EXPRESSION AND PURIFICATION OF *Arabidopsis thaliana* HETEROTRIMERIC G PROTEIN ALPHA SUBUNIT (GPA1) USING BACTERIA AND YEAST SYSTEMS

by

HAZAL BÜŞRA KÖSE

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
Spring 2014

EXPRESSION AND PURIFICATION OF *Arabidopsis thaliana* HETEROTRIMERIC G PROTEIN ALPHA SUBUNIT (GPA1) USING BACTERIA AND YEAST SYSTEMS

Hazal Büşra Köse

Department of Biological Science and Bioengineering, Sabanci University, Orhanli, Tuzla, 34956 Istanbul, Turkey

Keywords: G proteins, GPA1, different expression systems

Abstract

Heterotrimeric G-proteins mediate transmission of signals from G-protein coupled receptors to cell interior and activate signaling pathways in several organisms from yeast to mammals and plants. The heterotrimer consists of the alpha (Gα), beta (Gβ) and gamma (Gγ) subunits; Gα has GTP binding and hydrolysis activity and Gα and Gβ/Gγ dimeric complex interact with downstream effectors upon activation. G-protein subunits have been identified in several plant species and were shown to be involved in growth and in responses to light and environmental stress factors including draught and pathogens [1]. Although the crystal structure data at 2.34 Å resolution is available for *A. Thaliana* Gα [2] but direct structural data on the Gβ/Gγ dimer and the heterotrimer as a whole are lacking in the literature. The mechanism of activation for the plant heterotrimer is generally inferred by assuming analogy with the mammalian complex. However, recent studies indicate that the plant α subunit may possess a self activation mechanism not observed in the mammalian system which sets a limit to the extent of the analogy [3].

Previously in our group we cloned and expressed *Arabidopsis thaliana* α subunit (GPA1) using *Pichia Pastoris* and β (AGB1) andγ (AGG2) subunits using *E.coli* systems [4, 5] with the aim structure-function studies on the individual subunits and the reconstituted complex. However, since expression and purification of the recombinant protein from yeast are more time and resource consuming compared with E.coli, we investigated possibilities for cloning the GPA1 sequence using alternative methods. In this study, results of cloning of GPA1 using pETM41 and pQE80L vectors are given

together with investigation of expression. In different *E.coli* host cells, after optimizing the GPA1 expression, recombinant GPA1 was purified and biochemically characterized. Preliminary structural characterization of *E.coli* produced GPA1 was also conducted by circular dichroism spectropolarimetry and dynamic light scattering measurements.

Results show that GPA1 can be expressed in the Rosetta strain of E.*coli* as a fusion with maltose binding protein (MBP) giving a yield of 1.2 mg of protein from 1 L of culture. However, during cleavage of MBP tag with tobacco etch virus (TEV) protease, precipitation results in the loss of more than 70% of the purified protein with the remaining part of GPA1 being aggregated. We suggest that the one possible cause of precipitation is the N-terminal flexible region of GPA1 consisting of 36 aminoacids which may disrupt the stability. Another possible reason is the lack of post-translational modifications in *E.coli*. We did not observe any aggregation in GPA1 samples produced in P.*pastoris* system. Structural characterization experiments showed that the secondary structure content of *P.pastoris* GPA1 is consistent with the crystal structure data of mammalian GPA1. Further experiments are required to both improve the purification results to produce more GPA1 and for structural characterization of the protein.

A.thaliana G-PROTEİNİ ALFA ALTBİRİMİ (GPA1)' IN BAKTERİ VE MAYA SİSTEMLERİNDE EKSPRESYONU VE SAFLAŞTIRILMASI

Hazal Büşra Köse

Biyoloji Bilimleri ve Biyomühendislik, MSc Programı, 2014

Tez Danışmanı: Prof. Zehra Sayers

Anahtar Kelimeler: G proteinleri, GPA1, farklı ekspresyon sistemleri

ÖZET

Heterotrimerik G proteinleri, G-protein eşli reseptörlerden (GPCR) hücre içerisine sinyal iletimine aracılık eder ve maya, memeli ve bitki gibi birçok sistemde bulunan sinyal iletim yollarını aktifleştirir. Heterotrimer, alfa (Gα), beta (Gβ) ve gama (Gγ) olmak üzere üç altbirimden oluşur; Gα GTP bağlanma ve hidroliz aktivitesine sahiptir, GTP bağlanmasının üzerine Gα ve Gβ/Gγ dimer kompleksi aşağı yönde, hücre içine doğru efektör moleküllerle etkileşim içine girer. G- protein altbirimleri birçok bitki türünde tanımlanmış ve kuraklık, patojenler gibi çevresel stres faktörleri ve büyüme ile ilişkili olduğu gösterilmiştir [1]. *A. Thaliana* Gα için 2.34 A^o 'luk kristal yapı datası olmasına rağmen [2], Gβ/Gγ dimerin ve heterotrimerin bir bütün olarak yapısal dataları literatürde eksiktir. Bitki heterotrimeri için aktivasyon mekanizması, genel olarak genetik sekanstaki korunmuş bölgeler sebebiyle memeli kompleksiyle analoji oluşturarak yorumlanır. Buna rağmen, yakın zamanda yapılan çalışmalar bitki α altbiriminin kendine özgü, memeli sisteminde görülmeyen türde ve analojiyi limitleyen bir aktivasyon mekanizması olabileceği yönünde ipuçlarını göstermektedir [3].

Daha önce, *Arabidopsis thaliana* α altyapısı (GPA1), *Pichia Pastoris* kullanılarak, β (AGB1) ve γ (AGG2) altyapısı ise *E.coli* sistemleri kullanılarak klonlanmış ve ekspresyonları, her bir altbirimin ve bir araya getirilmiş kompleksin yapı-fonksiyon ilişkisi hedeflenerek gerçekleştirilmiştir [4, 5]. Buna rağmen, ekspresyon ve saflaştırma işlemi maya sistemi kullanıldığında, bakteri sistemine kıyasla çok daha fazla zaman ve kaynak kullanımına sebep olduğundan, GPA1'ın maya dışında alternatif methodlar kullanılarak klonlanması ve ekspresyonunu araştırılmıştır. Bu çalışmada, GPA1'ın pETM41 ve pQE80L vektörleri kullanılarak gerçekleştirilen klonlama sonuçları, bu vektörler kullanılarak elde edilen ekspresyon sonuçlarıyla birlikte verilmiştir. Farklı *E.coli* konak hücrelerinde GPA1 ekspresyonunun optimizasyonu yapıldıktan sonra,

rekombinant GPA1 saflaştırılıp biyokimyasal analizleri yapıldı. *E.coli* ile üretilen GPA1'ın öncül yapısal karakterizasyonu "Circular Dichroism" ve "Dynamic Light Scattering" ölçüm methodları kullanılarak belirlenmiştir.

Elde edilen sonuçlar, GPA1 *E.coli'* nin Rosetta konak hücre çeşidi ile MBP' ye (Maltose Binding Protein) bağlı olarak 1L'lik kültürden 1.2 mg miktarında üretilebildiğini gösterdi. Buna rağmen, MBP işaretinin Tobacco Etch Virus (TEV) proteazı ile kesilmesi, GPA1'ın yaklaşık %70'lik kısmını çökmesi, kalan kısmının da kümeleşmesiyle sonuçlandı. Çökmenin muhtemel sebebinin, GPA1'ın N-bitiş kısmındaki 36 aminoasitlik esnek bölgesinin proteinin kararlılığına zarar vermesi olabileceğini düşünüyoruz. Bir başka muhtemel sebep ise *E.coli'nin* translasyon sonrasındaki modifikasyonları gerçekleştirememesi olabilir. Maya sistemi kullanılarak üretilen GPA1 örneklerinde herhangi bir kümeleşmeye rastlanmadı. Bununla birlikte, yapısal karakterizasyon deneyleri maya kullanılarak üretilen GPA1 örneklerinin, GPA'ın kristal yapı datalarıyla tutarlı olduğunu gösterdi. Hem saflaştırma deneylerini geliştirmek için hem de üretilen GPA1 miktarını yapısal karakterizasyon deneylerinde kullanmak adına arttırmak için daha fazla deney yapılması gerektiğine karar verildi.

“Virtue is under certain circumstances merely an honorable form of stupidity: who could be ill-disposed toward it on that account? And this kind of virtue has not been outlived even today. A kind of sturdy peasant simplicity, which, however, is possible in all classes and can be encountered only with respect and a smile, believes even today that everything is in good hands, namely in the "hands of God"; and when it maintains this proportion with the same modest certainty as it would that two and two make four, we others certainly refrain from contradicting. Why disturb this pure foolishness? Why darken it with our worries about man, people, goal, future? And even if we wanted to do it, we could not. They project their own honorable stupidity and goodness into the heart of things (the old God, deus myops, still lives among them!); we others — we read something else into the heart of things: our own enigmatic nature, our contradictions, our deeper, more painful, more mistrustful wisdom.”

— Friedrich Nietzsche, *The Will to Power*

ACKNOWLEDGEMENTS

I would like to thank Zehra Sayers, my supervisor, for everything. She believed in me and she helped me when I jumped into the Biology ocean as a physics student. Whenever I doubted myself, she believed in me even more and took my self esteem from the ground. I hope that I can be open to my students in the future as much as she was to me.

Sandra Quarantini, the things that I learned from you were not only the experimental methods we used in the lab, but also how to have fun when you are abroad. Thank you for both being a selfless teacher and a friend. I had great times with you and your cats!

I would like to thank Emel Durmaz for kindly providing me TEV protease vector and answering all my questions whenever I faced with the dead end and helping me finding the way out.

I would like to thank each and every member of my family for being always with me and supporting all my decisions without a doubt.

There are people in my life who deserve to be in this page more than anybody but I feel like if I write their names, it is going to be sort of a good bye and this is not even the last thing that I want to do. If I say "I would like to thank Ayşegül Güvenek and Enes Karaboğa whom I spent my last year quite closely", it may seem like everything has ended and we are going to share nothing anymore. So, I refuse to thank those two people. Because I know that we will continue to contribute to our lives in many ways.

Ahmet Sinan Yavuz, who is one of the best graduate students in Sabanci University, has helped me a lot both when I first started to this school and when I am leaving now. I sincerely hope that he will never lose his enthusiasm about what he wants for his life and I know that he will get what he truly deserves.

Yusuf Talha Tamer, your sabotage will not be forgotten and forgiven. Yet, thank you for your patience about my tough jokes on you.

I would like to thank all the members of the Toprak Lab, both graduates and undergraduates, for creating such a friendly environment in the lab. The positive effect of a nice environment and seeing your friends instead of hostile, competitive people are priceless.

TABLE of CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST of ABBREVIATIONS

AGB1: Arabidopsis thaliana heterotrimeric G protein β subunit

AGG1: Arabidopsis thaliana heterotrimeric G protein γ subunit-1

AGG2: Arabidopsis thaliana heterotrimeric G protein γ subunit-2

AGG3: Arabidopsis thaliana heterotrimeric G protein γ subunit-3

AMP: Ampicilin

BME: β-merchaptoethanol

BMGY: Buffered glycerol-complex medium

BMMY: Buffered methanol-complex medium

BSA: Bovine Serum Albumin

cAMP: Adenylyl cyclase pathway

CD: Circular Dichroism

DLS: Dynamic Light Scattering

DTT: Dithiothreitol

ECL: Enhanced luminol-based chemiluminescent

EFPI: EDTA-Free Protease Inhibitor

FPLC: Fast Protein Liquid Chromatography

FRET: Fluorescence resonance energy transfer

FT: Flow Through

GAP: GTPase Activating Protein

GDI: Guanosine nucleotide dissociation inhibitors

GDP: Guanosine di-phosphate

GEF: Guanosine Nucleotide Exchange Factor

GPA1: Arabidopsis thaliana heterotrimeric G protein α subunit

GPCR: G Protein Coupled Receptor

GTP: Guanosine tri-phosphate

His: Histidine

IPTG: Isopropyl β-D-1-thiogalactopyranoside

KAN: Kanamycin

LB: Luria-Bertani

MBP: Maltose Binding Protein

MW: Molecular weight

MWCO: Moelcular Weight Cut-Off

NAD^+: Nicotinamide adenine dinucleotide

PB: Purification Buffer

PCR: Polymerase Chain Reaction

PDB: Protein Data Bank

PL: Pellet

PMSF: Phenylmethanesulfonyl Fluoride

RGS: Regulator of G proteins

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SN: Supernatant

TAE: Tris-acetate-EDTA

TB: Terrific Broth

TBS: TRIS Buffered Saline

TBS-T: TRIS Buffered Saline-Tween

TEV: Tobacco Etch Virus

XLG1: Extra Large G protein gene-1

XLG2: Extra Large G protein gene-2

XLG3: Extra Large G protein gene-3

YNB: Yeast Nitrogen Base

YPD: Yeast extract-Peptone-Dextrose

EXPRESSION AND PURIFICATION OF *Arabidopsis thaliana* HETEROTRIMERIC G PROTEIN ALPHA SUBUNIT (GPA1) USING BACTERIA AND YEAST SYSTEMS

1 INTRODUCTION

In 1970, the first step towards the discovery of the importance of G proteins was made by the work of Sutherland and his coworkers, which was on the stimulation of the production of cyclic AMP (cAMP) via the enzyme mono-ADP- ribosyl-transferase, which catalyzes the transfer of NAD^+ to protein substrate. Eventually, this discovery ended up with a Nobel Prize in Physiology and Medicine in 1971 for Sutherland for his work on cAMP. Funnily it was, the discovery was the result of an honest mistake. The bacterial exotoxins produced by *Vibrio cholerae* causing elevation of cAMP levels in the mouse lymphoma cell line S49, were thought to be ATP-dependent but the ATP used in the experiments was contaminated with GTP [6]. Later, with the treatment with radioactive [^{32}P] NAD^+ and cholera toxin of S49 cell line and its mutant cyc^-, which are unable to generate cAMP in response to the beta-adrenergic agonist isoprenaline, but still had a binding site similar to β_2-adrenoceptor, a 45 kDa polypeptide was identified. After purification of this polypeptide, it was understood that it was not alone but complexed with 35 kDa and 8-10 kDa polypeptides. These proteins were then labeled as α- subunit (45 kDa), β-subunit (35 kDa) and γ-subunit (8-10 kDa) of functional heterotrimeric G proteins [6]. Alfred Gillman and Martin Rodbell won Nobel prize for Physiology and Medicine in 1994 for this study together with other complementary studies to the function on G proteins [7].

1.1 G Protein Coupled Receptors

G protein coupled receptors (GPCR's) are one of the largest protein families. They are classified under five subgroups, namely Rhodopsin (683 members), Adhesion family (24 members), Frizzled/taste family (24 members), Glutamate family (15 members) and Secretin family (15 members) [3, 4]. A recent study by Krishnan *et al.* in 2012 revealed that these five families have a longer evolutionary history than it has been thought and

four of them (Rhodopsin, Adhesion, Frizzled and Secretin) have a common origin with cAMP receptors [8] . GPCR's are involved in many signaling pathways and The Nobel Prize in Chemistry 2012 is won by Brian K. Kobilka and Robert J. Lefkowitz for studies of G-protein–coupled receptors.

1.1.1 Structure of GPCRs

Main structural features of GPCR's can be seen in Figure 1-1. All GPCR's have seven transmembrane regions, which mostly conserved over years and among species, with an extracellular amino- and intracellular C terminal. Exceptionally, Adhesion and Glutamate family members are recognized with relatively longer N termini [10].

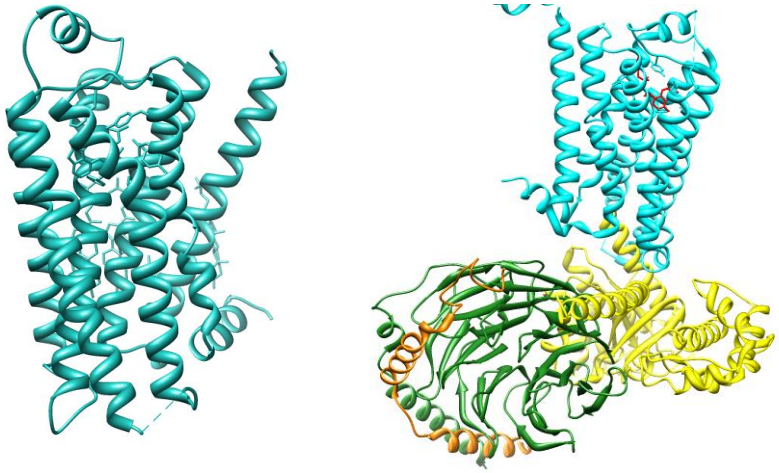


Figure 1-1: Crystal Structure of GPCR and together with heterotrimeric G protein. (left) Crystal structure of human β2 adrenergic receptor (one of the members of GPCR family), PDB code: 2RH1, (right) human β2 adrenergic receptor together with mammalian heterotrimeric G protein. Color code: GPCR: cyan, Gα: yellow, Gβ: green, Gγ: orange and ligant: red (PDB code: 3SN6).

GPCR's have two kinds of heterogeneity. The first one is structural heterogeneity caused by posttranslational modifications such as glycosylation, phosphorylation and palmitoylation. These modifications can be minimized if GPCR can be expressed in bacteria [11]. The other source of heterogeneity comes from conformational changes. GPCR's are highly flexible and it is functionally very important because these structural changes may be required for binding of signal molecules. These heterogeneities make GPCR's hard to crystallize and/or even if they are crystallized, flexible regions have poor resolutions which lead to poor understanding of the structure and the function of

these domains. It has been shown that flexibility of GPCR's can be reduced by using specific ligands [12]. There is no need to mention that transmembrane proteins are already quite hard to crystallize because of the fact that detergents required in their purification steps make the protein almost impossible to crystallize due to the large enclosing micelles.

1.1.2 Mechanisms of GPCRs

There are several evidences showing that GPCR's form oligomers [8–10]. Although the mechanism leading to oligomer formation is not yet completely understood, it is suggested that oligomerization might be essential for activation and signal transduction. Especially, the idea that GPCR's may be dimerized to complex with G proteins was suggested after realizing that a pentameric complex forms when leukotriene is reconstituted with purified G protein α-subunit (Baneres & Parello 2003).

A large number of studies have been conducted to understand the kinetics of GPCR's upon binding to a ligand or as in some cases diffusing into an unliganded receptor [17]. The most common and well known model to explain the mechanism of GPCR activation is the two-state model, depicted in Figure **1**-**2**, in which there is basically an active and inactive state of the GPCR. When there is an agonist in the environment, it binds to the GPCR and stabilizes its active state more. The activated GPCRs act as a guanine nucleotide exchange factor (GEF) and activate downstream elements such as G proteins to transmit the signal. In case of an inverse agonist, the inactive state is stabilized. It has been reported that there are partial agonists leading an in-between state where GPCR can be found both active and inactive states [18]. Although the two model system explains a vast majority of the GPCR's behaviors, there is a new trend in experiments showing that multiple conformational states are available instead of one active and one inactive state [19]. Based on these different suggestions based on logical evidences, more experiments such as fluorescence assays, FRET experiments for dynamic movements of different domains are required to understand the GPCR protein family which has too many members and all of them may have different conformations upon activation and deactivation. The bottom line that we can come up with based on these studies is that there is more work to do compare with what have been done so far.

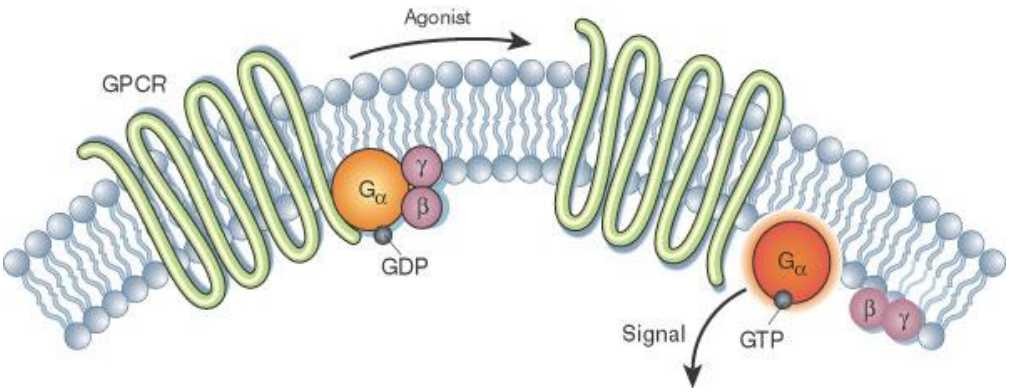


Figure 1-2: The model of GPCR with heterotrimeric G proteins and conformational changes of G protein upon an agonist stimulation. Li, J. *et al*. The Molecule Pages database. Nature 420, 716-717 (2002).

1.2 RGS Proteins

The first time that RGS (regulator of G protein signaling) proteins came onto stage is when researchers showed evidence that there is an enormous difference between the rates of Gα- subunit's GTP to GDP hydrolysis *in vivo* and *in vitro*. Although they observed a rapid hydrolysis *in vivo*, when the rates of purified Gα- subunit's GTP hydrolysis to GDP *in vitro* were measured, it was found out that purified Gα has a lower rate compared with *in vivo*. So, it was obvious that there are other proteins involved in the hydrolysis step of G protein activation/deactivation cycle in the cell [20]. The general name given for these proteins is GTPase activating proteins (GAPs). It did not take for a long time to discover RGS protein family with approximately 30 members. Contrary to GPCR's which has a role in activation of G proteins, RGS proteins stimulate GTPase activity and increase the hydrolysis rate of Gα- subunit from GTP to GDP on the Gα subunit around thousand fold and brings G proteins to their inactive state again [16, 17].

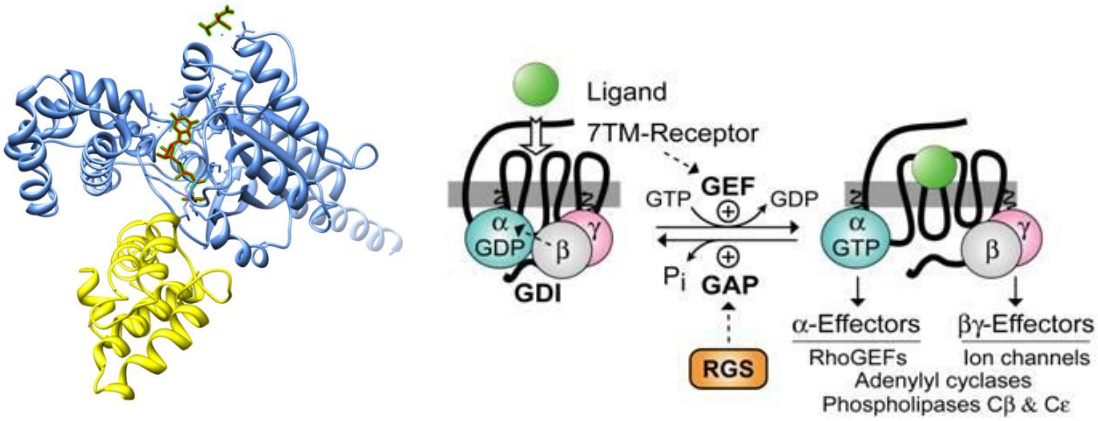


Figure 1-3: Crystal structure of RGS protein bund to Gα and their part in the activation mechanism. (left) Crystal structure of RGS4+Gαi1. RGS4 is shown blue and Gαi1 is shown yellow. The ligand which is shown in red is GDP. PDB code: 1AGR. (right) Generic model of G protein mediated signaling pathways and types of proteins including GEF, GAP etc. involved. D. P. Siderovski and F. S. Willard, "*The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits.*" Int. J. Biol. Sci., vol. 1, pp. 51–66, 2005.

From the crystal structures of RGS boxes together with G protein α subunit shown in Figure **1**-**3**, it came our attention that RGS interacts with the switch regions of Gα where it binds to GTP. In Tesmer JJ *et al*. 1997, it has been clearly shown that RGS4, one of the members of RGS protein family, binds to Gα switch regions and doesn't contribute to catalytic residues that are bound to GDP or AlF_4^- that mimics the transition state of hydrolysis. The reason why Gα hydrolyses GTP rapidly in presence of RGS is because it stabilizes these switch regions of Gα [23]. These experiments are also consistent with the idea that RGS protein family has a role in GTPase activity of Gα subunit of heterotrimeric G protein. Considering the fact that there are more than 20 Gα proteins and 30 RGS proteins, it is still remained in dark how Gα and RGS proteins are paired up in cell. Although some studies showed that they selectively binds to each other, other studies revealed that the selectively binding Gα by RGS proteins is occurred to be exceptional [22].

