83 (A) and not-furin cleaved dPA83-E (B) protein samples. As seen from the figures, any heptamer/or ring-like structures were not observed. Figure 4.15 showes the dPA83-E proteins cleaved with furin, the heptamer $(PA63)_7$ fractions isolated by gel filtration chromatography. The examined samples were detected large numbers of ring-shaped multisubunit structures in every preparation of the heptamer $(PA63)_7$ protein samples (Figure 4.15.)

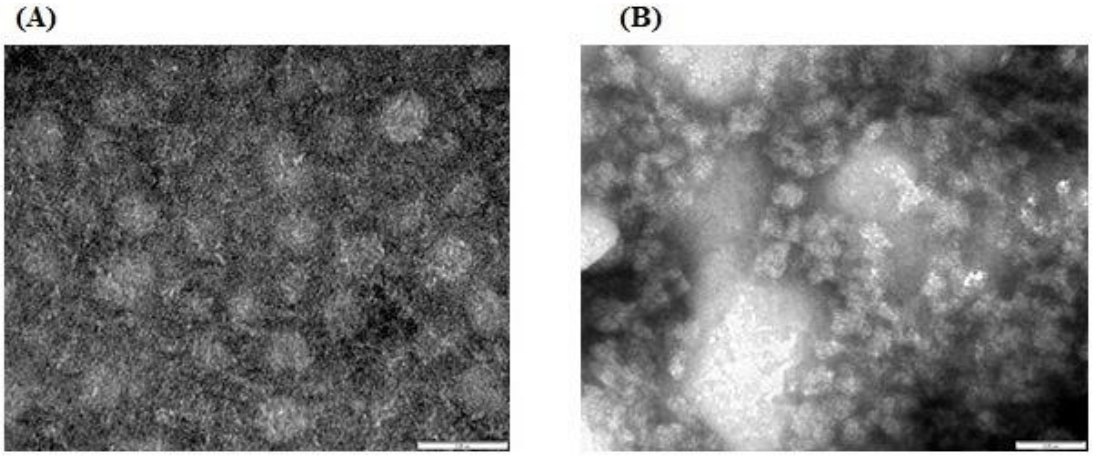


Figure 4.14. The results of transmission electron microscopy (TEM). **A)** Gel filtrated furin cleaved gPA83 protein sample; **B)** Gel filtrated not-furin cleaved dPA83-E protein sample. Protein samples were negatively stained with 1% uranyl acetate (pH: 4.5)

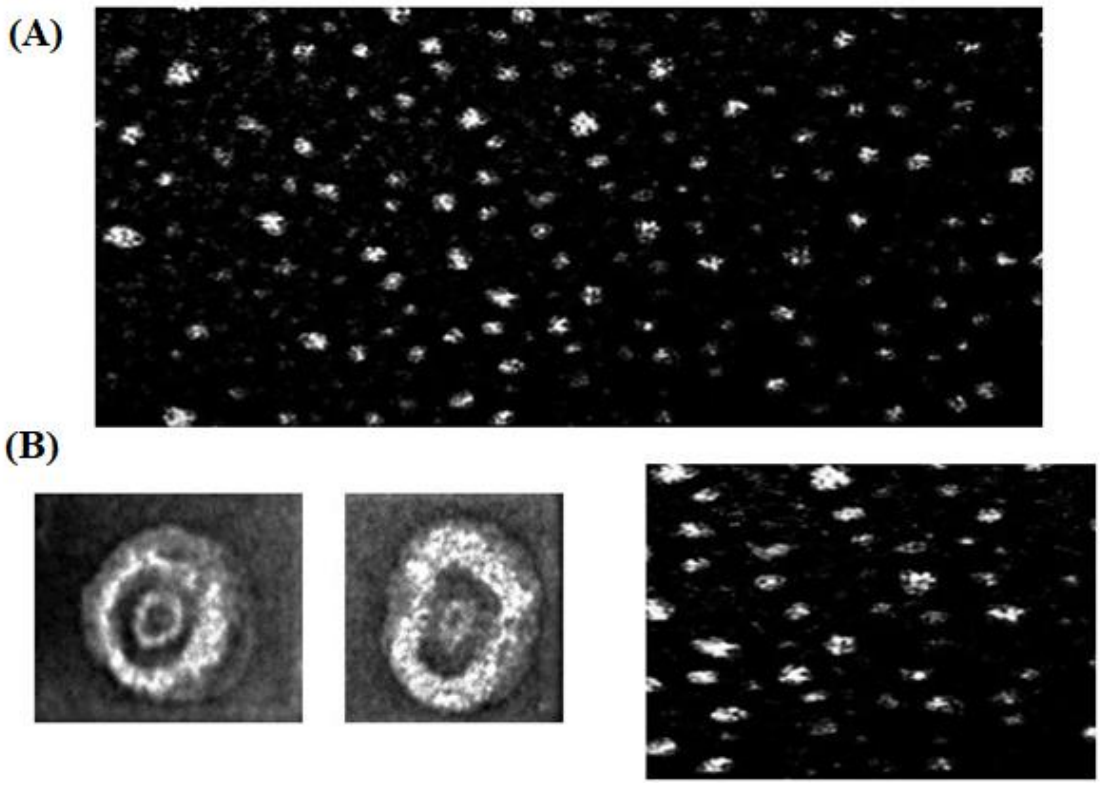


Figure 4.15. The results of transmission electron microscopy (TEM). **A)** Gel filtrated, furin cleaved dPA83-E protein; **B)** Heptamer $(PA63)_7$ proteins ring structures. Protein samples were negatively stained with 1% uranyl acetate (pH: 4.5)

4.5. Mass Spectrometer Analysis

The Mass MS/MS scanning was performed in the first five intense peaks in MS scan with LIFT method (accelerating voltage 20 KV; UV Laser at 337 nm; Laser shoots 3000). The spectra's were obtained using the Bruker flex Analysis Program. The SwissProt (Protective antigen OS=Bacillus anthracis OX=1392 GN=pagA PE=1 SV=2; Score: 33, Expect: 1.5e+02; Monoisotopic mass (Mr): 85759; Calculated pI: 5.88; Taxonomy: *Bacillus anthracis*) was used as a database.

