CLONING AND EXPRESSION OF BETA SUBUNIT (AGB1) OF HETEROTRIMERIC G-PROTEIN COMPLEX FROM *A.thaliana*

by

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ABSTRACT

Heterotrimeric guanine nucleotide-binding proteins(G proteins) act as molecular switches in signaling pathways by coupling the activation of receptors at the cell surface to intracellular responses.The heterotrimeric G protein complex consists of alpha, beta and gamma subunits. G proteins are the most prevalent signaling systems in mammalian cells, playing a role in the regulation of sensory perception, cell growth and hormonal regulation. In plants, G proteins play regulatory roles in multiple developmental processes ranging from seed germination and early seedling development to root development and organ shape determination. Our group is involved in structural studies of G proteins from *Arabidopsis thaliana* and the work presented in this thesis contributes to the development of purification process of AGB1 protein in *E.coli.*

The aim of this study was to express the *A.thaliana* beta subunit (AGB1) in *E.coli* using pMCSG7 vector. In this system the protein is expressed with a his-tag which can be removed after digestion with tobacco etch virus (TEV) protease at the TEV cleavage site introduced from the vector. For cloning AGB1 gene into pMCSG7 vector, ligation-independent cloning (LIC) which allows insertion of DNA fragments independent of restriction sites and ligases was used. The AGB1 gene (in pMCSG7 vector) was expressed in Top10, DH5α and BL21plus* *E.coli* cells. The optimum protein expression was observed in BL21plus* cells. Efforts focused on optimization of protein expression and on obtaining AGB1 in a pure state by developing a purification protocol involving affinity, ion exchange and size exclusion chromatography.

Another aim of this study was to establish the protocols for expression and purification of Tobacco Etch Virus (TEV) protease in our lab in order to cleave the his-tag from AGB1

efficiently in a cost effective way.pMHTDelta238 vector (with TEV protease gene) was introduced intoBL21De3 cells. His tagged TEV protease was expressed and isolated with affinity chromatography. The activity of TEV protease was confirmed using a control protein called BTL2 and western blot analysis.

The purified AGB1 protein was cleaved with TEV protease to remove the his-tag. Results were observed by western blot and the efficiency of cleavage with TEV protease was verified by comparing uncleaved and cleaved AGB1 protein samples.

ÖZET

Heterotrimerik G proteinleri, hücre zarı yüzeyinde bulunan reseptörlerin uyarılmasıyla hücre içinde tepki oluşumuna neden olarak sinyal iletiminde moleküler anahtar olarak görev yaparlar(Oldham, 2006). Heterotrimerik G proteinleri alfa, beta ve gama alt birimlerinden oluşur. Memeli hücrelerinde G proteinleri en yaygın sinyal iletim sistemlerini oluştururlar. Duyuların algılanması, hücre büyümesi ve hormonal düzenlemede rol alırlar. Bitkilerde ise G proteinleri, tohum çimlenmesi ve fide gelişiminden kök gelişimi ve organların şekillerinin belirlenmesine kadar birçok gelişimsel süreçlerde rol alırlar(Chen, 2008).

Bu çalışmada, *A.thaliana* heterotrimerik G-proteini beta altbiriminin(AGB1) pMCSG7 vektörü kullanılarak *E.coli* hücrelerinde ekspres edilmesi amaçlanmıştır. Bu sistemde, protein bir his etiket(his-tag)ile ekspres ifade edilir ve sonrasında vektörden gelen TEV(Tobacco etch virus) tanıma bölgesinden TEV proteaz ile kesilerek histidin etiket bölgesi uzaklaştırılır. AGB1geninin pMCSG7 vektörüne yerleştirilmesinde LIC (ligation independent cloning) klonlama yöntemi kullanıldı. Bu yöntem genin restriksiyon bölgelerinden ve ligazdan bağımsız olarak klonlanmasını sağlar.AGB1 geni (pMCSG7 vektöründe) Top10, DH5α ve BL21plus* *E.coli* hücrelerinde ifade edildi ve optimum gen ifadesi BL21plus* hücrelerinde görüldü.Burada beklenti, bu ekspresyonu optimum hale getirip proteini daha saf halde elde etmekti.AGB1 proteinin saflaştırılmasında afinite, iyon değişimi ve moleküler elek kromatografisi yöntemleri kullanıldı.

Bu çalışmaya ek olarak laboratuvarımızda TEV proteazın ifadesi ve saflaştırılması ile ilgili protokolün geliştirilmesi de amaçlanmıştır. TEV proteaz geni içeren pMHTDelta238 vektörü transformasyon yöntemi ile BL21De3 hücrelerine aktarıldı. Yapısında histidin etiketi bulunduran TEV proteaz ifade edilip afinite kromatografisi yöntemi ile saflaştırıldı. TEV proteazın aktivitesi kontrol BTL2 proteini ile western yöntemi uygulanarak doğrulandı.AGB1 proteini ayırma ve saflaştırma yöntemleri sonucunda TEV proteaz ile kesilerek histidin etiketi uzaklaştırılmaya çalışıldı.Sonuçlar western yöntemi ile incelendi. TEV proteaz ile kesme işlemindeki verim western yönteminde protein örneklerinin yaptıkları ışımaya göre doğrulandı.

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ABBREVIATIONS

AGB1	Beta Subunit of *A. thaliana* G-Protein
bp	Base pair
BSA	Bovine serum albumin
CD	Circular dichroism
Da	Dalton
DNA	Deoxyribonucleic acid
d_{max}	Maximum distance
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFPI:	EDTA-Free protease inhibitor cocktail
FT:	Flow Through
GTPase:	Enzyme hydrolyzing GTP to GDP
HCl	Hydrochloric acid
HEPES	4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl-D-thiogalactoside
kDa	Kilodalton
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Milliliter
µl	Microliter
NaCl	Sodium chloride
Ni	Nickel
nm	Nanometer
N-terminus:	Amino Terminus
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonylfluoride
SDS	Sodium dodecyl sulfate

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1. Introduction

1.1.Heterotrimeric G proteins

1.1.1. Heterotrimeric G protein structure and Mechanism of signaling

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are molecules that participate in transmission of signals from outside into the cell. G proteins are activated by G protein coupled receptors (GPCRs), which are 7 transmembrane proteins (Temple, 2006). There are nearly 900 different heptahelical receptors known in mammalian systems (Fredriksson and Schioth, 2005), which interact with a relatively small number of heterotrimeric G proteins composed of α, β and γ subunits. It is shown that in humans there are 21 Gα subunits encoded by 16 genes, 6Gβ subunits encoded by 5 genes and 12 Gγ (Downes and Gautam, 1999).

G proteins act as molecular switches in signaling pathways by coupling the activation of heptahelical receptors at the cell surface to intracellular responses (Oldham, 2006). The activity is regulated by hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) at the α subunit. The activation of receptors induces the exchange of GTP for GDP on G_{α}. The G_{α} subunit has an intrinsic GTPase that can be accelerated by regulator of G protein signaling (RGS) proteins which can also block interaction between G_{α} and its effectors (Temple, 2006).

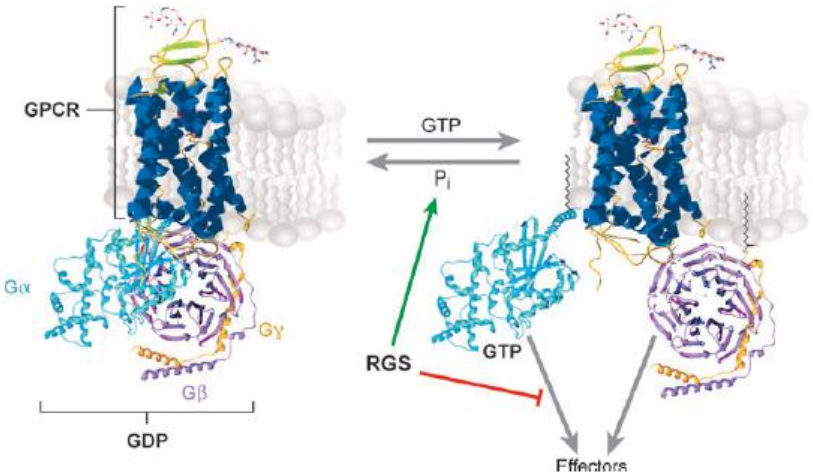


Figure 1.1 The classical model for G protein cycling in animals (Temple, 2006).

Left side in figure 1.1. represents the heterotrimer, GDP bound to G_{α} whereas on the right of figure 1.1.G_{α} becomes loaded with GTP and the heterotrimer dissociates into a free G_{α} subunit and a free Gβγ dimer.

When a ligand binds to the receptor, the alpha subunit becomes activated and separates from the beta-gamma subunits. This dissociation activates other proteins in the signal transduction pathway.

1.1.2. G protein α subunit

Heterotrimeric G proteins are typically divided into four main classes based on the primary sequence similarity of the Gα subunit: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (Simon et al. 1991). As shown in figure 1.2, structure of α-subunit consists of two domains: one is a Ras-like domain with six β strands surrounded by five α helices and the other is dominated by α helices and loop regions called the helical domain.

The members are composed of six stranded β sheet surrounded by five α helices.

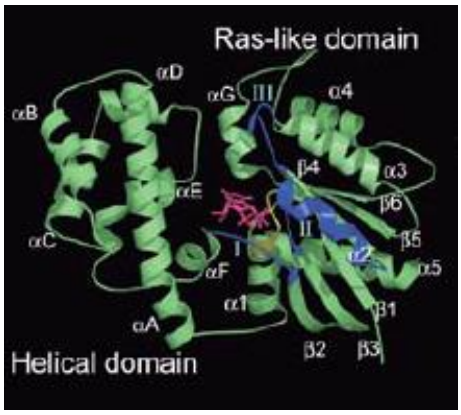


Figure 1.2 The crystal structure of Gα. (Cudden, 2004)

Functions of the helical domain include increasing the affinity of Gα for guanine nucleotides (Warner et al.1998 and Remmers et al. 1999a). Nucleotide binding resides in the cleft of the Ras like domain. The most highly conserved sequences are guanine nucleotide binding, the diphospate binding loop, the Mg^{2+} binding domain and the guanine ring binding motifs in the Ras-like domain (Oldham, 2006). The domain also contains three other flexible loops near the γ-phosphate binding sites. Here, significant structural differences between GDP-bound (Lambright et al. 1994 and Mixon et al. 1995) and GTP-bound (Noel et al. 1993 and Coleman et al. 1994) conformations of Gα are found. It is indicated that in *Arabidopsis thaliana*, GPA1-GDP is unstable relative to Gα-$GTP_\gamma S$ and Gβγ dimer stabilize GPA1-GDP (Jones, 2011). GTPγS is the non-hydrolysable form of GTP which maintains the activated conformation of Gα (Kato, 2004)

The GTPase domain with a site for hydrolysis of GTP contains also sites for binding to Gβα dimer, heptahelical receptors and downstream effector proteins. Functions of the helical domain also include increasing the GTP hydrolysis activity of the protein (Markby et al. 1993).This domain also is the most divergent among Gα subunits. This may also play a significant role in coupling specific G proteins to specific effectors (Liu *et al.* 1998). Less is known about the structure of other important regions of Gα.

1.1.3. G protein βγ dimer

In mammals, there are six Gβ subunits with ~36kDa and all share 50-90% sequence identity, with the long and short splice variants of $G\beta_5$ being the least similar to the other four members of the family(Downes and Gautam, 1999). All Gβ subunits contain seven WD-40 repeats, a tryptophan-aspartic acid sequence that repeats about every 40 amino acids, and forms small antiparallel β strands (Neer, 1994). As seen in figure 1.3, these seven repeats of the Gβγ subunit fold into a seven bladed β-propeller but the N terminus forms an α helix.

The N-terminal α-helical conformation that forms a coiled coil is essential for interaction with Gγ (Garritsen *et al.* 1993 and Sondek et al. 1996). In *Arabidopsis thaliana*, the removal of relatively short N terminus (coiled coil domain) from β subunit abolishes the interaction with the full length AGG2 (Mason, 2001). This interaction seems to be important for the proper folding and function of Gβ (Iniguez and Lluhi *et al.* 1992; Higgins and Casey, 1994).

Members of the Gγ family are small proteins, between 7 and 8kDa sharing 30-80% sequence identity (Downes and Gautam, 1999). As shown in figure 1.3, Gγ folds into two α helices; the N terminal helix forms a coiled-coil with the helix of Gβ, while the C terminal helix makes extensive contacts with the base of the Gβ torus (Lambright, 1996). The function of N-terminal helix is to interact with the N-terminus of Gβ, whereas the C-terminal binds to blades 5 and 6 of Gβ (Sondek *et al.* 1996).

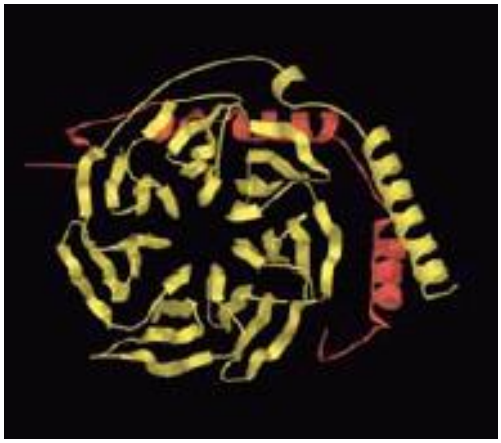


Figure 1.3 The structure of $G\beta_1\gamma_1$ dimer showing the seven-bladed propeller structure of $G\beta_1$ (yellow).Gγ(red) forms two alpha helices that bind to a single alpha-helix of Gβ and to several of the WD40 blades. The Gβγ complex is a functional heterodimer that forms a stable structural unit (Mc Cudden, 2004).

The G-protein β and γ subunits form a functional unit that do not dissociate except by denaturation (Schmidt *et al.* 1992). Most β subunits interact with γ subunits but not all of the possible combinations of dimers are actually formed (Clapham and Neer, 1997).For example, $G\gamma_1$ interacts only with $G\beta_1$ but not with $G\beta_2$ even though there is a 87% similarity between these beta and gamma subunits. In another example, $G\beta_2$ does not bind to $G\gamma_2$ despite it is 41% similar to $G\gamma_1$ (Pronin and Gautam, 1992; Schmidt et al. 1992; Garritsen and Simonds, 1994; Downes and Gautam, 1999). Unlike Gα subunit, the Gβγ complex does not change its conformation when it dissociates from the G protein heterotrimer (Sondek, 1996).Another important thing is that the association of Gβγ dimer with Gα prevents the dimer from activating its effectors. This suggests that the binding sites on Gβγ for Gα and the effectors of Gβγ are shared (Cudden, 2004). Several different Gβγ dimers can interact with the same Gα isoform (Graf et al. 1992).This suggests that different Gβγ dimers and subcellular localization may be an important determination of signaling specificity.

1.2.Regulation of G protein Signaling

The basic model of G-protein signaling can be expanded to include two additional regulatory mechanisms, non-receptor guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs) (Oldham, 2004). Several proteins are identified which increase the GTPγS binding to Gα and these proteins appear to be selective for $G\alpha_i$ family. GTPγS is the non-hydrolysable form of GTP which maintains the activated conformation of Gα

(Kato, 2004).As an example, RIC-8A plays a critical role in the asymmetric cell division during embryogenesis in *C.elegans* (Mc Cudden *et al.* 2005). Another example is AGS1 which plays role in the circadian cycle by processing signals in the suprachiasmatic nucleus (Sato *et al.* 2006).

GEFs cause GDP release whereas the class of GDI proteins inhibits GDP dissociation from $G\alpha_i$ family members (Mc Cudden *et al.* 2005). Progress is made but still additional work remains before the regulation of the GEF activity of these proteins are completely understood. The GDI proteins have been implicated as crucial players in asymmetric cell division during embryogenesis. These proteins contain motifs called GoLoco domains which consist of 19 amino acid sequences. They slow down the spontaneous GDP release from Gα (GDP).

1.3. G proteins in plants and mammalians

G proteins are the most prevalent signaling systems in mammalian cells. They play role in the regulation of sensory perception, cell growth and hormonal regulation. Whereas in plants G proteins play regulatory roles in multiple developmental processes ranging from seed germination and early seedling development to root development and organ shape determination (Chen, 2008). In mammals, several Gα and Gβγ interacting effectors were identified (Sunahara *et al*. 1996).None of these mammalian effectors were found in *Arabidopsis thaliana* (Jones and Assmann, 2004).The results of pharmacological and genetic studies have provided evidence that the plant heterotrimeric G protein is involved in the transmission of light (Warpeha, 1991) and hormone signals (Bowler, 1994) as well as regulation of ion channels (Wu, 1994).

Molecular modeling of the *Arabidopsis* G-protein complex based on an experimentally determined structure of a mammalian G-protein complex revealed known atomic interactions within the mammalian complex that are conserved in primordial G proteins. There are amino acid residues conserved within all the plant heterotrimeric complex model (Ullah *et al.* 2003). There are conserved residues that match in all mammalian Gα subunits. It was proposed that Gα evolution along the mammalian lineage consisted of gene duplications coupled with random mutations hitting at different residue positions, but at the same functional regions in different gene copies. Gene duplication along the plant lineage was a relatively rare event, with most plant species possessing a single Gα, a single Gβ, and only 2 Gγ subunits. Similar to plant Gα proteins, plant Gβ and Gγ subunits contain invariant, class-specific, and plant-specific amino acid residues when compared to the multigene families of mammalian subunits (Temple, 2006). There is a

much smaller number of genes encoding each of the different G protein components in plants than in other eukaryotes (Jones, 2002)

To get a better understanding of the role of G protein subunits in plants the role of these subunits were studied by studying the mutant forms of these proteins. *Arabidopsis gpa1* mutant plants, which lack the Gα protein exhibit reduced cell division during leaf formation (Ullah, 2001). Homozygous *gpa1* mutant plants are less sensitive to abscisic acid inhibition of stomatal opening (Wang, 2001).In contrast, *gpa1* mutant seeds show hypersensitivity to abscisic acid causing inhibition of germination suggesting they are more dormant. However overexpression of GPA1 causes ectopic cell division, including meristem proliferation (Okamoto, 2001). Recent studies revealed some major differences of these proteins from the animal system; GPA1 can activate itself without GPCR or other guanine nucleotide exchange factor (Johnston, 2007).

When plant and animal Gα subunits are compared the general amino acid sequence homology of NPGPα1 (*Nicotiana plumbaginifoliamaize)* to animal Gαs is not higher than 40% (Itoh, 1988; Thambi, 1989).When plant Gαs are compared significant homology is seen between plants; NPGPα1 (*Nicotiana plumbaginifoliamaize)* exhibits sequence identity of 75% to rice RGα1 (Seo, 1995) and 94,8% to tomato TGα1(Ma, 1990; Gotor, 1996)

It is also reported that GPA1 exist primarily in the monomeric state *in vivo* (Wang, 2008). These findings suggest that due to persistent self-activation, GPA1 may exist permanently in the dissociated and GTP-bound state.