1.3 Mammalian Heterotrimeric G Protein

The first detailed characterization and cloning of G protein α- subunit was done along with high-level expression of rhodopsin, G protein coupled photon receptor, due to its highly stable structure and retaining function under different conditions while other

GPCR's were denatured [24]. This was followed by cloning and early characterization of several other G proteins shown in Figure **1-4** and sometimes their truncated forms from different tissues that were involved in different signaling pathways.

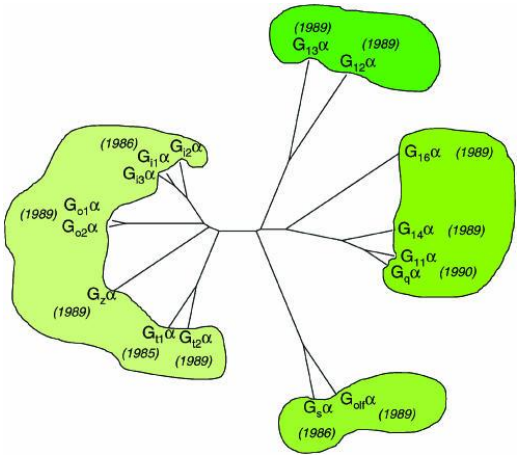


Figure 1-4: The unrooted homology tree of G protein alpha subunits together with their corresponding date of cloning of cDNAs. B. K. Kobilka, "G protein coupled receptor structure and activation.," *Biochim. Biophys. Acta*, vol. 1768, no. 4, pp. 794–807, Apr. 2007.

1.3.1 Structure of Mammalian G Proteins

There are four main families of Gα-subunit ($G_s\alpha$, $G_{i/o}\alpha$, $G_{q/11}\alpha$ and $G_{12/13}\alpha$) which then are divided into several subgroups, five Gβ ($G\beta_{1\text{-}5}$) and twelve different Gγ ($G\gamma_{1\text{-}12}$) subunits in mammalian system [6]. The alpha subunit is around 40-45 kDa consisting of mainly alpha helices and binds to the upstream receptors. β has a symmetrical seven bladed propeller structure and an alpha helix at the N terminus. Each blade consists of four antiparallel beta sheets as it is seen in its crystal structure given in Figure **1-5**. The γ subunit, on the other hand, relatively more flexible and a small protein which has a moleculer mass of 8-10 kDa and the bottom part of the propeller interacts with the γ subunit whereas the top surface of the propeller interacts with the α subunit. β and γ subunits form a tight dimer and when a signal arrives at GPCR, G proteins are activated and the βγ dimer dissociates from the α-subunit. The βγ complex cannot bind both Gα and another effector molecule when it is inactive. It was demonstrated that it sends another signal separately from alpha subunit when they are detached from each other upon activation of the α subunit and that if α is inactive, then it inactivates βγ complex and binds to it by showing the relation between phospholipase C activity and βγ

complex [25]. Briefly describing the experiment, βγ complex triggered phospholipase c activity instead of inhibiting basal activity which is a sign of activated Gα. Also, when purified Gα-GDP complex was added to the reaction more and more, βγ complex reversed its effect because it is more favorable for βγ to bind Gα-GDP instead of sending another signal.

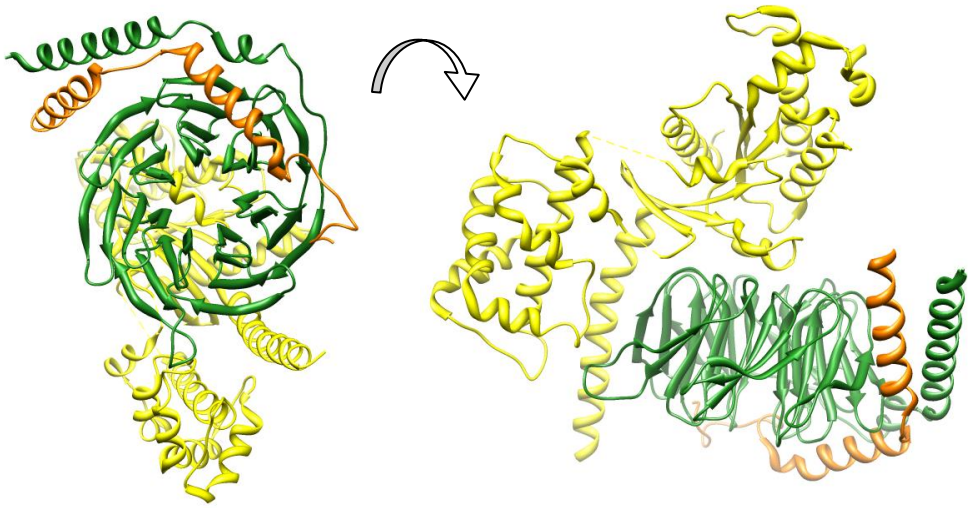


Figure 1-5: Mammalian heterotrimeric G protein. Alpha subunit is shown in yellow, beta subunit is shown in green and gamma subunit is shown in orange. (PDB code: 3SN6).

1.3.2 Signaling in Mammalian G Proteins

While transmembrane signaling systems include a stimulus and an effector protein to transfer the signal through the pathway, there are more elements such as heterotrimeric G protein itself as well as additional proteins or soluble. Briefly, in order to couple the receptors with an effector molecule, G proteins undergo several changes that can be defined as activation/inactivation cycles. In its inactive, so called basal state, Gα-subunit attached to the receptor is bound to the βγ-dimer complex and also to GDP (guanosine diphosphate) molecule. When a signal arrives to the receptor, the α- subunit exchanges GDP to GTP and dissociates from the βγ-dimer. Subsequently, the α- subunit hydrolyses GTP to GDP, which qualifies α- subunit as an effective GTPase, reattaches to the βγ-dimer and the receptor waiting for the next stimulus [26]. Although the activation mechanism was well understood, it took more time to understand the deactivation mechanism including GTPase activity of the α- subunit.

Cardiovascular system	**Endocrine System and Metabolism**	**Immune System**	**Nervous System**	**Sensory Systems**	**Development**	**Cell Growth and Transformation**
Autonomic control of heart function	Hypothalamo-pituitary system	Leukocyte migration/homing	Inhibitory modulation of synaptic transmission	Visual system	G_{11}-mediated signaling in embryonic angiogenesis	Cellular growth induced by G_{12}/G_{13}
Myocardial hypertrophy	Pancreatic β-cells	Immune cell effector functions	Modulation of synaptic transmission by the G_q/G_{11}-mediated signaling pathway	Olfactory/pheromone system	G_q/G_{11}-mediated signaling during embryonic myocardial growth	G_i-mediated cell transformation
Smooth muscle tone	Thyroid gland/parathyroid gland			Gustatory system	Neural crest development	The oncogenic potential of G_s
Platelet activation	Regulation of carbohydrate and lipid metabolism					

Table 1: Systems and functions that mammalian heterotrimeric G protein involved physiologically [27].

Functional significance of the animal heterotrimeric G proteins can be seen from the list of several systems that regulated by these proteins as shown in Table 1 [27]. Moreover it is estimated that about 50% of drug targets in the pharmaceutical industry are GPCR's [28].

1.4 Plant Heterotrimeric G Protein

1.4.1 Structure of Its Subunits

Two decades the discovery of Sutherland and his co-workers in 1971, that cAMP production requires nucleotide binding protein made by Sutherlands and his colleagues in 1970, plant G proteins (starting from α subunit) were cloned for the first time to show that there are conserved regions between mammalian and plant systems in 1990s [29]. In contrast to the mammalian systems, there are not too many variations of G protein subunits in plants. The plant complex also consists of three subunits, namely α, β and γ subunits but heterotrimeric G proteins have not had any crystal or dynamic structure as a heterotrimer which causes a vague view of them compared with the mammalian G proteins. Number of different types of subunits found in plants is as following:

α: 1 in *Arabidopsis thaliana* (AtGPA1), up to 4 in some other plants (*Glycine max*) has been identified [3],
β: 1 in *Arabidopsis thaliana* (AGB1), up to 4 in some other plants (*Glycine max*) has been identified [3],
γ: 3 in *Arabidopsis thaliana* (AGG1, AGG2, AGG3) and up to 8 subunits has been identified in other plants, particularly in *Glycine max* and *Manihot esculenta* [25, 26].

As it is mentioned above, there are conserved regions in between plant and mammalian G proteins and an alignment of these regions are shown in Figure 1-6. Briefly, A. *thaliana* G protein α subunit (which will be referred as GPA1) has similarities with mammalian G protein such as a glycine residue at position 2 which is thought to be a potential N-myristoylation site and an arginine residue that is a potential site for ADP-ribosylation. However, there is no C-terminal cysteine which provides ADP-ribosylation by pertussis toxin as it is in mammalian system [31]. Similarly, heterotrimeric G protein β subunit also showed significant conservation between plants and animals through the analysis done by ClustalW2 [32]. (The multiple alignment result of β subunit can be found in Appendix A).

Figure 1-6: Multiple sequence alignment result of heterotrimeric G protein α subunit found in different species. *A. thaliana* (gb|AEC07820.1), *S. lycopersicum* (tomato-P26981.1), *Xenopus laevis* (gb|AAI70080.1) and *Mus musculus* (gb|AAA37713.1). (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

A 2.34 A^{o} resolution crystal structure of A. *thaliana* G protein α subunit (AtGPA1) which lacks the last 36 aminoacids from the N-terminal was introduced in J. C. Jones *et al.* 2011 and the structure of α-subunit particularly GDP/GTP binding domains was revealed. Based on the crystal structure, approximately 49% of AtGPA1 is alpha helices, 41% is random coil and 10% is extended strand. It comprises of two main domains as Ras domain and helical domain in between where binding occurs. After mutagenesis experiments made on the suspected regions such as the switch regions and guanine nucleotide–binding pocket domains of the subunit, Jones and colleagues

demonstrated that AtGPA1 helical domain is more mobile compare to Ras domain whereas both helical and Ras domains of animal Gα have vigorous mobility upon binding. Furthermore, helical domain of AtGPA1 was found to be more mobile than animal Gα helical domain, too. This high mobility led to the hypothesis that the helical domain is where self-activation occurs in the plant Gα. To prove this hypothesis, intrinsic fluorescence measurements with GTP-γ-S and GTP were performed. Nucleotide exchange with not only AtGPA1 but also with chimeras formed by AtGPA1 and animal GPA1 helical domains were formed to see how helical domain changes upon binding. Based on the results obtained, it was decided that helical domain is sufficient to exchange GDP to GTP [2].

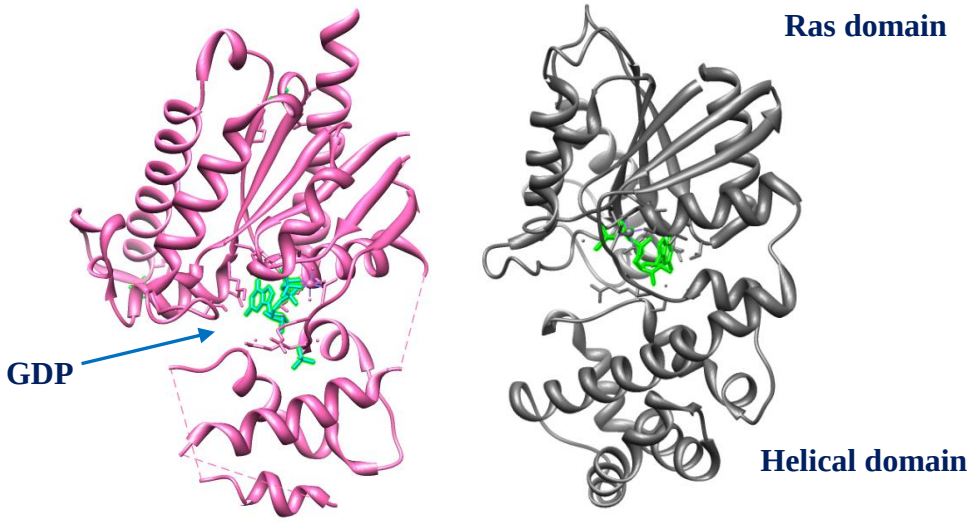


Figure 1-7: Comparison of the crystal structures of *A. thaliana* G protein alpha subunit (AtGPA1) and mammalian $G_{i\alpha1}$ alpha subunit. AtGPA1 is shown in pink, $G_{i\alpha1}$ is shown in gray and GDP is shown in green in both of the structures. PDB code of AtGPA1: 2XTZ, PDB code of $G_{i\alpha1}$: 1GIA [29, 30].

β subunit of plant G protein, namely AGB1, on the other hand, has N-terminal coiled coil structure and WD40-repeated seven bladed propeller structure on the C-terminal as it is in the animal Gβ subunit [34]. From its coiled coil structure, it binds to alpha subunit while WD40 repeating units have an interface with gamma subunit. Again, as it is in mammalian system, β subunit can transmit signals independently to different effectors when it is detached from alpha subunit, but it is impossible to both be bound to alpha subunit and involve another pathway simultaneously [32].

The smallest subunit of the heterotrimer, the γ subunit (named as AGG1, AGG2, AGG3 in A. *thaliana*) has a molecular weight around 8-10 kDa and are sub grouped under three classes. Type A γ subunit has an isoprenylation site which is defined as cysteine, two aliphatic residues followed by any residue (CaaX) at C-terminus, type B lacks this site and in type C, there are high number of cysteine residues at its C-terminus [1]. Since there is no isoprenylation site in animal γ subunit, it forms the major difference between plant and mammalian γ subunits. *A. thaliana* contains from both type A and type B. AGG1 and AGG2 is classified as type A, whereas AGG3 is classified as type C due to its cysteine rich domain. There is no type Bγ subunit of *A. thaliana* [30].

1.4.2 Signaling Mechanism of Plant G Proteins

As it comes to functions of each subunit, alpha subunit plays the major role in mediating signal transmission from receptors to their effectors. For a long time, mammalian system has been used as an analog for plant heterotrimeric G proteins assuming that they have similar mechanisms. However, recent studies led to the fact that the plant α subunit shows signatures pointing that it has a self activation mechanism unlikely to mammalian system. When it is bound to GDP, it is referred as the inactive state of GPA1 and with GTP, it is referred as the active state. What creates the difference according to the suggested model is that it spontaneously releases GDP and binds to GTP and it mostly prefers to be bound to GTP [3]. Furthermore, instead of having GPCR as a receptor, RGS proteins which accelerate the hydrolysis of GTP to GDP are found in plant system. In fact, they are a major element in the self activating mechanism in that GPA1 would stay bound to GTP for much longer time if RGS proteins did not stimulate hydrolysis. When there is a ligand in the environment, it binds to RGS and inhibits its function which eventually leads alpha subunit to dissociate from βγ dimer and stay bound to GTP, that is the active state and transmit the signal to its effectors. When ligand disappears from the environment, RGS goes back to its primary function which is acceleration of GTP hydrolysis, alpha subunit stops signaling, performs its GTPase activity and hydrolyzes GTP to GDP and finally it binds the βγ dimer resuming its inactive state. Memebers of the RGS protein family are the major GTPase activating proteins (GAPs) but GAPs don't necessarily have to be RGS proteins, other GAP proteins have the same function in plant system (This model has been suggested by Daisuke Urano and the colleagues and reviewed in [1]).

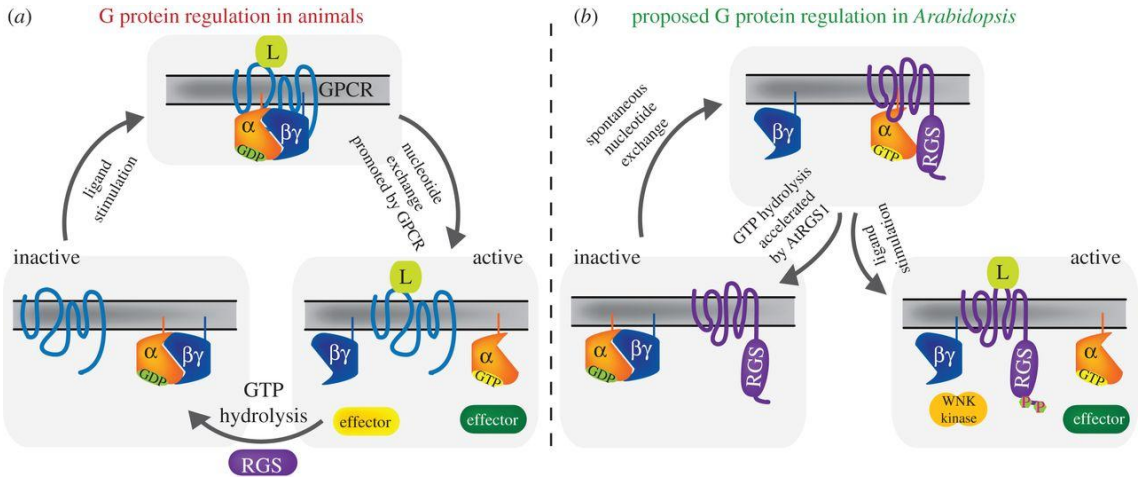


Figure 1-8: Proposed mechanism of the self activation of Gα in both mammalian and plant systems demonstrating GPCR and RGS proteins. D. Urano, J. Chen, J. R. Botella, and A. M. Jones, "*Heterotrimeric G protein signalling in the plant kingdom Heterotrimeric G protein signalling in the plant kingdom*," no. March, 2013.

A recent finding, the new three extra large Gα genes (XLG1, XLG2 and XLG3) has brought new impetus. Their N terminal sequence is highly similar to AtGPA1 and the C-terminal includes cysteine rich residues which differ from AtGPA1. Although there is not yet enough work to understand their structure and function thoroughly, what obtained so far is that XGL proteins are also capable ofguanine nucleotides hydrolysis, binding to Gβ and they require very low amount of Ca^{++} concentration instead of Mg^{2+} which is the case for AtGPA1 [30, 32].

Finally, plant heterotrimeric G proteins are also involved in several systems and numerous signaling pathways. Their functions in plants are growth and morphology, hormone and glucose responses, stomatal movements and ion channel regulation and pathogen resistance which are given in great detail in the recent review published by Urano *et al*. 2013. Since there are many issues that have not been addressed in plant G proteins compared with mammalian G proteins, the list of signaling pathways that G proteins have role may get longer with the future studies.

1.5 The Aim of the Study

The suggested self activation mechanism of heterotrimeric G-proteins in plants, which distinguishes them from their mammalian counterparts is not yet fully understood. There is limited evidence in the literature towards this model. In order to provide insight in to the less known aspects of the mechanism and fuction of plant heterotrimeric G proteins, it is necessary to express, purify and obtain adequate amounts of each three subunits of the heterotrimer so that reconstitution can be performed *in vitro* and dynamic data on the structure and structural changes can be obtained. Our ultimate goal is to determine 3D structure of the heterotrimeric G protein of *A.thaliana* as a whole by reconstituting the complex of the three subunits *in vitro*. In this study, we investigated the expression of the alpha subunit of *A. Thaliana* (AtGPA1) heterotrimeric G protein in *E.coli* in order to optimize the time and resource. Aims of the work presented in this were:

- Cloning of GPA1 by using two different vectors, namely petM41 and pQE80L which would produce the recombinant protein as fusion with his-tag and maltose binding protein (MBP) and his-tag respectively,
- Optimization of protein expression using different host *E.coli* strains and varying environmental conditions,
- Purification of the fusion proteins from *E.coli* by applying different chromatography methods,
- Cleavage of the tags, to obtain the native proteins,
- Characterization of the purified GPA1 by DLS (dynamic light scattering), absorption spectroscopy and SDS- and native-PAGE.

Additionally towards further biophysical characterization of GPA1 expressed *in P. pastoris* circular dichroism (CD) measurements were carried out to show that the secondary structure content is consistent with that obtained from the crystal structure.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Chemicals used through the project time were supplied from different companies which are Invitrogen, Sigma Aldrich, peQLAB, ThermoScientific, Roche, Qiagen, Fermentas, Miltenyi Biotechnology MACS, AppliChem, Amresco, MERCK, Molekula and Biolife.

2.1.2 Vectors

petM-41 and pQE80L vectors were used to clone GPA1 gene. Vectors were already stocked by former members of the lab and vector maps can be found in Appendix D. In Figure 2-1, more simplistic versions of petM-41 and pQE80L are represented to show the tags which are used to recognize GPA1 in cells and also further confirmation that it is GPA1 but not another random protein with similar molecular weight.

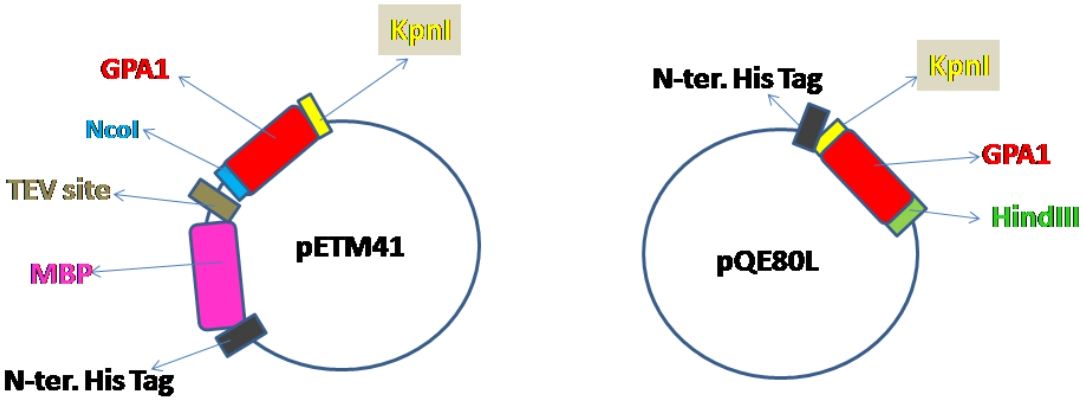


Figure 2-1: The vectors used to clone GPA1 and their tags inside the vector sequence.

Shortly, there two N-terminal tags in pETM-41 which are MBP (maltose binding protein) tag that corresponds to incrase solubility and has 45-kDa molecular weight and Histidine-tag that is six consequtive Histidine aminoacid for affinity chromatography. There is also TEV cleavage site which is comprised of “Glu-Asn-Leu-Tyr-Phe-Gln-Gly” aminoacid sequence and it is used for removal of MBP and leaving GPA1 without a tag. pETM-41 has Kanamycin resistance sequence in it. On the other hand, pQE80L has only one N-terminal tag which is His-tag for affinity chromatography. pQE80L has Ampicilin resistance sequence in it.

2.1.2.1 Primers designed for these vectors

Here are given the sequences of all the primers used for amplification of GPA1 gene, primers corresponding to cloning of GPA1 in pETM-41 vector and primers corresponding to cloning of GPA1 in pQE80L vector in Table 2.

	Sequences		Restriction enzyme
GPA1	Forward	5'-ATGGGCTTACTCTGCAGTAGA-3'	-
	Reverse	5'-TTATTATCATAAAAGGCCAGCCTCCAG-3'	-
GPA1-pETM41	Forward	5'-CATGCCATGGGCTTACTCTGCAGTTAC-3'	NcoI
	Reverse	5'-CGGGGTACCTCATCATAAAGGCCAGCCTC-3'	KpnI
GPA1-pQE80L	Forward	5'-GGGGTACCGGCTTACTCTGCAGTAGA-3'	HindIII
	Reverse	5'-CCAAGCTTTTATCATAAAAGGCCAGCCTC-3'	KpnI

Table 2: Primers used to insert GPA1 gene into pQE80L and pETM-41 vectors.

2.1.3 Enzymes

As it is listed in Table 2, restriction enzymes used to cut vector in their introduced sites are NcoI, KpnI for pETM41 and HindIII, KpnI for pQE80L. Ligation enzyme was always T4 Ligase, and Taq Polymerase was used to amplify the templates by PCR reaction.

2.1.4 Cell Lines

BL21(DE3) and Rosetta are the *E.coli* strains that were used to express GPA1 in high amounts and Top10 strain of *E.coli* was used to transform vectors into bacteria in the first step. Rosetta is a specific strain which includes the codons for rare tRNAs that are AUA,AGG, AGA, CUA, CCC and GGA.

For yeast expression, *P. pastoris* was used.

2.1.5 Culture Media

2.1.5.1 *E.coli*

In all transformation and expression trials, LB (Luria-Bertani) Broth was used. Ingredients are 10 g Trptone, 5 g yeast extract and 5 gr NaCl for 1 L medium. For solid medium that are used to inoculate cells after transformation, 15 g of LB-Agar was added to1 L LB medium with required antibiotics which are Kanamycin and Ampicilin for pETM41 and pQE80L respectively.