The mass tolerance was assumed to be 0.5 Da, the cleavage enzyme chosen which cuts the C-term side of the KR unless the next residue is P, missed cleavages were allowed as 1. After all the adjustments protein sequences were obtained and identified by MASCOT scanning (Apweiler et al. 1999).

Gel pieces were cut from 10% acrylamide-gels. All procedures were performed as previously shown in Materials and Methods. SDS-PAGE results from Heptamer $(PA63)_7$ protein gel are shown in Figure 4.16 and 4.17.

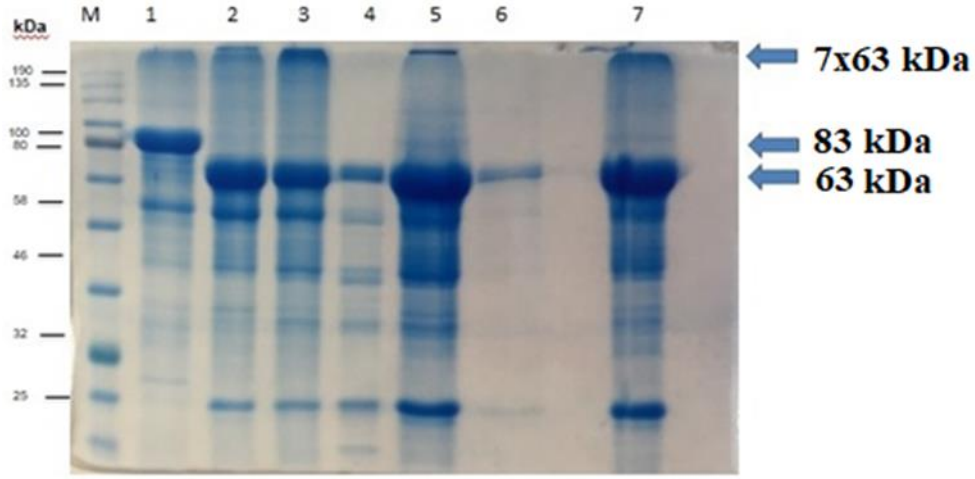


Figure 4.16. The SDS-PAGE result of heptamer $(PA63)_7$ proteins for MS analysis preparation

Figure 4.17. The proteins, gel pieces, cut from the gels (Figure 4.16.) for sending to MS/MS

71 peaks were identified in MS spectrum and the 11 of these were matched with analyzed heptamer form of PA63 protein (Figure 4.18).

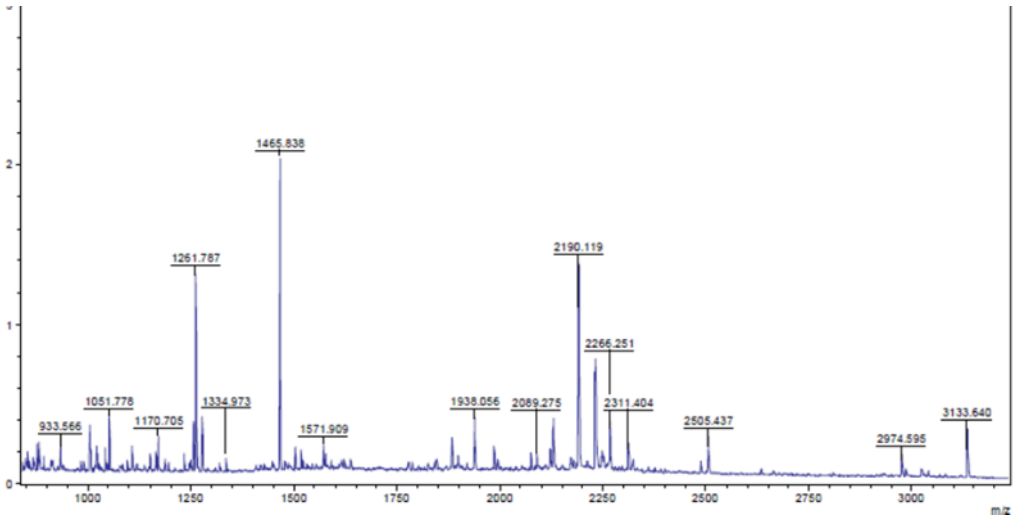


Figure 4.18. Heptamer $(PA63)_7$ protein MS result

The matched 11 peaks were performed MS/MS and 5 of these peaks; 1261; 1276; 1465; 2190 and 2231 were matched with heptamer $(PA63)_7$ protein spectrums (Figure 4.19). The 2190; 1465; 1261; 2330 and 1276 peaks matched with *B. anthracis* protein PA.