To better understand the role of Gβ, mutant Gβ Arabidopsis were studied. Results showed that mutant *agb1*plants lacking Gβ showed alterations in leaf, flower and fruit development and decreased hypocotyl cell division (Wang, 2006; Chen, 2006). Recent studies showed that important surface residues of AGB1, which were deduced from comparative evolutionary approach, were mutated to dissect AGB1-dependent physiological functions. Results revealed AGB1 residues critical for specific AGB1-mediated biological processes, including growth architecture, pathogen resistance, stomata-mediated leaf-air gas exchange (Jiang *et al*. 2012). Also G protein β subunit cDNAs of maize (ZGβ1) and *Arabidopsis thaliana* (AGB1)were translated. They shared strong sequence similarities with each other suggesting that these proteins have the same function(s) in these different plants (Weiss *et al.*1994). In general, plant G protein β subunits share significant homologies with each other. For example, *NPGPB1* (*Nicotiana plumbaginifoliamaize)* shares the following homologies; with maize ZGβ1 77,7%(Weiss, 1994), rice RGβ178,2% (Ishikawa, 1995) and *Arabidopsis thaliana* AGB1

80,4%(Weiss, 1994).However the homology with animal Gβs is low, extending not more than 48% identity (Codina, 1986).With respect to their number of amino acid residues plant Gβ sequences(about 41kDa) are about 40 amino acid residues longer than those of most animal Gβs(35-36kDa) (Kaydamov, 2000).

As mentioned before, there are two Gγ subunit genes in *Arabidopsis*; AGG1 and AGG2.The mutant analysis indicates that each Gγ subunit participates in a subset of G_{β} related developmental processes (Trusov, 2006).

1.4. Objective of the Study

The aim of this study was to clone and express the *A. thaliana* beta subunit (AGB1) in *E.coli* cells using pMCSG7 vector. Using this ligation independent cloning (LIC) vector AGB1was expressed with his-tag and a TEV protease cleavage site which facilitates the removal of the his-tag after purification. In a more general context, the expectation was to optimize the expression of the protein and to obtain large quantities of pure stable AGB1 for in vitro complexation with alpha and gamma subunits and for investigation of the structure of the subunits and the heterotrimeric complex by solution X-ray scattering.

Another aim of this study was to express TEV protease in *E.coli* and to purify the protein for cleavage of his-tag from AGB1 to obtain the recombinant protein in a more native state.

2. Materials and Method

2.1. Materials

2.1.1. Buffers and Solutions

Binding buffer for HisTrap Chromatography: 50 mM NaPO4, pH7.4, 150 mMNaCl, 2 mM $MgCl_2$

Binding buffer for QTrap Chromatography: 50 mMTris –HCl pH: 8.0

Coomassie Staining Solution: 0.1 % (w/v) CoomassieBrillant Blue R-250, 40 % (v/v) Methanol, 10 % (v/v) Glacial Acetic acid in ddH_2O.

Destaining Solution: 4 % (v/v) Methanol, 7.5 % (v/v) Glacial Acetic acid, completed to 1 L.

Dialysis buffer before QTrap chromatography: 50 mMTris –HCl pH: 8.0

Dialysis and Running buffer for Size Exclusion Chromatography (Hepes Buffer): 20 mMHepes, 50 mMNaCl, 1mM PMSF

Elution buffer for HisTrap chromatography: 50 mM NaPO4, pH7.4, 150 mMNaCl, 2 mM $MgCl_2$ and 1 M Imidazole

Elution buffer for QTrap Chromatography: 50 mMTris –HCl pH: 8.0 + 700 mMNaCl

Elution buffer for Ni-NTA column: 20 mM Tris pH 8.0, 150 mMNaCl, 400 mM imidazole, 2 mM BME, 10 % glycerol

High Salt buffer: 20mM Tris pH 8.0, 1M NaCl, 1mM imidazole, 2mM BME

50mM imidazole: 20mM Tris pH 8.0, 150mM NaCl, 50mM imidazole, 2mM BME

Lysis buffer for AGB1 purification: 50 mM NaPO4, pH7.4, 150 mMNaCl, 2 mM $MgCl_2$ 1X EDTA Free Protease Inhibitor, 5% glycerol and 2 mM PMSF/2mg/ml lysozyme and 25µl/ml DNAase are added just before usage

Lysis buffer for TEV protease purification:20mMTris pH 8.0, 150 mMNaCl, 1 mM imidazole, 2 mM BME/ 2mg/ml lysozyme and 25µl/ml DNAase are added just before usage

Wash buffer for HisTrap Chromatography: 50 mM NaPO4, pH7.4, 150 mMNaCl, 2 mM $MgCl_2$ and 10 mM Imidazole

SDS-PAGE Running Buffer: 25 mMTris, 192 mM Glycine, 0.1 % (w/v) SDS in ddH_2O.

Transfer buffer: 14.41 g. Tris base, 3.028 g. Glycine and 200 ml methanol in 1 L

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 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 ddH_2O.

6X SDS gel loading buffer: 125mM Tris-HCl pH6.8, 2% SDS, 20% glycerol, 0.2%bromophenol blue, 10% (v/v) β-mercaptoethanol

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

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

All buffers and solutions, except those provided by commercial kits were prepared according to (Sambrook, 2000).

2.1.2. Cell lines

BL21plus*, DH5α, and TOP10 *E.coli* strains were used (EMBL, Hamburg)

2.1.3. Chemicals and Enzymes

All chemicals were supplied by Stratagene, QIAGEN, Merck (Germany), Bioron, Fermentas, Riedel, Amresco, AppliChem, and SIGMA (USA).

For restriction BamHI, NcoI, and SspI enzymes, for ligation T4 DNA polymerase enzyme, for LIC T4 DNA polymerase enzyme and for PCR reactions Taq polymerase enzymes were used. All were purchased from Fermentas.

2.1.4. Commercial kits and protein purification columns

Qiaquick PCR Purification, Qiaquick Gel Extraction and Qiaprep Spin Miniprep Kits (QIAGEN) were used in recombinant DNA manipulations and molecular screenings.

HisTrap 5 ml Column (GE Healthcare) was used for affinity chromatography

The ion exchange column QTrap 5 ml (GE Healthcare) and size exclusion column

HiLoad 16/60 Superdex75pg(GE Healthcare) were used.

2.1.5. Culture Media and Antibiotics

LB broth (Luria-Bertani): 5 g Yeast extract, 10 g Tryptone, and 5 g NaCl in 1 liter.

LB Broth Agar: 15g agar-agar for 1 liter of LB Broth medium.

Terrific Broth (TB):12 g Tryptone, 24 g yeast extract, 4 ml glycerol, 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 per 1 liter.

Kanamycin and Ampicillin antibiotics were prepared with a final concentration of 50µg/ml

2.1.6. Equipments

Equipments used are listed in the Appendix G

2.1.7. Primers

Primers for the cloning of AGB1 for pMCSG-7 vector was designed according to literature (Swarbreck, 2000).

		Primer sequences
Primers for gene amplification from pQE80 vector	Forward AGB1F	**5' – TCTGTCTCCGAGCTCAAA – 3'**
	Reverse AGB1R	**5' – TCAAATCACTCTCCT – 3'**
Primers for pMCSG7 LIC Cloning	Forward LICF	**5' –TACTTCCAATCCAATGAAATGTCTGTCTCCG– 3'**
	Reverse LICR	**5' – TTATCCACTTCCAATGAATCAAATCACTCTC – 3'**

Table 2.1 Primers designed for PCR

Primers used for LIC site addition were designed according to literature (Doyle Sharon A. (ed.), *Methods in Molecular Biology: High Throughput Protein Expression and Purification, vol. 498, 2009)*

Maps of vector pMCSG7 is given in Appendix A

2.1.8. Vectors

AGB1 gene was initially obtained from pQE80-L vector.(Kaplan, 2009) (Appendix A) Map of pMCSG7 (Harvard) vector can be found in AppendixA. pMCSG-7 vector contains 6 His tag for affinity chromatography. pMHTDelta238 vector was used for TEV protease expression (Appendix B) which has also a his-tag.

2.2. Methods

2.2.1. Ligation independent cloning of AGB1 using pMCSG7 vector

AGB1 gene had previously been cloned in the group using the pQE80-L vector (Kaplan, 2009). Here, the gene was cloned into pMCSG7 vector

2.2.1.1 Amplification of AGB1 gene with PCR

A two-step PCR was used to clone this gene using the pMCSG7 vector which encodes a TEV protease site for removal of tags that facilitate protein purification (his-tag). In the first step, the gene was amplified from the pQE80 construct using gene specific primers corresponding to the exact sequences at 5' and 3' and of AGB1.This amplified fragment was used as template for further PCR. Details of the PCR mixture and PCR conditions are given in tables 2.2. and 2.3.

Master mix	5µl
Primers(each)	1 µl *1/5 dilution with dH_2O, 100mM is changed into 20mM
Taq polymerase	1 µl
template	1 µl
dH_2O	16 µl
Total:	*24µl*

Table 2.2 PCR ingredients and their amounts

Annealing temperature for AGB1R/F PCR was 47.5C

Annealing temperature for LICBR/F PCR was 58.4C

95°C,	5 minutes	
95°C,	1 minute	32 cycles
47.5°C, or 58.4°C,	45 sec	
72°C,	45 sec	
72°C,	5 minutes	
4°C		Stored for use

Table 2.3 PCR cycles for AGB1 gene amplification

After analysis of PCR products on gel, the samples were purified with ethanol precipitation method. EtOH precipitation of PCR product: The 144 µl reaction volume was increased to 200 µl by ddH_2O.21.4 µl 3M NaOAc, pH 5.2, 535 µl isopropanol and 10.7 µl %0.5 LPA were added to the tube. The tubes were incubated at -80°C o/n. Samples were centrifuged for 15 min at max speed and resuspended in 250 µl 70% EtOH. The resuspended samples were centrifuged for 5 min at max speed and dried in laminar flow. Finally the samples were resuspended in 25 µl ddH_2O.

2.2.1.2. Preparation of pMCSG7 vector

o/n of 5ml of cells containing pMCSG7 plasmid in LB medium were purified by Qiaspin Miniprep Kit. SspI digestion was performed. SspI enzyme cuts the AATATT pallindromic sequence bluntly which enables the enzyme to be exposed to the activity of T4 DNA Polymerase enzyme.

SspI	1µl
template	X (10 µg)
dH_2O	Y
Fast digest buffer	3 µl
Total:	*30µl*

Table 2.4 SspI Digestion of pMCSG7 plasmid

Digestion took place at 37C for 16 hours o/n. The digested vectors were examined by %1agarose gel. For obtaining purified digested vectors Qiaquick Gel Extraction Kit was used. Initial weight of 4 eppendorf tubes were measured and written down. The bands in gel containing pMCSG7 plasmid were cut under UV light. Final weights of eppendorf tubes (containing gel) were measured. Qiagen gel extraction protocol was followed. The plasmid was concentrated in 2 columns at the end. Final elution was done with 25 µl for each column.

2.2.1.3. T4 polymerase reaction

T4 DNA Polymerase has two functions: formation of a phosphodiester bond from 5' to 3' and exonuclease activity from 3' to 5'; both cutting and pasting nucleotides. When the enzyme recognizes a nucleotide, the polymerase function gets activated, if not, the enzyme simply acts as an exonuclease. Only one type of dNTP is added to the reaction tube so the enzyme simply creates single strand DNA's out of any blunt-ending oligonucleotide it encounters that does not possesses that specific dNTP. When T4 Polymerase reaches the nucleotide complementary to the dNTP, the enzyme both adds and cuts the same nucleotide over and over again so a flanking single strand DNA of interest can be obtained (Stols, 2002).

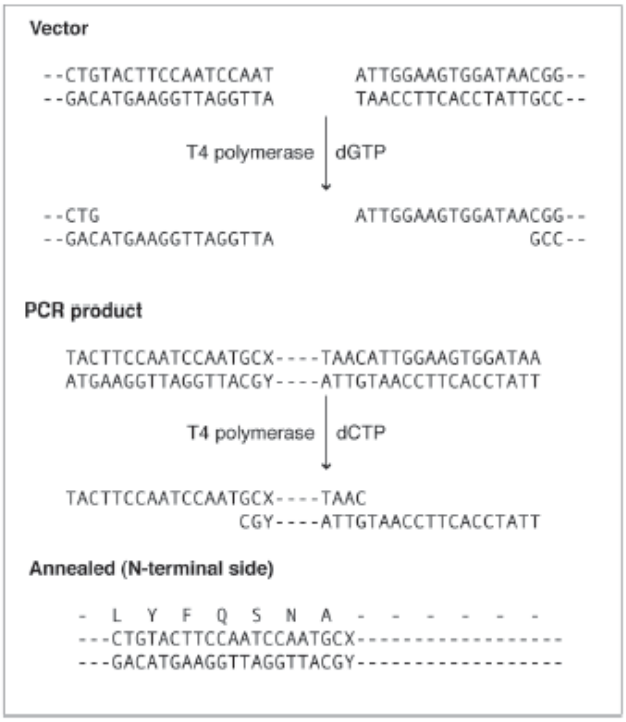


Figure 2.1 Cleavage with *Ssp*I (a blunt cutter) followed by treatment with T4 DNA polymerase (Doyle Sharon A. (ed.), *Methods in Molecular Biology: High Throughput Protein Expression and Purification, vol. 498., 2009)*

Insert reaction	
dd H_2O	2µl
5X buffer	14 µl
d CTP	1.75µl(35nmol)
insert	50 µl
T4 DNA polymerase	2 µl
	70 µl

Vector reaction	
dd H_2O	2µl
5X buffer	14 µl
d GTP	1.75 µl(35nmol)
Vector	50 µl
T4 DNA polymerase	2 µl
	70 µl

Table 2.5 Insert and Vector reaction during T4 DNA Polymerase reaction

*d GTP and d CTP are taken from 100µM stock

*reaction lasted for 50min at 20^0C followed by 30min at 70^0C

The T4 DNA polymerase reaction samples were purified withEtOH precipitation: The reaction products were precipitated by ethanol. The precipitation was done for both vector and insert in separate tubes.

The 70µl reaction volume was increased to 100µl by ddH_2O. 5µl 3M NaOAc, pH 5.2, 250µlEtOH (100%) and 5µl %0.5 LPA were added to the tubes. The tubes were incubated at -80^oC for 1 hour. Then they were centrifuged for 15 min at max speed.250µl 70% EtOH (cold) was added to pellet (pellet was not resuspended). 5 min max speed centrifuge and the tubes were air dried (no liquid was left behind). Both vector and sample were resuspended in 12µl ddH_2O.

2.2.1.4. Annealing of insert and vector

Concentration determination of insert and the vector were determined by running them on a %1 agarose gel. The samples and the DNA ladder mix were loaded as 1µl and 2µl. By comparison the bands of marker and samples concentrations were determined.

The concentrations of insert and vector were determined by molar ligation ratio calculation:

$$\left(\frac{(ng\ vector) * (kb\ size\ of\ insert)}{(kb\ size\ of\ vector)}\right) * (Molar\ ratio\ of\ insert/vector) = (ng\ insert)$$

Vector: insert ratio	1-3	1-10
Amount of vector µl	X	X
Amount of insert µl	Y	Y
dd H_2O µl	As needed	As needed
Total volume µl	10	10

Table 2.6 Vector-insert amounts for annealing process

The insert and vector were placed in a tube according to the molar ratio defined as 1/3 and 1/10, and were incubated o/n at room temperature.

2.2.1.5. Preparation of competent cells

For the preparation of competent cells $CaCl_2$ solution was prepared.15ml 2M $CaCl_2$, 10ml 0.5M PIPES pH 7.0 and 86ml 87% glycerol were mixed and topped up to a volume of 500ml by addition of dH_2O. The solution was filtered. o/n of cells was prepared. 50ml LB was inoculated with cells taken from -80^{0}C in a 200ml flask. Next day, OD 600 value was measured. 4ml of this culture was added into 400 ml LB medium in a sterile 2L-flask in the hood. The culture was grown with moderate shaking 250 rpm at 37°C until OD_{600} ~ 0.375. Eight 50 ml pre-chilled (on ice) sterile polypropylene tubes were prepared and the tubes were left on ice for 5-10 min. Cells were kept cold for all subsequent steps. Cells were centrifuged for 10 min at 1600 g (3000 rpm), 4°C (in the cold room). The supernatant was poured off and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution. Cells were centrifuged for 5 min at 1100 g (2500 rpm), 4°C Supernatant was discarded and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution. Resuspended cells were kept on ice for 30 min. Eppendorf tubes were prepared on ice. Cells were centrifuged for 5 min at 1100 g, 4°C. Supernatant was discarded and each pellet was resuspended in 2 ml ice-cold $CaCl_2$ solution. Cells were pooled and mixed to a total volume of 16 ml in a polypropylene tube.150 µl cells were aliquoted in eppendorf tubes and freezed in liquid nitrogen immediately. Competent cells were stored at -80°C.

2.2.1.6. Transformation of cells

BL21plus*, DH5α andTOP10 cells were transformed with the ligated samples. Competent cells were taken from -80^{0}C, thawed on ice. 10µl of construct were added to the tubes of competent cells. After 30 min incubation on ice, the cells were heated to 42^{o}C for 90 seconds, placed on ice for 2 min. 800µl LB was added to the tubes and the cells were incubated at 37^{o}C for 1.5 hours. Incubated cells were centrifuged at 10,000 rpm for 5 min, 700µl was discarded and the rest was resuspended in 200 µl medium and spread on ampicillin LB agar plates. The cells were incubated at 37^{o}C o/n.

2.2.1.7. Verification of annealing by PCR screening

Samples from well-grown colonies were picked and incubated in liquid 5ml LB +amp (50µg/ml) o/n. Cells were prepared for plasmid isolation. Plasmid isolation was done with Qiaprep Spin Miniprep Kit (QIAGEN).

The isolated plasmids were tested for verification of the annealing process. Both primers were used in the PCR reactions.

Master mix	5µl
Primers(each)	1 µl *1/5 dilution with dH_2O, 100mM is changed into 20mM
Taq polymerase	1 µl
template	Colony sample taken with tip
dH_2O	16 µl
Total:	*24µl*

Table 2.7 Content of PCR tubes used in verification of annealing

The content of the reaction tubes were run on 1% agarose gel. The gels were examined under UV light with the help of ethidium bromide.

2.2.1.8 Verification of annealing by digestion of pMCSG7 plasmid

Samples from well-grown colonies were picked and incubated in liquid 5ml LB +amp (50µg/ml) o/n. Cells were prepared for plasmid isolation. Plasmid isolation was done with Qiaprep Spin Miniprep Kit (QIAGEN). Digestion was performed in the following amounts.

BamHI buffer 10X	3µl
KpmI	4µl
BamHI	2µl
Template x	2µg
dH_2O y	16 µl
	X+y=21
Total:	*30µl*

Table 2.8 amounts of construct and restriction enzymes used during digestion

PCR products and purified plasmids were analyzed by 1% agarose gel electrophoresis. Samples were mixed with 6X loading buffer (final loading buffer concentration 1X) and gels were run at 100 mV for 40 minutes. DNA Mass ruler (Fermentas) was used as DNA ladder and samples were visualized by using ethidium bromide.