Additionally, Terrific Broth (TB) medium was used only in TEV protease expression. The content of TB was 24 gr Tryptone, 12 gr yeast extract, 0.17 M KH_2PO_4 (in 100 ml), 0.72 M K_2HPO_4 (in 100 ml), 0.12% glycerol and 2 mM $MgSO_4$ for 1 L medium.

2.1.5.2 *P. pastoris*

YPD (Yeast extract-Peptone-Dextrose) Broth was used for inoculation on solid agar medium and starter culture (5 ml) with required antibiotic which is Zeocin. Ingredients of YPD are given as, 10 gr yeast extract, 20 gr peptone and 20 gr agar for solid medium only.

After dissolving the above in 900 ml dH_2O, it is autoclaved and then 100 ml of filter-sterilized 20% dextrose is added for 1 L YPD medium.

BMGY and BMMY media are used to make larger culture. Ingredients for 1 L are 10 gr yeast extract and 20 gr peptone are dissolved in 700 ml dH_2O autoclaved. Then, 100 ml 1M sterilized potassium phosphate (pH=6.0), 100 ml 10X YNB stock and 1ml 500X Biotin are added. Finally, for BMGY,100 ml 10X glycerol and for BMMY 100 ml 10X methanol instead of glycerol are added to media.

2.1.6 Buffers

2.1.6.1 *E.coli* system

After obtaining cell pellet at the end of expression step;

Lysis buffer: 100 mM NaCl, 50 mM TRIS pH=7.5, 50 µg/ml PMSF, Protease Inhibitor Tablet, 0.5 mg/ml Lysozyme from chicken egg white,

Affinity (HiTrap) buffer (Buffer A): 50 mM TRIS, 300 mM NaCl, 1% glycerol, 10 mM imidazole, 50 μM GDP, 5 mM $MgCl_2$, 30 μM $Al_2(SO_4)_3$ and 20 mM NaF,

Elution buffer (Buffer B): 50 mM TRIS, 300 mM NaCl, 1% glycerol, 1 M imidazole, 50 μM GDP, 5 mM $MgCl_2$, 30 μM $Al_2(SO_4)_3$ and 20 mM NaF,

Desalting buffer: 50 mM TRIS pH=7.4,

Anion Exchange (QTrap) buffer (A): 50 mM TRIS pH=7.4

Anion Exchange (QTrap) elution buffer (B): 50 mM TRIS pH=7.4, 700 mM NaCl buffer,

Size Exclusion Chromatography (SEC) buffer: 25 mM TRISpH=7.4, 100 mM NaCl, 50 μM GDP, 1 mM DTT.

2.1.6.2 *P. pastoris* system

Lysis buffer: 10 mM $MgCl_2$, 0.1% Triton-X-100, 2 mM PMSF, 1X EDTA-Free Protease Inhibitor Tablet.

10X Purification Buffer: 500 mM TRIS, pH 8.0, 100 mM BME (β-merchaptoethanol), 3 M NaCl, 200 mM imidazole, 50 mM $MgCl_2$, 2 mM PMSF, 300 μM $Al_2(SO_4)_3$, 100 mM NaF, 1X EDTA-Free Protease Inh. Tablet, 500 μM GDP.

Dialysis buffer: 50 mM TRIS, pH=8.0, 1 mM DTT, 5% glycerol with CelluSep Renegenerated tubular membrane with molecular weight cutoff (MWCO) 6000-8000 and MWCO 12000-14000 Da.

Anion Exchange (QTrap) Start Buffer: 50 mM TRIS, pH: 8.0

Anion Exchange (QTrap) Binding Buffer: 50 mM TRIS, 700 mM NaCl.

Size Exclusion Chromatography (SEC) buffer: 50 mM TRISpH=7.4, 150 mM NaCl, 50 μM GDP, 1 mM DTT.

All buffers used in chromatography steps were prepared with dH_2O, filtered with 0.22 μm Millipore filter paper and degassed before using them.

Buffers used in electrophoresis, SDS-PAGE, Native-PAGE, Western Blotting, etc. can be found in Appendix E.

2.1.7 Columns

For affinity chromatography, HisTrap 1ml and 5 ml columns depending on the amount of supernatant, for desalting, HiPrep 26/10 Desalting column, for ion exchange chromatography, QTrap 5 ml column and for size exclusion chromatography, HiLoad 16/60 Superdex 75pg and HiLoad 16/600 Superdex 200pg columns were used. All of them were supplied by GEHealthcare. Also, for GPA1 in *P. pastoris* purification, instead of automated system, batch mode affinity chromatography was applied with QIAGEN Ni-NTA agarose slurry.

In between chromatography steps and at the end of each purification cycle, concentrators with MWCO=10000 and MWCO=30000 (Millipore Centrifugal Filter Unit, Sartorious Biolab Product) were used to concentrate depending on the molecular weight of the protein.

2.2 METHODS

2.2.1 Cloning GPA1 gene into vectors

In order to clone GPA1 gene into vectors (pETM-41 and pQE80L), PCR was applied with primers having restriction enzyme recognition sites given in Table 2.

Chemicals	Amount (µl)
Forward primer (20 mM)	1
Reverse primer (20 mM)	1
Template (GPA1)	1
$MgCl_2$ (25 mM)	2,5
Taq Polymerase	1
Taq poly buff w/o $MgCl_2$	1,5
dNTPs	1
dH_2O	15
Total	25

Table 3: Chemicals used in PCR reaction and the temperatures of PCR reaction carried out in thermal cycler.

PCR conditions (pETM-41)	Time	
95° C	5’	
95° C	45’’	34 cycles
50° C	45’’	
72° C	45’’	
72° C	5’	
4° C	∞	

PCR conditions (pQE80L)	Time	
95° C	5’	
95° C	45’’	34 cycles
52° C	45’’	
72° C	45’’	
72° C	5’	
4° C	∞	

Table 4: PCR conditions applied for amplification of GPA1 gene with primers specially for pETM-41 and pQE80L vectors.

After each PCR reaction, 1% agarose gel electrophoresis was applied for 1 hour with 1X TAE buffer (ingredients/preparation of the buffer can be found in Appendix E) to visualize the PCR products and see if their molecular weight that is number of approximate base pair is consistent with the expected molecular weight. 6X loading dye was added to each sample to visualize them with the help of ethidium bromide (EtBr) staining. Molecular weight estimation of DNA fragments was made by using MassRuler

DNA ladder mix (ThermoScientific). If DNA fragment is consistent with the length of GPA1 with restriction sites, the fragment was cut out of the gel and it was purified from the gel by using Gel Extraction Kit (QIAGEN).

2.2.2 Restriction Digestion with Corresponding Restriction Enzymes

As it is stated in Table 2, we used NcoI and KpnI restriction sites of pETM-41 and designed primers according to their recognition sites. For pQE80L, HindIII and KpnI restriction sites were used in the vector. That is why before ligating GPA1 into the vectors, vector and the gene (GPA1) have to have the sticky complementary sites to each other. After applying PCR reaction with corresponding primers, GPA1 is supposed have the sites that restriction enzymes can recognize. Since these sites are already introduced in the vector, when restriction enzymes are applied, it is supposed to cut the region in between two restriction sites so that GPA1 can be ligated to that region by complementing the single stranded hanging region of restriction site.

Restriction reaction took place in room temperature for overnight. The next day, reaction mixture was applied for agarose gel electrophoresis so that if any kind of changes happened in the length of DNA fragments and also to see if there is any DNA fragments with unexpected length which points that restriction might have happened on multiple sites.

KpnI buffer	3 µl
KpnI	0,4 µl
NcoI	0,8 µl
Insert (GPA1 w/rest. sites)	25 µl (~1 µg)
H_2O	0.8 µl
TOTAL	30 µl

KpnI buffer	3 µl
KpnI	0,4 µl
NcoI	0,8 µl
Vector (petM41)	17 µl (~1.5 µg)
H_2O	8.8 µl
TOTAL	30 µl

Table 5: Restriction reactions of GPA1 having corresponding restriction sites and pETM-41.

KpnI Buffer	3,5 µl
KpnI	0,4 µl
HindIII	1.6 µl
Insert (GPA1 w/rest. sites)	24 µl (~1 µg)
H_2O	1 µl
TOTAL	33 µl

KpnI Buffer	3 µl
KpnI	0,4 µl
HindIII	1.6 µl
Vector (pQE80L)	13 µl (~1.5 µg)
H_2O	12 µl
TOTAL	30 µl

Table 6: Restriction reactions of GPA1 having corresponding restriction sites and pQE80L.

If the DNA fragments are consistent with the expected length, again, corresponding bands seen in agarose gel was cut out of it and gel extraction was applied to prepare the sample for the next step.

2.2.3 Ligation of GPA1 into pETM-41 and pQE80L

After restriction reactions took place for both the vectors and the insert (GPA1), they are supposed to have complementary sites hanging from the 5'- and 3'- ends of GPA1 gene sequence. Ligation was performed by using T4 Ligase enzyme and its buffer. Instead of using only one molar ratio, three different molar ratios was applied. Molar ratios are determined based on the following equation:

$$\left[\frac{(\text{ng vector})\times(\text{kb size of insert})}{(\text{kb size of vector})}\right]\times\left(\text{Molar ratio of}\frac{\text{insert}}{\text{vector}}\right)=\text{ng of insert}$$

As molar ratio, 1/3, 1/6 and 1/9 (vector/insert) were chosen and based on these ratios reaction was started with buffers and amount given below in Table 7 at 16^{o}C and overnight.

	1:3 (v:i)	**1:6**	**1:9**
GPA1 (i)	5 µl	10 µl	15 µl
PetM41 (v)	6 µl	6 µl	6 µl
T4	2 µl	2 µl	3 µl
T4 Buffer	2 µl	2 µl	3 µl
H_2O	5 µl	-	4 µl
TOTAL	20 µl	20 µl	30 µl

	1:3 (v:i)	**1:6**	**1:9**
GPA1(i)	5 µl	10 µl	15 µl
pQE80L	6 µl	6 µl	6 µl
T4	2 µl	2 µl	3 µl
T4 Buffer	2 µl	2 µl	3 µl
H_2O	5 µl	-	4 µl
TOTAL	20 µl	20 µl	30 µl

Table 7: The amounts of buffers, insert (GPA1) and vectors according to the molar ratios used in ligation reaction.

2.2.4 Transformation

After ligation which means GPA1 is supposed to be in the vectors, it is needed to transform them into bacteria so that they can be multiplied via bacteria growth. Plasmids including GPA1 insert were transformed into Top10 *E.coli* strain which are competent cells prepared before. For transforming the plasmid into bacteria, 10 µl of ligated sample were added to 90 µl competent cell aliquots and waited on ice for 30 minutes. After 30 minutes, 42^{o}C heat shock was applied for 1 minute and then samples were put onto ice immediately. 250 µl LB broth was added to each sample and they were left for growing for 1 hourin 37^{o}C shaker. At the end of 1 hour, they were spread onto solid LB

agar medium including the corresponding antibiotics which are Kanamycin and Ampicilin for pETM-41 and pQE80L respectively. Agar plates were incubated overnight at 37^{o}C. And the next day, colony formation was observed.

2.2.5 Colony Selection

After observing colonies on plates, single well grown colonies from each plate were picked and inoculated in 5 ml LB media including respective antibiotics stated above to increase their number of copies so that plasmid isolation can be applied. 5 ml cultures were left in shaker for growing overnight at 37^{o}C and 250 rpm.

2.2.6 Plasmid Isolation

At the end of overnight growth, 5 ml cultures were taken and in order to isolate the plasmids inside bacteria cells, QIAprep Spin Miniprep Kit (QIAGEN) was used. The final concentration after Miniprep was measured by using ND 1000 Nanodrop Spectrophotometer.

2.2.7 Control PCR

In order to confirm that isolated plasmids have GPA1 gene inside, control PCR was applied. Isolated plasmids were used as template and corrsponding primers given in Table 2 were also added to the reaction mixture to understand whether GPA1 sequence is inside the plasmid or not. After PCR reaction, DNA fragments were visualized 1% agarose gel electrophoresis by running it with 1X TAE buffer for 60 minutes.

2.2.8 Verification of GPA1 Sequence by Restriction Digestion

Another method to confirm that GPA1 is indeed inside of the plasmid is to digest the plasmid with restriction enzymes whose recognition sites were introduced to 5'- and 3'-end of GPA1 gene. The reaction was given below in Table 8:

Reaction mixture	Amounts
KpnI	2 µl
NcoI	0,4 µl
Tango Buffer	1 µl
ddH_2O	1,6 µl
TOTAL	10 µl

Table 8: Reaction mixture for restriction digestion applied for confirmation of GPA1 being inside the plasmid.

2.2.9 Sequence Verification

The sequence of plasmid (pETM-41+GPA1) were verified by the company named Eurofins.

2.2.10 Gene Expression

After confirming the presence of GPA1 in the plasmid by sequencing it, GPA1 expression was initiated. Plasmids were transformed into different competent cells having different features in order to optimize the expression. These cells are BL21(DE3) and Rosetta strains of *E.coli*. After transformation of GPA1 to these strains, single colony from LB+KAN solid agar plates was picked and 5 ml culture with KAN (50 µg/ml) was started overnight at 37^{o}C and 250 rpm. Next day, 5 ml culture was used to inoculate 300 ml LB medium and cell growth continued at 37^{o}C, 250 rpm. When OD value reached 0.6, 1 mM IPTG was added to the culture to initiate induction. In order to avoid any harms to protein expressed in cells, temperature was dropped to 27^{o}C because when cells are dividing too fast, it is highly probable that they may not allow proteins to express and fold properly and also may lead to protein degradation. In order to avoid this, lowering temperature is the best way to apply so that cell growth is decelerated. Since the temperature is lowered and cell growth is slowed down, the induction duration was prolonged to 6 hours to increase the expression of GPA1. At the end of 6 hours, cells were pelleted by using centrifuge at 3000g for 30 minutes by using Sorvall Centrifuge.

Cell pellets were lysed with lysis buffer (100 mM NaCl, 50 mM TRIS pH=7.5, 50 µg/ml PMSF, Protease Inhibitor Tablet, 0.5 mg/ml Lysozyme from chicken egg white) with a ratio of 10 ml/ per 1 gram of pellet. 6X SDS loading dye was added to the samples and they were boiled at 95^{o}C for 5 min. Expression of the gene was monitored by 12% SDS polyacrylamide gels. Gels were run always at constant 30 Ampere (30 A).

Samples were run until the dyes of the samples were at the lower border of the gel. Protein molecular weight markers (Fermentas, pqeLAB) were loaded in each gel to identify the molecular mass of the proteins in the samples and at the end of each run protein bands were visualized by coomassie blue staining.

2.2.11 Large Scale Expression

2.2.11.1 *E.coli* system

After confirming that GPA1 gene is expressed in E.*coli* cells, in order to find the optimum conditions for expression, different conditions of IPTG (isopropyl-beta-D-thiogalactopyranoside) and temperature were applied for two different *E.coli* strains that are BL21(DE3) and Rosetta. In each case, a small culture (5 ml) of LB+KAN (Kanamycin with the concentration of 50 μg/ml) was inoculated with a single colony grown on the LB plate after transformation overnight at 37^{o}C and 250 rpm. Next day, observably grown cultures were used to inoculate a larger one (generally it varied between 1-2 L cultures) and their OD_{600} (Optical density, 600 nm) measurements were performed to monitor how fast the cells are growing. When they reached OD=0.6 value, induction was started by adding IPTG (0.5 mM, 0.8 mM and 1 mM depending on temperature) and lowering the temperature from 37^{o}C to 20^{o}C, 25^{o}C and 27^{o}C. After the induction (induction time is also varied from 6 hours to 18 hours depending on temperature), cells were pelleted by using centrifuge at 3000g for 30 minutes by using Sorvall Centrifuge and pellets were kept at -80^{o}C until purification took place.

2.2.11.2 *P. pastoris* system

Additionally, the expression of GPA1 was also monitored in yeast system. For this, former construct and its -80^{o}C stocks were used [4]. The optimized conditions defined in Kaplan, 2009 were used to express GPA1 in yeast.

2.2.12 Purification

2.2.12.1 Affinity Chromatography

2.2.12.1.1 Ni-Affinity HisTrap Columns

After harvesting cells from large scale expressions, they were lysed with lysis buffer as it is described in Section 2.1.6.1. After lysis, sonication was applied with parameters of 38% amplification, 7 sec pulse on, 20 sec pulse off for 30 min. At the end of sonication,

it is expected to see clearer lysate if sonication worked well. After sonication, in order to have the supernatant which includes all the contents of cells, centrifugation was applied at 15000 rpm for 30 min at 4^{o}C with Sorvall SS-34 centrifuge rotor. At the end of centrifugation, supernatant was kept on ice and only a small portion of pellet was kept seperately in order to load it to SDS-PAGE to visualize GPA1 remained in the pellet if any.

Before loading the supernatant into His-Trap column which is connected to AKTA FPLC (Fast Protein Liquid Chromatography), the column was washed with dH_2O to eliminate the EtOH used to store the column. Depending on the volume of supernatant, either 1 ml or 5 ml HisTrap column was used. After UV line is stabilized, pumps were washed with affinity buffers described in Section 2.1.6 and then column was washed wtih buffer A (binding buffer). By using appropriote sized loop, supernatant was loaded into column to allow GPA1 which has N-terminal His_6- tag to bind Ni ions available in the column. When all the sample was loaded into column, elution was started by applying a gradient of elution buffer which includes high concentration of imidazole that competes with His_6- molecules and binds Ni ions instead of GPA1. When an increase was observed in UV spectrum, fractions were collected and 12% SDS-PAGE was loaded with these fractions together with FT (flow through), supernatant and pellet samples.

2.2.12.1.1.1 Desalting

Based on the SDS result, desalting was applied to eliminate high concentration of imidazole and salts to prepare the sample for the next purification which is to eliminate further proteins came together with GPA1. For desalting, HiPrep 26/10 Desalting column was used. Again, first the column was washed with dH_2O to eliminate the EtOH used to store the column and then washed with desalting buffer (50 mM TRIS, pH=7.4). The sample was loaded to the column and when UV values started to increase, fractions were collected.

2.2.12.1.2 Batch mode

Instead of using AKTA FPLC system, a slurry of Ni-NTA agarose was used to elute GPA1 from the supernatant obtained from *P. Pastoris* cell lysate. First, cells were lysed with lysis buffer described in Section 2.1.6.2 (10 ml/gr pellet) and Then, re-pelleted at

5000 rpm for 15 minutes. Re-pelleted sample was lysated in 60 ml (5 ml/gr) lysis buffer in which EFPI tablet was added. Instead of sonication, zirconia beads and beater were used were used to break the cells mechanically. After 10-12 cycles, cells were observed via light microscope and they were disrupted. Centrifugation at 5000 rpm for 10 minutes was applied in order to eliminate beads and high speed centrifugation at 23000g (~13400 rpm) for 30 minutes was applied and supernatant was kept for purification. Supernatant was equilibriated with purification buffer described in Section 2.1.6.2. Supernatant with PB was mixed with resin for binding and waited for at least 60 minutes on moving table. 20 ml plastic column was filled with the mixture and seperated from flowthrough by using pump with a speed of 1 ml/min. When flowthrough was finished, washing step was started. Column was washed with 1 column volume of 1X PB for 4 times after incubating for 5 minutes. After washing step, elution was started. Elution buffer is slightly different from washing buffer, it includes higher concentration of imidazole (300 mM) to elute the protein bound to resin. Then, 5 ml of elution buffer was added to column for two times and collected seperately.Again, at the end of purification, flow through (FT), supernatant, pellet, wash and elution samples were loaded into 12% SDS-PAGE gel to visualize GPA1 through the steps.

2.2.12.1.2.1 Dialysis

In order to eliminate high concentration of imidazole, dialysis was applied. All eluates were pooled in CelluSep Regenerated Celluose Tubular Membrane with a molecular weight cut off value of either 6000-8000 Da or 12000-14000 Da both of which is acceptable since GPA1 has a molecular weight of 45000 Da (45 kDa). Then, membrane was left in 50 mM TRIS, pH=8.0 overnight.

2.2.12.2 Anion Exchange Chromatography by QTrap Column

After desalting/dialysis, anion exchange chromatography was applied to further eliminate the proteins came along with GPA1 during affinity chromatography. A similar procedure was applied: The column was connected to the FPLC system, first washed with dH_2O, then with buffer A (same with desalting/dialysis buffer). After loading the sample to the QTrap column (5 ml) and observing a steady UV spectrum, a gradient of buffer B including high concentration of salt (50 mM TRIS pH= 7.4 or 8.0, 700 mM NaCl) was applied to elute the bound GPA1 on the column. Fraction starting from the point that UV started to increase were collected.

At the end of purification step, before dialysis/desalting sample, after dialysis/desalting sample and fractions collected were loaded to 12% SDS-PAGE to visualize how the purity of GPA1 was changed in between steps.

2.2.12.3 Size Exclusion Chromatography (Gel Filtration)

In order to seperate proteins based on its molecular weight, size exclusion chromatography was used. This method is useful for both increasing the purity and confirming that the purified protein in former steps is the protein that was aimed to be purified by analyzing it via its molecular weight. It is also practical to see if proteins form oligomers instead of being as monomer. For size exclusion chromatography, HiLoad 16/60 Superdex 75 and HiLoad 16/600 Superdex 200 pg were used. Since the maximum volume that can be loaded into SEC columns is 5 ml, samples were concentrated at 3000 rpm at 4^{o}C until they reach 5 ml or less. Concentration was performed by using Millipore Centrifugal Filter Units or Sartorious Biolab Products concentrators with MWCO=10000 Da or 30000 Da depending on which construct of GPA1 was purified. After connecting the column to the FPLC system, it is washed first with dH_2O and then equilibriated with SEC buffer described in Section 2.1.6. Then the sample is loaded and again fractions starting from the point that UV started to increase were collected until UV value reached its the lowest value and was stabilized.

At the end of SEC, fractions were loaded to 12% SDS-PAGE and dyed with comassie blue after running them at 30 A. Concentration measurements in between steps were done by using the absorbance of the samples at 280 nm (A280) by NanoDrop and the concentration of the samples were determined by the extinction coefficient (GPA1=0.93, GPA1 with MBP tag=1.24).

2.2.13 TEV Protease Digestion

2.2.13.1 Expression, Purification and Storage of TEV Protease

TEV protease expression, purification and storage was performed as it was described in [36]. Briefly, pMHT238Δ vector which has His_6-tag TEV protease gene was transformed into BL21 *E.coli* cells and spreaded onto LB-KAN solid agar medium since the vector is KAN resistant. After observing colony formation, single colony was picked and and 5 ml Terrific Broth (TB) medium was inoculated with it overnight at 37^{o}C, 250 rpm. Next day, the small culture was used to inoculate 1 L TB medium.

Expression was started at 37^{o}C, 250 rpm and when OD reached 0.6 value, 0.5 mM IPTG was applied to induce TEV expression and the temperature was dropped to 30^{o}C. After 3 hours, cells were harvested by centrifugation at 4000g for 20 min and the pellet was kept at -80^{o}C until purification took place.

To purify TEV protease, 6 ml/per pellet gr lysis buffer (50 mM Na_2HPO_4+NaH_2PO_4, 300 mM NaCl, 0.3 mM DTT, EDTA-Free Protease Inh.) was used to resuspend the pellet and then 0.5 mg/ml lysozyme was added and let it wait for 1 hour in cold room shaking. After lysis, sonication was applied with parameters of 38% amplification, 7 sec pulse on, 20 sec pulse off for 30 min. In order to have the supernatant which includes all the contents of cells, centrifugation was applied at 15000 rpm for 50 min at 4^{o}C with Sorvall SS-34 centrifuge rotor.
Since TEV protease has a His_6-tag, affinity chromatography took place by using AKTA FPLC system. As it was described above, first HisTrap column was washed with water, then binding buffer (20 mM Na_2HPO_4+NaH_2PO_4, 350 mM NaCl, 0.3 mM DTT).Supernatant obtained after centrifuge was loaded to the column and then it was washed with binding buffer to eliminate unbound proteins. To elute TEV protease, elution buffer (50 mM Na_2HPO_4+NaH_2PO_4, 300 mM NaCl, 0.3 mM DTT, 500 mM imidazole) was passed through the column with a linear gradient. Fractions starting from the point that UV started to increase were collected. To visualize the TEV protease, collected fractions were loaded into 12% SDS-PAGE and after running it, comassie staining was applied.

To remove imidazole and store TEV protease in more stable conformation, desalting was applied with storage buffer (25 mM TRIS-HCl, pH=8.0, 50 mM NaCl, 0.25 mM DTT) by using HiPrep 26/10 Desalting column. At the end of desalting, TEV protease was concentrated by using concentrators with 10000 Da MWCO to reach a concentration higher than 1 mg/ml. The final concentration was 1.35 mg/ml and aliquots of TEV protease was stored with 50% glycerol in -80^{o}C.