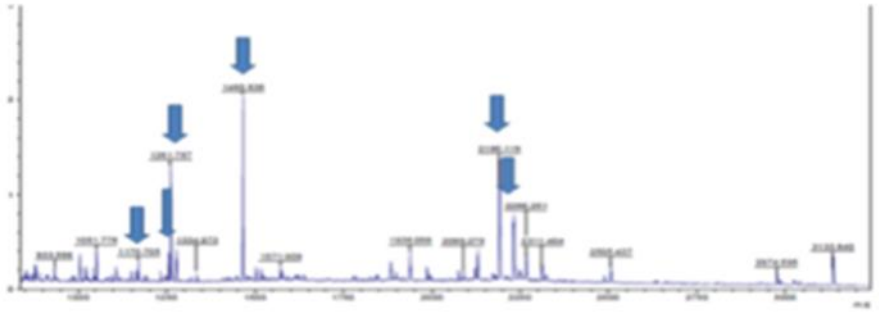


Figure 4.19. The applied MS/MS peaks on the 11 matched peaks of the heptamer $(PA63)_7$ protein

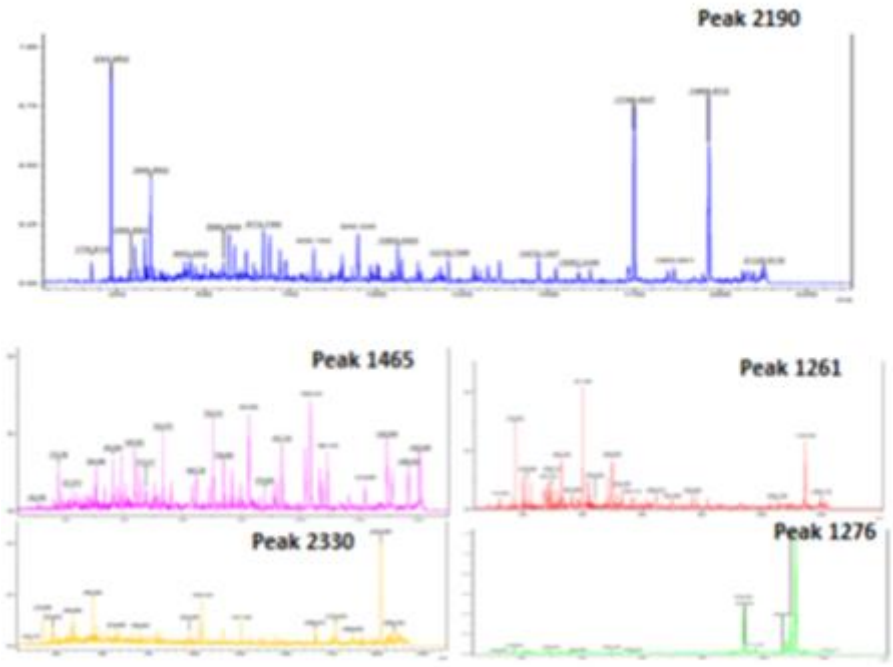


Figure 4.20. Eleven matched masses peaks of heptamer $(PA63)_7$ protein which applied MS/MS. The matched 2190; 1465; 1261; 2330 and 1276 are the peaks with *B. anthracis* protein PA

In consequences, the 71 mass values were searched and 11 of them were matched with our analyzed protein. Matching 18th line peptides consists of 764 amino acid sequences, 152 amino acid sequences were paired with the analyzed heptamer form of PA63. All over, matched protein sequences coverage was 19%. If we remove first 29 amino acid sequences, which are signal sequences, this value will be approximately 20.68 % coverage (Figure 4.20.). The matched peptide sequences were shown on Table 4.2 and unmatched peptide sequence on Table 4.3.

Analyzed protein sequences were:

MKKRKVLIPLMALSTILVSSTGNLEVIQAEVKQENRLLNESESSSQGLLGYYFSD
LNFQAPMVVTSSTTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKSDEYTFATS
ADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTEKGLDFKLY
WTDSQNKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEV
EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSGPTVPDRDNDGIPDSLEVEGYT
VDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV
SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSQTRTISKNTSTSRTHTSE
VHGNAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTA
DTARLNANIRYVNTGTAPIYNVLPTTSLVLGKNQTLATIKAKENQLSQILAPNN
YYPSKNLAPIALNAQDDFSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIATYN
FENGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTK
PDMTLKEALKIAFGFNEPNGNLQYQGKDITEFDFNFDQQTSQNIKNQLAELNAT
NIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVISSTEGL

LLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYND
KLPLYISNPNYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG.

Table 4.2. The matched peptides sequences' informations

Start	End	Observed	Mr (exp)	Mr (calc)	Delta (M)	Peptide
189	194	6.783.311	6.773.239	6.773.456	0.02181	K.SSNSRK.K
197	226	31.336.936	31.326.323	31.324.735	0.1588 1	R.STSAGPTVPDRDNGIPDSLFEVEGYTVDVK.N
230	242	15.719.093	15.709.020	15.708.144	0.08760	R.TFLSPWISNIHEK.K
389	394	7.002.863	6.992.790	6.994.027	0.1237 0	R.LNANIR.Y
479	497	21.901.189	21.891.116	21.890.025	0.1091 0	R.LDTDQVYGNIATYNFENGR.V
498	519	24.863.897	24.853.825	2485,2561	0.12641	R.VRVDTGSNWSEVLPQIQETTAR.I
500	519	22.312.120	22.302.047	22.300.866	0.11810	R.VDTGSNWESEVLPQIQETTAR.I
625	629	7.373.702	7.363.630	7.363.293	0.03370	R.FHYDR.N
647	665	21.292.506	21.282.433	21.281.375	0.10581	R.EVINSSTEGLLLNIDKDIR.K
667	688	25.054.375	25.044.302	24.043.010	0.12931	K.ILSGYIVEIEDTEGLKEVINDR.Y
683	698	19.380.560	19.370.487	19.369.676	0.08111	K.EVINDRYDMLNISSLR.Q