2.2.1.9. Colony selection

Samples from well-grown colonies were picked and incubated in liquid 10ml LB +amp (50μg/ml) o/n. Cells were prepared for glycerol stock

2.2.1.10. Sequencing of construct for verification

Plasmids were purified with QIAGEN Plasmid Mini Kit (QIAGEN) and were sequenced by Refgen Company (Ankara)

2.2.2. Expression of AGB1 protein

2.2.2.1. Expressing AGB1 protein in different cells

BL 21 plus*, DH5α and Top10 glycerol stock cells were taken from -80°C and prepared for o/n growth (37°C and 270 rpm) in 3 different medium; LB, SOC medium and terrific broth.5ml liquid medium and 5μl ampicillin(50μg/ml)(final dilution 1/1000) was inoculated with cells. The next day, OD_{600} (600nm optical density) values were measured. Cells were placed into fresh 50ml medium with ampicillin with a final OD_{600} value of 0,15.Cells were incubated at 37°C and 270rpm conditions until the OD_{600} value reached 0,8. After this point the cells were induced for protein expression by 1mM IPTG and were incubated at 27°C and 250rpm conditions. This would slow down the harm to cells during the overexpression of protein. Every 40min the OD_{600} values were measured for 4hours. By using these measurements growth curves were analyzed so to understand the optimum cell culture for the protein expression. When all three types of cells were compared BL 21 plus* cells showed the highest expression.

At the end of the 4 hours the cells were pelleted by centrifugation. Pellets were lysed by using lysis buffer consisting of 25mM tris, 10mM EDTA, 50mM glucose and 1mg/ml lysozyme. The samples were prepared for SDS Page analysis. Gels were run at 100V for 45min. By coomassie blue staining the protein bands were analyzed. Protein ladder (Fermentas) was used for identifying the molecular weight of the protein. The band showing protein of interest was compared in the three different cells. BL 21 plus* cells showed the optimum protein expression.

2.2.2.2. Culture growth for AGB1 protein purification

100ml terrific broth and ampicillin (50mg/ml) was inoculated with BL21 plus* cells and incubated at 37°C and 270rpm.The next day, OD_{600} values were measured. The cells were transferred into 4x500ml terrific broth (+ampicillin) medium with a starting optical density of 0,

15. Cells were incubated at 37°C and 270rpm until OD_{600} value reached 0, 8. Then, 1mM IPTG was added for overexpression of protein at 27°C and 250rpm for 4,5hours. This would slow down the harm to cells during the overexpression of protein. A total of 2l medium was centrifuged in 6 large sorvall tubes at 9700rpm for 15min at $+4^0$C. The supernant was discarded. Pellet was resuspended in 60ml supernant, put into two falcon tubes and centrifuged at 4750rpm for 30min at $+4^0$C. The supernant was discarded and the pellet was put into -80°C.

2.2.3. Purification of AGB1 protein

Pellet of cells were taken from -80°C and resuspended in lysis buffer (25mM Tris, 10mM EDTA, 50mM glucose and 1mg/ml lysozyme). Sonication (8 second of pulse and 9 second rest period, total 15minutes) was applied to cells at 4 °C for further lysis. TritonX-100 was added so that the final concentration was %1 and the cells were stored at 4 °C for 1 hour. These cells were then centrifuged at 14000rpm for 1 hour at 4 °C.

2.2.3.1. Affinity Chromatography

Meanwhile the nickel affinity chromatography column histrap (GE Lifesciences) was prepared. The column was first washed with dH_2O and then with binding buffer. If the column was before, the column was also washed with elution buffer (preparation of buffers are explained in part 2.1.1. The lysate was injected into the system (about 100µl was set apart for gel analysis) and flow through (FT) was collected. After this point, the column was washed with wash buffer and collected. Finally 30ml elution buffer was given to the system which reaches %100 at the end of 30ml. Elution was also done without a gradient. When both applications of elutions were compared, it was concluded that without gradient the protein came more pure. This was understood by analysis of the 12% SDS –page results. 1ml of elution fractions were collected.

All fractions including lysate, FT and wash were loaded to 12%SDS page and analyzed. The protein content of fractions were measured by looking to their optic density OD_{600} values at 280nm.The fractions with high protein content were dialyzed against 20mM Tris pH8.0 with 1mM PMSF. Dialysis was performed o/n at 4 °C and the buffer was refreshed after 4hours.

2.2.3.2. Anion exchange chromatography

To get rid of any aggregates the dialysis samples were centrifuged at 13000rpm for 15minutes.The optic density OD_{600} values at 280nm were measured again. The anion exchange column Q-trap (GE Lifesciences) was used. It was washed first with dH_2O, then with Q-trap

elution buffer and finally with Q trap binding buffer. The protein sample was injected to the system (approximately was injected into the system (about 100µl was set apart for gel analysis) and flow through (FT) was collected. After this point, the column was washed with Q trap binding buffer. Finally, 20ml of elution buffer was applied with a gradient reaching %100 at the end of 20ml.

All fractions including before dialysis, after dialysis, FT and wash were loaded to 12%SDS page and analyzed. The protein content of fractions were measured by looking to their optic density OD_{600} values at 280nm.The fractions with high protein content were dialyzed against Hepes. Dialysis was performed o/n at 4 ^{o}C and the buffer was refreshed after 4hours.

2.2.3.3. Size Exclusion Chromatography

To get rid of any aggregates the dialysis samples were centrifuged at 13200rpm for 20minutes.The absorption value at 280nm were measured again. HiLoad 16/60 Superdex 75pg (GE Healthcare) was used for size exclusion chromatography. The system was washed with 2 column volumes of dH_2O and then with Hepes buffer. 2ml of protein (~15mg) was loaded to the system. Here, the flow rate was 1ml/min and fractions of 600µl were collected.

2.2.4. Analysis of AGB1 protein

2.2.4.1. Absorbance spectroscopy

For the concentration determination of protein Nanodrop Spectrophotometer (Thermo) was used. Measurement at 280nm was used for the calculation of protein concentration. The following formula was used for concentration determination.

$$\varepsilon * 10^{-4}(M^{-1}cm^{-1}) = \frac{A}{c*l} * 10^{-4}$$

($\mathcal{E}$: extinction coefficient,*A*:absorbance value, *c*: concentration in molar, *l*: pathlength in cm)

2.2.4.2. SDS polyacrylamide gel electrophoresis

The preparation of SDS-PAGE is given in Appendix D and 6X SDS gel loading buffer is described in part 2.1.1.20µl of protein sample was mixed with 6µl 6X SDS gel loading buffer(final 1X concentration).The samples were boiled at 95 ^{o}C for 3min. 10µl sample was loaded into SDS-PAGE. The gel was run using 1X SDS buffer AT 100V until the loading dye reached the bottom of the gel. For examining the protein bands the gels were stored in commasie blue o/n. Then, gels were destained in dH_2O.

2.2.4.3. Western Blotting

To analyze the protein bands with western blot the proteins were loaded into a SDS-PAGE. After the gel has run the protein bands were transferred onto a PVDF membrane (Thermo). Semi –dry Western Blot method was used. The system was as following:
top of the system-2 watman paper-transfer paper-gel-2watman paper-bottom of the system. The system was run at constant 265Amper for 70 minutes.

Milk solution was prepared for preventing unspecific binding of proteins in the membrane. For one paper the milk solution was prepared as 50ml 1X TBS and 2,5gr Non-fat dried bovine milk (sigma) were mixed. The membrane was put into the milk solution and waited for 1 hour. The transfer membrane was subjected to antibody 6His (Roche) and waited for 1 hour at room temperature. The membrane was washed with 1X TBS buffer 3times for 15minutes each. Then, the membrane was subjected to pierce ECL western blotting substrates (Thermo). In the dark room, kodak exposure films were exposed to the signals on the membrane. The blotted films were examined.

2.2.4.4. Circular dichroism spectropolarimetry

Circular dichroism (CD) is an excellent method for the study of the conformations adopted by proteins and nucleic acids in solution. Although not able to provide the beautifully detailed residue-specific information available from nuclear magnetic resonance (NMR) and X-ray crystallography, CD measurements have two major advantages: they can be made on small amounts of material in physiological buffers and they provide one of the best methods for monitoring any structural alterations that might result from changes in environmental conditions, such as pH, temperature, and ionic strength (Martin and Schilstra, 2008).

Circular dichroism (CD) refers to the differential absorption of left and right circularly polarized light (Atkins, 2005).It is exhibited in the absorption bands of active chiral molecules. It has a wide range of applications in different fields. UV CD is used to investigate the secondary structure of proteins (Nakanishi, 1994).

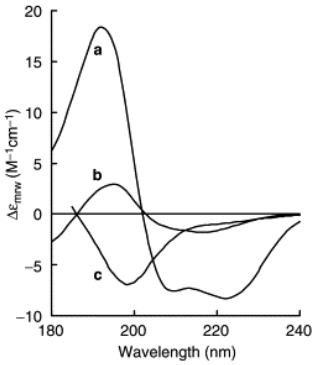


Figure 2.2.CD Spectra of three different conformations **a**) myoglobin (α),**b**) prealbumin (β) and **c**)acid denatured staphylococcal nuclease at pH 6.2 and 6 °C (unordered) (Martin and Schilstra, 2008).

1mm quartz cuvettes were used.200µl of protein samples were analyzed by CD spectropolarimetry (JascoJ-815 CD Spectropolarimeter). The samples were first diluted by 1/100 with dH_2O and to minimize the effect of hepes which has high salt concentration, it was diluted 1/100 with dH_2O. Spectra Manager software was used for the measurements.

2.2.4.5 Dynamic light scattering (DLS)

Dynamic light scattering is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution(Berne, 2000).

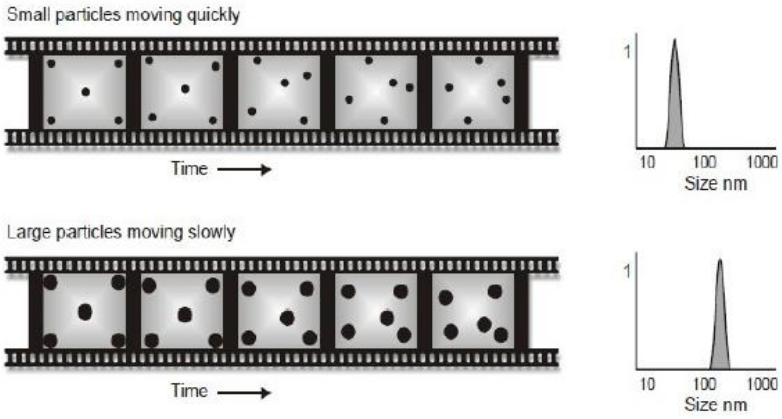


Figure 2.3. Determination of size of the particles in dynamic light scattering (Arzensek, 2000)**.**

The particles in a liquid move about randomly and their motion speed is used to determine the size of the particle.

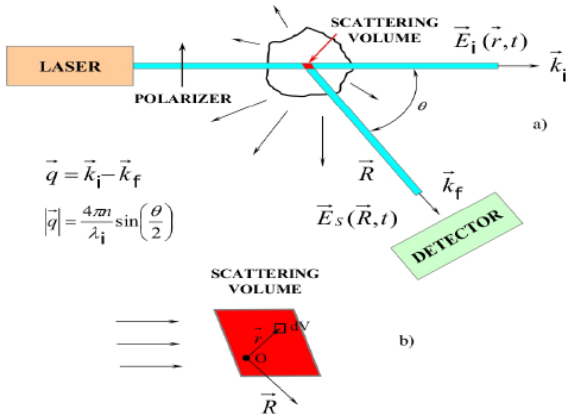


Figure 2.4a) Sheme of a typical light-scattering experiment, b) expanded view of the scattering volume(Kozina, 2009)

Depending on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium, the mean effective diameter of the particles can be determined if the system is monodisperse. If the system is monodisperse, there should only be one population, whereas a polydisperse system would show multiple particle populations. The diffusion coefficient of particles can be determined as DLS essentially measures fluctuations in scattered light intensity due to diffusing particles. Zetasizer (Malvern Instruments) machine was used for DLS measurements.

2.2.5. Expression of TEV protease

TEV protease is a very useful reagent for cleaving fusion proteins. TEV protease utilizes a 'catalytic triad', the three amino acid residues found inside the active site of certain protease enzymes, of residues to catalyze peptide hydrolysis (Waugh, 2010).The structure of TEV protease is similar to those of serine proteases like chymotrypsin (Phan *et al.* 2002).

2.2.5.1. Transformation of cells with TEV plasmid

BL21De3 competent cells were used in the transformation process. Preparation of competent cells was explained in part 2.2.1.1.5. Competent cells were taken out of -80^{o}C and thawed on ice. 10 µl of pMHTDelta238 TEV plasmid was added to the tubes of competent cells. The tubes were left on ice for 30 min. After cold incubation, the tubes were placed on a dry heat block at 42^{o}C for 90 sec. The tubes were placed on ice for 1 – 2 min. 800 µl LB was added on the cells and were incubated at 37^{o}C for 60min. Incubated cells were centrifuged for 5 min at 10.000 rpm. 700µL was discarded and rest was resuspended. 150 µL of bacteria was spread onto agar

plates (containing kanamycin). Cells were incubated at 37^{o}C overnight. Two additional control groups were also prepared; Competent BL21De3 cells were spread (150 µL) onto LB plate, competent BL21De3 cells were spread (150 µL) onto kanamycin containing LB plates.

2.2.5.2. Expression of TEV protease

100 mL LB with was inoculated with TEV-BL21De3 cells. 100µl kanamycin (50µg/ml) was added and grown overnight at 37 ^{o}C, 270 rpm. The next day, OD 600 value of culture was measured.4 x 2L flasks each containing 500 ml LB with 500µl kanamycin were inoculated with preculture with a starting OD_{600} value of 0,2. Cells were grown at 37^{o}C for 2.5 hours, 270 rpm. OD 600 value was measured. It should be around 0,8.1 mM was added and cells were grown at 25 ^{o}C for 5 hours. OD 600 value was measured. Cells were harvested at 9000 rpm for 15 minutes at 4^{o}C. Supernatants were removed; pellets were resuspended in a total of 30 ml LB. The resuspended cells in 30 ml were put into 2 50 ml falcon tubes and were harvested at 5000rpm for 30 min at 4^{o}C in the cold room. Supernatants were removed and pellet was obtained. Pellets were stored at -20^{o}C.

2.2.5.3. Purification of TEV protease

For purification of TEV protease 5ml Ni-NTA column was used. First lysis buffer was prepared (explained in part 2.1.1.) and for Ni-NTA column high salt buffer, 50mM imidazole buffer and elution buffer was prepared (explained in part 2.1.1.).

First the lysate was prepared. Pellets were taken from -20^{o}C and resuspended in 80ml lysis buffer and prepared for sonication process. Sonication: 5 sec on/5sec off /total duration 20 min. Lysate was kept on ice for 20 min at 4^{o}C in cold room. Then, lysate was centrifuged at 13000rpm for 20minutes.Supernatant was taken and divided into 2 falcon tubes. The content of the Ni-NTA column was divided equally into these tubes and put onto the shaker in the cold room and waited for 1hour.Column was first washed with dH_2O. Column was washed with 25ml lysis buffer. When there was 5ml lysis buffer left in the column the flow was stopped. All the steps are performed in cold room at 4^{o}C. Lysate was given to the column and flow through was collected(1ml was set aside for gel analysis).Column was next washed with 25ml lysis buffer and then washed with 25ml high salt buffer, wash 1 was collected. Next, the column was washed with 50ml 50mM imidazole buffer, wash 2 collected. Finally, 10ml elution buffer was added, put onto the shaker in cold room at 4^{o}C and waited for 30min.Fractions of 2ml were collected. All

fractions were analyzed on %12 SDS PAGE.TEV protease was stored as 500µl aliquots at -80 ^{0}C in TEV buffer; 20Mm Tris, 150mM NaCl, %50 glycerol.

2.2.6. Cleavage of AGB1 protein with TEV protease

For the control of AGB1 cleavage with TEV protease, BTL protein (a thermoalkalophilic lipase originating from *Bacillus thermocatenulatus*) was used as a control which has a TEV site with a his tag . This protein was obtained from Emel Durmaz from Sabancı University. Different concentrations and incubation times were performed. Optimum concentration of enzyme and protein are determined as the followings;

Cleavage of AGB1 with TEV protease:

TEV amount(µl) (O,5mg/ml)	AGB1(µl) (1,6mg/ml)	10X TEV buffer(µl)	dH_2O(µl)
0	20	4	16
1	20	4	15
2	20	4	14
8	20	4	8

Table 2.9 Amounts of AGB1 protein and TEV protease used during cleavage process samples were incubated at room temperature for 4 hours

Cleavage of AGB1 with TEV protease:

TEV amount(µl) (O,5mg/ml)	BTL2(µl) (1,58mg/ml)	10X TEV buffer(ul)	dH_2O(µl)
0	20	4	16
1	20	4	15
2	20	4	14
8	20	4	8

Table 2.10 Amounts of BTL2 protein and TEV protease used during cleavage process samples were incubated at room temperature for 4 hours

Preparation of 10X TEV reaction buffer:

500mM Tris.Cl pH 7,4

50mM EDTA

10mM DTT

The result of cleavage was examined on SDS PAGE and Western blot.

3. RESULTS

3.1 Cloning AGB1 gene into pMCSG7 vector

pMCSG7 vector is a vehicle for ligation independent cloning. Due to the advantages of this system over restriction enzyme dependent cloning, we worked to adapt the known protocol for routine use in our laboratory as explained in detail below.

3.1.1. Amplification of AGB1 gene

AGB1 gene was amplified by PCR with LIC site insertion primers (Table 2.1.) using the pQE80 construct as template. This construct had been developed in our lab previously (Kaplan, 2009). The gene was purified from the contaminant coming from PCR mixture by ethanol precipitation as explained in part 2.2.1.1.

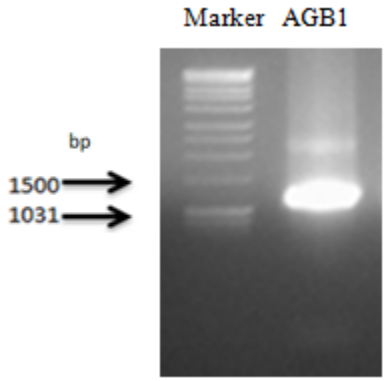


Figure 3.1AGB1 gene amplified with primers introducing the LIC site

The size of AGB1 is about 1100bp and the amplified gene was verified by analysis on 1% agarose gel. As it can be seen in figure 3.1, the amplified gene was observed just above 1031kDa.

3.1.2. Preparation pMCSG7 vector and annealing of AGB1

The isolated pMCSG7 plasmids (explained in part 2.2.1.2) were digested with SspI to obtain the linear form with blunt ends. These blunt ends are necessary to obtain LIC sites after T4 DNA polymerase reaction. The digested plasmid was examined on %1 agarose gel (shown in figure 3.2.)