2.2.14 Applying TEV to GPA1

In order to remove N-terminal MBP (maltose binding protein) tag of GPA1 coming after N-terminal His_6-tag, TEV protease was used since there is a TEV site between MBP site and GPA1 sequence. After SEC, TEV protease reaction was performed as it is described in [37]. Briefly, the buffer that GPA1 was in it was modified to be resembled

to TEV protease reaction buffer. TEV protease is not active in salt concentrations higher than 500 mM. The recommended TEV reaction buffer is 50 mM TRIS pH=8.0, 1 mM DTT and 0.5 mM EDTA. GPA1 final buffer included 50 mM TRIS pH=7.5, 1 mM DTT, 100 mM NaCl and 50 μM GDP.In order to make it more similar to TEV reaction buffer, 0.5 mM EDTA was added. For 1 mg of GPA1, 30 μg of TEV protease was used in the reaction. The reaction took place at 4^{o}C to avoid possible precipitation of GPA1. The reaction was checked at each hour and taken 20 μl of samples. At the end of 4 hours, precipitation was observed and the reaction was stopped by applying affinity chromatography. As binding buffer, TEV reaction buffer was used and as elution buffer, TEV reaction buffer plus 500 mM imidazole was used. Affinity procedure was the same as described above in the Section 2.2.12.1. The important part is that since GPA1 has no tags after TEV protease reaction, it was supposed to come in the flow through part because GPA1 is not supposed to bind to the column. Thus, all the flow through was collected and when UV profile was stabilized, a gradient of elution buffer was applied and again fractions starting from UV increase point were collected. At the end of affinity chromatography, 12% SDS-PAGE was run to see if GPA1 was indeed in the flowthrough part without any tags.

2.2.15 Western Blotting

Western Blotting with required antibody (anti-His antibody) was performed to confirm that the purified protein was indeed GPA1. After running 12% SDS-PAGE with the samples to be visualized, PDVF Transfer Membrane (0.45 μm) having the same size with the SDS gel was prepared by immersing it into MetOH for 1 minute. The western sandwich was prepared with sponges and filter papers including SDS gel and transfer membrane on it. It was run until reaching 300 mA then the transfer membrane was taken out. It is washed with TBS (25 mM TRIS- HCl pH= 8.5, 200 mM Glycine) and ponceus stain is used to visualize whether transfer is completed or not. Ponceus stain was washed with dd H_2O and TBS until there is no ponceus stain on the membrane. Then it was left with blocking buffer (10 ml TBS-T, 5% nonfat dry milk) overnight at 4^{o}C. The next day, the membrane is washed thrice with TBS-T and twice with TBS. Then, membrane is left with anti-His solution (TBS-T, 3% BSA) together with anti-His antibody for 1 hour at room temperature. At the end of 1 hour, the membrane is washed with TBS-T and TBS. To visualize it, ECL solution (TRIS-HCL pH=8.8, 90 mM Cumeric acid, 250 μM Luminol and H_2O_2) was prepared and the rest of the procedure is

taken place in dark room. Membrane was washed with ECL solution and then put into a casket. X-Ray Films are cut according to the size of the membrane and put onto it. Then, casket is closed and wait ~1-2 minutes. X-Ray films were washed with KONIX Developer and Fixer solution until black bands become visible. At the end, films are washed with water.

2.2.16 Sample Analysis

In between each step, concentration measurement was made by using the absorbance of the samples at 280nm (A280) by NanoDrop and the concentration of the samples were determined by the extinction coefficient (GPA1=0.93, GPA1 with MBP tag=1.24). Also, 12% SDS-PAGE was used to confirm the presence of proteins with their estimated molecular weights.

8% Native-PAGE was used to visualize if the sample was in a good conformation or it has any oligomer forms. In the case of having high concentration of samples (more than 1.5 mg/ml), DLS (Dynamic Light Scattering) was applied to determine the quality of the sample in terms of being aggregated. CD (Circular Dichroism) measurements took place when we have samples with a concentration higher than 4 mg/ml in order to make the secondary structure analysis.

3 RESULTS

3.1 Cloning GPA1 into vectors

In order not to damage DNA fragments in the middle steps of cloning, they were not exposed to UV under which EtBr is visible to take a picture of them. So, after plasmid isolation, control PCR was applied to visualize GPA1 if it is inside the pETM-41 and pQE80L. The result is given in Figure 3-1. The length of GPA1 is 383 aminoacids corresponding to 1149 bp.

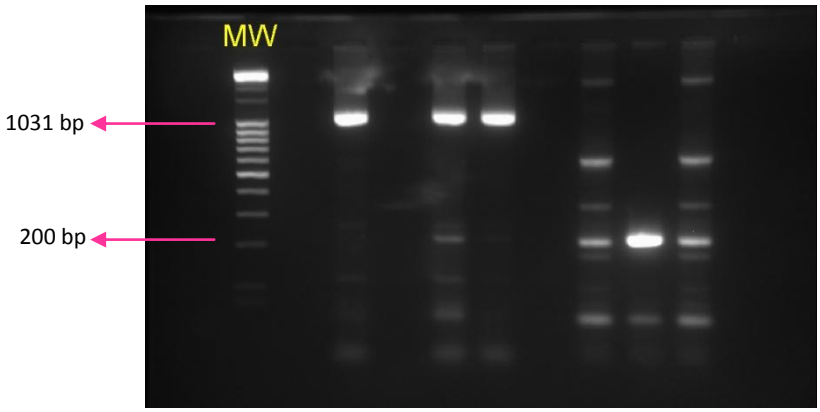


Figure 3-1: The control PCR results of GPA1 gene when isolated plasmids were used as template and primers having the restriction enzyme recognition sites were used as primers. From left to right, 1st lane: GPA1-pETM-41 1/3 ligation construct, 2nd lane: GPA1-pETM-41 1/6 ligation construct, 3rd lane: GPA1-pETM-41 1/9 ligation construct, 4th lane: GPA1-pQE80L 1/3 ligation construct, 5th lane: GPA1-pQE80L 1/6 ligation construct, 6th lane: GPA1-pQE80L 1/9 ligation construct.

It is obvious that pQE80L construct did not work and GPA1 gene is not in it because there is no band around ~1000 bp marker. On the other hand, inserting GPA1 gene into pETM41 worked well because there are not multiple bands resulted at the end of PCR. The size of the fragment is also consistent with the length of GPA1. It is above the 1031 bp molecular marker band.

To further confirm that GPA1 is inside pETM-41 vector, restriction digestion was applied. As it is known, NcoI restriction enzyme recognition site was introduced before the N-terminal region of GPA1 and KpnI restriction enzyme recognition site was introduced after C-terminal region of GPA1. Thus, if GPA1 is indeed in the plasmid, then it means that there should be ~1150 bp long DNA fragment visible after applying both of the restriction enzymes to the plasmid. In Figure 3-2, the result of 1% agarose gel electrophoresis run after restriction reaction was shown. And in Figure 3-3, it is

shown that there is only one recoginition site for each restriction enzyme in pETM-41 which means that if NcoI and KpnI were cut a fragment, that fragment corresponds to GPA1, not any other non specific part of the vector.

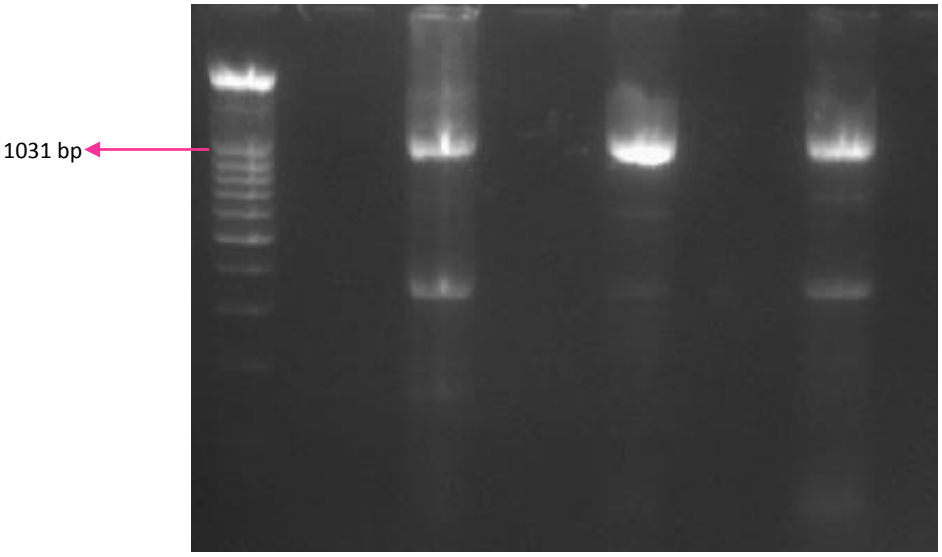


Figure 3-2: After restriction digestion was applied to GPA1-pETM-41 plasmids with NcoI and KpnI enzymes. From left to right; 1st lane: 1/3 ligation construct, 2nd lane: 1/9 ligation construct and 3rd lane: 1/6 ligation construct.

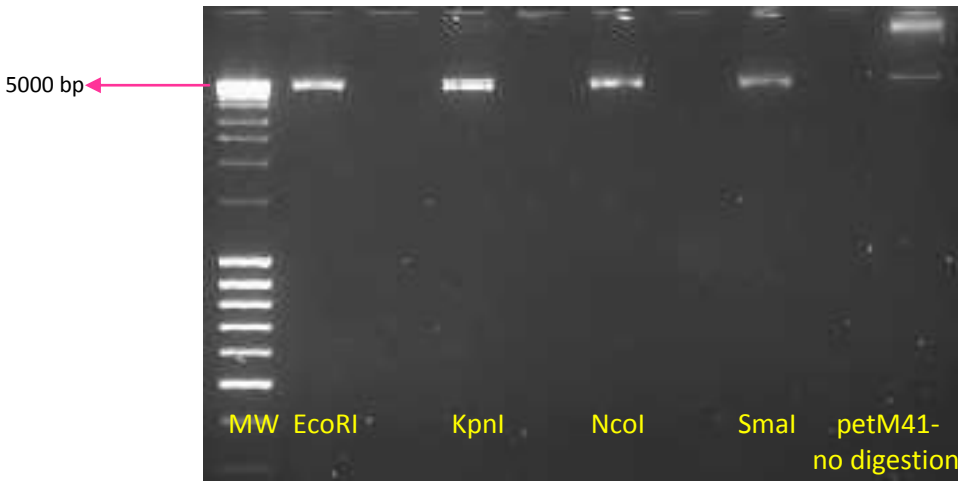


Figure 3-3: Restriction digestion of pETM-vector with single restriction enzymes to show that there are no multiple recognition sites.

In Figure 3-1, since the cleanest construct is the one with 1/9 molar ratio (described in Methods Section 2.2.3), the remaining experiments were performed by using that construct. This construct was also sent for sequencing to Eurofins and the presence of GPA1 sequence in pETM-41 vector was further verified.

To insert GPA1 into pQE80L, different temperatures in the the annealing step of PCR were applied but none of them had a succesful positive result.

3.2 Verification of GPA1 Gene Expression in BL21(DE3) Competent Cells

In order to verify that GPA1 gene is translated and GPA1 protein is expressed in *E.coli* cells, small scale expression experiments were performed as they were described in Section 2.2.10. The SDS-PAGE is given in Figure 3-4. At the end of 6 hours induction with 1 mM IPTG, the obtained cell pellet was resuspended in lysis buffer and left for lysis for 1.5 hours with lysozyme (1 mg/ml). Sonication was applied with 38% amplification, 6 sec pulse on, 25 sec pulse off for 15 min. Then, it was centrifuged at 15000 rpm for 45 min at 4^{o}C. Instead of applying purification, SDS and Western blotting were performed to see whether GPA1 was expressed in cells or not.

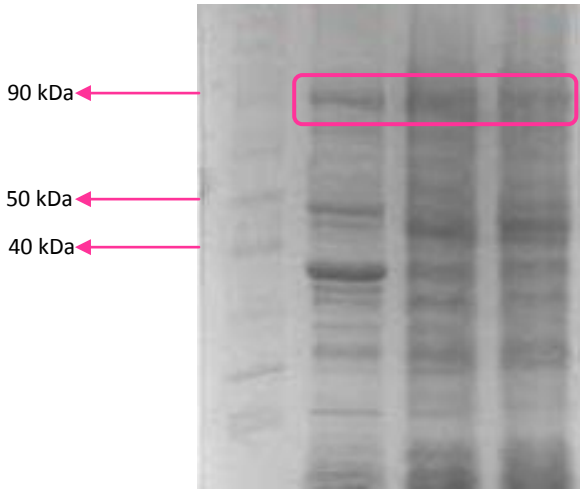


Figure 3-4: SDS-PAGE result to verify GPA1 expression in BL21(DE3) cells. From left to right: 1st lane: Molecular weight (MW), 2nd lane: 1:3 diluted supernatant, 3rd lane: supernatant and 4th lane: pellet.

Since there is N-terminal MBP tag available in pETM-41 vector, it is expected to see a band around ~90 kDa because MBP has a molecular weight of 45 kDa and GPA1 has a molecular weight of 44 kDa. In order to verify that what is seen around 90 kDa is indeed GPA1 itself, Western blotting with anti-His antibody was applied and the result is given in Figure 3-5.

Figure 3-5: Western blotting result to verify GPA1 expression in BL21(DE3) cells.1st lane: 1:5 diluted supernatant, 2nd lane: supernatant, 3rd lane: 1:5 diluted pellet, 4th lane: pellet and 5th lane: Negative control with empty BL21 cell pellet.

As it is seen from the results, GPA1 gene is expressed in BL21(DE3) cells together with high amount of another 45 kDa protein which has also His-tag otherwise they would not be visualized with anti-His antibody. GPA1 is found in supernatant, not in the pellet. From these results, it was decided that large scale expression of GPA1 can be performed with BL21 (DE3) cells if the optimum conditions are adjusted. In order to find the optimum conditions of GPA1 to express it in possible highest amounts in BL21 cells, different IPTG concentrations and temperatures were tried.

3.3 IPTG and Temperature Effects on the Expression of GPA1 in BL21 Cells

In order to find the best condition to express the highest amount of GPA1, two different IPTG concentrations and two different temperatures when expressing the GPA1 were tested. Four equal volumes (25 ml) of cell cultures were induced with 0.5 mM IPTG (2 of them) and 1 mM IPTG (the other two of them) after reaching OD=0.6. After adding IPTG, one 1 mM IPTG-induced and one 0.5 mM IPTG-induced cultures were taken to 25^{o}C and other two were taken to 27^{o}C shaker and left for 6 hours of induction. In the first, third and the sixth hours, 1 ml of samples were taken to visualize how much GPA1 was expressed in each of them. The samples were centrifuged at 13000 rpm for 1 min and then resuspended in required amount of lysis buffer described above (Section 2.1.6.1) based on their OD values. In order to compare them efficiently, the number of cells should be equal in each sample. In Figure 3-6, SDS-PAGE of these samples is shown.

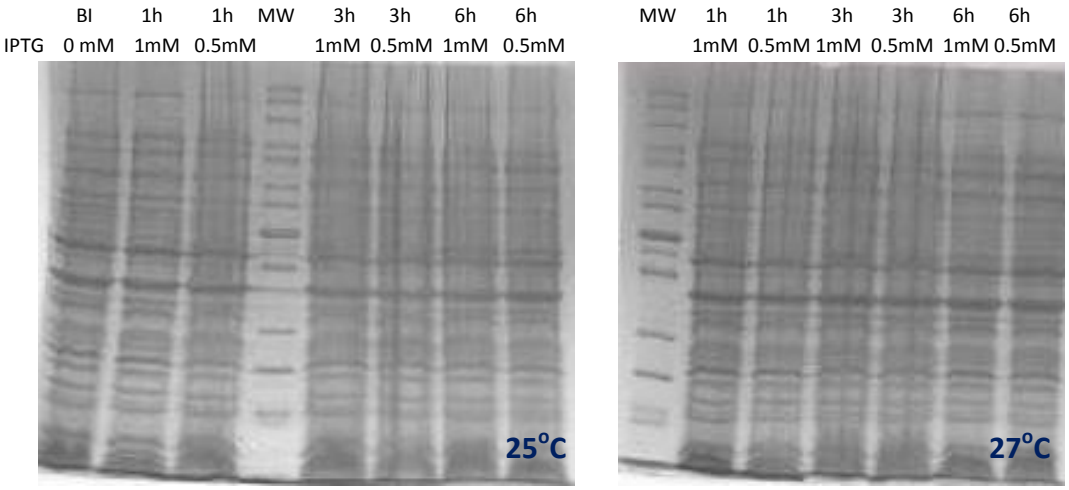


Figure 3-6: SDS-PAGE results of pellets harvested from cell cultures induced with different IPTG concentrations (indicated above) and grown in different temperatures (indicated right bottom). BI means "before induction" which means before IPTG was added.

Since pellets include several other proteins, Western blotting is a logical method to determine how much GPA1 is available in these samples. So, Western blotting was applied by using the same SDS-PAGE gels. The result is shown in Figure 3-7.

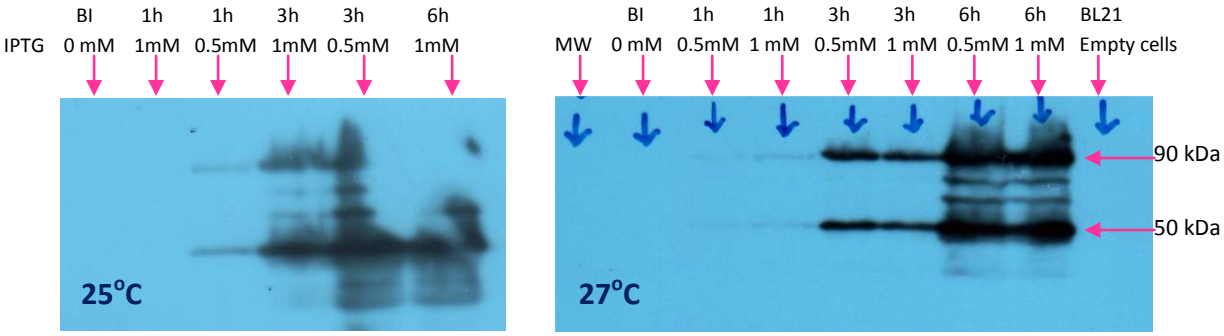


Figure 3-7: Western blotting performed with the samples defined in Figure 3-6 by using anti-His antibody staining.

Although the GPA1 amounts were not enough to see by comassie staining applied to SDS-PAGE gels, they were enough to visualize by using anti-His antibody binding. From the results shown in Figure 3-7, it is clear that 27°C and 1 mM IPTG conditions are more convenient to enhance the GPA1 amount expressed in BL21 cells.

3.4 Purification of GPA1 Expressed in BL21 Cells

3.4.1 Affinity Chromatography

Since the optimal condition was decided, GPA1 was expressed in large scales (2 L) in these conditions and then harvested cells were lysed and purified by using affinity chromatography method with 5 ml HisTrap column (GEHealthcare) as it was described in Section 2.2.12.1.1 The UV profile and SDS-PAGE result run with the fractions obtained through the affinity chromatography were given in Figure 3-8.

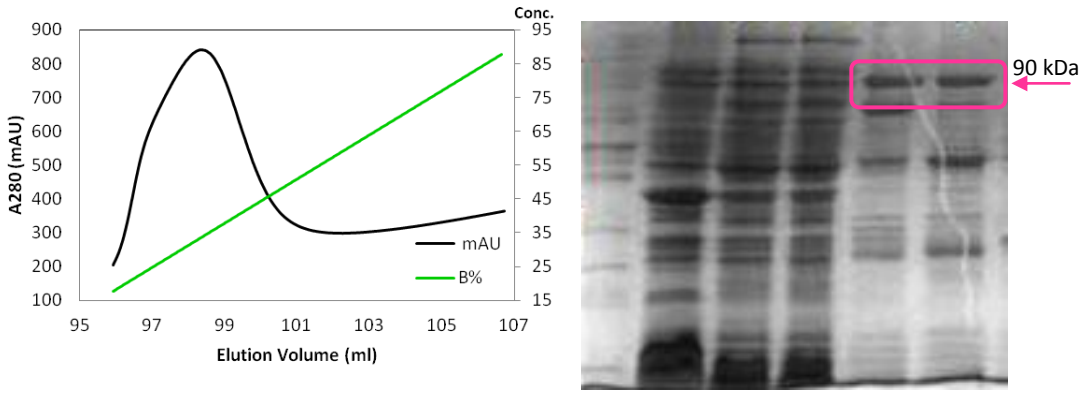


Figure 3-8:(left) UV profile obtained in affinity chromatography, (right) SDS-PAGE result run with the fractions (7th and 8th) collected in affinity. Bands encirculated around ~90 kDa corresponds to GPA1.

According to UV profile and concentration gradient, GPA1 started to elute around ~170 mM imidazole because it is seen that the point that UV started to increase is around 17% of buffer B in which there is 1 M imidazole and 17% of it equals to 170 mM imidazole. SDS-PAGE result clearly showed that GPA1 was expressed in the cells but it needs further purification steps to eliminate the other proteins came along with GPA1 during affinity chromatography.

3.4.2 Anion Exchange Chromatography

Before applying the second chromatography, first imidazole and most of the salt concentration should be eliminated from the samples. So, all the fractions of GPA1 was pooled and changed the buffer by desalting as described in Section 2.2.12.1.1.1. At the end of desalting step, the final buffer of GPA1 was 50 mM TRIS, pH=7.5 which is the start buffer of Anion exchange. 5 ml QTrap column (GEHealthcare) was used for Anion

exchange chromatography and a gradient of NaCl concentration was applied to elute the bound GPA1 as described in Section 2.2.12.2. The UV profile and SDS-PAGE result run with the fractions obtained through the affinity chromatography were given in Figure 3-9.

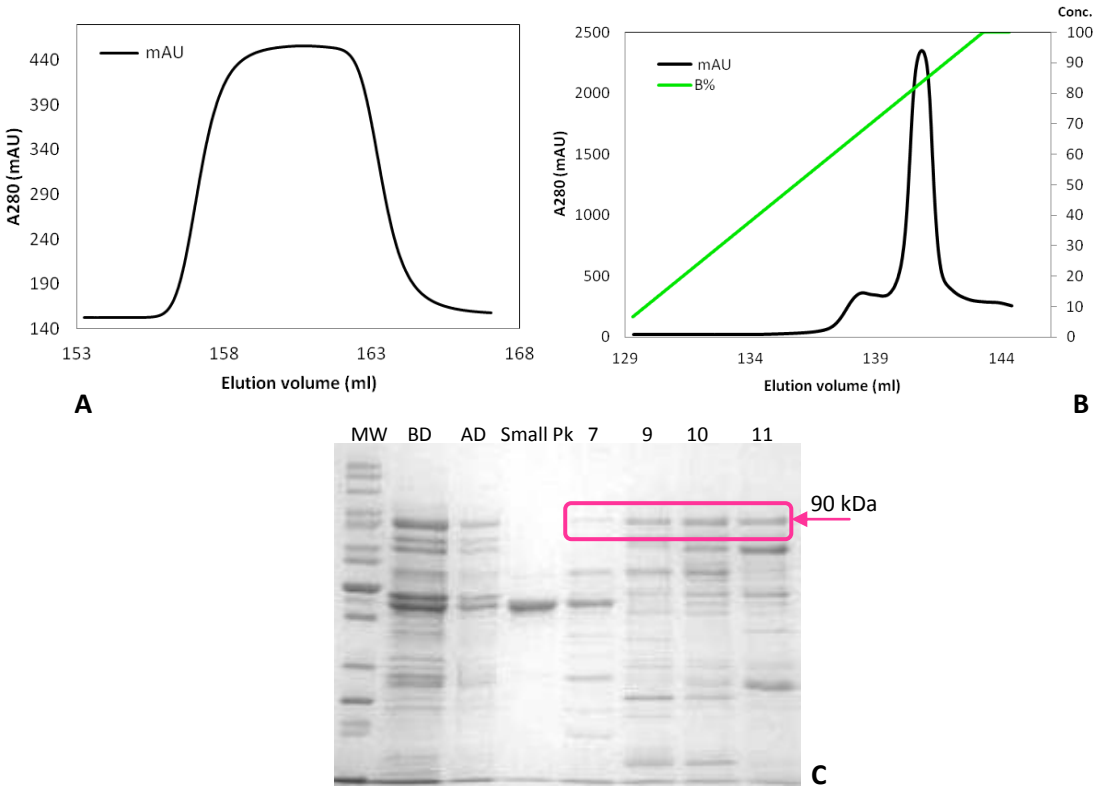


Figure 3-9: (A) UV absorbance profile obtained in the desalting step. (B) UV absorbance profile of anion Exchange chromatography.(C) SDS-PAGE result which was run with the samples named above the image. BD: before desalting, AD: after desalting, Small pk: fraction collected from the small peak coming before the actual one, 7, 9, 10 and 11 are the numbers of fractions collected through the big peak.