Table 4.3. The unmatched peptides

632.2822, 634.2980, 646.2940, 651.2764, 660.3195, 662.3311, 663.3424, 680.2213,
684.3128, 712.2781, 752.3881, 768.5760, 804.3188, 832.3504, 833.3559, 834.3601, 854.3305,
868.6006, 876.3102, 880.4976, 882.6171, 892.2930, 933.5661, 1004.5802, 1005.6615, 1021.5862,
1042.5719, 1051,7780, 1095.8076, 1106.5942, 1151.7999, 1165.8153, 1170.7051, 1187.7206,
1233.6890, 1248,5712, 1256.7304, 1261.7865, 1276.6068, 1278.6254, 1334.9728, 1465.8375, 1502.9342, 1516,8509, 1575.9353, 1620.9556, 1883.0719, 1898.0237, 1985.0966, 1994.0826,
2075.2588, 2089,2752, 2120.9986, 2173.0951, 2248.1788, 2250.7724, 2266.2506, 2311.4037, 2974.5948, 3022,6003.

4.7. Toxin- Neutralization Assay (TNA)

TNA titers were measured using a lethal toxin (LeTx) neutralization assay as mentioned previously (Mamedov et. al. 2016) and expressed as the GMT of the Effective Concentration 50 (EC50) values per group. Briefly, protein samples were titrated on a plated mouse macrophage cell line (J774A.1 cells, ATCC TIB-67, Manassas, VA) (Figure 4.21.) culture in the presence or absence of LeTx. After incubation, cell viability was accessed by the addition of WST-1 (Roche Applied Sciences, Indianapolis, IN), a proliferation reagent, followed by a spectrophotometric measurement at 450 nm.

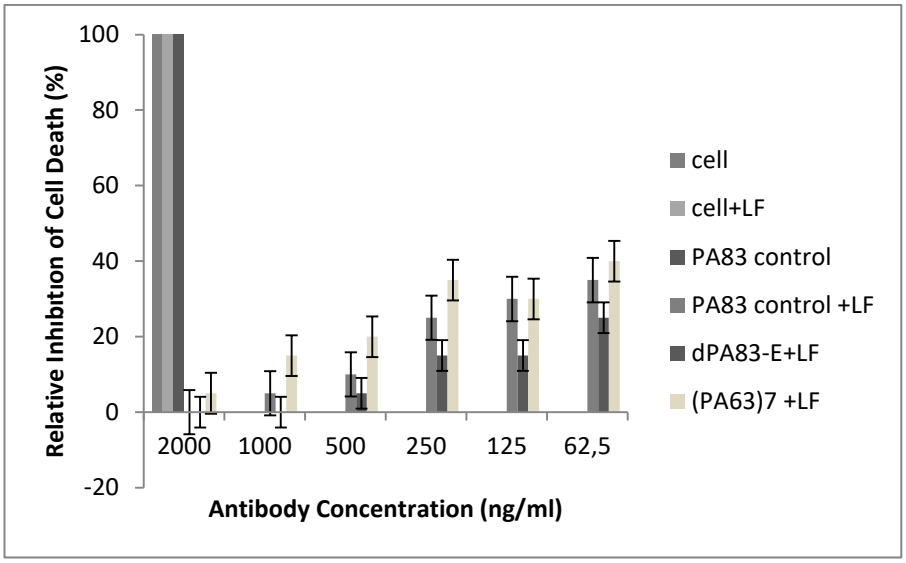


Figure 4.21. Percentage of the relative inhibition of a cell death as result of toxin neutralization assay (TNA) by dPA83-E and $(PA63)_7$. Statistical significance was calculated using the one-way ANOVA ($p<0.05$)

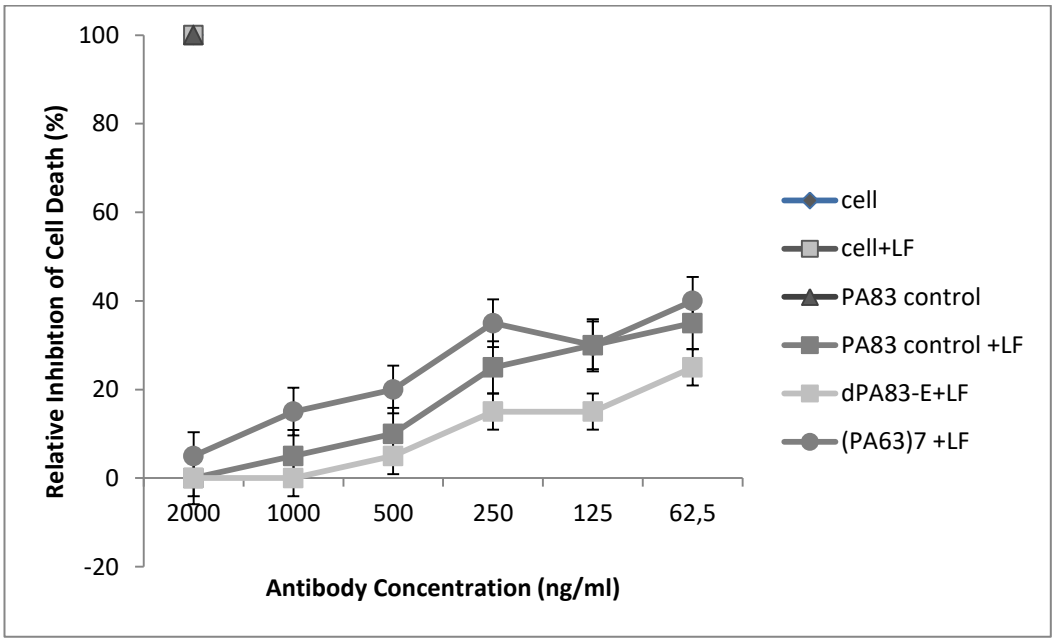


Figure 4.22. The result of the relative inhibition of a cell death of toxin neutralization assay (TNA) by dPA83-E and $(PA63)_7$. Statistical significance was calculated using the one-way ANOVA ($p<0.05$)