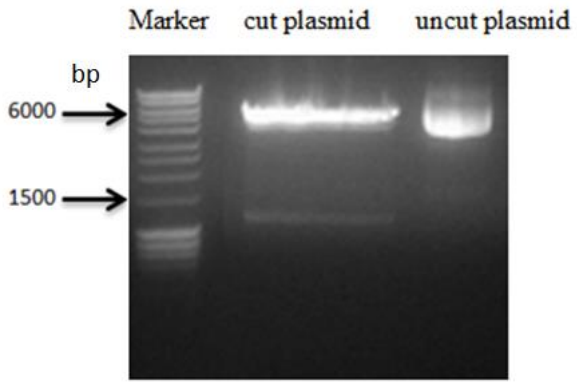


Figure 3.2. SspI digestion of pMCSG7 vector

A total of 72µl digested plasmid was loaded into 3 wells for purification as well as for monitoring digestion. It is seen from figure 3.2 that there is a significant difference between mobilities of the uncut and cut plasmids. The supercoiled uncut plasmid migrates faster than the digested linear plasmid.

The digested plasmid was purified from the gel to remove contaminants and both this vector DNA and amplified gene were digested by T4 DNA polymerase (explained in 2.2.1.1.3.). Concentrations of both insert and vector were determined by comparing band intensities on the gel in order to set several annealing reactions at different vector/insert ratios.

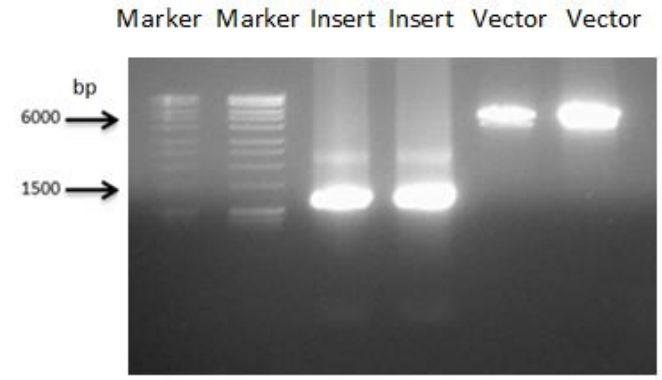


Figure 3.3 Concentration determination of T4 DNA polymerase digestion

The concentration of AGB1 was 500ng/µl whereas the concentration of pMCSG7 plasmid was 225ng/µl. For annealing of AGB1 into pMCSG7 vector specific ligation calculations were done (explained in 2.2.1.4).

3.1.3. Transformation of *E.coli* with pMCSG7 and AGB1

For transformation different types of *E.coli* were tried in order to find out which provided a better host for AGB1 expression. These were BL21plus*, DH5α and Top10 cells. The transformed cells were spread on LB agar plates (50µg/ml ampicillin) and cells were grown at 37^0C o/n. Colonies were observed and cells were stored at 4^oC for verification of the presence of the annealed construct and for preparation of glycerol stocks.

3.1.4. Verification of cloning

For the verification of annealing both colony PCR and restriction analysis of isolated plasmids were done.

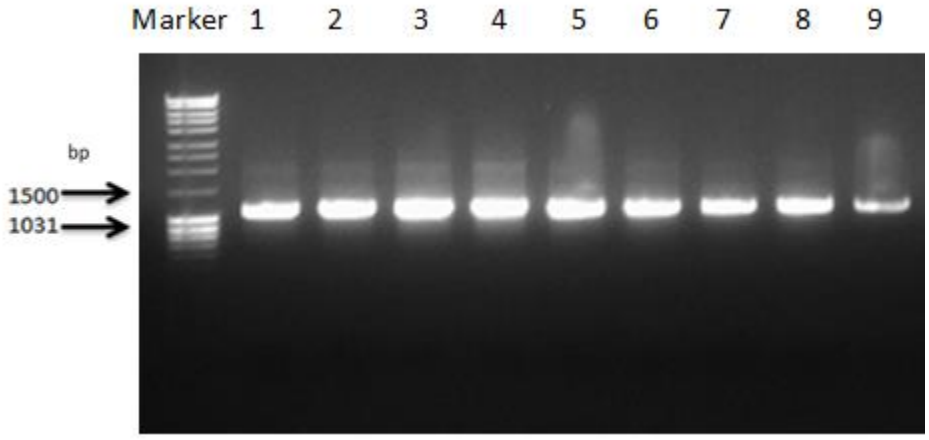


Figure 3.4 Results of verification of annealing by colony PCR with AGB1primers (Lanes1-3 show amplified AGB1 from BL21plus* cells, lanes 4-6 show amplified AGB1 gene from DH5α cells, lanes 7-9 show amplified AGB1 gene from Top10 cells.)

Colony PCR was performed using AGB1F/R primers and results shown in figure 3.4 indicate that all cell types contained the AGB1 gene. For further verification, constructs were isolated from cells for digestion of the insert. Plasmids were digested with restriction enzymes; BamHI and KpnI which have sites located in the LIC cloning region. Results of agarose gel analysis of digestion of isolated constructs are shown in Figure 3.5.

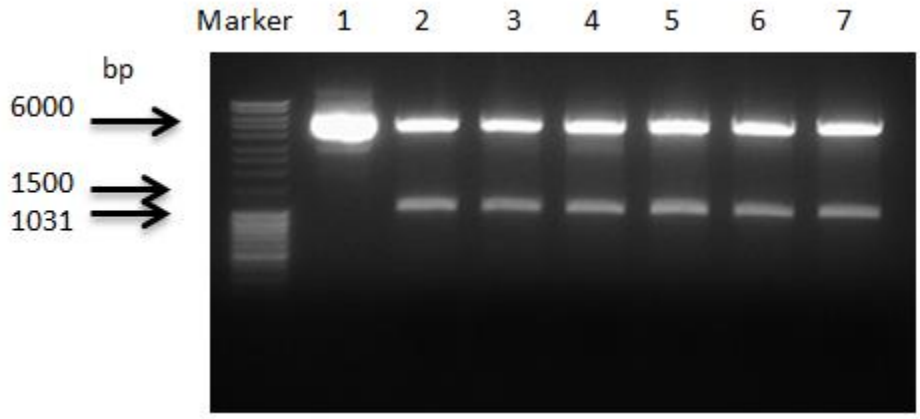


Figure 3.5 Results of digestion of pMCSG7+AGB1 constructs. Lane 1: undigested construct, Lanes 2-3 show digested plasmids isolated from BL21plus* cells, Lanes 4-5 show digested plasmid isolated from DH5α cells and lanes 6-7 show digested plasmid from Top10 cells.

Colony PCR results and digestion results demonstrate that the AGB1 gene has been inserted into the pMCSG7 plasmid. The bands observed on the agarose gel at about 1100bp correspond to the expected size of AGB1 gene. Bands observed at about 5000bp in figure 3.5 correspond to the linearized pMCSG7 plasmid which is about 5300bp long.

The verified constructs were sent to Refgen Company (Ankara) for sequence verification. Sequence data of AGB1 gene from literature and the data obtained from sequencing were compared. The results showed that the AGB1 gene was inserted into the plasmid (Appendix C). The results were analyzed by the program CLC Main Workbench.

3.1.5. Expression of AGB1 protein

The expression of AGB1from the pMCSG7+AGB1 construct was monitored in different types of *E.coli* cells; BL21plus*, DH5α and Top10. Using untransformed cells as controls and also by comparing the growth of non-induced and induced transformed cells we tried to identify the cell type with maximum protein expression. For the induced samples 1mM IPTG was added to cell cultures at the beginning of the experiment and OD_{600} values were measured every 40 min for determination of the growth curves for these cells.

The expression of protein was investigated by determining the growth curves of all types of cells and the extracted protein was analyzed in all cell types.

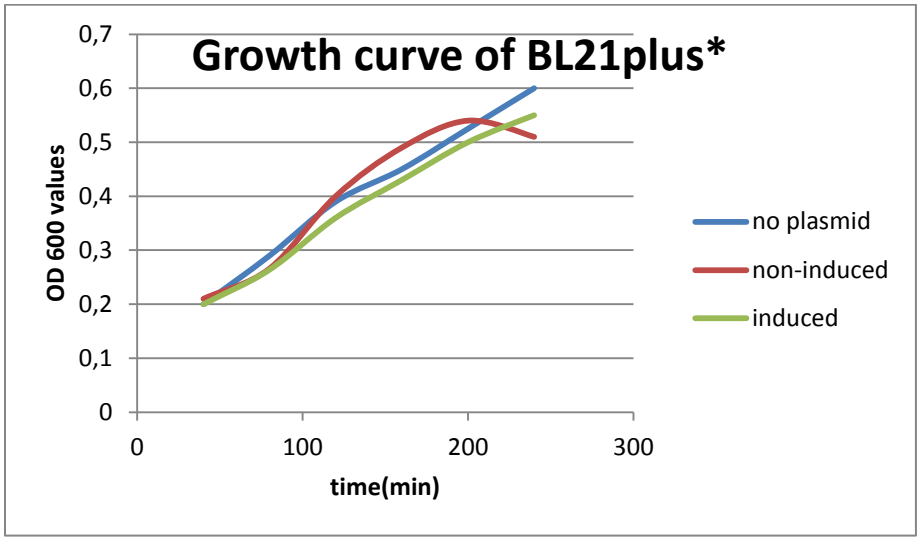


Figure 3.6: Growth curve of BL 21 plus* cells (grown in terrific broth) carrying the pMCSG7+AGB1 construct

Figure 3.6 shows the growth curve of BL 21 plus* cells expressing AGB1 protein in terrific broth medium. The induced cells show slightly lower level of growth when compared to the controls. This is interpreted as AGB1 expression hindering the growth in BL 21 plus* cells but the growth level is comparable to that observed with untransformed cells which increased to about OD600 0,6.

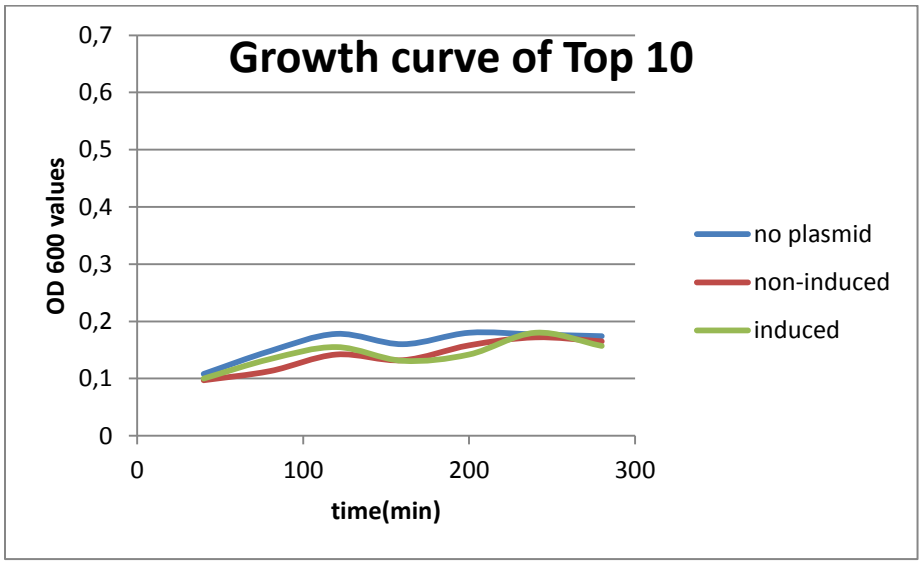


Figure 3.7: Growth curves of Top10 cells (grown in terrific broth) carrying the pMCSG7+AGB1 construct

When growth curve of TOP10 cells is examined in figure 3.7, it can be seen that the cell growth is again lower in the induced cells. The growth curve of BL 21 plus* cells reached a maximum growth level of 0,6 whereas the maximum growth in TOP10 cells was only 0,16. So, it was decided that Top10 cells were not suitable for high cell biomass production.

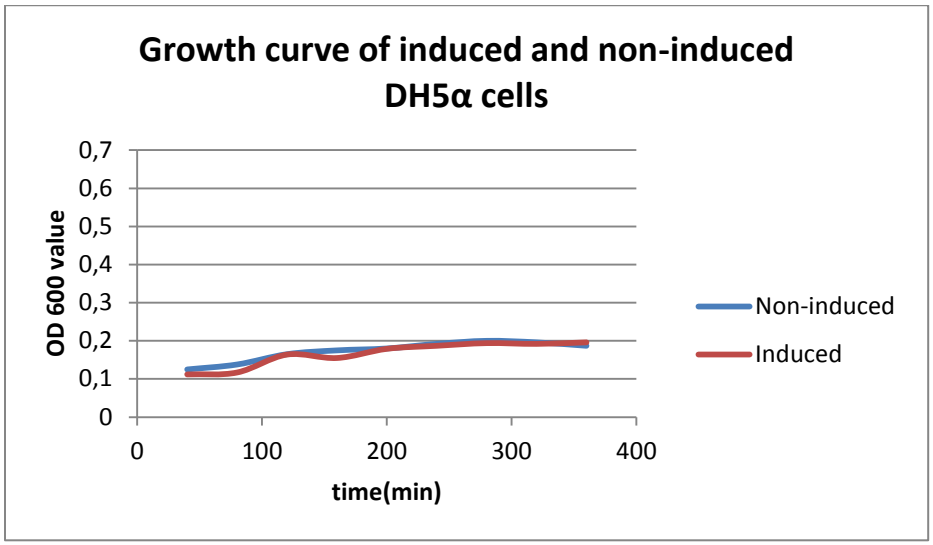


Figure 3.8: Growth curves of DH5α cells (grown in terrific broth) carrying the pMCSG7+AGB1 construct

In figure 3.8, DH5α cells showed a similar growth curve to that of Top10 cells. Here, it was seen again that the level of growth was very low. At this level, low growth rate also indicates low protein expression. When growth curves of all three cells were compared, it was seen that the highest growth rate was in BL 21 plus* cells. Therefore BL 21 plus* cells were used for developing a purification procedure for AGB1.

Protein expression during the time period of the growth curves was analyzed by SDS-PAGE. Results of these analyses for BL 21 plus* cells is shown in Figure 3.9.

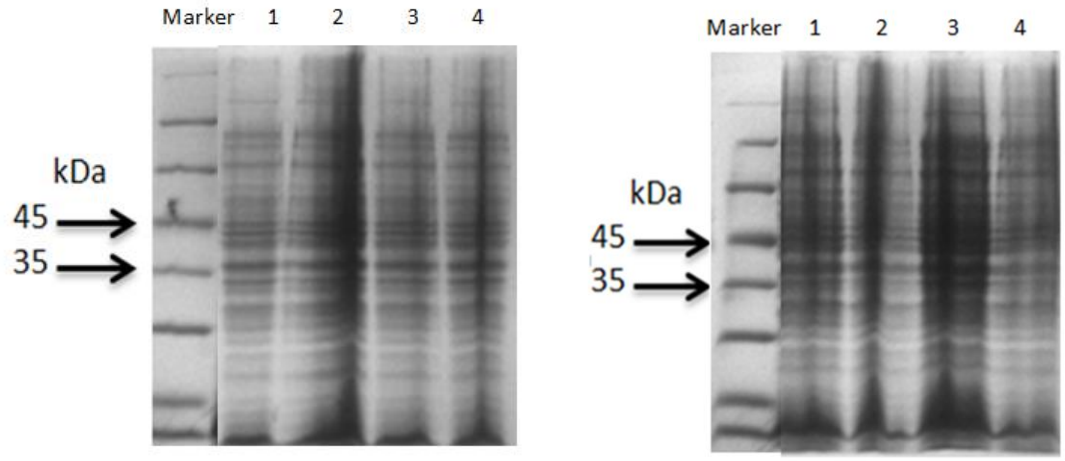
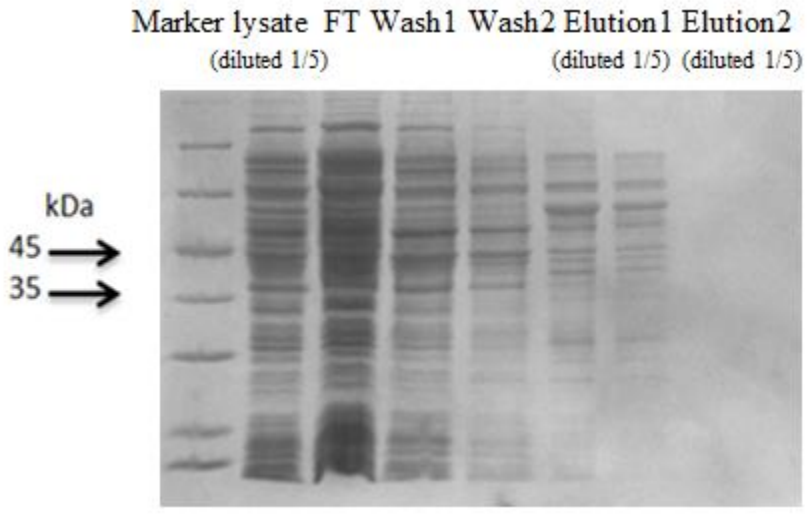
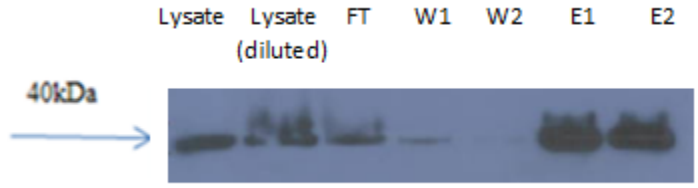


Figure 3.9 Comparison of AGB1 protein expression in non-induced (left) and induced (right) BL 21 plus* cells. Total cell extracts (lysates) were analyzed on SDS-PAGE %12 polyacrylamide gels. Cells were lysed. (Lane 1: after 240minutes, Lane 2: after 280minutes, Lane 3: after 320minutes, Lane 4: after 360minutes).

When the SDS-PAGE results of induced and non-induced samples are compared in figure 3.9, it was not possible to detect a significant increase in the amount of protein around 40kDa, which is the molecular mass of AGB1.



(A)



(B)

Figure 3.10 (A) SDS- PAGE results of batch purification and western blot results obtained from induced BL 21 plus* cells. **(B)** Western blot results showing AGB1 expression obtained from induced BL 21 plus* cells. FT: flow through, W1: first wash, W2: second wash, E1: elution fraction 1, E2: elution fraction2.

In figure 3.10, it can be seen that elution fractions contain high protein yield. Western blot results were compared with different cells' western blot profiles and highest protein expression was observed in BL 21 plus* cells.

Batch purification results indicated that AGB1 was being synthesized and based on this we proceeded with large scale preps to purify AGB1.

3.2. Purification of AGB1 protein

Highest biomass of BL 21 plus* cells was obtained by using terrific broth (TB) and this medium was used to grow cells for purification.

The purification process involved 3 steps; Nickel Affinity Chromatography, Ion exchange Chromatography and Size exclusion chromatography. Elution from the affinity column was varied in order to increase the protein yield; either a linear or a step gradient was applied.