Although there were still other proteins need to be eliminated, since GPA1 amount is not so high, SEC chromatography was not applied. Instead it was concentrated by using a concentrator with a MWCO=10000 Da and then MBP-tag was tried to be eliminated from the N-terminal of GPA1 by applying TEV protease produced as described in Section 2.2.13.1.

3.5 Elimination of MBP-tag with TEV Digestion

GPA1 was in the buffer which is similar to TEV reaction buffer. So, by adding 1 mM DTT and 0.5 mM EDTA, the missing ingredients of TEV reaction buffer was completed and TEV protease was added with a 1:30 ratio to GPA1 at the end. At the end of TEV reaction, precipitation occurred, thus sample was centrifuged and the precipitated part

was kept for further investigation. The second affinity chromatography was applied to eliminate MBP which has a His_6-tag in its N-terminus. Since GPA1 is supposed to have no tags after TEV reaction, it should come in the flow through part. So, flow through was collected and then gradient of elution buffer including imidazole was used to elute MBP tags. The UV absorbance profile and the SDS-PAGE result is given in Figure 3-10.

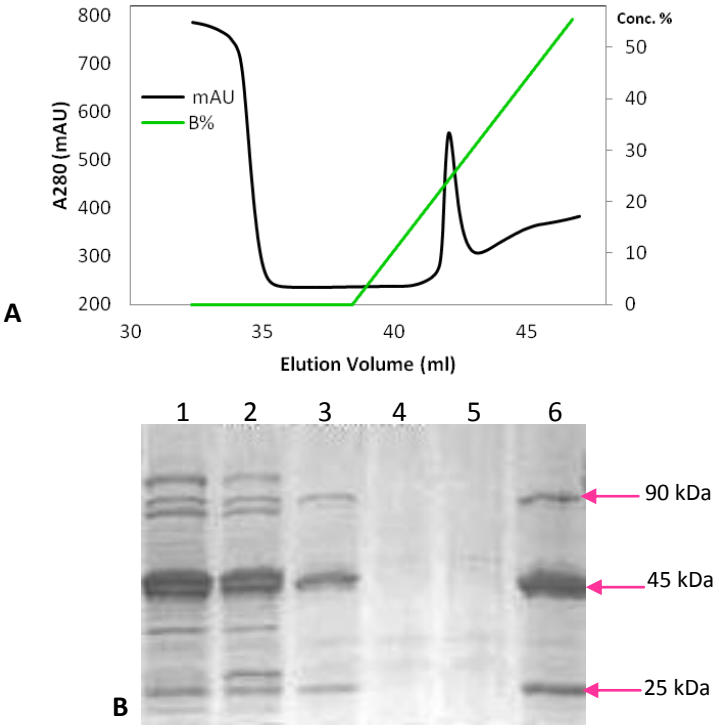


Figure 3-10: Affinity chromatography applied after TEV reaction to eliminate GPA1 which has no tag from the His-MBP-tag. (A) UV absorbance profile obtained, (B) SDS-PAGE run after affinity with the samples numbered as 1: Before desalting, 2: After desalting, 3: After TEV reaction, 4: Flow through-1, 5: Flow through-2 and 6: Elution.

Since no band is visible in flow through samples, Western blotting was performed to determine if GPA1 is available in flow through in low concentrations that commassie dying cannot detect but antibody binding can. Western blotting results are given in Figure 3-11. After collecting flowthrough, it was concentrated by using concentrators with MWCO=10000 Da. Concentration measurement was made by Nanodrop spectrophotometer and it was found as 0.13 mg/ml which was quite insignificant for further characterizations.

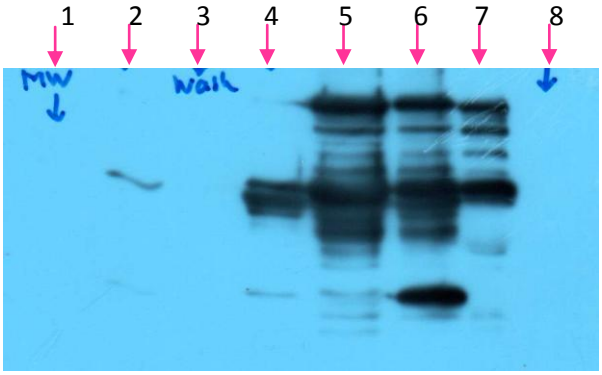
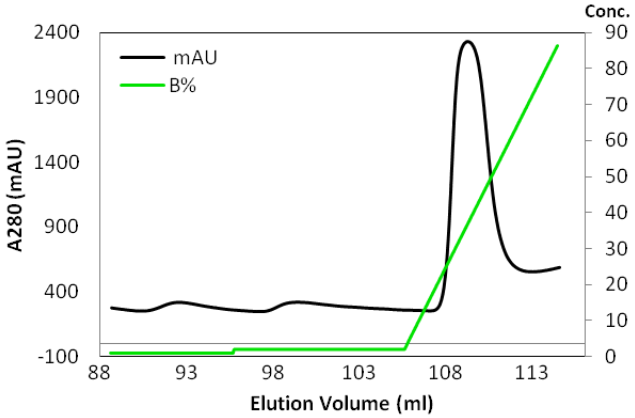


Figure 3-11: Western blotting after TEV digestion. Numbers correspond to 1: Molecular weight, 2: Flow through (concentrated), 3: Flow through, 4: Elution, 5: Before TEV reaction, 6: After TEV reaction 7: Precipitated part, 8: BL21 empty cells for negative control.

3.6 Expression of GPA1 in Rosetta Cells

Transformation of pETM-41+GPA1 plasmid into Rosetta cells were performed as it was described in Section 2.2.4. The optimum conditions determined for BL21 (DE3) cells were applied to compare the performance of two competent cells. At the end of expression, cells were harvested by centrifugation at 3000g for 30 minutes at 4^{o}C. Harvested cells were lysed with the lysis buffer described in Section 2.1.6.1 and then sonicated and centrifuged. The pellet obtained at the end of centrifugation was kept for further investigation. The supernatant was loaded into 5 ml HisTrap column (GEHealthcare) and affinity chromatography performed as it was described in Section 2.2.12.1. The UV absorbance profile and SDS-PAGE result which was run with the fractions obtained in the chromatography were shown in Figure 3-12.

A



MW PL 2 3 4 5 6 7 8 9

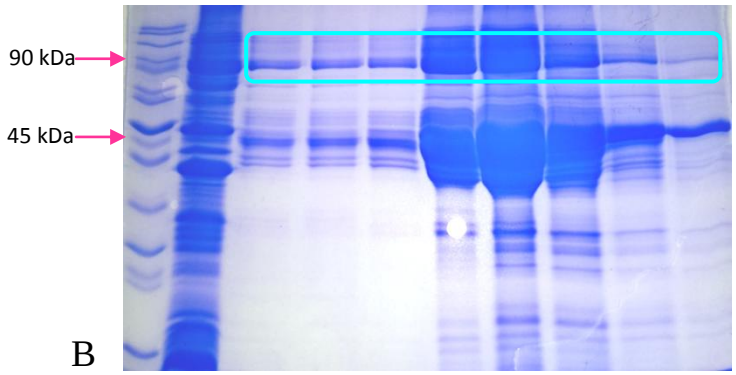


Figure 3-12: Affinity chromatography applied to GPA1 expressed in Rosetta cells (A) UV absorbance profile, (B) SDS-PAGE run with the fractions collected through the affinity chromatography. The lanes correspond to MW: Molecular Weight, PL: Pellet, 2, 3, 4, 5, 6, 7, 8 and 9 are number of fractions collected in the purification step.

As it is seen clearly, the amount of GPA1 expressed in Rosetta cells are significantly higher than BL21 cells. The amount of GPA1 obtained in this trial expression made with 500 ml culture medium was higher than the amount obtained in 2 L of expression performed with BL21 cells.

3.7 Temperature and IPTG Effects on the Expression of GPA1 in Rosetta Cells

Although the GPA1 amount was dramatically increased compared to BL21 cells, different IPTG concentrations ans temperatures were also tried to see how they effect the expression amount in Rosetta cells. Thus, lower IPTG concentration at 27^{o}C and the same IPTG concentration (1 mM) at lower temperature (20^{o}C) were applied to 1 L cultures to see if GPA1 expression will be increased or decreased in any of the conditions. Induction time was 6 hours for culture grown at 27^{o}C, 18 hours for the other culture grown at 20^{o}C. As it is described above, harvested cells were lysed, sonicated and centrifuged to obtain supernatants. Then, affinity chromatography was performed for both of the samples. The UV absorbance profiles and SDS-PAGE results are given in Figure 3-13 together.

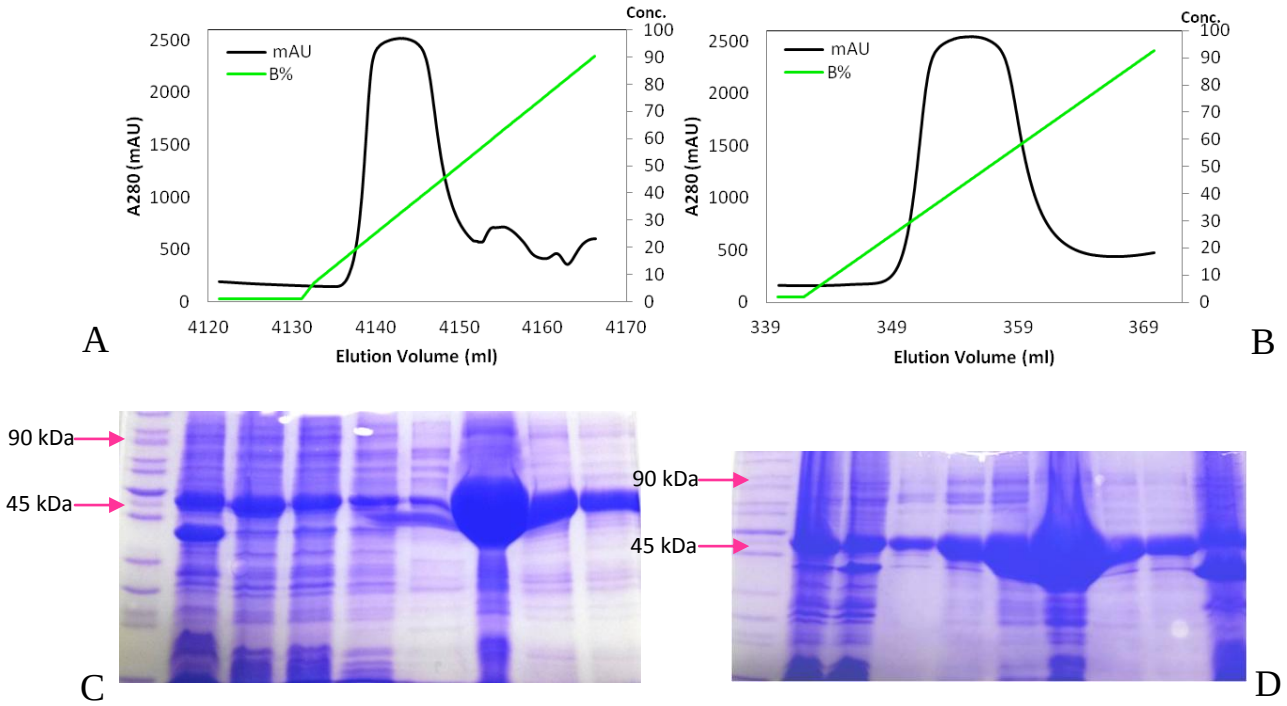


Figure 3-13: IPTG and temperature effects on GPA1 expression in Rosetta cells. (A) UV absorbance profile obtained from the cells grown at 20°C with 1 mM IPTG, (B) UV absorbance profile obtained from the cells grown at 27°C with 0.8 mM IPTG, SDS-PAGE results (C) obtained from the fractions of the purification (20°C),(D) and 27°C.

So the results showed that neither ITPG concentration different than 1 mM, nor temperatures other than 27°C contributes to increase in GPA1 expression amount. In fact, GPA1 was nearly not expressed in the cells because no significant band was observed around 90 kDa in any of the gels. Thus, for the remaining part of the experiments, in order to express GPA1, cultures were grown at 27°C inducing with 1 mM IPTG.

3.8 Purification of GPA1 Expressed in Rosetta Cells

3.8.1 Affinity Chromatography

After setting the optimal temperature and IPTG concentration for expression of GPA1 in Rosetta, large scale expression (1 L) of GPA1 was performed. After obtaining supernatant, it was loaded into 5 ml HisPrep Ni affinity column and affinity chromatography was performed as explained in Section 2.2.12.1.1. The UV absorbance profile and the SDS-PAGE result is given in Figure 3-14.

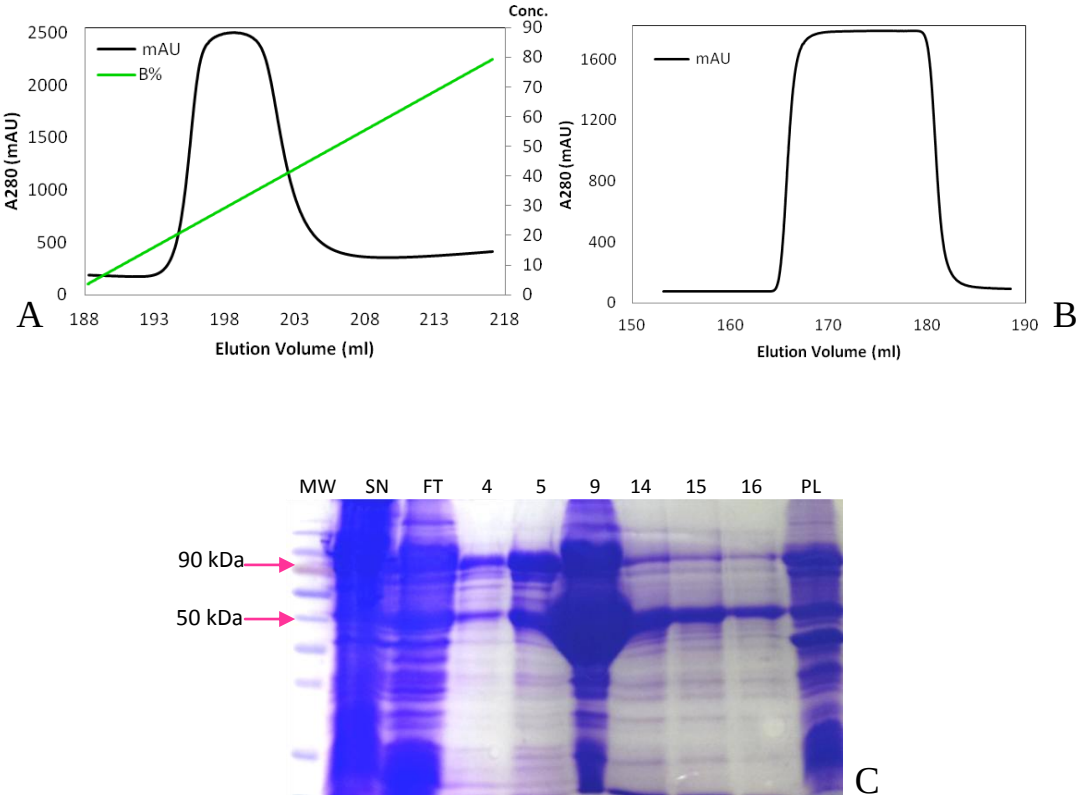


Figure 3-14: Affinity chromatography applied to GPA1 expressed in 1 L using Rosetta cells (A) UV absorbance profile, (B) UV absorbance profile obtained in desalting step, (C) SDS-PAGE run with the fractions collected through the affinity chromatography. The lanes correspond to MW: Molecular Weight, SN: Supernatant, FT: Flowthrough, 4, 5, 9, 14, 15 and 16 are number of fractions collected in different parts of the peak observed in UV profile, PL: Pellet.

Although the gel looked quite crowded due to other proteins, especially the one which has 45 kDa molecular weight, GPA1 amount expressed was significantly high. Thus, prior to anion exchange chromatography, fractions showing the presence of GPA1 in the gel was pooled and desalting was applied to eliminate imidazole and excessive salt included in purification buffer given in Section 2.1.6.1. Desalting procedure is the same with what was applied previously and the result of it is given in Figure 3-14B. Briefly, by using HiLoad 26/10 desalting column and 50 mM TRIS, pH=7.4, the buffer of GPA1 was changed.

3.8.2 Anion Exchange Chromatography

Again, fractions collected in the desalting step were loaded into QTrap 5 ml in exchange column and anion exchange chromatography was applied as described in Section 2.2.12.2. The UV absorbance profile showed two different peaks which means that we

were able to seperate GPA1 and the other 45-kDa protein that was observed in SDS-PAGE gels after affinity.Figure 3-15 shows the UV absorbance and SDS-PAGE result.

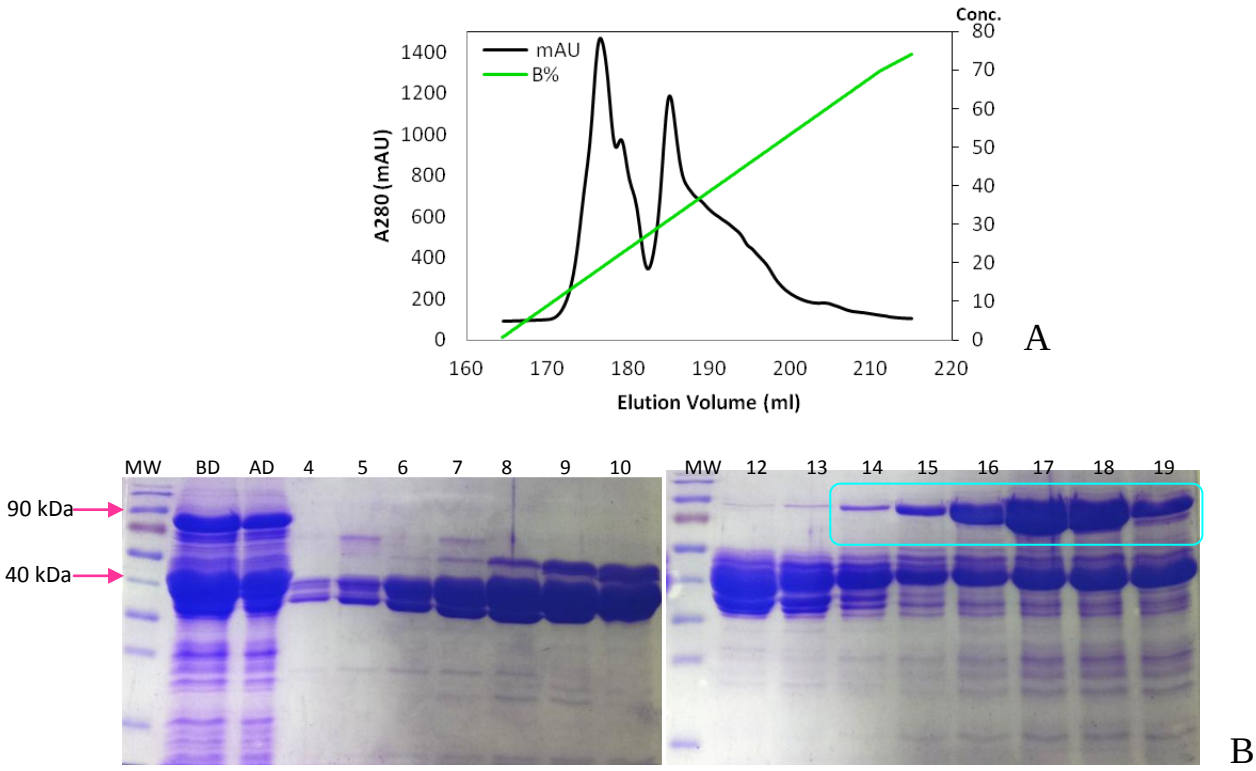


Figure 3-15: Anion exchange chromatography applied to GPA1 after affinity chrom. and desalting (A) UV absorbance profile, (B) SDS-PAGE run with the fractions collected through the affinity chromatography. The lanes correspond to MW: Molecular Weight, BD: Before desalting, AD: After desalting, 4, 5, 6, 7, 8, 9 and 10 are the fractions collected through the first peak, 12 and 13 are the fractions in between two peaks, 14, 15, 16, 17, 18 and 19 are the fractions collected in the second peak observed in UV profile.

Compare to the SDS-PAGE gel result, we succesfully eliminated most of the proteins and also, significant amount of 45-kDa protein coming along with GPA. To further eliminate that protein to have more pure GPA1 sample and estimate the molecular weight by SEC, size exclusion chromatography was applied as the next step.

3.8.3 Size Exclusion Chromatography

The fractions that GPA1 was observed pooled and concentrated by using concentrators with MWCO=30000 Da at 3000 rpm for 10 minutes until it reached a volume less than 5 ml. Because the maximum amount that can be loaded into SEC chromatography column is 5 ml. After equilibriating the HiLoad 16/600 Superdex 200 pg SEC column

with the buffer (25 mM TRIS, pH=7.4, 100 mM NaCl, 1 mM DTT, 50 µM GDP), concentrated sample was loaded into column and the the fractions were collected after UV started to increase. The UV absorbance profile is given in Figure 3-16 together with SDS-PAGE result.

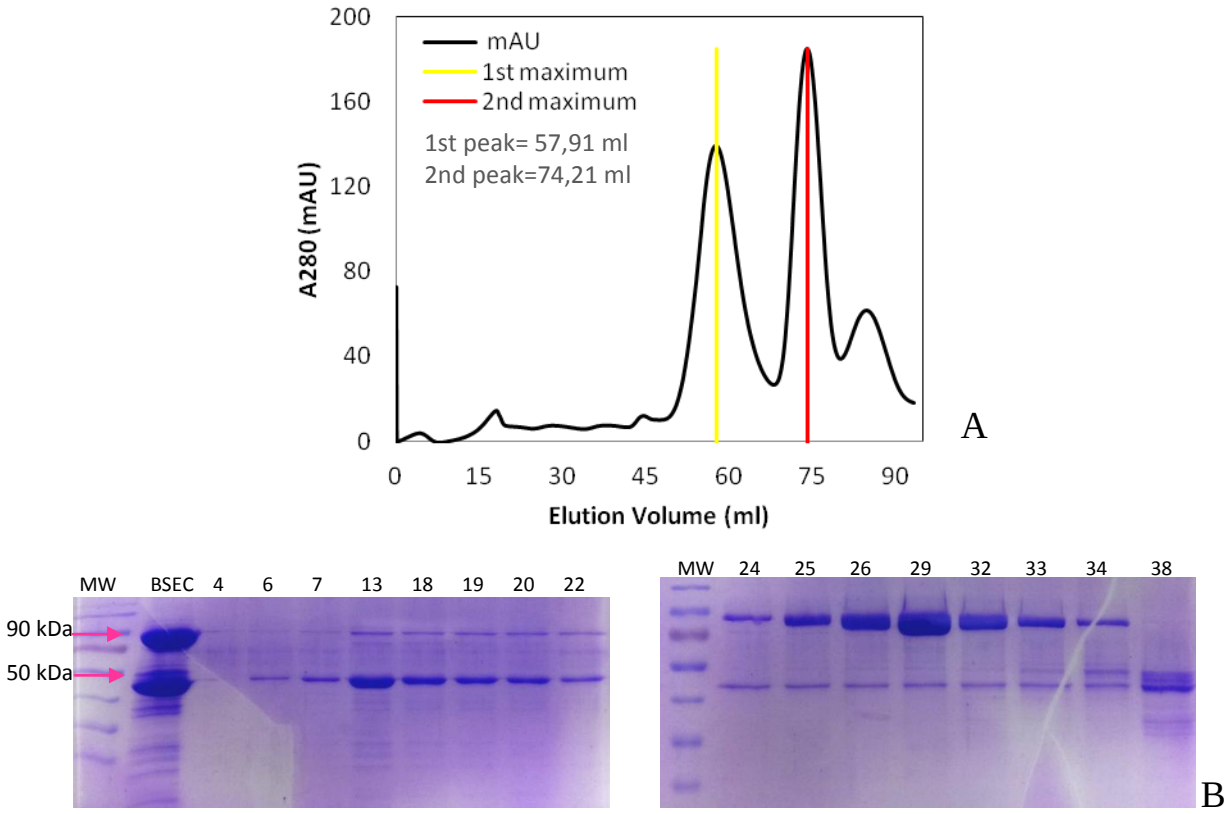


Figure 3-16: Size exclusion (SEC) chromatography applied to GPA1 after anion exchange chrom. (A) UV absorbance profile, (B) SDS-PAGE run with the fractions collected through the affinity chromatography. The lanes correspond to MW: Molecular Weight, BSEC: Before SEC applied (pooled sample), 4, 6, 7, 13, 18, 19, 20 and 22 are the fractions collected through the first peak, 24, 25, 26, 29, 32, 33 and 34 are the fractions collected in the second peak observed in UV profile. 38 is the fraction collected from the small bump after the second peak.