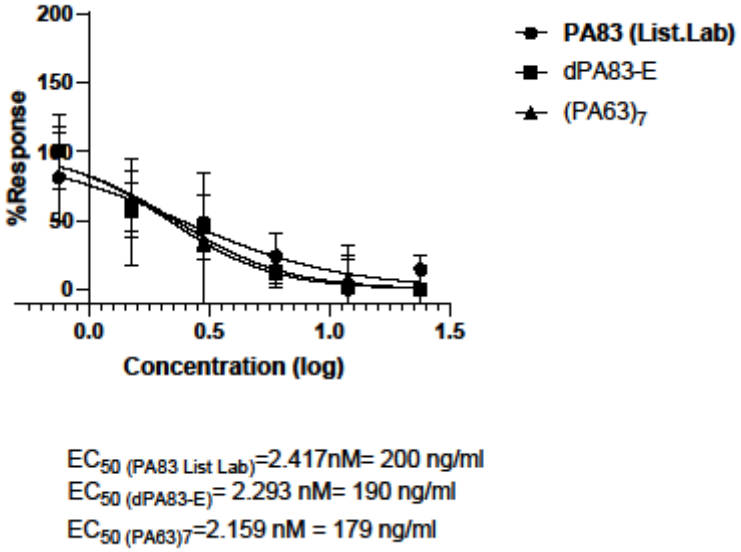


Figure 4.23. Dose-response curves of the neutralization of protein sample and PA83 control (PA83 from *B. antracis* List. Biol.) in the J774A.1 (ATCC TIB-67, Manassas, VA) cell's TNAs. Statistical significance was calculated using the one-way ANOVA ($p<0.05$)

The results show that plant-produced gel-filtered (PA63)7 can combine with LF to induce cell death and form LeTx (Figure 4.21 and 4.22.). $(PA63)_7$ were able to neutralize toxin activity similar to dPA83-E and rPA positive control variant. In addition, the EC50 value of the heptamer form was close to deglycosylated dPA83-E and recombinant PA positive control.

The inflection point for each curve from this model was reported as the effective concentration at 50% inhibition (EC50) for the corresponding protein samples. Figure 4.23 describes representative dose-response data from tested protein samples. The control protein used for this study is rPA from *B. anthracis* (List Biological Lab). The tested and controlled proteins' effective concentration was 50% inhibition (EC50) shown at Table 4.4.

Table 4.4. Measuraments of the tested proteins EC50

Samples	**EC_{50} (ng/ ml)**
PA83 (control rPA)	200
dPA83-E	190
$(PA63)_7$ (gel filtrated)	179

4.8. Immunization of Animals with Target Antigens and Elisa Analysis

Immunogenicity experiments were performed in seven to eight week old mice (6 animals/ group). Mice were immunized on study days 0 and 21 IP with 5 µg of plant-produced dPA83-E and $(dPA63E)_7$ (with Alhydrogel.) proteins. Serum samples were

collected on day 42 of the study (post 2nd vaccination) and analyzed for anti-PA IgG responses by ELISA. For ELISA, 0.5 mg plant produced, dPA83-E protein was coated into a plate and different dilutions of serum immunized with dPA83-E and $(dPA63E)_7$ proteins were added to test the binding affinity of the plant that produced dPA83-E, $(dPA63E)_7$ and PA83 (negative control) to anti-PA IgG antibody in the serum immunized with dPA83-E and $(dPA63E)_7$ proteins.

Figures 4.24 and 4.25 shows IgG responses in mouse obtained with monomer and heptamer variants of PA83 using Alhydrogel as adjuvant. As seen from Fig. 4.25, the immunized animals with 5 μg dPA83-E protein were detected with high Anti-PA IgG antibody titers. However, 5 μg heptamerized $(PA63)_7$ protein was able to induce higher titers of Anti-PA-specific antibodies compared to its monomer form of dPA83-E serum samples (Figure 4.24). The immunogenicity and protective efficacy of the heptamer form PA63 allows the idea that it may have the capability of new a recombinant vaccine against anthrax.