3.2.1. Nickel Affinity Chromatography with linear imidazole gradient

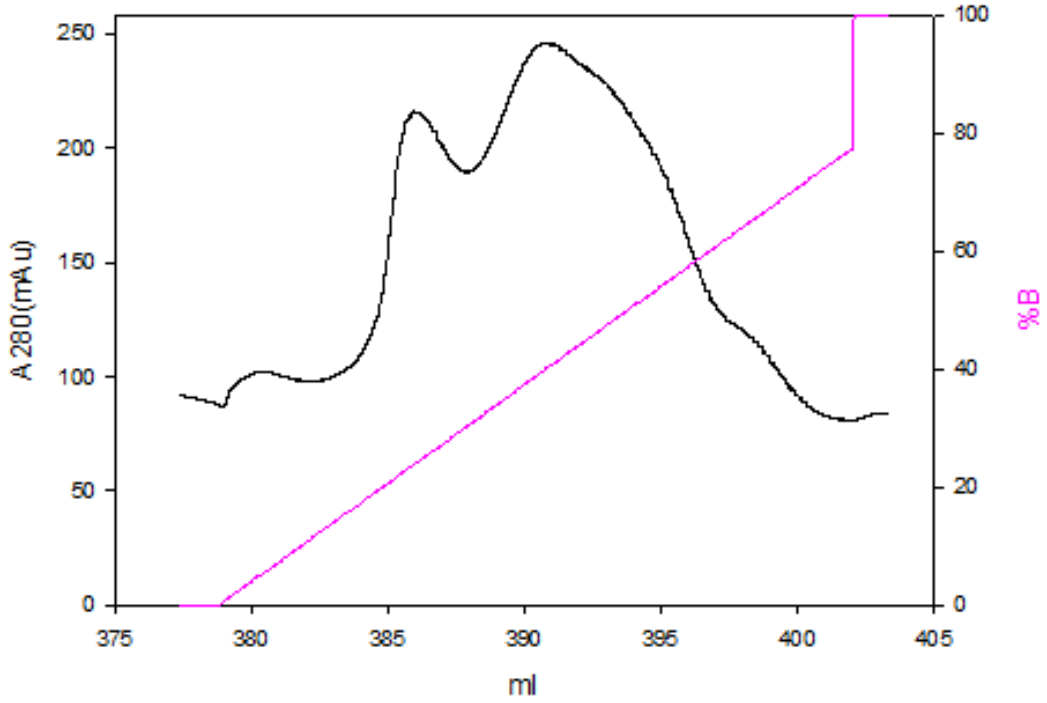


Figure 3.11 Nickel Affinity Chromatography results of isolation of AGB1 (using a linear gradient) (%B indicates the elution)

As shown in figure 3.11, when a linear gradient of 1M imidazole (reaching %100 in 30ml) was used, protein eluted in two main peaks. Previous experience had shown most of AGB1 in the second peak (Kaplan, 2009). But as these two peaks show overlapping regions it was clear that the isolated protein would not be pure.

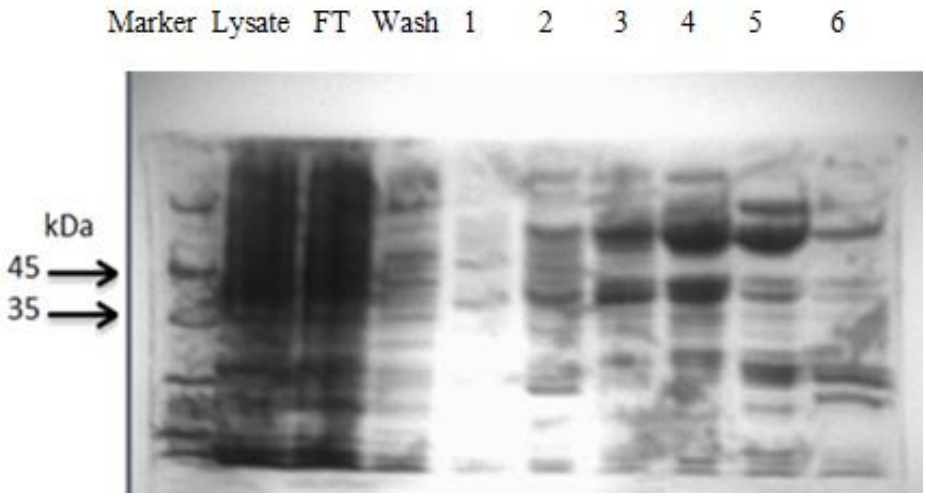


Figure 3.12%12 polyacrylamide SDS-PAGE analyses of early steps of AGB1 purification. Lysate is shown next to protein molecular weight marker. Flow through (FT) and wash fractions from Ni-affinity chromatography are indicated. Lanes 1-6 are fractions from second peak.

Figure 3.12 shows the results of SDS-PAGE analysis of fractions from the right of the second peak displaying concentrated protein bands. However, it is also seen from this figure that these fractions also contain high levels of contamination, particularly a component with about 60kDa molecular mass. In order to eliminate this component an ion exchange chromatography step was introduced after Ni-affinity step.

3.2.2. Q-trap ion Exchange Chromatography

Fractions were pooled (a total of 5ml) and dialyzed against Tris-HCl pH 8.0 in order to remove the imidazole and the sample was loaded on a 5ml Q-trap column. Elution was done with a linear NaCl gradient reaching 700mM in 30ml.

The protein sample was centrifuged at 13200rpm for 20min at 4^0C to remove any aggregates.

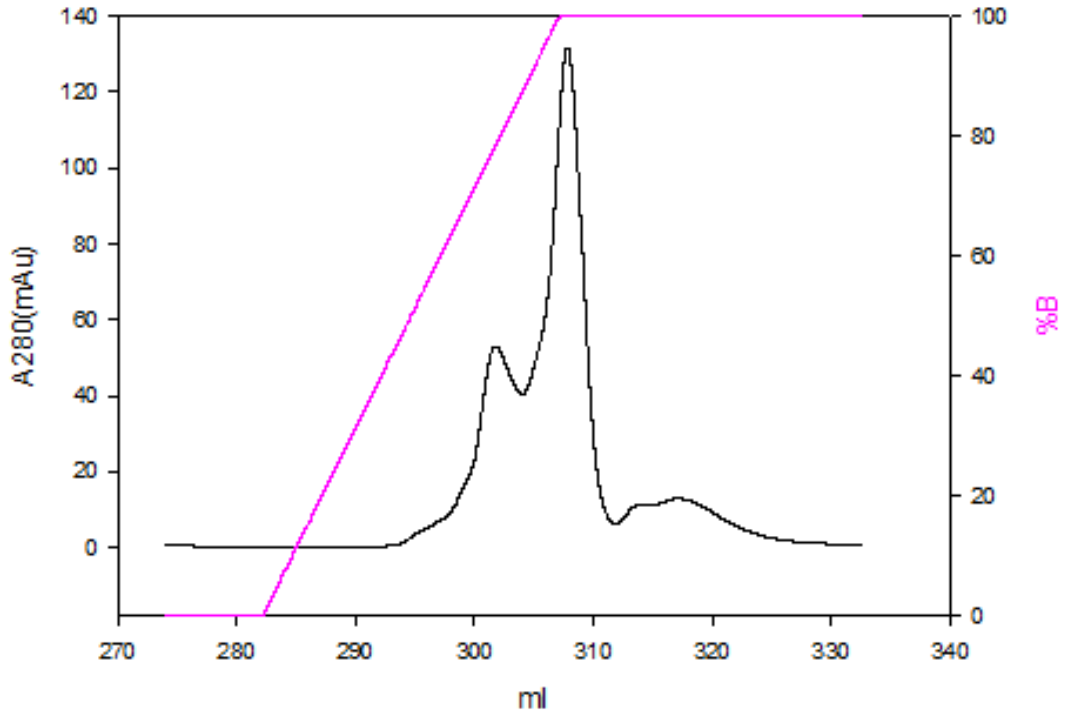


Figure 3.13 Elution profile of AGB1 from ion exchange column (%B indicates gradient)

As seen in Figure 3.13, two peaks one at about 50% and the other at about 100% of NaCl gradient was observed. The protein content of the peak fractions from peak 2 were examined by SDS-PAGE.

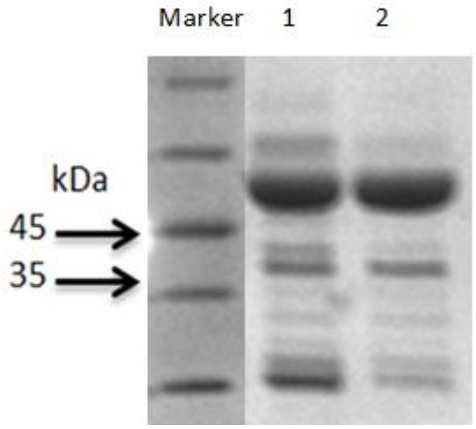


Figure 3.14%12 polyacrylamide SDS-PAGE analysis of top 2 fractions of peak 2 from the Q-trap column

In the SDS-PAGE analysis of the top fractions of Q-trap shown in figure 3.14, a cluster of bands with molecular weight around 42kDa was observed in significant quantity in the top

fractions of the ion exchange column, however the contaminating protein with 60kDa molecular mass was also present in these fractions in amounts which were much higher than AGB1. A final attempt with size exclusion chromatography was tried to eliminate this component.

3.2.3. Sephadex G75 Size Exclusion Chromatography

Size exclusion chromatography was performed on samples which were pooled after ion exchange chromatography and buffer was changed to 20mM Hepes pH 8.0. To ensure good separation less than 4mg protein was loaded on the column.

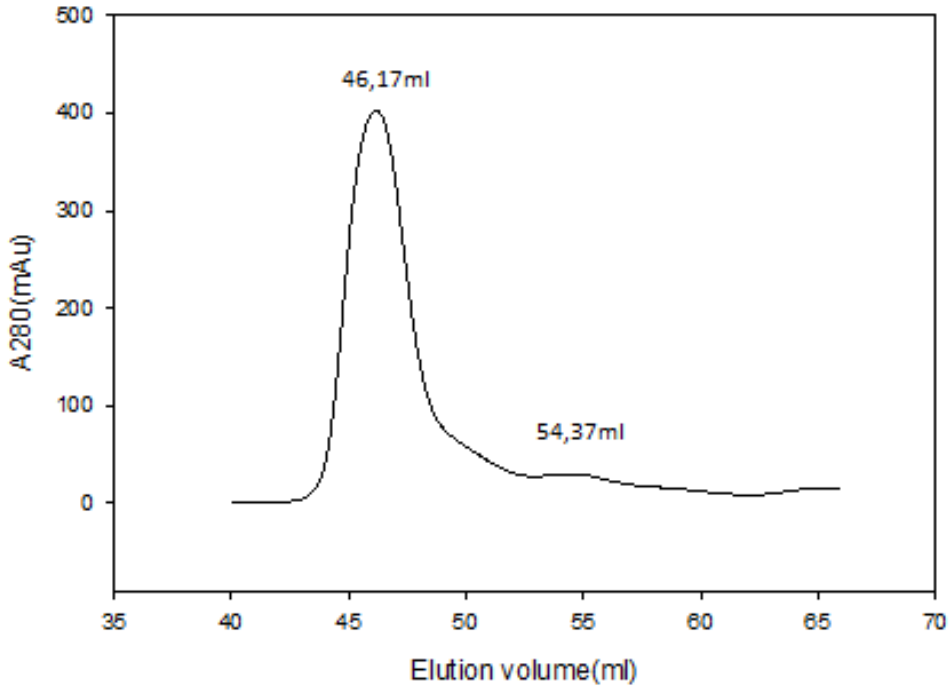
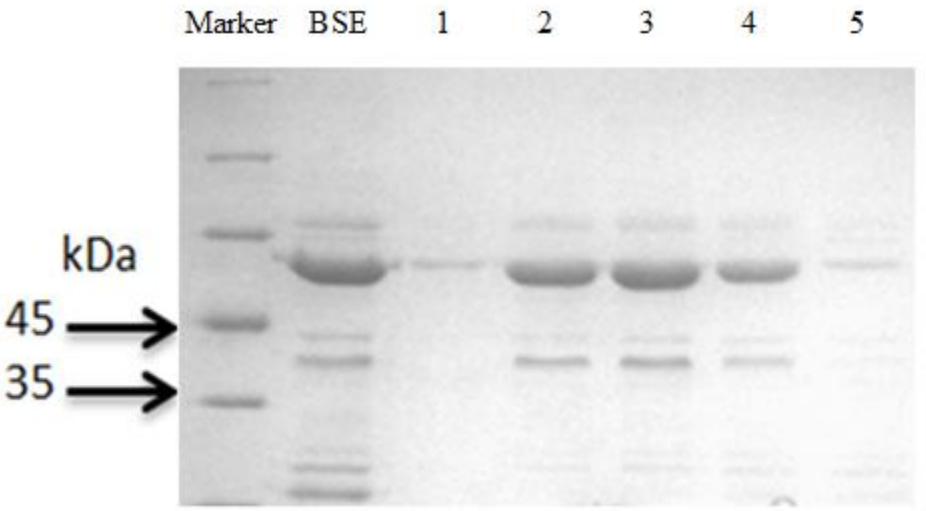


Figure 3.15 Elution profile of High load 16/60 superdex75pg Size Exclusion for AGB1

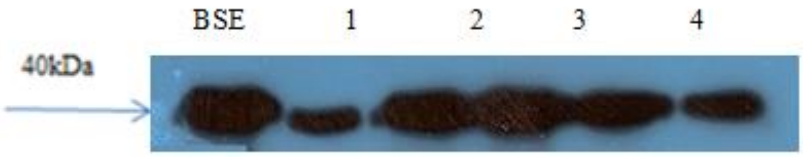
Chromatography results shown in Figure 3.15, display a prominent maximum at 46,17 ml, followed by a small peak at an elution volume of 54,37ml. Molecular masses corresponding to these elution volumes (calculated from the calibration curve of this column) correspond to 83 kDa and 45 kDa respectively.

To determine the homogeneity of the protein samples, corresponding fractions were analyzed by SDS PAGE and results of these were complemented by western blotting These results can be summarized as (1) according to the size exclusion analysis AGB1 solutions contain a major component with 80 kDa molecular mass and a minor component with 40 kDa molecular mass. (2) SDS-PAGE analysis of the 80 kDa component under denaturing conditions

gives bands in the vicinity of 60 kDa, 40 kDa and 25 kDa. (3) Western blotting with his-tag antibodies shows that band around 40 kDa is AGB1.



(A)



(B)

Figure 3.16 (A) %12 SDS PAGE analysis of fractions after size exclusion chromatography BSE: sample loaded on the size exclusion. Lanes 1-5: fractions of the 46ml peak.**(B)** Western blot analysis of AGB1 in fractions 1-5.

We expected that size exclusion chromatography would eliminate the 60kDa contamination observed with AGB1, however as it can be seen in figure 3.15 this is still visible together with AGB1.The results of size exclusion chromatography showed that there was a protein with a size of 83kDa co-purifying with AGB1 through the whole procedure. It appears that this protein yields a major 60kDa component and smaller bands around 20kDa on the denaturing gels. The bands observed between 35-45kDa, on the other hand, correspond to AGB1 protein.

Results given above showed that the additional chromatography steps performed after the Ni-affinity column were not effective in further separating AGB1 from the contaminating factors, and we were losing protein at each additional step. We decided to try the affinity step with a larger amount of cell mass to see if we could enrich AGB1 content at this step.

3.2.4. Nickel Affinity Chromatography with a step gradient of imidazole

In different attempts of AGB1 purification, biomass was increased to 35 gr corresponding to pellets from a total of 4 liter cultures. A further modification involved eluting the protein with a 1M imidazole step gradient from the Ni-affinity column after extensive washing.

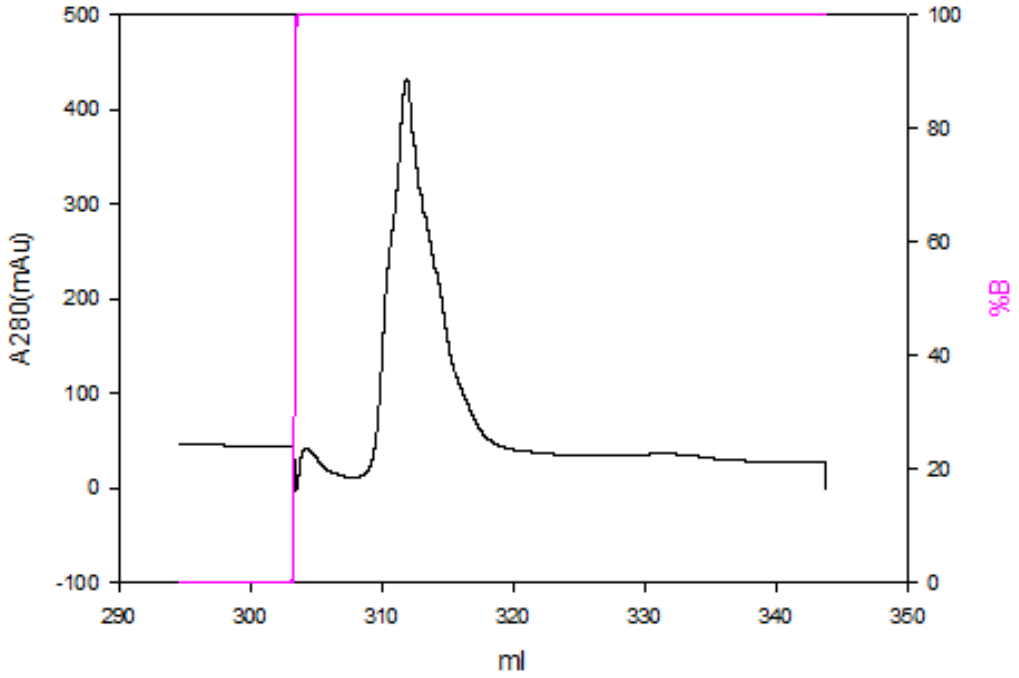


Figure 3.17 Affinity Chromatography result of AGB1 (step gradient)

These modifications resulted in obtaining a single peak from the column with high protein content. As can be seen in Figure 3.17 the peak displays a shoulder on the back part (right hand side) and SDS-PAGE analysis of the fractions of the peak, given in figure 3. 18, showed that AGB1 was enriched in the fractions in the vicinity of the shoulder.

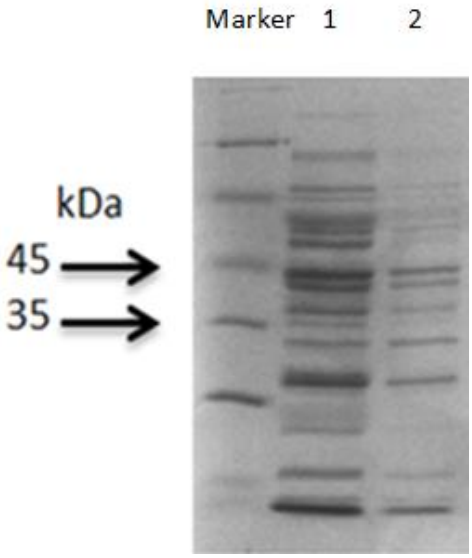


Figure 3.18%12 SDS-PAGE analysis of AGB1 purified by Nickel Affinity Chromatography (Lanes 1-2 are fractions from the shoulder on the right-hand side of the peak).