The two peaks were seperated well in the SEC and SDS-PAGE results reflected it by showing two completely seperated proteins. According to the SDS-PAGE result, 1st peak corresponds to 45-kDa protein and the 2nd peak corresponds to GPA1 even though GPA1 was the protein to be expected to elute first due to its higher molecular weight. It can be explained that 45-kDa protein is MBP which is expressed seperately in the cell and it dimerized and formed a 90-kDa oligomer which eluted right before 89-kDa GPA1 with MBP tag.

The estimated amount of GPA1 which is calculated approximately by integrating the corresponding peak using UNICORN Software was 1,2 mg in total. Fractions showing the presence of GPA1 were pooled and concentrated by using concentrators of MWCO=30000 Da at 3000 rpm for 10 minutes until it reached a volume less than 1 ml. Then concentration measurement was performed by using Nanodrop Spectrometer and the estimated extinction coefficient value of GPA1 with MBP tag. Extinction coefficient of GPA1+MBP is (0.1%) 1,24 and extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water. Measured concentration with these parameters was found as 6.35 mg/ml and is shown in Figure 3-17.

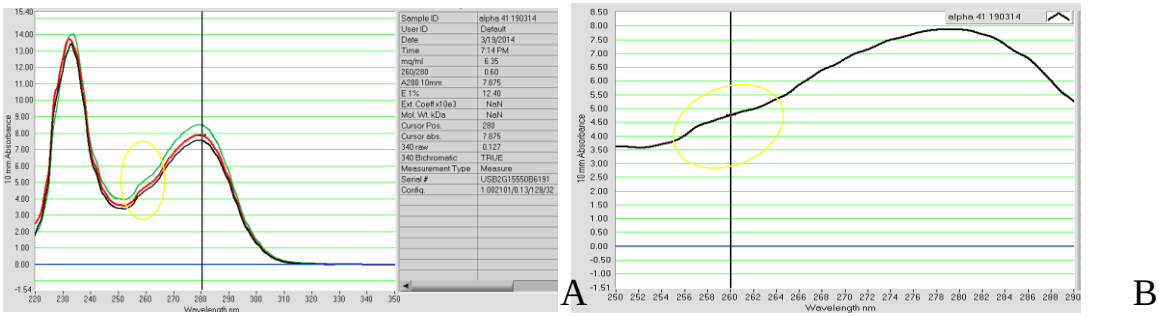


Figure 3-17: (A) Concentration measurements after the fractions obtained from SEC chromatography were pooled and concentrated. There is a bump which is encirculated with yellow elips visualized around 257 nm. (B) The same plot with a narrowed x-axis between 250-290 nm.

3.9 Applying TEV Digestion to GPA1

In order to eliminate MBP tag from GPA1 TEV digestion was performed as it was described in Section 2.2.14. Briefly, the buffer that GPA1 was in it was slightly modified to resemble TEV reaction buffer and for 1 mg of GPA1, 30 µg of TEV protease was used in the reaction. The reaction was performed at $4^{o}C$ to avoid precipitation and continued until any precipitation was observed. At the end of 4^{th} hour, precipitation was observed and the sample was filtered to eliminate precipitated part. The remaining part was subjected to affinity chromatography to remove tags. Again, since GPA1 is supposed to have no tags, it is expected to find it in flow thorugh part. So, flow thorugh part was also collected. And then, the column was washed with a linear gradient of elution buffer having high concentration of imidazole. The UV absorbance profile and the SDS-PAGE run after affinity with the fraction collected are given in Figure 3-18.

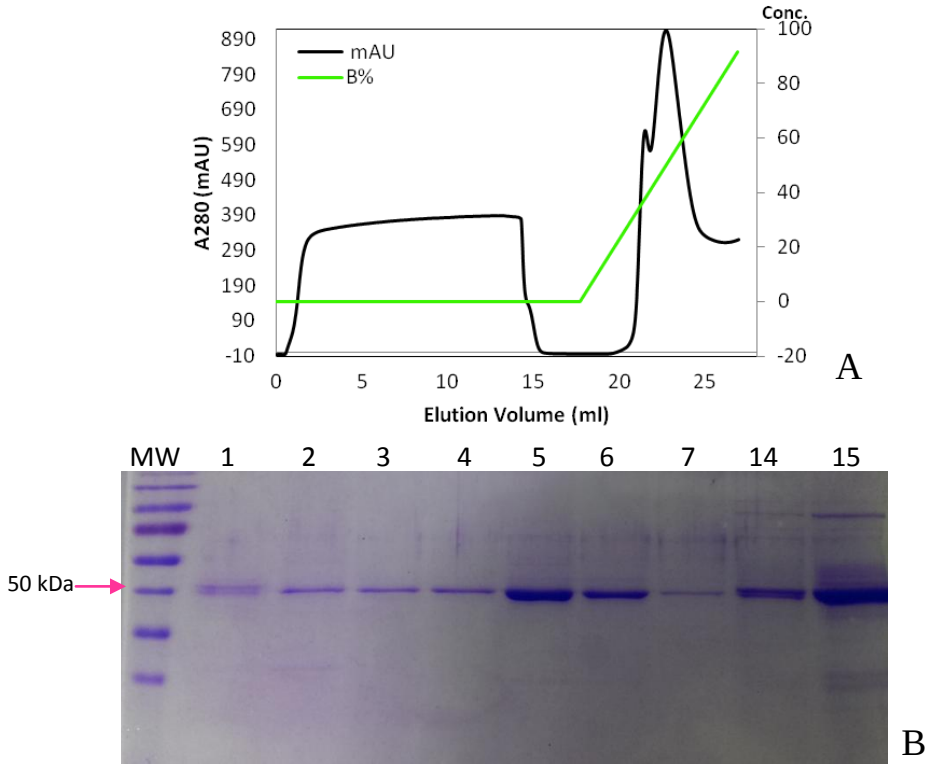


Figure 3-18: Affinity chromatography applied to GPA1 after TEV digestion reaction (A) UV absorbance profile, (B) SDS-PAGE run with the fractions collected through the affinity chromatography. The lanes correspond to MW: Molecular Weight, fractions from 1 to 14 correspond to flow through part, fraction #15 is the one collected around the maximum point of the peak after elution started.

From the SDS-PAGE result, it is clear that there is no ~90 kDa protein which means all GPA1 in the reaction was digested by TEV and the MBP-tag was removed from all of them. In order to be sure, Western blotting was performed to see if any anti-His signal is detected from the flow through part. If GPA1 has no tag, then no signal should be detected.

3.10 Western Blotting

As it is described in Section 2.2.15, Western blotting was applied to confirm that the protein washed in flowthrough and stained in SDS-PAGE was GPA1 without a tag.Western blotting result is given in Figure 3-19.

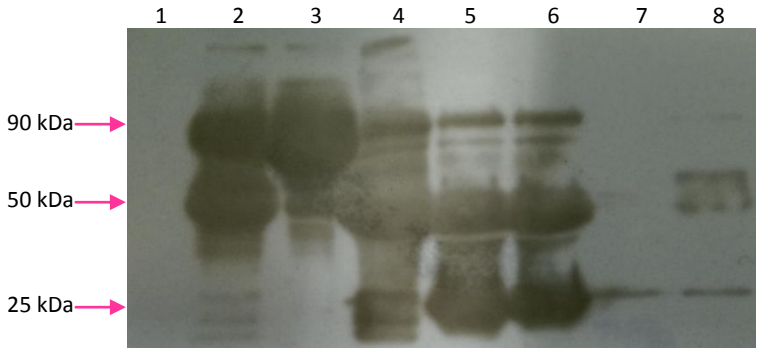


Figure 3-19: Western blotting result. Lanes correspond to 1: MW, 2: Before SEC pooled sample, 3: After SEC pooled sample, 4: After SEC 1st peak fractions, 5: TEV reaction 2nd hour, 6: TEV reaction 4th hour, 7: Flowthrough fractions pooled, 8: Elution fractions pooled.

From the Western blotting result, we confirmed that the 45-kDa protein washed in flow through and visualized in SDS-PAGE is GPA1 without a tag. Moreover, it verifies that the first peak coming in SEC chromatography is MBP with a His tag due to signal detected around ~50 kDa in the 4 th lane corresponding to 1st peak fractions of SEC. The signal detected around ~25 kDa corresponds to TEV protease which has also a His-tag and a molecular mass of 27 kDa.

The concentration of the flowthrough was measured with Nanodrop Spectrometer and this time since there is no tag available in GPA1, the actual extinction coefficient value which is 0,94 (0.1%, in units of M^{-1} cm^{-1}, at 280 nm). It was found as 0,62 mg/ml and the UV absorbance obtained from Nanodrop Spec. is given in Figure 3-20.

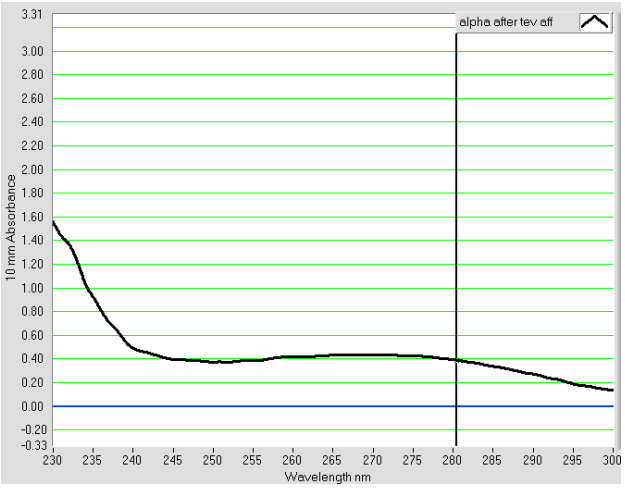


Figure 3-20: The concentration measurement of GPA1 obtained after TEV reaction. Unlike Fig. 26, the bump around 257 nm is not available in here.

3.11 Sample Analysis

In order to check the quality of GPA1 obtained in terms of aggregation or possible oligomer forms NATIVE-PAGE was performed. Native gel sample buffer was added to all samples and then loaded to the gel. It was run with constant 30 Ampere (A) until reaching the bottom part of the gel. The result is given Figure 3-21.

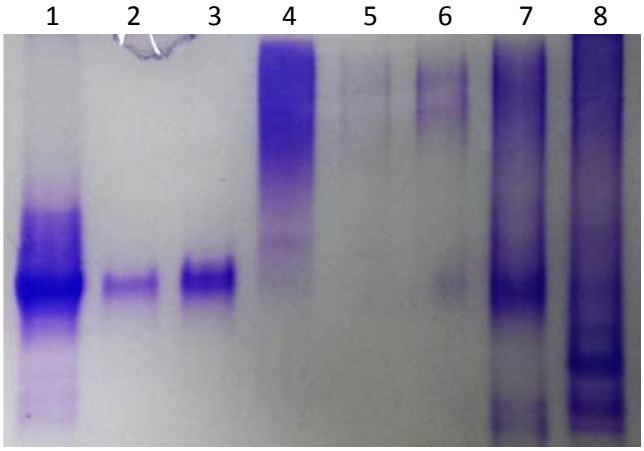


Figure 3-21: Native gel result. Lanes correspond to 1: GPA1+MBP (concentrated), 2:GPA1+MBP (before concentration), 3: Central fraction of the 2nd peak from SEC corresponding to GPA1+MBP, 4: Just MBP (concentrated), 5: Just MBP (before conc.), 6: Central fraction of the 1st peak from SEC corresponding to just MBP, 7: Before SEC, 8: Before anion Exchange.

Native gel result showed that no aggregation or oligomerization was present before concentration applied to the GPA1 with MBP tag. The idea of that MBP is dimerized is further verified with 4th lane in which a certain, single band is not available and this is the sign of being in oligomer forms, not in a monomer form.

To understand the whether there is aggregation in GPA1 without tag (obtained after TEV digestion), DLS (Dynamic Light Scattering) is another and more certain method to apply. DLS measurment was made by Malvern Zetasizer Nanoseries. It was performed to visualize possible aggregations present in GPA1 after TEV digestion. DLS results are given in Figure 3-22.

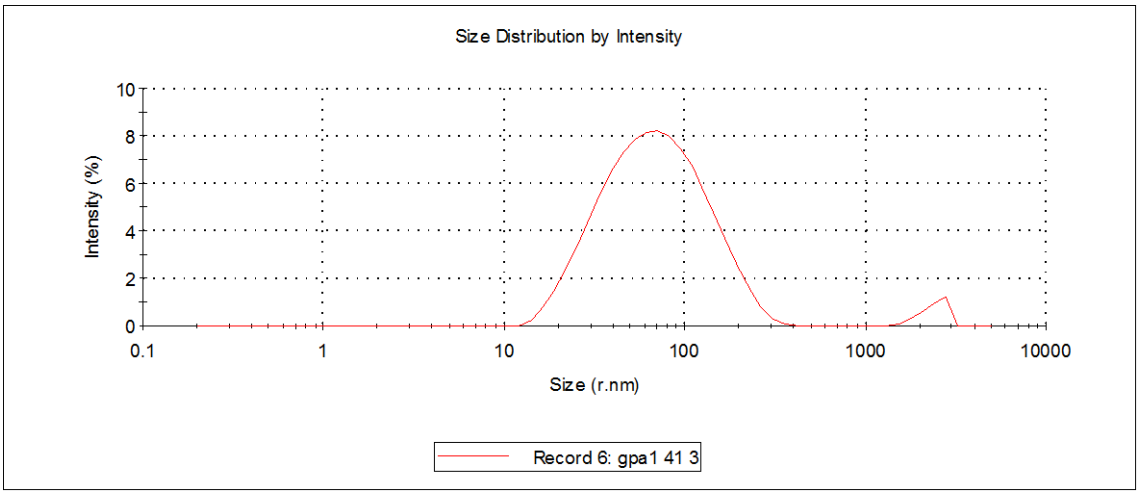


Figure 3-22: Intensity distribution of GPA1 after TEV digestion. The hydrodynamic radius is found to be 78 nm.

DLS results showes that GPA1 was aggregated and it is not a monodispersed sample which means the particles in the sample are not in a uniform size. The broad peaks observed in DLS correspond to aggregation generally.

3.12 Experiments with *P. Pastoris*

3.12.1 Expression of GPA1 in *P. Pastoris*

The large scale expression (2 L) of GPA1 was performed as it was explained in detail in [4] by using old stocks in -80^{o}C. At the end of expression, cells were harvested by centrifugation at 2500g for 30 minutes at 4^{o}C. The cell pellets were kept at -80 ^{o}C until futher use.

3.12.2 Purification of GPA1 Expressed in *P. Pastoris*

3.12.2.1 Affinity Chromatography in Batch mode

The lysis buffer and the lysis procedure is described in Section 2.2.12.1.2. To prepare the sample, briefly, at the end of the lysis, cells were centrifuged and the supernatant was equilibriated with purification buffer given in Section 2.1.6.2. It was mixed with Ni-NTA resin slurry and left for binding for 1 hour. After 1 hour, it was loaded to a plastic column and the flow through part was seperated. Then, the resin was washed for four times with purification buffer. Elution buffer having high concentration of imidazole was added and left on the rotating table for 20 minutes to elute the bound GPA1, then collected by gravity. Since it was batch mode, UV absorbance profile is not available. The gel result is given in Figure 3-23.

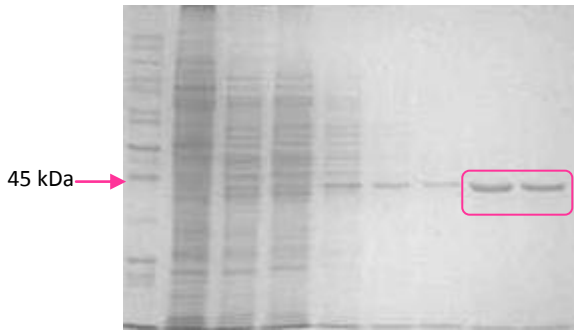


Figure 3-23: SDS-PAGE result after affinity chrom. of GPA1 expressed in *P.Pastoris*. Lanes correspond to MW: Molecular Weight, PL: Pellet, FT: Flow through, SN: Supernatant, W1: Wash 1, W2: Wash 2, W3: Wash 3, E1: Elution 1, E2: Elution 2.

SDS-PAGE result showed that GPA1 was expressed in the cells and it is in significantly pure form in 15 ml. In order to increase purity and concentration, anion Exchange chromatography was decided to apply. Before, GPA1 sample was pooled and dialysed as it is described in Section 2.2.12.1.2.1.

3.12.2.2 Anion Exchange Chromatography

After dialysis of GPA1, precipitation was observed and the precipitated part was eliminated by filtering the sample. Also, after dialysis, GPA1 was in the start buffer of anion Exchange and ready to be applied chromatography. Anion exchange was applied as it is described in Section 2.2.12.2. The UV profile and the SDS-PAGE result run after collecting the samples during the chromatpgraphy step is given in Figure 3-24.

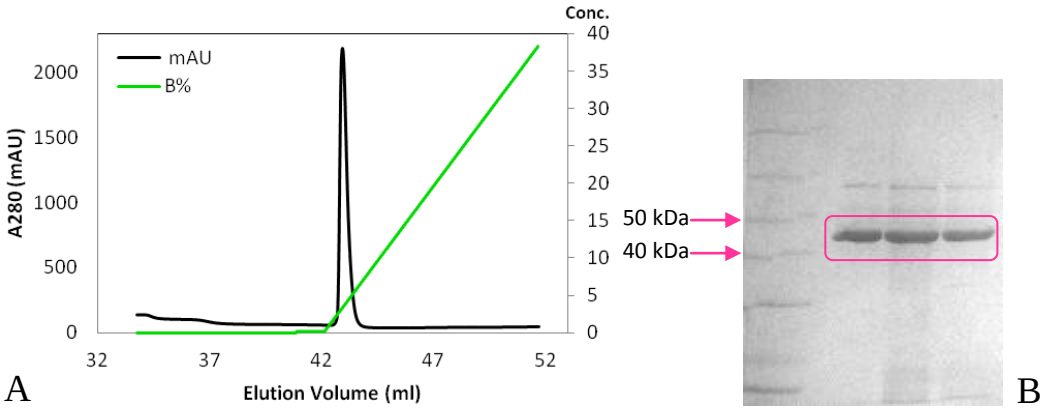


Figure 3-24: (A) Anion exchange UV absorbance profile and (B) SDS-PAGE result with collected fractions.

From the strength of the bands compare to SDS-PAGE result obtained after affinity (Figure 3-24B),it is clear that GPA1 is more concentrated.

3.12.2.3 Size Exclusion Chromatography

In order to show the consistency of molecular weight, size exclusion chromatography was performed. Fractions including GPA1 were concentrated at 2250 rpm for 5 minutes at 4^{o}C until reaching a volume less than 5 ml. During concentration, precipitation was occurred and the precipitated part was eliminated by filtration. Then, as it is described in Section 2.2.12.3, SEC was performed. The UV absorbance profile and the SDS-PAGE result is given in Figure 3-25.

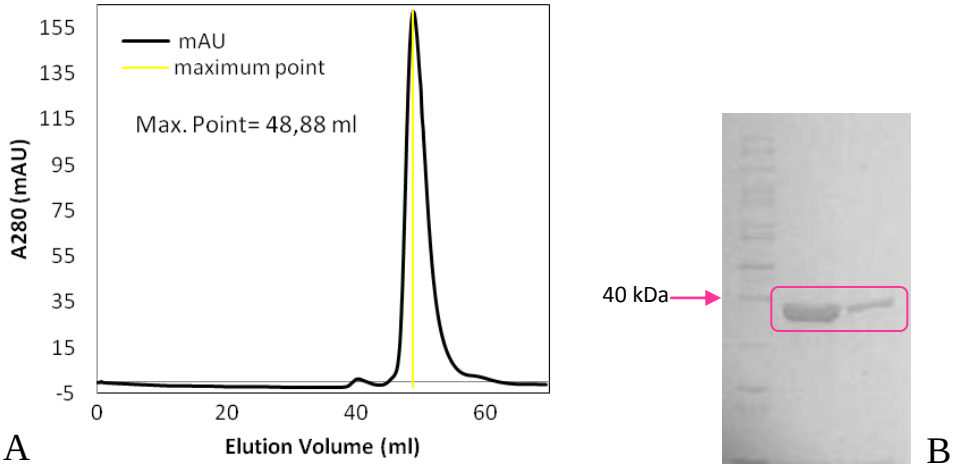


Figure 3-25: (A) The UV absorbance profile of SEC and (B) SDS-PAGE result obtained by using fractions collected during SEC.

The elution volume that GPA1 started to come and reached its maximum is consistent with the calibration curves showing approximate elution volumes for different sizes of proteins. SDS result showed that GPA1 is in its purest form because there is no other protein is available and seen in the gel.

3.12.3 Sample Analysis

3.12.3.1 Western Blotting

To further confirm that the visualized protein around 45 kDa is indeed GPA1, Western blotting with anti-His antibody was performed as it is described in Section 2.2.15. The result is given in Figure 3-26.

3.12.3.2 Native-PAGE Analysis

Native gel was run to see the quality of sample such as any oligomers or aggregation were available in GPA1. The result is shown in Figure 3-26B.

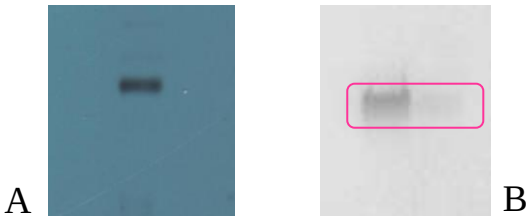


Figure 3-26: (A) Western blotting result confirming that what is seen in SDS-PAGE result is GPA1, (B) Native gel result showing that no aggregation occurred in GPA1.

3.12.3.3 DLS Results

Although Native gel result pointed that there is no aggregation or oligomer forms in GPA1, in order to further analyze the quality of sample DLS (Dynamic Light Scattering) was performed. DLS measurment was made by Malvern Zetasizer Nanoseries. The results are given in Figure 3-27.

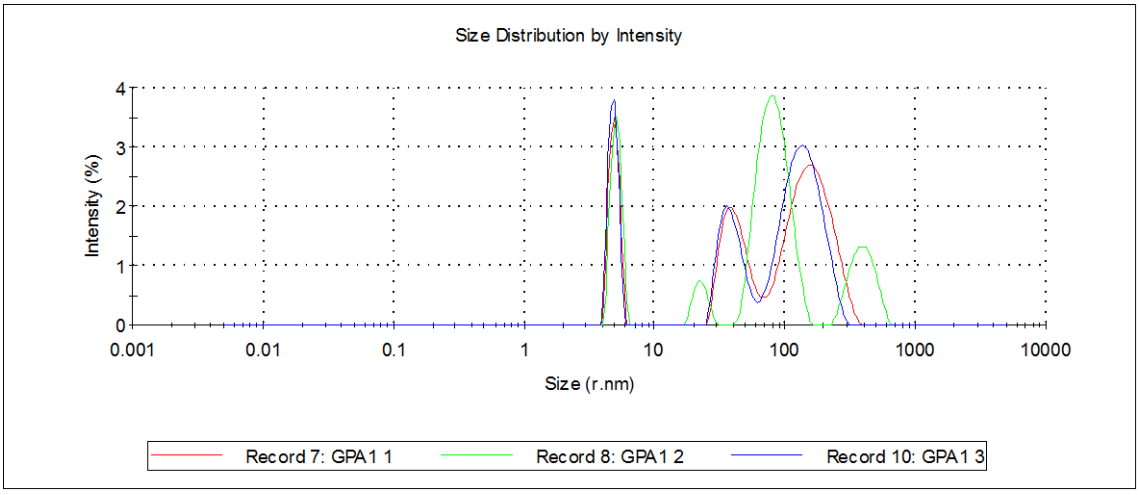


Figure 3-27: Intensty distribution of GPA1 for three measurements. The hydrodynamic radius is found to be 4.8 nm. The peaks occurred after 10 nm correspond to buffer and other particles, not GPA1.