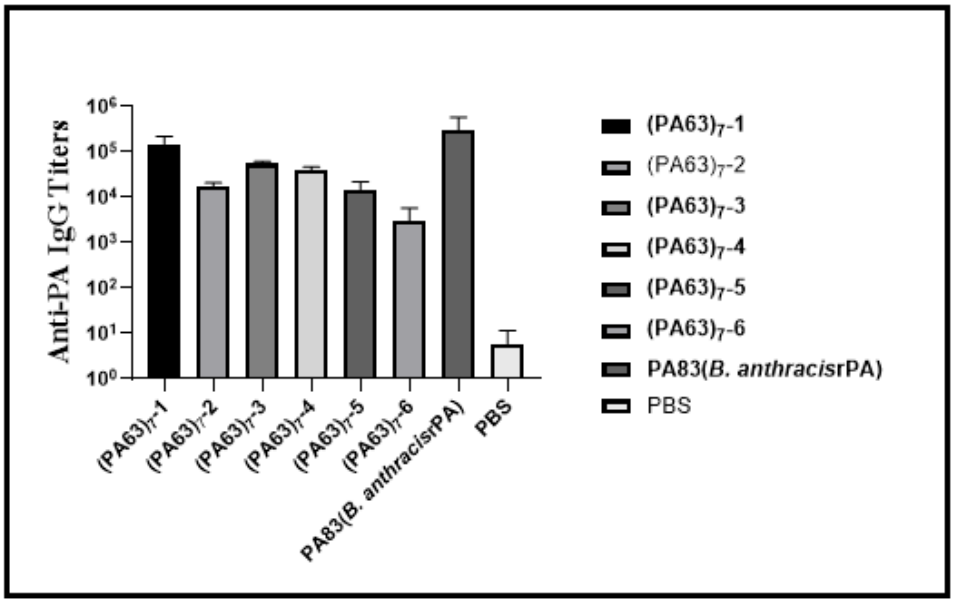


Figure 4.24. Comparative immunogenicity of plant produced 5 μg $(PA63)_7$ proteins' serum samples results in mice. Mice were immunized on study days 0 and 21. Serum samples were collected on study day 42 (post 2nd vaccination) and analyzed for anti-PA IgG responses by ELISA. Each point on the graph was derived from three replicas for each dilution. PA83 (control)- control PA83, the recombinant Protective Antigen from *B. anthracis* (rPA) (List Biological Laboratories, USA). One-way ANOVA test was used to compare antibody response of Anti-PA IgG induced serums and PBS to control. *$p < 0.05$; **$p < 0.01$ (n=6 mouse/group)

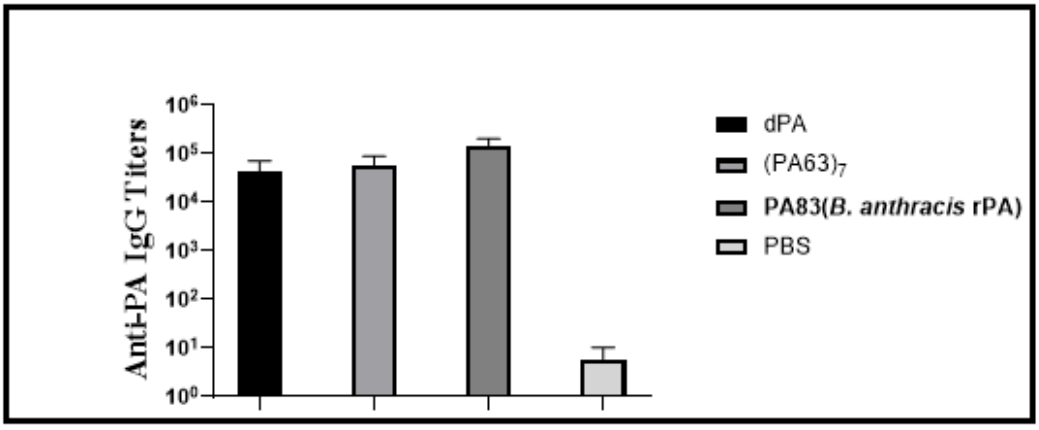


Figure 4.25. Comparative immunogenicity of plant produced 5 μg dPA83-E and $(PA63)_7$ proteins' serum samples results in mice. Mice were immunized on study days 0 and 21. Serum samples were collected on study day 42 (post 2nd vaccination) and analyzed for anti-PA IgG responses by ELISA. Each point on the graph was derived from three replicas for each dilution. PA83 (control)-control PA83, the recombinant Protective Antigen from *B. anthracis* (rPA) (List Biological Laboratories, USA). One-way ANOVA test was used to compare antibody response of the Anti-PA IgG induced serums and PBS as control. *$p < 0.05$; **$p < 0.01$ (n=6 mouse/group)

5. CONCLUSION

Anthrax is one of the most dangerous diseases in the world. This infection can cause different inhalational diseases with *Bacillus anthracis* spores. Vaccination is a major strategy to prevent the population from anthrax spreading accidentally. The several animal model studies have shown that the antibodies against PA can neutralize anthrax toxins. After proteolytic cleavage by the human furin protease enzyme, the 20 kDa fragment attached to the N-terminal end is a released terminal (Leppla et al. 1988; Singh et al. 1989; Klimpel et al.1992), and the 63 kDa fragment at the carboxyl end attached to the cell receptors forms the heptameric complex (Beauregard et al. 2000; Elliott et al. 2000; Mogridge et al. 2002). The heptamers combine with LF and EF (Mogridge et al. 2002) then enter the host cell by endocytosis (Abrami et al. 2003).

The protective antigen (PA) is important for the host cell intoxication to form the lethal toxin (LeTx; PA + LF) and edema toxin (ETx; PA+LF). Therefore, the protective antigen is seen as the main target of vaccine development against anthrax. Hence, developed vaccines should be stable and low-dose.

Currently developed live attenuated vaccine formulations contain one of the *B. anthracis* strains combined with PA and adjuvant aluminum hydroxide. The countries like USA and UK use non-bacterail vaccines, due to the concerns about the possibility of LF, EF, and other bacterial contaminations of live attenuated bacterial vaccines.