As mentioned before, AGB1 has a molecular weight of 42kDa and in figure 3.17 strong bands can be seen between 35-45kDa. Furthermore the ratio of 49kDa bands to the contaminating factor at 60kDa has also increased significantly indicating a decrease in the contamination of AGB1 fractions.

When results of the step gradient (figure 3.17) are compared with previous linear gradient results (figure 3.13), it appears that the step gradient helped to eliminate some of the large protein contaminants from the AGB1 sample. Since fractions 1 and 2 shown in Figure 3.18 were enriched in protein with 35-45kDa, these fractions were pooled together with the other fractions from the top peak for a second step gradient his-trap. Here, the expectation was to increase the purity of the protein sample. The pool had a protein concentration of 9,13mg/ml with a total volume of 12ml.

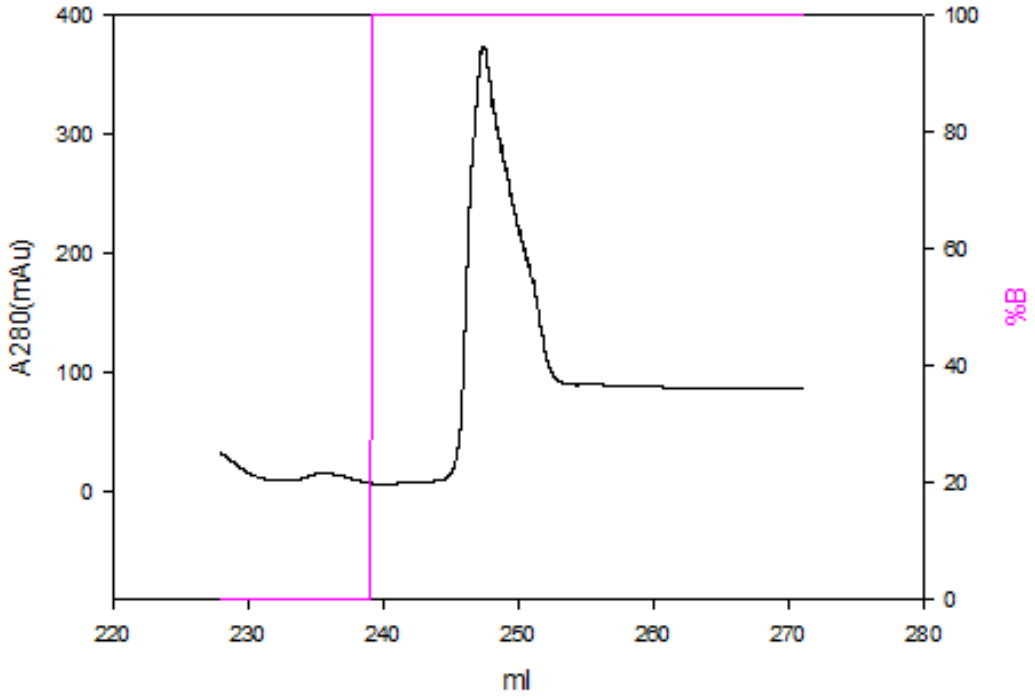


Figure 3.19 Second Affinity Chromatography result of AGB1 (step gradient)

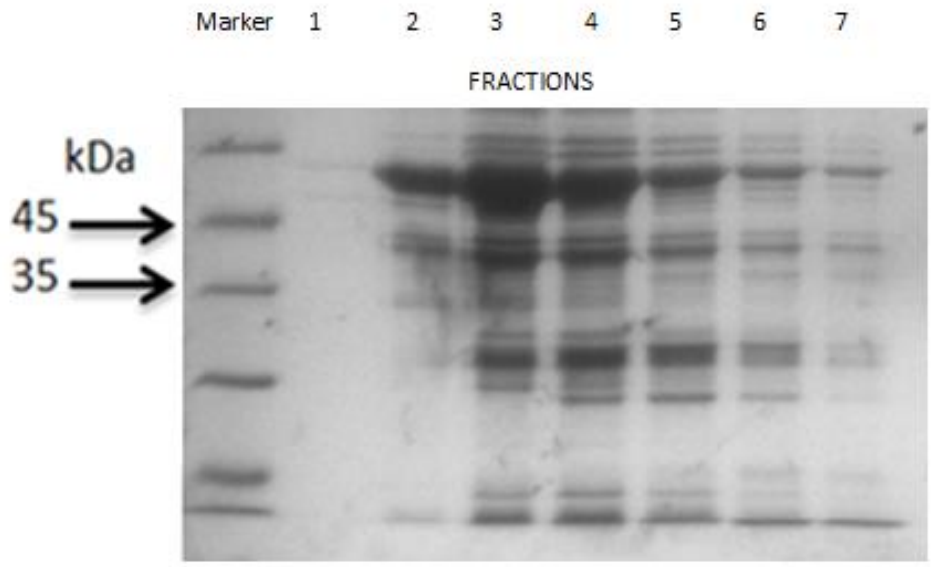


Figure 3.20%12 SDS-PAGE analysis of AGB1 purified by a second Nickel Affinity Chromatography

(Lanes 1-7 are fractions from the shoulder on the right-hand side of the peak).

The step gradient results showed that it may be possible to increase the protein yield. The expectation was to increase the protein yield and the purity of the protein with an extra step gradient but the results in figure 3.20 showed a similar profile as in linear gradient purification process.

3.2.5. Analysis of AGB1 protein by Western Blot

In the SDS PAGE results it could be seen that there were concentrated bands at about 42kDa which was the expected size of AGB1 protein. To understand the concentration difference among the samples western blot was carried out with his-antibodies. The results are seen in figure 3.21. All samples were diluted at 1/100 ratio. Fraction 2 showed the thickest band indicating that it contained more concentrated AGB1 protein.

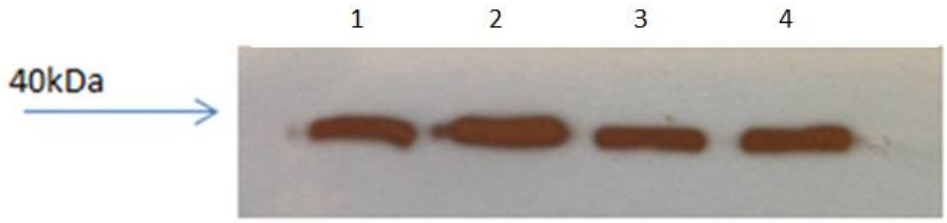


Figure 3.21 Western blot results of AGB1 protein after Ni-affinity chromatography (Samples were diluted 1/100 before loading on the gel). Fractions are taken from top peak.

Fractions 1-2 and 3-4 were pooled separately and dialyzed against Tris-HCl pH 8.0 buffer. The concentration of fractions 1-2 was 6,99ml/ml whereas the concentration of fractions 3-4 was 3,37mg/ml. When compared to previous experiments, there was 3 fold increase in AGB1 protein concentration.

3.2.6 Biophysical Characterization of AGB1 fractions

Cicular dichroism (CD) spectropolarimetry and dynamic light scattering (DLS) were used to determine some of the structural features of the protein in AGB1 fractions.

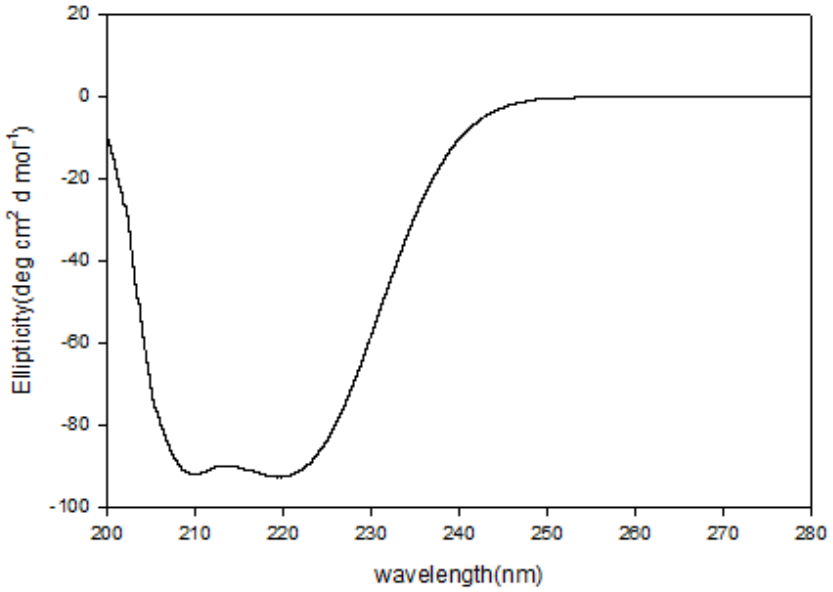


Figure 3.22 CD measurement result of AGB1. Top peak fraction of second his-trap was used.

Secondary structure elements of the protein were investigated using CD spectropolarimetry (CD). The CD profile of the AGB1 fraction shown in figure 3.19 displays features that are expected from a-helical secondary structure elements and is not in agreement with the expected beta-propeller structure of AGB1. This might have been caused because of contaminations or aggregation of proteins.

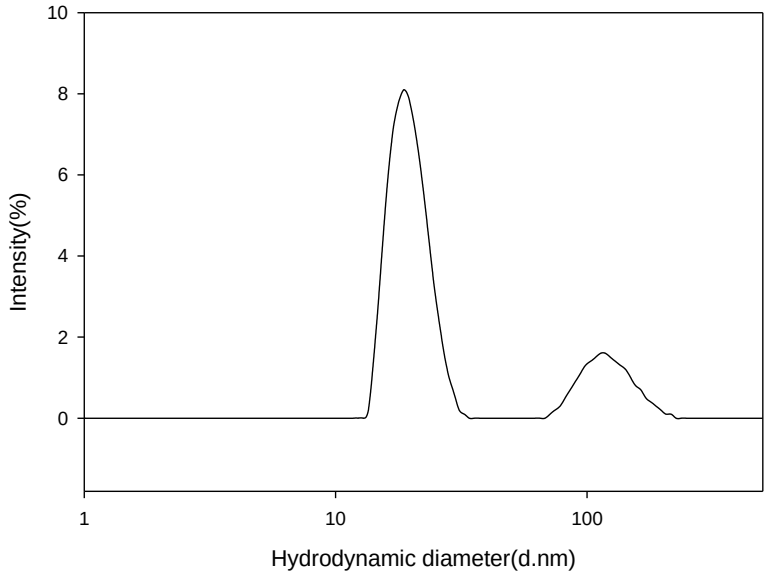


Figure 3.23 Value of the hydrodynamic diameter. Top peak fraction of second his-trap was used.

Dynamic light scattering measurements shown in figure 2.10 give a hydrodynamic diameter of about 19 nm for AGB1 in solution. Theoretically, a protein with a molecular weight of 42kDa

should give a diameter of about 12nm. These results show that the purified protein might have aggregated with other particles resulting in a molecule with higher diameter. In previous measurement, we were able to obtain protein samples more close to 12nm diameter (data not shown here).

3.2.7. Analysis of AGB1 fractions by mass spectrometry

Mass spectrometry analyses were carried out (TUBITAK-MAM) on protein solutions from the Q-trap column and also on protein bands after SDS-PAGE analysis of SEC fractions such as bands observed in Figure 3.16.

In all analyses major components were E. coli 60 kDa chaperonin 1 (A1AJ51) detected together with AGB1 (InterProt Accession number P49177). In Q-trap fractions, which could be better quantitated because the protein is in solution, the ratio of A1AJ51:P19177 was 5:1 chaperonin/AGB1(Appendix D).

3.3. Cleavage of AGB1 protein with isolated TEV protease

The aim of this study was to express the *A.thaliana* beta subunit (AGB1) in pMCSG7 vector, introducing a TEV site into the protein which allowed cleaving at the TEV site and removing his-tag after the purification process. TEV protease had also a his-tag. TEV protease was expressed and purified by affinity chromatography. Finally, TEV protease and purified AGB1 protein were brought together in different concentrations and different incubation times. Optimum concentrations and incubation time were determined.

3.3.1. Analysis of TEV plasmid

For analysis, the plasmid was isolated from *E.coli* by using a miniprep kit (Qiagen). The concentration of TEV plasmid was 271,2ng/µl. The plasmid had a size of 5777bp which can be seen in figure 3.23 close to 6000bp.

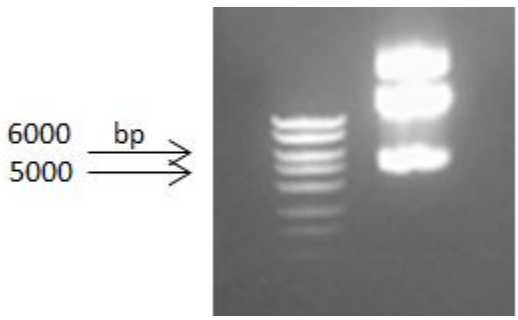


Figure 3.24TEV plasmid analysis on %1 agarose gel

3.3.2. Transformation of cells with TEV plasmid

TEV plasmid (pMHTDelta238 vector) (shown in Appendix B) was transformed into BL21De3 competent cells which showed optimum growth with this plasmid. Cells were grown in LB medium.

3.3.3. Expression and purification of TEV protease

BL21De3 cells containing TEV plasmid were grown in LB medium and a pellet of 5gr was obtained. The expressed TEV protease had an N-terminus his-tag (Appendix B). This feature allowed the use of a 5ml Ni-NTA column for the purification process.

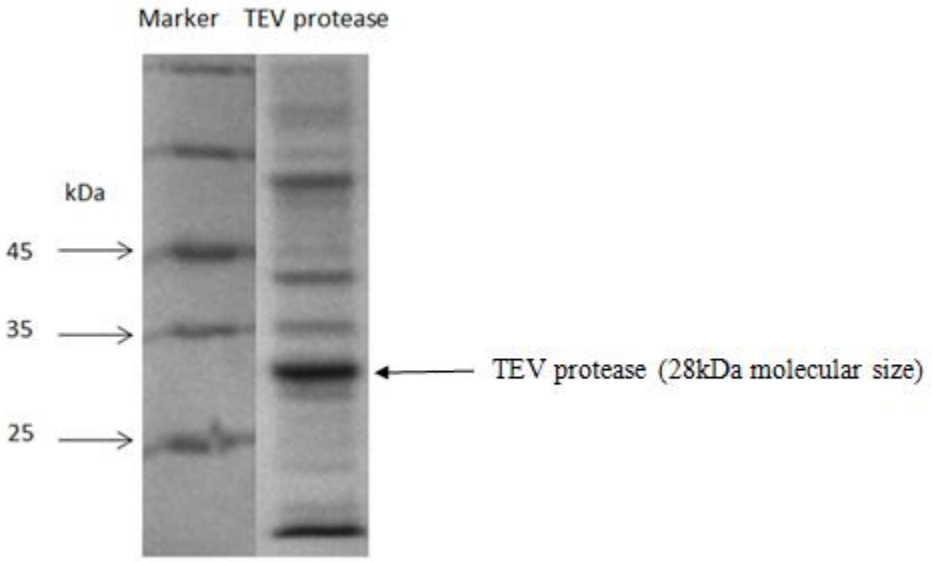


Figure 3.25 %12 SDS-PAGE analysis of isolated TEV protease

After elution, 6ml of volume was pooled and concentrated to a final volume of 1ml (10 MWCO used during the concentration process) .The final concentration of TEV protease was 5,06mg/ml. The isolated TEV protease was stored in TEV buffer at -80^{0}C.

The TEV protease has a size of 28kDa which can be seen in figure 3.25 and the concentrated protein was clearly visible below 35kDa in the form of two bands; one dark and one light band. This concentrated TEV protease was used for AGB1 cleavage.

3.3.4. Cleavage of AGB1 protein with TEV protease

The AGB1 protein isolated by second Ni-Affinity chromatography (step gradient) was used for the cleavage procedure. Final concentration of the AGB1 protein was 3,37mg/ml.

To ensure that TEV protease was functioning properly, a control protein was needed. BTL2 (a thermoalkalophilic lipase originating from *Bacillus thermocatenulatus*) with a histag next to a TEV site was used. BTL2 protein has a molecular weight of about 45kDa. TEV protease was going to remove histag from BTL2 protein and the cleavage would be observed on the gel.

The concentrations and amounts of samples used were explained in 2.2.6.

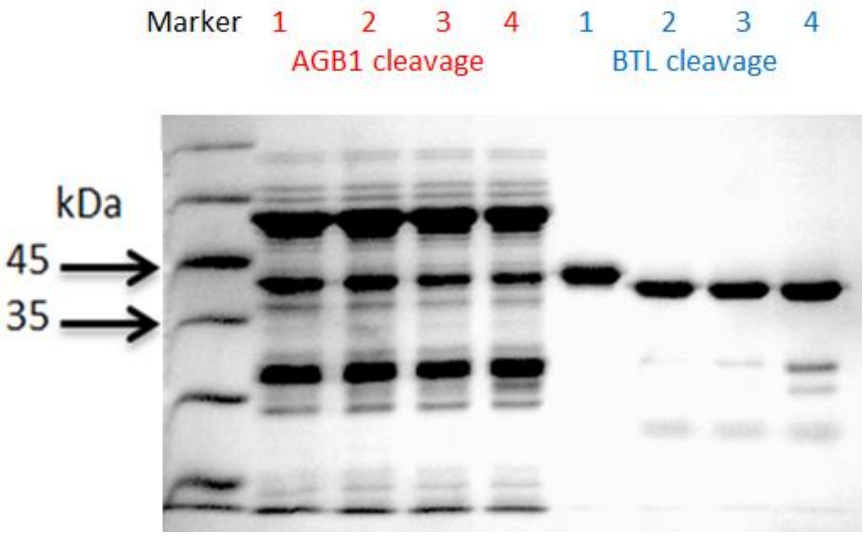


Figure 3.26 TEV cleavage results of AGB1 and control protein BTL2 (about 45kDa) analyzed by %12 SDS PAGE. Lanes 1: no TEV protease, lanes 2: 2µl TEV protease, lanes 3: 4µl TEV protease, lanes 4:8µl TEV protease.AGB1:1,6mg/ml and BTL: 1,58mg/ml.

.

To understand effect of TEV treatment on AGB1, western blot was applied to samples in figure 3.26 and results are shown in figure 3.27.