Based on DLS result, the hydrodynamic radius which was found to be 4.8 nm is consistent with the size of the GPA1 and the sample was found as monodisperse meaning there is no aggregation and particles are in uniform size in the sample.

3.12.3.4 CD Results

In order to analyze the secondary structure of GPA1, CD was performed. First, the buffer that GPA1 was inside was scanned and formed a baseline. Then samples were diluted at the final concentration of 0.1 mg/ml in buffer after dilution 1 to 2 with water (final concentration 20 mM NaCl, 25 mM TRIS pH 8) . Buffer was diluted in water at

the same ratio than the sample and subst to the sample. The Cd parameters were: Wavelength 260-190 nm, Data pitch: 0.2 nm, Bandwidth: 1, Sensitivity: standart 100 nm/min. The secondary structure of GPA1 was analyzed for two different conditions which are with 1 mM DTT and without DTT. Since all the samples included DTT after purification, in order to eliminate DTT, we applied dialysis with the same buffer without DTT and changed the buffer. The mean residue ellipticity was calculated by using Dichroweb Online tool and template method CDSSTR METHOD 7. The plots are given in Figure 3-28.

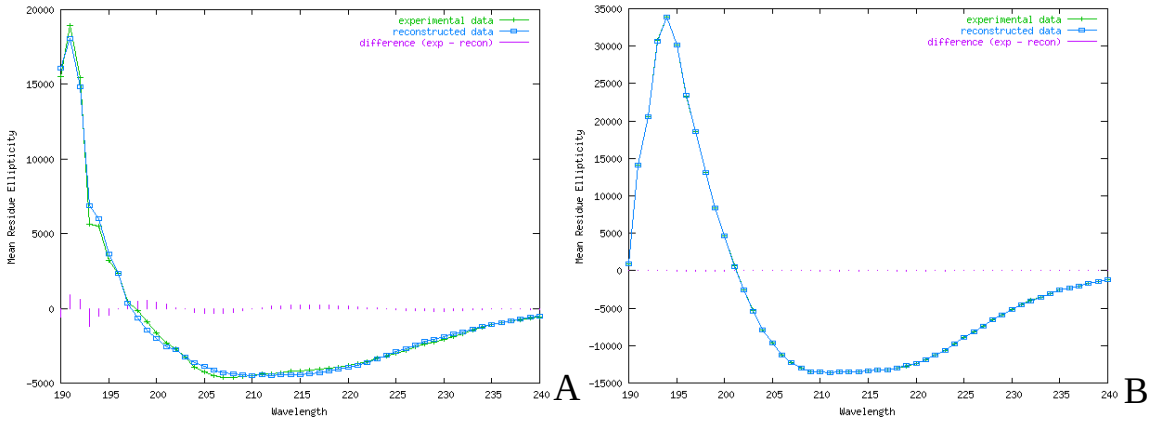


Figure 3-28: (A) CD data obtained from GPA1 without DTT, (B) GPA1 with 1 mM DTT.

According to these results, the percentage distribution of secondary structures are given in Table 9.

	Helices	**Strands**	**STD (%)**
GPA1 w/o DTT	2%	25%	0.2
GPA1 w/ DTT	42%	15%	0.3
Crys. Str. of GPA1	49%	10%	

Table 9: The comparison of the secondary structure distribution obtained from GPA1 without DTT, with 1 mM DTT and the crystal structure of GPA1.

The percentage distribution of secondary structure shows that GPA1 with DTT is more consistent with the crystal structure of GPA1. Moreover, after CD measurements, in order to compare GPA1 with DTT and without DTT, SDS-PAGE and Native-PAGE were run again. The results were given in Figure 3-29.

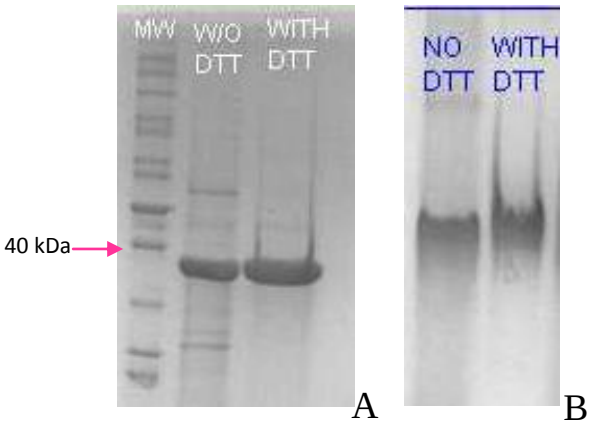


Figure 3-29: (A) SDS-PAGE result, lane names, MW: Molecular Weight, W/O DTT: GPA1 without DTT, WITH DTT: GPA1 with 1 mM DTT. (B) Native Gel result with the lanes corresponding to NO DTT: GPA1 without DTT, WITH DTT: GPA1 with 1 mM DTT.

The gel results also supported the CD results by showing more spreaded and blurry lane in Native gel for GPA1 without DTT.

4 DISCUSSION

The alpha subunit of the G proteins, namely GPA1, is the important part of the heterotrimer due to its interaction with regulatory upstream proteins and GTPase activity upon activation. Also, the least amount of variation is observed in plant G protein α subunit. However, the activation/deactivation mechanism is not well known and the structure of GPA1 is available only with the 2.34 A^o resolution crystal structure data which is not detailed enough [2]. GPA1 was formerly cloned and expressed using different a vector named pPROEXHT explained in Willard *et al*. in 2004. And all the other studies on GPA1 were referred Willard *et al*. without giving any explanation stating that cloning, expression and purification were performed as explained in Willard *et al*. In our studies, we also used the methods similar with Willard *et al*. but there are certain differences in our results compared with the literature. For instance, even though Jones *et al*. stated that they were used TEV protease for cleavage of MBP from GPA1, they did not mention any kind of precipitation. Here we discussed our result in detail in separate sections.

4.1 Cloning GPA1 into Vectors

Attempts were made to express GPA1 in *E.coli* using pQE80L and pETM41 vectors. These vectors were chosen because pQE80L has been previously used in expression of mammalian G protein subunits [38] and pETM41 is commonly used for insoluble proteins [39]. Insertion of the GPA1 sequence into pETM41 was achieved through utilization of NcoI and KpnI restriction sites whereas for pQE80L vector HindIII and KpnI sites were used. Control PCR and sequencing results showed that although GPA1 gene was introduced into pETM41 vector, we failed to clone it using the pQE80L vector. Different annealing temperatures and durations used did not change this result. The reason for this may be mistakes in primer synthesis which we could not be repeated due to lack of time. On the other hand, as it was stated before, pPROEXHT and pPROEx-Htb including MBP tag and TEV cleavage site were preferred to clone GPA1 in the literature. [2, 40].

pETM41 vector includes a His-tag followed by MBP (maltose binding protein) tag and a cleavage site for Tobacco Etch Virus (TEV) protease which removes MBP tag and leaves GPA1 without a tag. The presence of GPA1 in the pETM41 vector was

confirmed with control PCR and DNA sequencing. Sequencing results were noisy at some regions and possible mutations cannot be ruled out. The expressed protein was visualized by Western blotting using his antibodies. The protein band is seen at a position corresponding to its molecular weight. So, if there are mistakes in the GPA1 gene causing mutations that we are unable to detect, they may cause conformational changes on GPA1. Or, simply they may be silent mutations having no possible effect on the function and the structure of the protein.

For *P. pastoris*, we did not perform any cloning, the old stocks prepared previously were used [4, 5].

4.2 Expression and Purification of GPA1 in *E.coli* System

After confirming the insertion of GPA1 in pETM41, protein expression in different host cells was tried. The first host cells that we tried were BL21 (DE3). GPA1 expression in these cells did not result in a yield sufficient to go through all the purification steps to eliminate all contaminating proteins. An additional problem that we encountered with was the synthesis of a truncated version of GPA1 which was detected in Western blotting and SDS-PAGE results. The possible reason for incomplete synthesis of GPA1 in BL21 cells is likely to be the rare tRNA codons in BL21 cells. When the aminoacid sequence of GPA1 given below is studied we see that out of a total of 383 amino acids:

MGLLCSRSRHHTEDTDENTQAAEIERRIEQEAKAEKHIRKLLLLGAGESGKSTIFKQIKLLFQTGFDEGE
LKSYVPVIHANVYQTIKLLHDGTKEFAQNETDSAKYMLSSESIAIGEKLSEIGGRLDYPRLTKDIAEGIE
TLWKDPAIQETCARGNELQVPDCTKYLMENLKRLSDINYIPTKEDVLYARVRTTGVVEIQFSPVGENKKS
GEVYRLFDVGGQRNERRKWIHLFEGVTAVIFCAAISEYDQTLFEDEQKNRMMETKELFDWVLKQPCFEKT
SFMLFLNKFDIFEKKVLDVPLNVCEWFRDYQPVSSGKQEIEHAYEFVKKKFEELYYQNTAPDRVDRVFKI
YRTTALDQKLVKKTFKLVDETLRRRNLLEA

23 are Arginine (R), 10 are Proline (P), 39 are Leucine (L) and 22 are Isoleucine (I). These aminoacids have the so called rare tRNA codons and proteins having these aminoacids in their sequences in high numbers need to be expressed in specially modified *E.coli* strains containing additional rare tRNA codons. We suggest that the low yield and incomplete synthesis of GPA1 in BL21 cells is due to its sequence which contains 24.5% rare tRNA codon amino acids. The example of usage of these host cells including rare tRNA codons is available for both together with pEMT41 vector and also

other vectors [2, 39]. That is why we tried to express GPA1 in Rosetta cells which have rare tRNAs additionally.

When we changed the strain from BL21 to Rosetta, the yield of GPA1 (as MBP+GPA1 fusion) increased significantly. The highest yield that we obtained at the end of purification steps was 1.2 mg of protein from 1 L expression in LB medium. Despite the fact that the solubility of GPA1 was increased due to the MBP tag, MBP expression on its own also occurred at extremely high levels as it is seen from Figure 3-12, Figure 3-13 Figure 3-14. Even though MBP was expressed more than the fusion protein in cells, we were able to separate them and obtain a clean sample of MBP+GPA1 fusion protein. But the drawback of having excessive yield of MBP was that the culture volume could not be increased beyond 1 L. A scale up would result in a sample that would have too much contamination that would be hard to eliminate.

We did not observe any precipitation when GPA1 had MBP tag. It is an expected result due to the fact that MBP increases solubility [41]. We found out from the SEC results that MBP was dimerized and eluted before GPA1. It appears that MBP is not stable in monomeric form and dimerizes in solution. When it is bound to GPA1, on the other hand, it remains in the monomeric form.

Another issue that needs to be pointed out is that after TEV protease reaction, we observed precipitation and almost 70% of GPA1 was lost. Even worse, the remaining part of the GPA1 was aggregated according to DLS results. So, we can claim that GPA1 is not soluble enough and when there is no solubility agent like MBP, it precipitates. Also, we can say that GPA1 is not sufficiently stable when the βγ dimer is not in the environment. Jones *et al*. in 2011 reported that before they crystallized Gα subunit, they removed 36 aminoacids from the N-terminus stating that these do not have any contribution to the self activation mechanism which was demonstrated by intrinsic fluorescent measurement comparison between non-truncated and the truncated form of Gα, so structurally the protein structure remains unchanged [2]. In order to crystallize a protein, high concentrations of the purest form are required. When these 36 aminoacids were removed, GPA1 was expressed and purified with high yields, probably without precipitation. We may infer that these 36 aminoacids from N-terminal contributes to the flexibility of GPA1, which in turn, may cause precipitation since the stability of GPA1 may be reduced.

The observed aggregation may be caused of that there is no posttranslational modifications in E.coli. It has been reported that there are several posttranslational modifications such as myristoylation occuring at N-terminus and palmitoylation which involves the attachment of palmitic acid using the first cysteine aminoacid [41, 42] by N-myristoyl transferase (NMT) and palmitoyl transferase (PAT), respectively [43]. The posttranslational modifications occurred on Gα can be found in Appendix A. They may stabilize GPA1 and when such modifications cannot be made, it may cause aggregation.

4.3 Expression and Purification of GPA1 in *P. pastoris* System

When GPA1 was expressed and purified, the eluate was significantly more pure and cleaner compared with the eluate obtained in *E.coli* system. Unfortunately, since there is no MBP tag or any other solubility agent to increase solubility, more precipitation was observed. In this system, again, the highest yield of GPA1 obtained was ~1.5 mg per 20 gr of pellet. When DLS measurements were performed, aggregation was not observed and the sample was monodisperse. This allowed us to perform further measurements such as CD measurements as a biophysical characterization method to understand its secondary structure. Although the C-terminal His-tag was not eliminated, it was shown that the percentage of secondary structure distribution is consistent with crystal structure of GPA1. In the presence of DTT, GPA1 was more consistent to the crystal structure but when there is no DTT, its secondary structure was disrupted. So, we can say that DTT has positive effect on the structure of GPA1. Although *E.coli* system was easier to work with in terms of time and source comsumption, the quality of sample was higher in the yeast system.

5 CONCLUSION AND FUTURE WORKS

Cloning of the alpha subunit of heterotrimeric G protein, namely GPA1, into two vectors, pQE80L and pETM41, was investigated and the cloning was successfully accomplished in the latter. pETM41 cloning resulted in the synthesis of the MBP+GPA1 fusion protein. Different expression systems such as *E.coli* and *P.pastoris* were used to express the protein after optimizing the expression conditions in both of them. Although GPA1 was previously cloned and expressed in the yeast system previously, since it is more time and resource consuming compared with bacteria, we switched to the bacteria systems for GPA1 expression by using different strains of *E.coli.* It was found that, due to the presence of several rare codon in the GPA1 sequence Rosetta strain of *E.coli* is an efficient host to express GPA1 giving the yield of 1.2 mg/ 1 L expression.

Purification results showed that, although a significant amount of incomplete synthesis gives high quantities of MBP, it is possible to eliminate it from the samples and obtain pure form of GPA1 at the end of size exclusion chromatography. In between purification steps, no precipitation occurred in the bacteria system due to the fact that MBP increased the solubility of GPA1. However, precipitation was observed in several steps when purifying GPA1 expressed in the yeast system and most of the protein was lost in these steps.

After purifying GPA1, preliminary biophysical characterization of the samples obtained from both the bacteria and yeast systems was performed by using native page gel, CD (circular dichroism) and DLS (dynamic light scattering) measurements. Results showed that after eliminating MBP tag with TEV protease digestion from the N- terminal of GPA1, samples were aggregated. However, no aggregation was observed in the samples obtained from the yeast expression system.

As future works, firstly purification and cleavage conditions need to be optimized to eliminate aggregation of GPA1 after treatment with TEV. After obtaining sufficient amount of non-aggregated protein without a tag, further structural characterization such as secondary structure analysis using CD measurements can be performed. Additionally

functionality of GPA1 in terms of GTP and GDP binding and GTPase activity need to be tested. These functions can be monitored by intrinsic fluorescent measurements and real-time fluorescence-based assays as reported in the literature [2, 43, 44] Furthermore if sufficient amponts of protein can be obtaine it will be informative to perform SAXS measurements to follow dynamic changes in the structure upon binding different nucleotides and their analogues.

6 REFERENCES

[1] D. Urano, J. Chen, J. R. Botella, and A. M. Jones, “Heterotrimeric G protein signalling in the plant kingdom Heterotrimeric G protein signalling in the plant kingdom,” no. March, 2013.

[2] J. C. Jones, J. W. Duffy, M. Machius, B. R. S. Temple, H. G. Dohlman, and A. M. Jones, “The crystal structure of a self-activating G protein alpha subunit reveals its distinct mechanism of signal initiation.,” *Sci. Signal.*, vol. 4, p. ra8, 2011.

[3] D. Urano, J. C. Jones, H. Wang, M. Matthews, W. Bradford, J. L. Bennetzen, and A. M. Jones, “G protein activation without a GEF in the plant kingdom.,” *PLoS Genet.*, vol. 8, no. 6, p. e1002756, Jun. 2012.

[4] B. Kaplan, “Structural Investigation of G-Protein Signaling in Plants.,” 2009.

[5] A. Akturk, “Characterization of A. thaliana G-Protein Gamma Subunit (AGG2) and Investigation of DTT Effect on Its Oligomeric State,” 2012.

[6] G. Milligan and E. Kostenis, “Heterotrimeric G-proteins: a short history.,” *Br. J. Pharmacol.*, vol. 147 Suppl , pp. S46–S55, 2006.

[7] M. Rodbell, “Signal Transduction: Evolution of an idea,” pp. 220–237, 1994.

[8] A. Krishnan, M. S. Alm??n, R. Fredriksson, and H. B. Schi??th, “The origin of GPCRs: Identification of mammalian like rhodopsin, adhesion, glutamate and frizzled GPCRs in fungi,” *PLoS One*, vol. 7, no. 1, 2012.

[9] B. K. Kobilka, “G protein coupled receptor structure and activation.,” *Biochim. Biophys. Acta*, vol. 1768, no. 4, pp. 794–807, Apr. 2007.

[10] M. C. Lagerstrom and H. B. Schioth, “Structural diversity of G protein-coupled receptors and significance for drug discovery,” *Nat Rev Drug Discov*, vol. 7, pp. 339–357, 2008.

[11] V. Sarramegna, F. Talmont, P. Demange, and A. Milon, “Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification.,” *Cell. Mol. Life Sci.*, vol. 60, pp. 1529–1546, 2003.

[12] P. Ghanouni, Z. Gryczynski, J. J. Steenhuis, T. W. Lee, D. L. Farrens, J. R. Lakowicz, and B. K. Kobilka, “Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor.,” *J. Biol. Chem.*, vol. 276, pp. 24433–24436, 2001.

[13] S. Bulenger, S. Marullo, and M. Bouvier, “Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation,” *Trends in Pharmacological Sciences*, vol. 26. pp. 131–137, 2005.

[14] J. A. Javitch, “The ants go marching two by two: oligomeric structure of G-protein-coupled receptors.,” *Mol. Pharmacol.*, vol. 66, pp. 1077–1082, 2004.

[15] S. Angers, A. Salahpour, and M. Bouvier, “Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function.,” *Annu. Rev. Pharmacol. Toxicol.*, vol. 42, pp. 409–435, 2002.

[16] J. L. Baneres and J. Parello, “Structure-based analysis of GPCR function: Evidence for a novel pentameric assembly between the dimeric leukotriene B4 receptor BLT1 and the G-protein,” *J. Mol. Biol.*, vol. 329, pp. 815–829, 2003.

[17] T. Kenakin, “Efficacy at G-protein-coupled receptors.,” *Nat. Rev. Drug Discov.*, vol. 1, pp. 103–110, 2002.

[18] G. Swaminath, X. Deupi, T. W. Lee, W. Zhu, F. S. Thian, T. S. Kobilka, and B. Kobilka, “Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists.,” *J. Biol. Chem.*, vol. 280, pp. 22165–22171, 2005.

[19] T. Kenakin, “Ligand-selective receptor conformations revisited: The promise and the problem,” *Trends in Pharmacological Sciences*, vol. 24. pp. 346–354, 2003.

[20] C. Hapter, D. P. Siderovski, and T. K. Harden, “The RGS Protein Superfamily,” *Handb. Cell Signal.*, vol. 2, no. Chapter 225, pp. 631–638, 2003.

[21] J. R. Hepler, D. M. Berman, A. G. Gilman, and T. Kozasa, “RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, pp. 428–432, 1997.

[22] J. R. Hepler, “RGS protein and G protein interactions: a little help from their friends.,” *Mol. Pharmacol.*, vol. 64, no. 3, pp. 547–9, Sep. 2003.

[23] J. J. Tesmer, D. M. Berman, A. G. Gilman, and S. R. Sprang, “Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis.,” *Cell*, vol. 89, pp. 251–261, 1997.

[24] M. Pines, P. Gierschik, G. Milligan, W. Klee, and A. Spiegel, “Antibodies against the carboxyl-terminal 5-kDa peptide of the alpha subunit of transducin crossreact with the 40-kDa but not the 39-kDa guanine nucleotide binding protein from brain.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 82, pp. 4095–4099, 1985.

[25] D. T. Lodowski, J. A. Pitcher, W. D. Capel, R. J. Lefkowitz, and J. J. G. Tesmer, “Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma.,” *Science*, vol. 300, pp. 1256–1262, 2003.

[26] L. Birnbaumer, “Signal transduction by G proteins. basic principles, molecular diversity, and structural basis of their actions,” in *Handbook of Cell Signaling, 2/e*, vol. 2, 2010, pp. 1597–1614.

[27] N. Wettschureck and S. Offermanns, “Mammalian G Proteins and Their Cell Type Specific Functions,” *Physiol Rev.*, vol. 85, pp. 1159–1204, 2005.

[28] B. Ahrén, “Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes.,” *Nat. Rev. Drug Discov.*, vol. 8, pp. 369–385, 2009.

[29] H. Ma, M. F. Yanofsky, and E. M. Meyerowitz, “Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from Arabidopsis thaliana.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, pp. 3821–3825, 1990.

[30] Y. Trusov, D. Chakravorty, and J. R. Botella, “Diversity of heterotrimeric G-protein gamma subunits in plants,” *BMC Research Notes*, vol. 5. p. 608, 2012.

[31] H. Ma, M. F. Yanofsky, and E. M. Meyerowitz, “Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from Arabidopsis thaliana.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, no. 10, pp. 3821–5, May 1990.

[32] D. Chakravorty, Y. Trusov, and J. R. Botella, “Site-directed mutagenesis of the Arabidopsis heterotrimeric G protein β subunit suggests divergent mechanisms of effector activation between plant and animal G proteins,” *Planta*, vol. 235. pp. 615–627, 2012.

[33] D. E. Coleman, A. M. Berghuis, E. Lee, M. E. Linder, A. G. Gilman, and S. R. Sprang, “Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis.,” *Science*, vol. 265, pp. 1405–1412, 1994.

[34] D. Hackenberg, H. Sakayama, T. Nishiyama, and S. Pandey, “Characterization of the heterotrimeric G-protein complex and its regulator from the green alga Chara braunii expands the evolutionary breadth of plant G-protein signaling.,” *Plant Physiol.*, vol. 163, pp. 1510–7, 2013.

[35] Y. R. Lee and S. M. Assmann, “Arabidopsis thaliana ‘extra-large GTP-binding protein’ (AtXLG1): a new class of G-protein.,” *Plant Mol. Biol.*, vol. 40, pp. 55–64, 1999.

[36] P. G. Blommel and B. G. Fox, “A combined approach to improving large-scale production of tobacco etch virus protease.,” *Protein Expr. Purif.*, vol. 55, no. 1, pp. 53–68, Sep. 2007.

[37] D. S. Waugh, “TEV Protease FAQ,” no. September, 2010.

[38] P. Mega, O. Orun, C. Nacar, and U. Osman, “Structural characterization of recombinant bovine Go α by spectroscopy and homology modeling,” vol. 26, no. 1, pp. 213–229, 2011.

[39] C. Fladeby, E. S. Vik, J. K. Laerdahl, C. Gran Neurauter, J. E. Heggelund, E. Thorgaard, P. Strøm-Andersen, M. Bjørås, B. Dalhus, and I. Alseth, “The human homolog of Escherichia coli endonuclease V is a nucleolar protein with affinity for branched DNA structures.,” *PLoS One*, vol. 7, no. 11, p. e47466, Jan. 2012.

[40] F. S. Willard and D. P. Siderovski, “Purification and in vitro functional analysis of the Arabidopsis thaliana regulator of G-protein signaling-1.,” *Methods Enzymol.*, vol. 389, no. 1995, pp. 320–38, Jan. 2004.

[41] J. D. Fox and D. S. Waugh, “Maltose-binding protein as a solubility enhancer.,” *Methods Mol. Biol.*, vol. 205, pp. 99–117, Jan. 2003.

[42] M. J. Lee, “Activation of G protein signaling by a non-receptor exchange factor,” 2008.

[43] M. J. W. Adjobo-Hermans, J. Goedhart, and T. W. J. Gadella, “Plant G protein heterotrimers require dual lipidation motifs of Galpha and Ggamma and do not dissociate upon activation.,” *J. Cell Sci.*, vol. 119, no. Pt 24, pp. 5087–97, Dec. 2006.

[44] R. E. Muller, D. P. Siderovski, A. J. Kimple, N. Carolina, and C. Hill, “Using Intrinsic Tryptophan Fluorescence to Measure Heterotrimeric G-Protein Activation,” vol. 7, 2009.

[45] R. J. Kimple, M. B. Jones, A. Shutes, B. R. Yerxa, D. P. Siderovski, and F. S. Willard, “Established and emerging fluorescence-based assays for G-protein function: heterotrimeric G-protein alpha subunits and regulator of G-protein signaling (RGS) proteins.,” *Comb. Chem. High Throughput Screen.*, vol. 6, no. 4, pp. 399–407, Jun. 2003.