Various studies in animal models have shown that the human licensed anthrax vaccine absorbed (AVA, BioThrax) in the USA and the anthrax vaccine precipitated in the UK (AVP) are both protective against anthrax at repeated doses. The negative effects of both vaccines, such as the length of the vaccination schedule and doses, and the instability of PA, have led to the development of existing and new vaccine candidates based on recombinant PA (rPA) (Geirer et al. 2002).

Recombinant subunit vaccines are a promising alternative to live attenuated vaccines. Currently, several recombinant expression systems have been developed and used for expressing various types of vaccines, called a protein based, new generation subunits vaccines. *E. coli* expressed rPA-based anthrax candidate vaccines have demonstrated protection against lethal aerosolized challenge in different animal models.

Plant expression systems became a viable alternative system for animal cell factories for the production of therapeutic proteins. In addition, plant expression platforms provide cost-effective, scalable, safe and high production capacity for the production of recombinant mAbs, therapeutic proteins and vaccine antigens.

In its native host the PA protein of *B. anthracis* does not carry the N-linked glycans in its native glycans form. However, it has some possible glycosylation sites which can be abnormally glycosylated in eukaryotic expression systems. The aberrant glycosylation could be the cause of an incorrect folding of proteins; which can also mask the epitopes of target proteins. Recently, Mamedov et al. (2016, 2017), developed an *in vivo* deglycosylation of plant-made recombinant proteins, by co-expressing with bacterial deglycosylation enzymes PNGaseF (Mamedov et al. 2016) and EndoH (Endo-β-N-acetylglucosaminidase) (Mamedov et al. 2017) using plant transient expression

systems. The results have demonstrated that PNGaseF *in vivo* deglycosylated proteins are highly immunogenic and appear to be more stable than their glycosylated counterparts (Mamedov et al. 2016).

It should be noted that PNGaseF removes N- glycans from glycoproteins, causing amino acid changes in the expressed proteins due to the deamidation reaction (Mamedov et al. 2017). On the other hand, EndoH catalyzes the bond between the two acetylglucosamine residues of the N-linked oligosaccharides while preserving the native protein structure without amino acid changes in the produced proteins (Mamedov et al. 2017). Moreover, EndoH *in vivo* deglycosylated proteins have superior properties compared to PNGaseF counterparts and glycosylated form of the same protein (Mamedov et al. 2016, 2017). For example, EndoH *in vivo* deglycosylated form of Protective antigen (PA) of *Bacillus anthracis* was more stable, active and highly immunogenic compared to its PNGaseF deglycosylated counterpart (Mamedov et al. 2016, 2017). Using this expression strategy, a number of difficult-to express proteins such as; human Furin, Factor IX, full length Pfs48/45 protein of Plasmodium falciparum (Mamedov et al. 2017, 2019b), binding domains (RBD) of Spike protein of SARS-CoV-2 (Mamedov et al. 2021) and other complex proteins have been successfully produced in the *N. benthamiana* plant.

Previous studies have shown that tomato, potato and tobacco plants were the first plants to be able to express recombinant PA. Aziz (2002, 2005), expressed recombinant PA for the first time, despite the low expression efficiency and toxin neutralizing antibodies in transgenic tomato and tobacco plants. Watson (2002, 2005), showed that the expression of PA in tobacco plant chloroplasts can be increased by 360 million doses. Studies have shown that oral immunization of a mouse with plant chloroplast-derived rPA survives the challenge of a deadly toxin (Koya et al. 2015).

The full-length produced plant PA83 was expressed using vector-based transient plant expression system (Chichester et al. 2013). However, the glycosylated plant produced PA83 was elicited TNA titers in mice and rabbits because this protein was not able to form LeTx.

In this study, we aim to produce the *in vivo* heptamerized form of PA83 in *N. benthamiana* plants using the plant expression system. It is necessary to produce stable and low effective dose vaccine candidates. We had hoped that PA63 might be more stable than monomer PA83 variants due to its heptamer structure since this ability is essential for disease control in pandemic period.

In addition, furin processing was applied for the production of heptamerized form of the PA83 *B. anthracis* of PA. Most therapeutic proteins usually require at least a proteolytic cleavage for their activation and other functions. PA of *B. anthracis* must cleave with furin to become an active form. Furin is too expensive and difficult to get it an active form. Mamedov et al. (2019a) demonstrated the production of a highly soluble and functionally active form of furin by the transient *in vivo* co-expression system in *N. Benthamiana* plants for the first time.

Our results show that, the *in vivo* enzymatic deglycosylation (with EndoH and PNGaseF) and proteolytic cleavage processing (with furin) PA83 of the proteins were

accomplished successfully in the *N. Benthamiana* plants. The data's were confirmed with a SDS-PAGE and Western Blot analyses. The deglycosylation enzymes EndoH and PNGaseF were perfectly deglycosylated in a plant produced PA83 (Fig. 4.1. lane1, 2). A high molecular band was observed with Endo H and PNGase F deglycosylated PA83 (Fig 4.2, lane 4, 5), but not with glycosylated PA83 (Fig 4.2, lane 6), suggesting that formation of the heptamerized form of glycosylated PA63 is probably blocked by plant N-linked glycans in the plants. The purification of the confirmed proteins performed with IMAC (Ni-NTA) purification method and all data's were confirmed by SDS-PAGE and Western blot analysis.