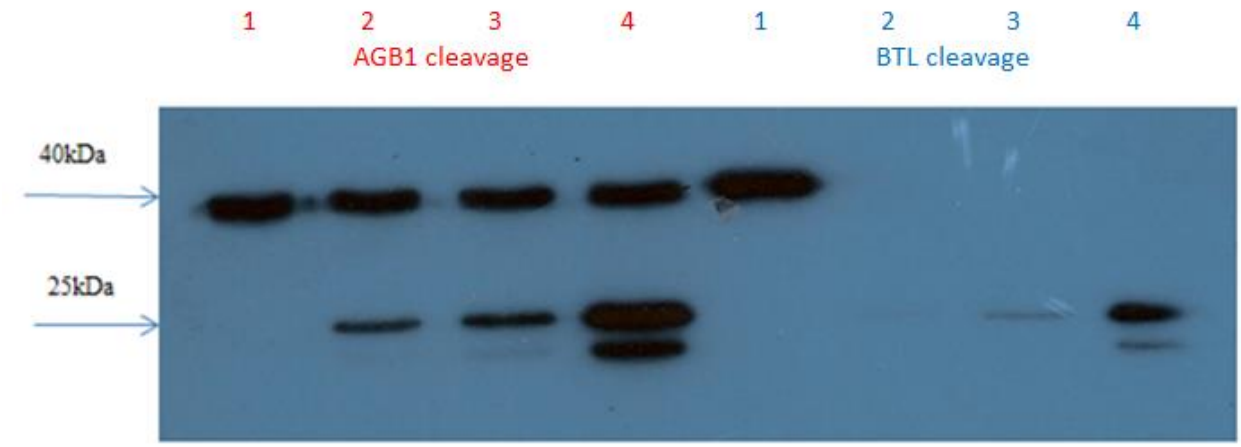


Figure 3.27 Western Blot analysis for AGB1 and BTL control protein cleavage (Samples were diluted to 1/10, final protein concentration ~0,08mg/ml for both AGB1 and BTL proteins, Lanes 1: no TEV protease, Lanes 2: 2µl TEV protease, Lanes 3: 4µl TEV protease, Lanes 4:8µl TEV protease).

4. Discussion

4.1. Cloning of AGB1

When different studies in literature are examined, it could be seen that β subunit was cloned into different vectors but frequently expressed in *Arabidopsis thaliana* plant cells (Jiang, 2012), yeast cells (Mason, 2001) or even insect cells (Robishaw, 1992). *E.coli* based methods have several advantages which makes them preferable to other cell-based methods. They show higher transformation efficiency and faster rate of growth. Therefore, in this study, we aimed to clone β subunit AGB1in *E.coli* cells and improve the expression of AGB1 in these cells.

AGB1 was previously cloned using pQE80-L plasmid (Kaplan, 2009), expressed and partially purified in our lab. In this study, the aim was to develop a better system for expression of the protein so that it could be purified in a more homogeneous state and in large quantities. As the pQE80-L plasmid did not contain a site for removal of 6histidine (his-tag), it was also decided to use another plasmid which would allow the removal of his-tag. For this purpose, the AGB1 gene was cloned using pMCSG7 plasmid which contained his-tag (6 His) and a cleavage site for TEV protease. Ligation independent cloning by using vector pMCSG7was preferred because it enables effective high-throughput cloning and purification of recombinant proteins. Based on homology between vector and insert, it provides cloning of only specific products. After having cloned and expressed AGB1, cleaving AGB1 protein with TEV protease and removing his-tag from the protein, it would be possible to obtain the protein in a more native state. Sequencing results of the construct confirmed that we cloned the AGB1 into the pMCSG7 vector (Appendix C).

4.2. AGB1 expression and purification

As mentioned before, there are studies where $G_{\beta}$was expressed in different cells; plant, yeast and insect. What we share in common with these studies is that we used similar chromatography methods for purification; affinity, anion exchange and size exclusion chromatography. There are examples where Gα subunits are expressed in *E.coli* cells(Wall, 1995, Jones, 2011)and the protein is purified by anion exchange and size exclusion chromatography(Wall, 1995, Wise, 1997) but expression of Gβ was usually in eukaryotic cells (Jiang, 2012, Mason, 2001, Robishaw, 1992) in which Gβ was used in co-expression with Gγ. Production of βγ protein was accomplished by baculovirus expression system using fall

armyworm (Sf9) cells (Wall, 1995, Jones, 2011).Again the association between AGG2 and AGB1 was observed in vivo using yeast two-hybrid system(Mason, 2001). In previous studies performed in our lab, expression of AGB1 in yeast cells was tried but the results showed that expression in yeast cells was not successful. So, based on the fact that *E.coli* show higher transformation efficiency and faster rate of growth, we aimed to improve a system for expression of AGB1 protein in *E.coli* in our lab.

The AGB1 gene was expressed in three different types of *E.coli;* Top10, DH5α and BL21plus* cells. Cells were initially grown in LB medium but in addition cells were also grown in TB and SOC medium. The highest biomass of cells were obtained from BL21plus* cells in TB medium. When the protein expression profiles were compared, again highest protein yield was obtained from BL21plus* cells. So, we decided to use BL21plus* cells in further protein expression and purification processes.

For the purification of AGB1 protein affinity, anion exchange and size exclusion chromatography were used. When previous studies are examined, it can be seen that similar chromatography methods were used (Wall, 1995, Wise, 1997, Jones, 2011).We first used affinity chromatography with his-tagged AGB1 protein. As this method was specific for the protein, the expectation was to obtain the protein in pure state. The bands shown in figure 3.12 were obtained after the affinity chromatography. As it can be seen there were many other strong bands showing that there was contamination. Findings show that stable interaction between the expressed fusion protein and bacterial proteins such as GroEL do occur (Rohman, 2000). GroEL molecules which belong to chaperonin family are required for the proper folding of protein. These results showed that this purification method was not sufficient for purifying the AGB1 protein. So, we decided to continue the purification process with further chromatography methods. In Jones 2011 study, the purification of G_{α} from *E.coli* cells started with affinity chromatography, then continued with size exclusion chromatography. We preferred to add an additional purification; ion exchange chromatography method because from previous attempts we knew that there was contamination and this might help in elimination of the contaminants in our lysate. Figure 3.13.shows the results obtained from ion exchange chromatography. Again we could see other dark bands showing that the contamination still existed. To optimize the purification of AGB1 and get rid of the GroEL subunits, size exclusion chromatography was applied so that these large molecules were going to be removed. In figure 3.16, there are strong bands between 35-45kDa which show the existence of AGB1 protein. However the large bands were still visible. Molecular weight of the protein was calculated from the calibration curve. The results showed that the first peak in

figure 3.15 contained protein with a molecular weight of 83kDa showing the existence of contamination whereas the second peak in the same figure contained protein with 45kDa which was more close to the size of AGB1 protein but with very low concentration.

Three different chromatography methods were performed and the protein could not be obtained in pure state. As using several chromatography types one after another decreased the protein yield, it was decided to use only one of these chromatography methods. So, we would not be able to eliminate the contaminants but we would provide high yield of protein which was going to be used in the cleavage process.

Affinity chromatography is more specific for AGB1 protein than the other methods because of the presence of his-tag, so it was decided to continue the purification process with this system but with some differences in the elution step. In affinity chromatography, the elution was obtained with a linear imidazole gradient. In our next attempt, we applied step gradient for elution. Additionally, we used two pellets (each obtained from 2L cell culture) in order to increase protein yield and concentration. Results were surprisingly better. Figure 3.18 shows the results obtained from this purification method. We could see strong bands at about 42kDa, which is the size of AGB1 protein. In addition to this, we were able to eliminate the larger bands which were thought to be GroEL molecules from the sample. The final concentration of protein obtained from this purification process was 9,13mg/ml which was the highest concentration we obtained.

We tried to determine the size of AGB1 protein by dynamic light scattering (DLS). According to the hydrodynamic radius from DLS measurements shown in figure 3.23, the corresponding protein molecular mass was larger than the expected molecular mass. Heterotrimeric G proteins are conserved signaling components across diverse eukaryote species (Jones& Assmann, 2004). It is known that Gγ and Gβ subunits are usually co-expressed (Robishaw, 1992). Some resources state that, according to hydrodynamic studies, without γ subunits, the β subunits are not stable but tend to aggregate into high molecular weight complexes (Schmidt, 1991).This might explain the reason for observing such large molecules. We also analyzed the structure of AGB1 protein by circular dichroism measurements. The results as given in figure 3.22 indicate that the structure showed highly alpha-helix structure which did not confirm the beta-propeller structure of AGB1. Here, we concluded that unstable AGB1 protein might have aggregated in the sample.

For verification of AGB1 protein we did western blot. Figure 3.21 shows the AGB1 protein obtained from the final purification method. The thickness of the bands showed the

difference between the concentrations of fractions. For further verification mass spectrometry results were analyzed. The results supported the expression of AGB1. Figure in Appendix D shows different samples taken from the gel. Sample 3 which showed closest size to AGB1 protein was confirmed by mass spectrometry analysis to be AGB1. Other fractions were also analyzed and results showed that in addition to sample 3, sample 1 and 4 did also contain AGB1 protein. Here, AGB1 was observed in bands with a molecular size higher than 100kDa. This event can be explained by Schmidt Carl which reports that unstable AGB1 proteins aggregate resulting in large molecules.

4.3. Cleavage of AGB1 protein with TEV protease

In the last part of our study, we aimed to further purify AGB1 protein by cleaving it with TEV protease which is a very useful reagent for cleaving fusion proteins (Waugh D., 2010). It is stated that the homogeneity of expressed and purified G_{α} has been achieved by TEV protease cleavage (Willard, 2004). Similarly, as we cloned AGB1 gene into pMCSG7 vector, it contained his-tag next to a TEV site which could be cleaved by TEV protease. Figure 3.26 shows the cleavage results of AGB1 and control protein BTL2.

The TEV protease was going to cleave only the His tag (6 His) in AGB1 and as this fragment was very small, it was not expected to see a dramatic change in the positions of bands. But still as the TEV protease amount increased, a decrease in the thickness of the bands in AGB1 (with a size of 42kDa) could be observed on the gel in figure 3.26. This made us think that high amount of his-tag removal may have caused this change. The best way of understanding the cleavage efficiency would be the analysis of these samples with western blot.

In BTL2 control protein, his tag was going to be removed by TEV protease. The cleavage of BTL2 could be detected clearly when the gel in figure 3.26 was examined. The right side of the gel, shows the TEV protease activity on BTL2 control protein. A clear difference between the uncleaved and cleaved protein was examined. To better understand the efficiency of cleavage in AGB1, western blot was applied. Figure 3.26 shows the western blot results of TEV cleavage for both AGB1 and BTL control protein. As AGB1 protein was cleaved, the his-tag was going to be removed from the protein. This would cause less emission of light during the chemiluminescent reaction. So, a change in the intensity of the bands would indicate that cleavage of his-tag has occurred. When western blot results were examined, a slight difference between uncleaved AGB1 (lane1) and cleaved (lane2-4) could be observed. It could not be said that cleavage occurred but still it could be concluded that cleavage might have occurred at a

certain level (the concentration in all samples were same, shown in 2.2.6). The BTL2 protein cleaved with TEV protease was again used for controlling the activity of TEV protease.

As we used the AGB1 protein obtained from the last step gradient his-tap, we were able to conclude that the contamination of AGB1 was less when compared to previous protein samples we obtained. The tendency of AGB1 forming aggregation (Schmidt Carl J., 1991) may have interfered with the cleavage process. Also AGB1 is a quite large protein (42kDa) and due to its unstable state in the absence of G_{γ}, it may have caused improper folding. Due to this fact, his-tag may not be exposed so that TEV protease could act on it.

1. Conclusion and future works

In this study, the aim was to express AGB1 protein with a his-tag and TEV protease cleavage site with effective high-throughput cloning and purification. By introducing the protein a his-tag next to a TEV site, TEV protease was used for the cleavage of his-tag enabling the removal of the his-tag after purification.

In our lab, previous attempts for AGB1 purification were done by using different vectors and cells. In these early studies, AGB1 gene was cloned into pGEX-4T-2 and pQE-80L vectors together with GST, resulting in a recombinant fusion protein with a total size of 69kDa. The expression of AGB1 was investigated in BL21 (DE3) cells and in Rosetta (DE3) cells. It was indicated that the yield was very low for all the expression and purification conditions (Kaplan, 2009).

In our study, we aimed to increase the expression of AGB1 protein. Using the pMCSG7 vector, we investigated the expression in three different cells:Top10, DH5α and BL21plus* where the last cell type showed highest biomass and expression of protein. For the purification process, best results were obtained from step gradient affinity chromatography. Figure 3.18 shows the fractions obtained from this experiment. The results showed that we could mostly eliminate the large protein particles which were thought to be GroEL molecules with an increased protein expression of about 3 folds when compared to our previous attempts.

Another important thing was to obtain the protein in a more pure state. For this purpose, the purified AGB1 were treated with TEV protease. The results in figure 3.26 show that cleavage occurred partially with low efficiency. Here, it was concluded that this may have been caused because of the tendency of unstable AGB1 forming aggregation. Another explanation could be that AGB1 protein is a quite large protein making it difficult to say that all his-tag terminals were exposed to TEV protease. Therefore all his-tags could not be removed.

We aimed to eliminate large protein molecules by applying step gradient elution during affinity chromatography. This method could be developed in further AGB1 purification studies. The study of Rohman M.,(2000) shows a useful tool for fusion protein expression in *E.coli*. It is stated that when fusion proteins are incubated with urea-denatured proteins(obtained from bacterial lysate) and ATP, these proteins compete for GroEL binding, therefore releasing the GroEL contaminants from the expressed fusion protein (Rohman M., 2000).This finding should be used in further AGB1 purification attempts using *E.coli.*

For further investigations including dimerization of β and γ subunits and crystallization of AGB1, it is important to obtain the protein more pure and in high yield.

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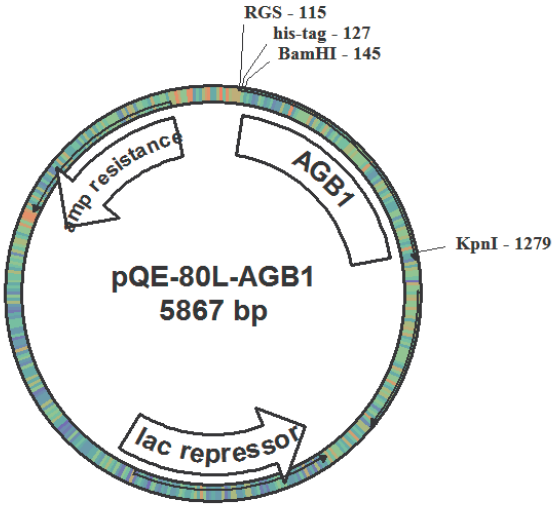
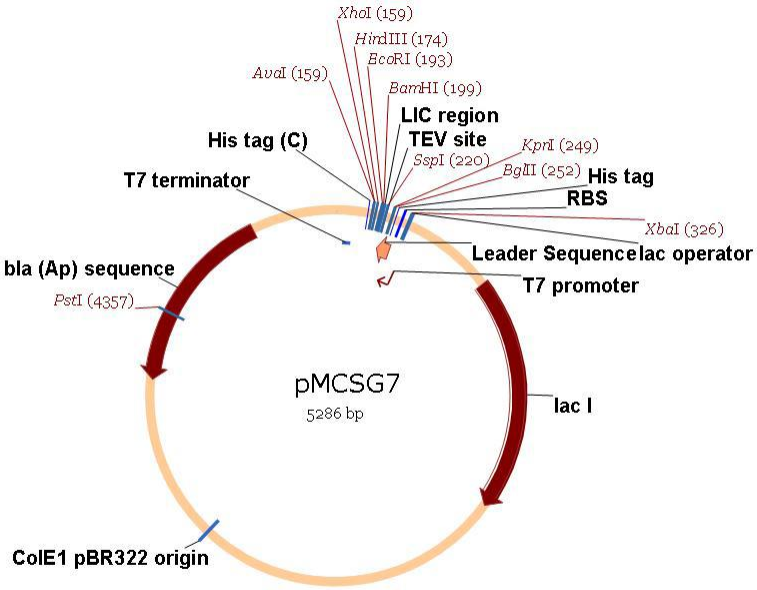
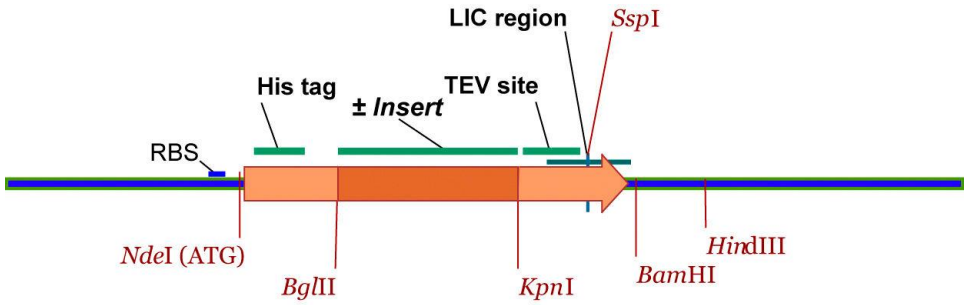
APPENDIX A



pQE80-L vector (Kaplan, 2009)