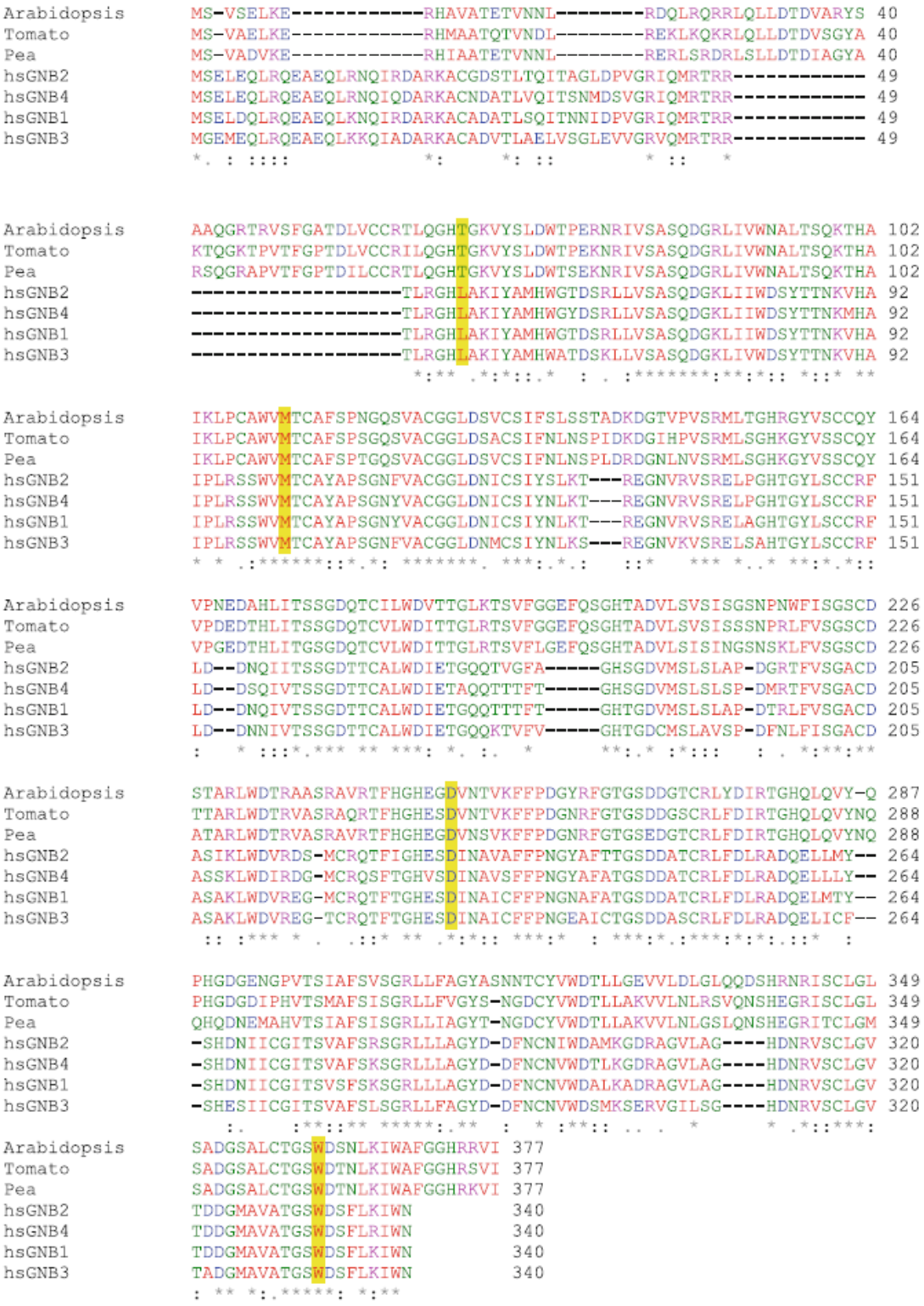
APPENDIX LIST

APPENDIX A

The post translational modifications occurred in GPA1 [43].

G protein	Lipid modification	Motif*	References
Gα subunits			
*Hs*G$\alpha_{i/o/z}$	*N*-myristoylation	MG**C**xx**S**	(Galbiati et al., 1994; Grassie et al., 1994; Parenti et al., 1993; Wilson and Bourne, 1995)
	S-palmitoylation	where x=TL/TV in α_i x=TL in α_o x=RQ in α_z	
*Hs*Gα_s	*N*-palmitoylation *S*-palmitoylation	MG**C**LGNS	(Kleuss and Krause, 2003)
*Hs*Gα_q	*S*-palmitoylation	MTLESIMA**C**C	(Wedegaertner et al., 1993)
*Hs*Gα_t	*N*-myristoylation	MGAGAS	(Yang and Wensel, 1992)
*Hs*Gα_{12}	*S*-palmitoylation	MSGVVRTLSR**C**	(Ponimaskin et al., 1998)
*Hs*Gα_{13}	*S*-palmitoylation	MAD-^{14}**C**FPQ**C**^{18}-^{37}**C**LS	(Ponimaskin et al., 2000)
*Sc*Gpa1	*N*-myristoylation *S*-palmitoylation	MG**C**TVS	(Song and Dohlman, 1996; Song et al., 1996)
*At*GPα1		MGLL<u>C</u>S	
Gγ subunits			
*Hs*Gγ_1	Farnesylation	KELKGG**C**VIS	(Sanford et al., 1991)
*Hs*Gγ_2	Geranylgeranylation	REKKFF**C**AIL	(Sanford et al., 1991)
*Sc*STE18	*S*-palmitoylation Farnesylation	NSNSV**C**CTLM	(Manahan et al., 2000; Whiteway and Thomas, 1994)
*At*AGG1		GGEG<u>C</u>RCLIL	
*At*AGG2		EAKR<u>C</u>G<u>C</u>SIL	

*Amino acids are indicated by their single letter code. Lipidated amino acids are in bold. Underlined amino acids are subject of this study.



Multiple alignment result of β subunit of G proteins in between Arabidopsis thaliana, Tomato, Pea and human [32].

APPENDIX B

CHEMICALS

Merck

$MgCl_2$

NaCl

Na_2HPO_4

NaH_2PO_4

$Al_2(SO_4)_3$

Ethanol

Tween-20

Agar-agar

2-Mercaptoethanol

Hydrochloric acid (37%)

Applichem

TRITON

Non-fat dried milk powder

Tryptone

Peptone

Yeast Extract

Glycine

PMSF (Phenylmethylsulphonylfluoride)

QIAGEN

Ni-NTA Agarose Slurry

QIAquick Gel Extraction Kit

QIAquick PCR Purification Kit

QIAprep Spin Miniprep Kit

Sigma Aldrich

Trizma Base

Imidazole

NaF

Methanol

NaOH

Isopropanol

1.1.1 TEMED (Tetramethylethylenediamine)

APS (Amonium per sulfate)

Acrylamide-bis-acrylamide 30% Solution

Lysozyme from chickedn egg white

Kanamycin sulfate

1.1.2 GDP (Guanosine 5′-diphosphoglucose sodium salt)

Albumin from bovine serum

ThermoScientific

MassRuler DNA Ladder Mix

PVDF Transfer Membrane 0,45 µm.

GEHealth Care

HisTrap HP, 1-5 ml immobilized metal ion affinity columns (IMAC)

Qtrap HP 1-5 ml anion exchange columns

HiLoad 16/60 Superdex 75 SEC column

HiLoad 16/600 Superdex 200 pg SEC column

HiPrep 26/10 Desalting Column

Fermentas

BamHI

NcoI

KpnI

HindIII

T4 Ligase

Taq. Polymerase

dNTPs

Pre-stained Protein Molecular Marker

AMRESCO

SDS (Sodium dodecyl sulfate)

TRIS

CelluSep

Regenerated Cellulose Tubular Membrane

peqGOLD Lab

Protein Marker II Unstained 10-200 kDa

Universal Agarose

Duchefa Biochemie

Ampicillin

Roche

Complete Protease Inhibitor Tablets

Complete EDTA-Free Protease Inhibitor Tablets

ITPG (Isopropyl-β-D-thiogalactoside)

Fluka

1,4-Dithiothreitol (DTT)

Coomassie Brillant Blue R-250

FUJI

Medical X-Ray Films

BioSpec Products

0.5 mm Zirconia/Silica Beads

Millipore

Membrane Filters (0.22, 0.45 μm)

Miltenyi MACS Biotechs

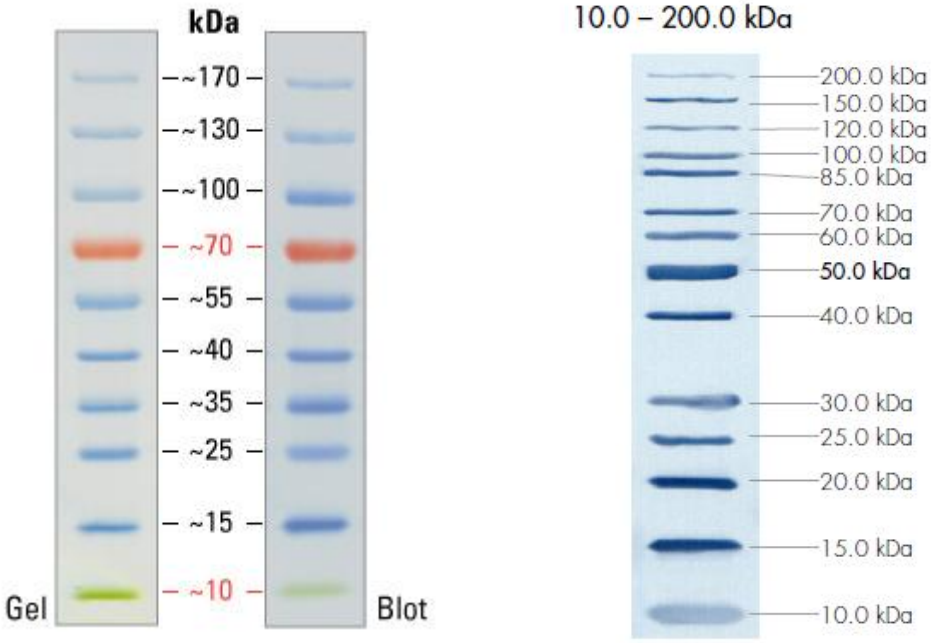
Anti-His antibody

KONIX

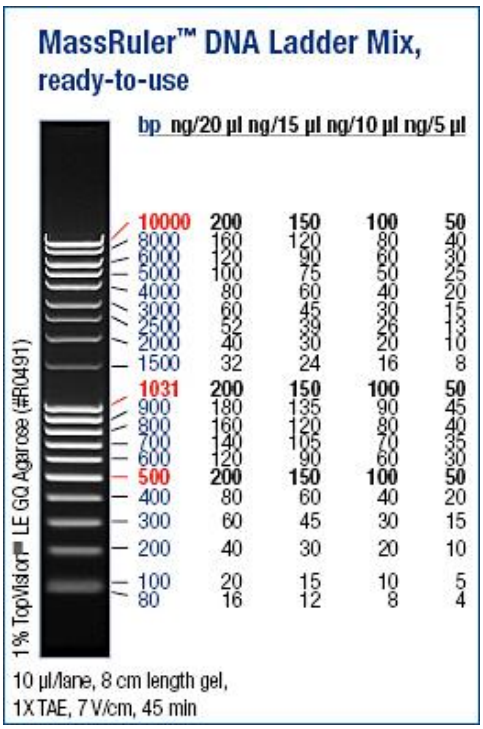
Developer, Fixer

APPENDIX C

MOLECULAR WEIGHT MARKERS



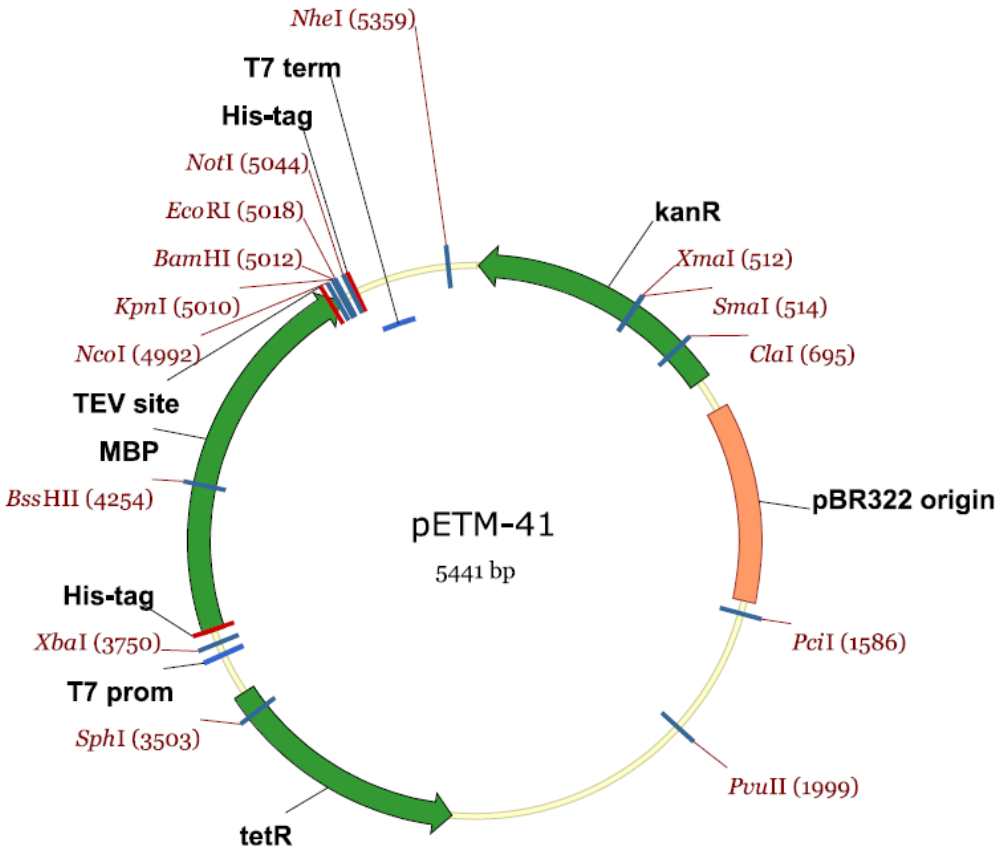
Fermentas pre-stained molecular weight marker (left) and peqGOLD unstained molecular marker (right).



MassRuler DNA Ladder Mix.

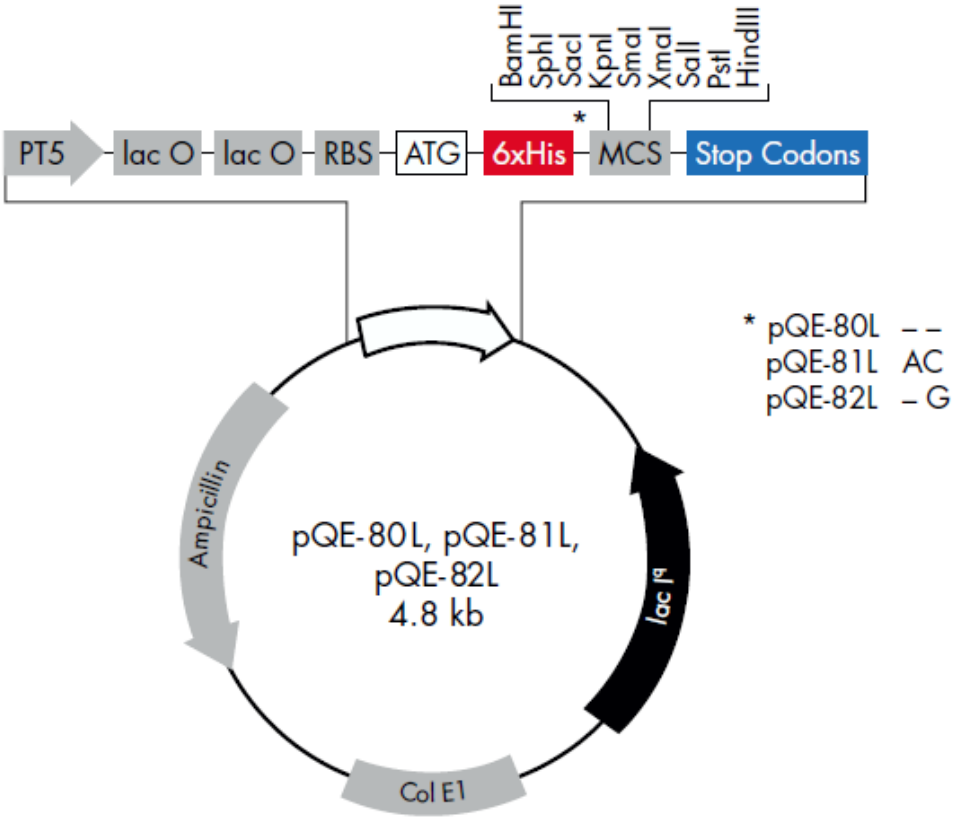
APPENDIX D

Vector Maps

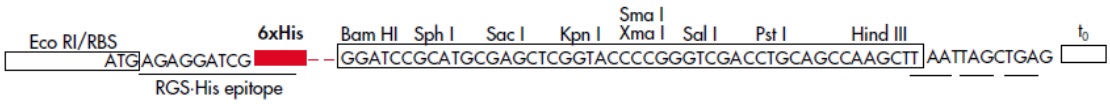


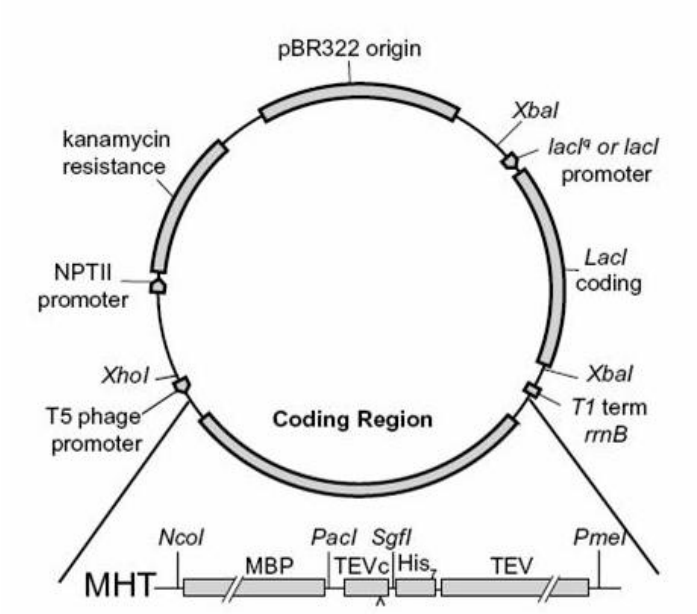
pETM-41

```
                                    MBP
          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                                    TEV site
                                                                   ------------
                                                                    E   N  L  Y
4921      TCGAGCTCGA ACAACAACAA CAATAACAAT AACAACAACC CCATGAGCGA GAATCTTTAT
          AGCTCGAGCT TGTTGTTGTT GTTATTGTTA TTGTTGTTGG GGTACTCGCT CTTAGAAATA

           TEV site                  KpnI        EcoRI
          ---------                 ~~~~~~~     ~~~~~~~
                       NcoI                BamHI
                      ~~~~~~                ~~~~~~
           F  Q  G  A   M  D  *   *
4981      TTTCAGGGCG CCATGGACTA ATGAGGTACC GGATCCGAAT TCGAGCTCCG TCGACAAGCT
          AAAGTCCCGC GGTACCTGAT TACTCCATGG CCTAGGCTTA AGCTCGAGGC AGCTGTTCGA

            NotI              His-tag
           ~~~~~~~~          ---------- *
5041      TGCGGCCGCA CTCGAGCACC ACCACCACCA CCACTGAGAT CCGG
          ACGCCGGCGT GAGCTCGTGG TGGTGGTGGT GGTGACTCTA GGCC
```



pQE-80 L





Vector map of pMHTDelta238 (used in TEV expression)

APPENDIX E

Buffers and Solutions

Tris Acetate EDTA Buffer (TAE) (50X): 121.1 g Tris Base, 28.55 ml Glacial Acetic acid, 7.3 g EDTA, completed to 500 ml.

2X Native Sample Buffer: 200 mM Tris-HCl pH 7.5, 20 % (v/v) Glycerol, 0.05 % (w/v) Bromophenol Blue in ddH2O.

Native-PAGE Running Buffer: 25 mM Tris, 192 mM Glycine in ddH2O

2X SDS Sample Buffer: 4 % (w/v) SDS, 20 % (v/v) Glycerol, 0.004 % (w/v) Bromophenol blue, 10 % (v/v) 2-mercaptoethanol, 0.125 M Tris-HCl, pH 6.8 in ddH2O.

SDS-PAGE Running Buffer: 25 mM Tris, 192 mM Glycine, 0.1 % (w/v) SDS in ddH2O.

Coomassie Staining Solution: 0.1 % (w/v) Coomassie Brillant Blue R-250, 40 % (v/v) Methanol, 10 % (v/v) Glacial Acetic acid in ddH2O.

10X Transfer Buffer: 1,92 M Glycine, 250 mM Tris Base in 1 L. 200 ml Methanol is added to the solution containing 1X Transfer Buffer

10X TBS Solution: 500 mM Tris Base, 45% NaCl, pH: 8.4. 500µl Tween-20 is added to 1X TBS buffer.

NATIVE-PAGE

8% Seperating

	1 gel	2 gels	[Final]
dH_2O	3 ml	6 ml	
3 M TRIS pH=8.9	1.25 ml	1.25 ml	3.75 mM
30% Polyacrylamide	1.335 ml	2.67 ml	8%
20% APS	37.5 µl	75 µl	0.075 %
TEMED	2.5 µl	5 µl	0.05%

3% Stacking

	1 gel	2 gels	[Final]
dH_2O	2.1 ml	4.2 ml	
1 M TRIS pH=6.8	125 µl	250 µl	50 mM
30% Polyacrylamide	255 µl	510 µl	3%
20% APS	18.75 µl	37.5 µl	0.075 %
TEMED	1.25 µl	2.5 µl	0.05%

SDS-PAGE GEL

12% Seperating

	1 gel	2 gels	[Final]
dH_2O	2.31 ml	4.62 ml	
3 M TRIS pH=8.9	625 ml	1.25 ml	3.75 mM
30% Polyacrylamide	2 ml	4 ml	12%
20% SDS	25 µl	50 µl	0.1%
20% APS	37.5 µl	75 µl	0.075 %
TEMED	2.5 µl	5 µl	0.05%

8% Stacking

	1 gel	2 gels	[Final]
dH_2O	1.925 ml	3.85 ml	
1 M TRIS pH=6.8	125 µl	250 µl	50 mM
30% Polyacrylamide	425 µl	850µl	8%
20% SDS	5 µl	10 µl	0.1%
20% APS	18.75 µl	37.5 µl	0.075 %
TEMED	1.25 µl	2.5 µl	0.05%

APPENDIX F

Equipments

AKTA Prime: GE-Healthcare, SWEDEN

Autoclave: Hirayama, Hiclave HV-110, JAPAN, Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA

Centrifuge: Eppendorf, 5415D, Eppendorf, 5415R, GERMANY, ThermoScientific, SORVALL RC6+ Centrifuge, USA

Circular Dichroism: JASCO J-810 Spectometer, USA

Dynamic Light Scattering: Malvern, Zetasizer Nano-ZS, UK

Deepfreeze: -80^{o}C, Kendro Lab. Prod., Heraeus Hfu486, GERMANY, -20^{o}C, Bosch, TURKEY

Distilled water: Millipore, Elix-S, Millipore, MilliQ Academic, FRANCE

Electrophoresis: Biogen Inc., Biorad Inc., USA

Ice Machine: Scotsman Inc., AF20, USA

Incubator: Memmert, Modell 300, Modell 600, GERMANY

Laminar Flow: Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY

Magnetic Stirrer: Witeg, WiseStir Magnetic Stirrer, GERMANY

Microliter Pipette: Gilson, Pipetman, FRANCE

Microwave Oven: Bosch, TURKEY

pH Meter: WTW, pH540 GLP MultiCal, GERMANY

Power Supply: Biorad, PowerPac 300, USA

Refrigerator: +4^{o}C, Bosch, TURKEY

Shaker: Forma Scientific, Orbital Shaker 4520, USA, GFL, Shaker 3011, USA, New Brunswick Sci., Innova 4330, USA

Sonicator: BioBlock Scientific, Vibracell 7504, FRANCE

Spectrophotometer: Nanodrop, ND-1000, USA

Thermocycler: BIORAD, C1000 Touch Termal Cycler, USA