Gel filtrations analysis was performed to plant produced, *in vivo* glycosylated, deglycosylated and furin cleaved forms of the PA83 variants. As shown in the graphics, the *in vivo* deglycosylated, furin cleaved and non-furin cleaved forms of the elution fractions are observed approximately in same peaks. However, the first separated heptamer form of PA63 was the furin cleaved proteins especially deglycosylated with EndoH deglycosylation enzyme, but furin cleaved PNGaseF deglycosylated proteins heptamer form was not separated, which might be related with its low concentration. The graphs also show the separation of the 20 kDa fragment from the heptamer form. All gel filtrated proteins' concentrated fraction elutions were analyzed by Western blot and SDS –PAGE. The results show that in all the tested proteins proper bands and heptameric form of PA63 protein was successfully seperated from PA20 kDa component.

The gel filtrated, heptamer $(PA63)_7$, form was examined with negatively staining transmission electron microscope to confirm the heptamer ring structures. All the tested heptamer form of the protein samples was observed in ring-like structures, and this means that we successfully isolated the heptamer form from the protein mix. In tested glycosylated PA83 variants heptamer ring structures were not observed.

As a the result of MS and MS/MS, it was found that the heptamer form of PA63, and native PA83 *Bacillus anthracis* protein in the 18th line matched, and it had a similarity of about 20.68% with the native recombinant form.

Immunization analysis on the mice shows that the heptamer form of the PA63 are produced high anti-PA IgG titers in all of the animals in the groups. These results presents that the heptameric form of the PA83 protein can elicit higher antibody titers compare to its monomer forms.

The TNA results show that plant produced, gel filtration $(PA63)_7$ was able to combine with LF to form LeTx and induce cell death. $(PA63)_7$ was able to neutralize toxin activity in a similar dose-dependent manner for dPA83-E and recombinant PA variant yielding in full sigmoid neutralization curves that had similar slopes and asymptotes.

Finally, our results demonstrate the practicality of expressing heptamer form of the PA83 protein in *N. benthamiana* plants in high level and purification yield. *In vivo* processing of furin, with deglycosylated PA83 protein variants in plants, allows the development of a new promising cost-effective, safe and highly immunogenic vaccine candidate against anthrax based on heptamerized PA63, that can be produced *in vitro*

(expensive commercial cleavage enzymes, i.e. furin or trypsin). This protein could be more stable than its monomer form, because of its structure.

In addition, the study also confirms the promising use of plant-based expression systems for the production of target proteins to provide safer and cheaper products in a shorter time, which is important for mass vaccination during the pandemic period. Using plant transient expression systems allows getting safe and cost-effective products as an alternative to the existing protein expression systems.

Recent studies show that there is a relationship between N-glycosylation capacities of cell proteins, drug sensitivities and susceptibility to infection. Understanding N-glycosylation molecular points has helped the association between N-glycosylation deficiencies and human diseases.

In over all, the possibility of using N-glycan expression as potential determinants of disease duration on cells has been highlighted. Therefore, information on the regulatory properties of N-glycosylation may be useful in the design of new therapeutic proteins (Kukuruzinska and Lenon 1998). Applications of this technology have great advantages in molecular industrial agriculture, the production of medicinally therapeutic proteins, subunit vaccines, and the production of antibodies for protecting against different diseases.

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Publications

Articles published in international refereed journals

1. Nilüfer Gün, Tarlan Mamedov. Flexible Approaches are Required for Successful Production of Recombinant Proteins in Plants, Medicine Science (International Medical Journal), Accepted not Published.

2. Gulshan Mammadova, Irem Gurbuzaslan, Damla Yuksel, Nilufer Gun, Nedim Mutlu, Gulnara Hasanova, Tarlan Mamedov. Engineering, production, and immunogenicity studies of a truncated form of rabies virus glycoprotein produced in Nicotiana benthamiana plant, Accepted not Published.

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Abstract Papers

1. Nilufer Nagizade, Zakir M. O. Rzayev, Esra Karaca, Demet Erdönmez, Necdet Sağlam. 2013. Fabrication and Characterization of Antimicrobial Nanofiber Webs From Binary PVA/ODA-MMT/Silver Nanoparticle and Poly(MA-alt-1-Octadesene)-g-PLA Blend System By E-spun Method, 44TH WORLD CHEMISTRY CONGRESS, 11-16 August, Istanbul, Turkey.

2. Bayram Ali Göçmen, Zakir M. O. Rzayev, Nilüfer Nagizade (2013). Fabrication and Characterization of polymer Silicate Nanocomposites From EPDM/PP-g-MA/Polylactide/Organo-MMT Blends in Melt By Reactive Extrusion, 44TH WORLD CHEMISTRY CONGRESS, 11-16 August, Istanbul, Turkey.

Poster Presentations

1. D. Erdönmez, K. Erkan, Z. M. O. Rzayev, N. Sağlam, K. Salimi, N. Nagizade NanoFabrication and Characterization of Antimicrobial PVA–Organofiller/Ag Nanocoatings for Nanomedicine Applications, Poster presentations / Current Opinion in Biotechnology 24S, Page S109, 16-18 May, Bratislava, Slovakia, 2013. https://doi.org/10.1016/j.copbio.2013.05.335.

2. K. Erkan, D. Erdönmez, Z. M. O. Rzayev, N. Sağlam, K. Salimi, N. Nagizade, E. Pişkin, Antifungal Activity of Ag-carrying Polymer Layered Silicate Composites in Broth Medium and Solid State: Effects of Structural Factors and Silver Loading, Poster presentations / Current Opinion in Biotechnology 24S, Page S110, 16-18 May, Bratislava, Slovakia, 2013. https://doi.org/10.1016/j.copbio.2013.05.339.