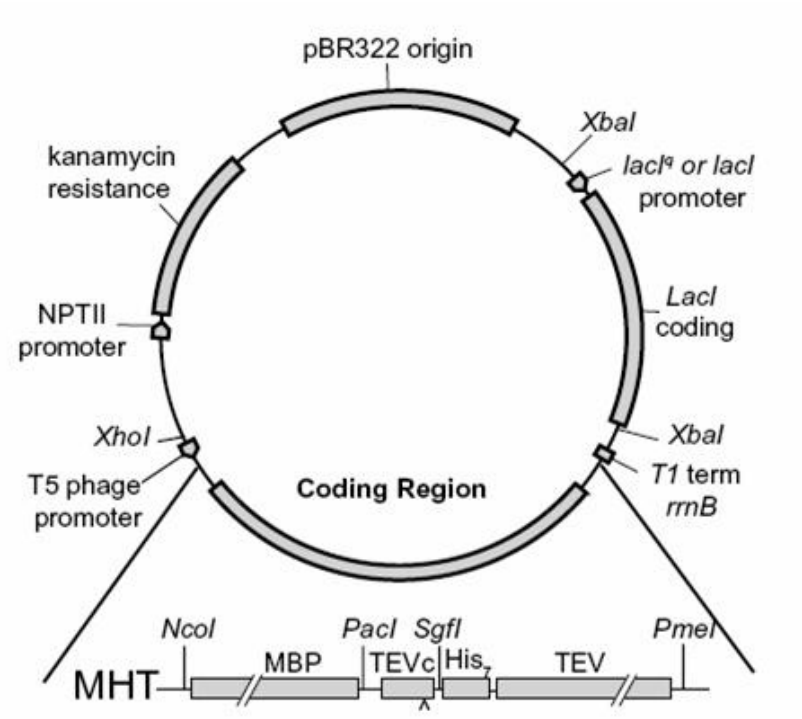


pMCSG7 vector map and LIC site showing the restriction sites



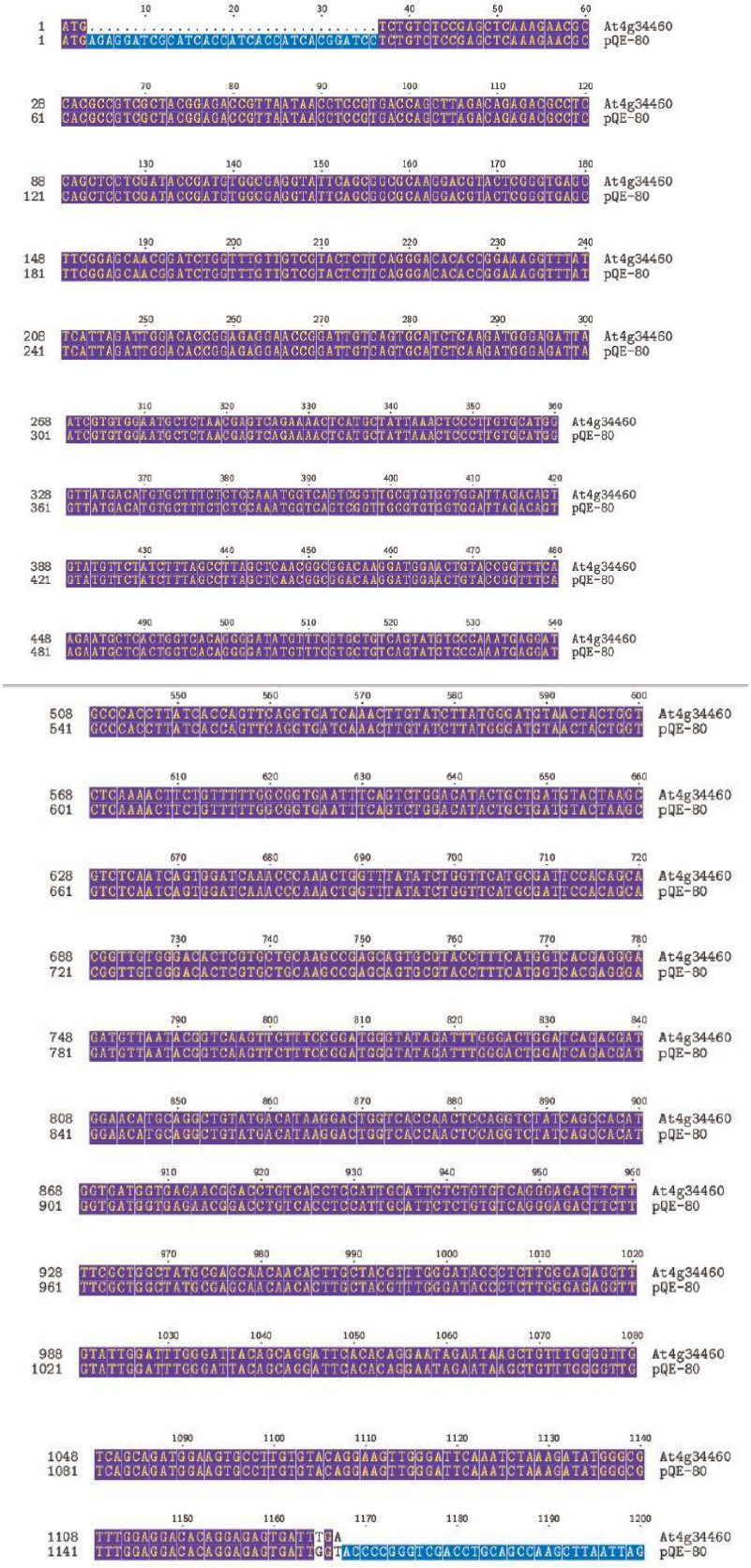
LIC sites showing the available restriction sites

APPENDIX B

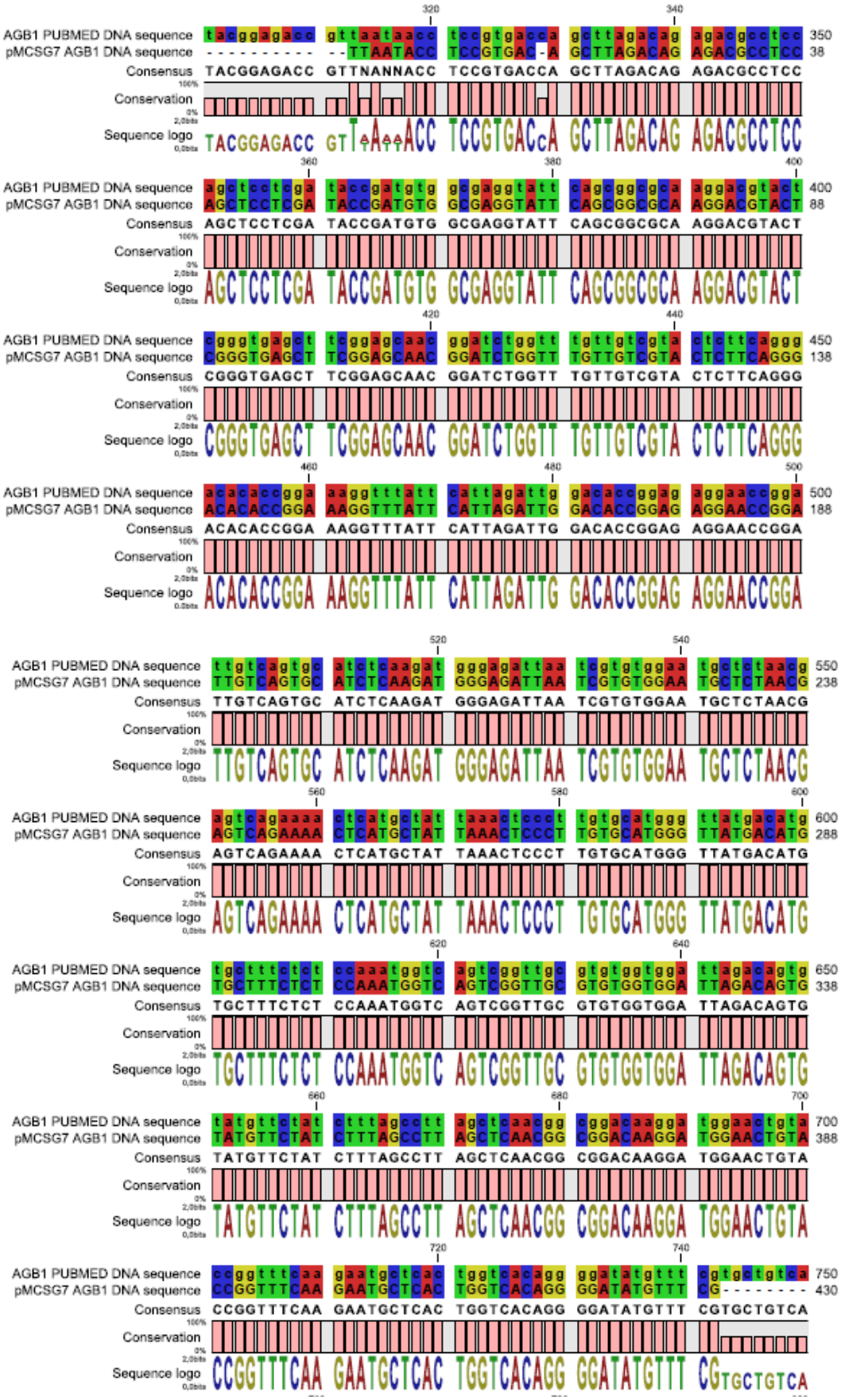


Map of vector pMHTDelta238

APPENDIX C

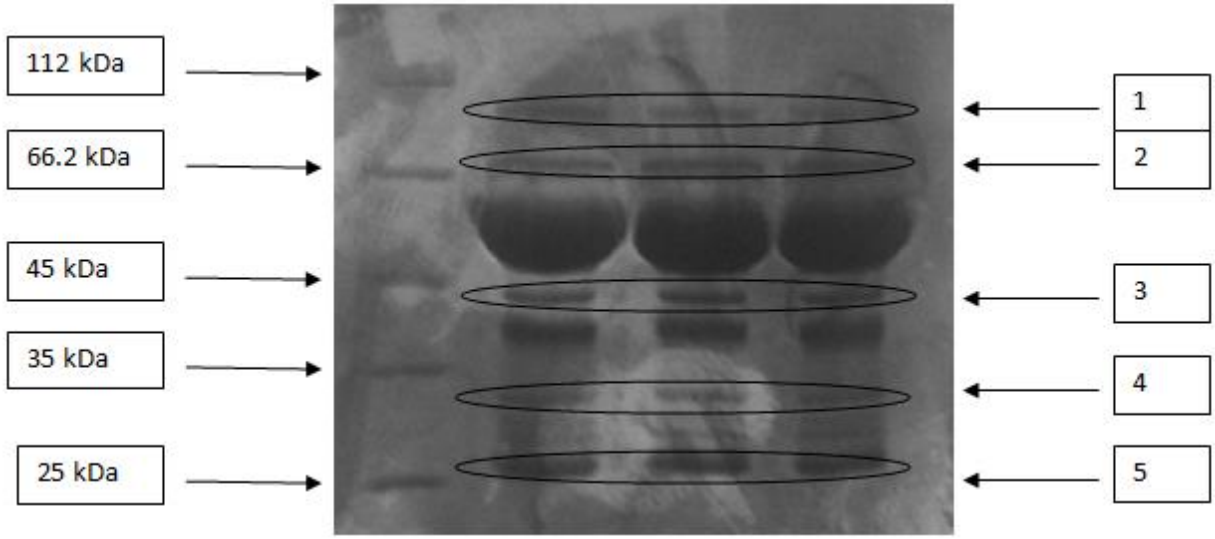


AGB1 DNA Sequence



Verification of AGB1 sequence

APPENDIX D



Probability Legend:
over % 95
% 80 to % 94
% 50 to % 79
% 20 to % 49
0% to % 19

Bio View:
Identified Proteins (22)
Including 0 Decoys

#	Visible?	Starred?	Accession Number	Molecular Weight	Protein Grouping Ambiguity	Taxonomy	Biological Proc...	Cellular Comp...	Molecular Functi...	gel s...	gel s...	gel s...	gel s...	gel s...
							biological regulation, cellular process, establishment of localization, localization, metabolic process, response to stimulus	cytoplasm, intracellular organelle, membrane, organelle part, plasma membrane, ribosome	binding, catalytic activity, electron carrier activity, molecular function, structural molecule activity, transcription regulator activity, translation regulator activity	BioSample 1	BioSample 2	BioSample 3	BioSample 4	BioSample 5
1	☑	☆	RPOL BPT7 DNA directed RNA poly... P00573	?		unkno...				% 100	% 99			
2	☑	☆	CH601 ECOK1 60 kDa chaperonin ... A1AJ51 (+...	?		unkno...				% 100	% 100	% 100		
3	☑	☆	ARNA ECOLC Bifunctional polymyx... B1IXT2 (+3)	?	★	unkno...				% 100	% 100			
4	☑	☆	NUOG ECOLI NADH quinone oxidor... P33602	?		unkno...				% 100				
5	☑	☆	ODO1 ECOLI 2 oxoglutarate dehyd... P0AFG3 (+2)	?		Escheri...				% 100				
6	☑	☆	EFTU1 ECOK1 Elongation factor Tu ... A1AGM6 (+...	?		Escheri...						% 100	% 100	
7	☑	☆	GBB ARATH Guanine nucleotide bi... P49177 (+1)	?		unkno...				% 100		% 100	% 100	
8	☑	☆	DNAK ECOK1 Chaperone protein d... A1A766 (+9)	?		Escheri...					% 100			
9	☑	☆	GLND ECO5E Protein PII uridylyltr... B5Z0E5 (+1)	?		Escheri...				% 100				
10	☑	☆	GLMS ECOLI Glucosamine fructose... P17169 (+3)	?		Escheri...					% 100			
11	☑	☆	HFQ ECOK1 Protein hfq OS Escheri... A1AJ78 (+...	?		unkno...				% 100	% 100			
12	☑	☆	LACI ECOLI Lactose operon repres... P03023	?		Escheri...						% 100		
13	☑	☆	NAGC ECOLI N acetylglucosamine ... P0AF20 (+3)	?		Escheri...						% 100		
14	☑	☆	CYSD ECO24 Sulfate adenylyltrans... A7ZQJ6 (+...	?		Escheri...							% 100	
15	☑	☆	YHBW ECOLI Uncharacterized prot... P0ADV5 (+1)	?		Escheri...							% 100	

Verification of AGB1 by mass spectrometry

APPENDIX E

SDS-PAGE:

12 % Separating Gel:

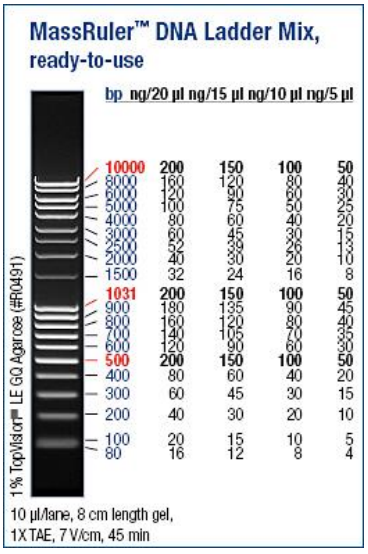
	for 2 gels	for 1 gel	[final]
dH_2O	4.62 ml	2.31 ml	
3 M Tris, pH 8.9	1.25 ml	625µl	3.75 mM
30 % Acryl-0.8 % Bisacryl	4 ml	2 ml	12 %
20 % SDS	50 µl	25 µl	0.1 %
10 % APS	75 µl	37.5 µl	0.075 %
TEMED	5 µl	2.5 µl	0.05 %

5 % Stacking Gel:

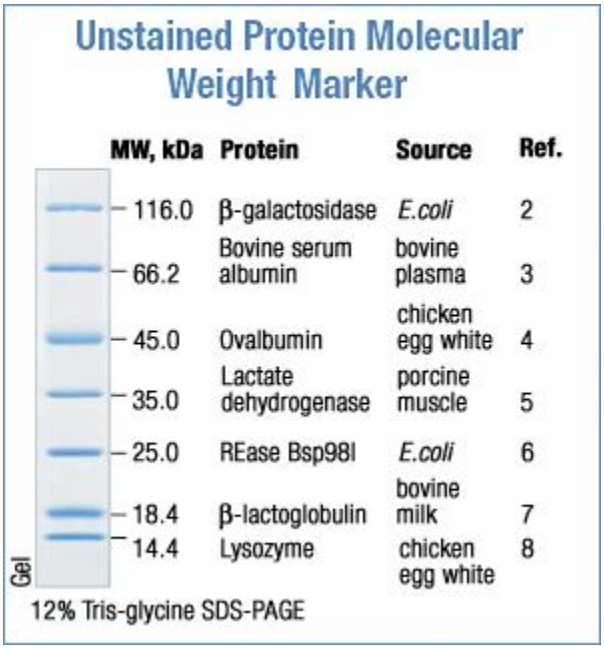
	for 2 gels	for 1 gel	[final]
dH_2O	3.850 ml	1.925 ml	
3 M Tris, pH 8.9	250 µl	125 µl	50 mM
30 % Acryl-0.8 % Bisacryl	850 µl	425 µl	5 %
20 % SDS	10 µl	5 µl	0.1 %
10 % APS	37.5 µl	18.75 µl	0.075 %
TEMED	2.5 µl	1.25 µl	0.05 %

APPENDIX F

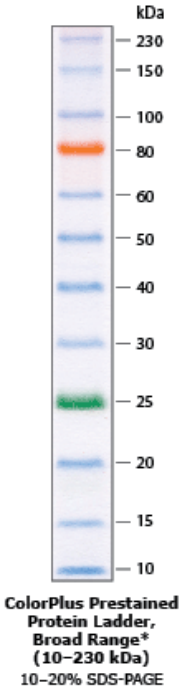
Molecular Weight Markers



Mass Ruler DNA Ladder Mix (Fermentas)



Unstained Protein Molecular Weight Marker (Fermentas)



BioLabs Color PlusPrestained Protein Ladder

APPENDIX G

CHEMICALS

Acetic acid (glacial)	Riedel-de Haen, Germany	27225
30 % Acrylamide-0.8 % Bisacrylamide	Sigma, Germany	A3699
BamHI	Fermentas, Germany	ER0051
Bromophenol blue	Applichem, Germany	A3640
CoomassieBrillant Blue R-250	Fluka, Switzerland	27816
EDTA- Free Complete Protease Inhibitor Cocktail Tablets	Roche	11873580001
dNTP mix	Fermentas, Germany	R0241
1,4-Dithiothreitol	Fluka, Switzerland	43815
*Eco*RI	Fermentas, Germany	ER0271
Ethanol	Riedel-de Haen, Germany	32221
Ethylenediaminetetraaceticacid	Riedel-de Haen, Germany	27248
Glycerol (87 %)	Riedel-de Haen, Germany	15523
Glycine	Amresco, USA	0167
HEPES	Fluka, Switzerland	54461
Hydrochloric acid (37 %)	Merck, Germany	100314
Imidazole	Sigma, USA	I5513
IPTG	Fermentas, Germany	R0392
KH_2PO_4	Amresco, USA	0781
K_2HPO_4	Amresco, USA	0782
Lysozyme	Sigma, USA	L7651
MassRuler DNA Ladder Mix	Fermentas, Germany	SM0403
2-Mercaptoethanol	Aldrich, Germany	M370-1

Methanol	Riedel-de Haen, Germany	24229
Na_2HPO_4	Duchefa, Germany	S0522
NaH_2PO_4	Duchefa, Germany	S0521
NcoI	Fermentas, Germany	ER0571
Non-Fat Bovine Milk Powder	Sigma, USA	C3400
PageRuler protein ladder	Fermentas, Germany	SM0661
Phenylmethylsulphonylfluoride	Amresco, USA	0754
2-Propanol	Merck, Germany	100996
Protein Molecular Weight Marker	Fermentas, Germany	SM0431
PVDF Membrane	Sigma, USA	P2938
Qiaprep Miniprep Kit	Qiagen, USA	19064
Qiaquick PCR Purification Kit	Qiagen, USA	28104
Qiaquick Gel Extraction Kit	Qiagen, USA	28704
Sodium Chloride	Riedel-de Haen, Germany	13423
Sodium dodecyl sulphate	Sigma, Germany	L-4390
SspI	Fermentas, Germany	ER0771
T4 DNA Ligase	Fermentas, Germany	EL0011
T4 DNA Polymerase	Fermentas, Germany	EP0061
Taq polymerase	Fermentas, Germany	EP0401
Tris	Fluka, Switzerland	93349
Triton X-100	Applichem, Germany	A1388
Tryptone	Sigma, Germany	61044
Tween-20	Sigma, USA	P1379
Yeast Extract	Sigma, Germany	Y1625

APPENDIX H

EQUIPMENTS

AKTA Prime:	GE-Healthcare, SWEDEN
Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Cenrifuge:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, 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, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
Microwave Oven:	Bosch, TURKEY
pH Meter:	WTW, pH540 GLP MultiCal, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4^{o}C, Bosch, TURKEY
Semi-Dry Western Transfer Machine	Novex, USA

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
Speed Vacuum:	Savant, Speed VacPlus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	Eppendorf, Mastercycler Gradient