REPUBLIC OF TURKEY
ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

DEVELOPMENT OF HIGH THROUGHPUT METHODS FOR HIGH CAPACITY EXPRESSION AND PURIFICATION OF NOVEL ANTIMICROBIAL PEPTIDES TO BE USED IN MEDICINE

GİZEM BOSKAN

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SUPERVISOR

Assoc. Prof. Dr. Ozge Can

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T.C.
ACIBADEM MEHMET ALİ AYDINLAR ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

İLAÇ OLARAK KULLANILMAK ÜZERE TASARLANAN ANTİMİKROBİYAL PEPTİTLERİN YÜKSEK KAPASİTEDE EKSPRESYONLARI VE SAFLAŞTIRILMALARI İÇİN YÖNTEMLERİN GELİŞTİRİLMESİ

GİZEM BOSKAN

YÜKSEK LİSANS TEZİ

TIP MÜHENDİSLİĞİ ANABİLİM DALI

DANIŞMAN

Doç. Dr. Özge Can

İSTANBUL-2020

DECLARATION

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
LIC	Ligation independent cloning
PCR	Polymerase chain reaction
GST	Glutathione-S-transferase
Trx	Thioredoxin
MIC	Minimal inhibitory concentration
SPPS	Solid-phase peptide synthesis
DMF	Dimethylformamide
IB	Inclusion body
MBP	Maltose-binding protein
NusA	N-utilizing substance A protein
Sumo	Small ubiquitin-related modifier
GFP	Green fluorescent protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IMAC	Immobilized metal affinity chromatography
CNBr	Cyanogen bromide
TEV	Tobacco etch virus
RP-HPLC	Reverse-phase high performance liquid chromatography
SEC	Size exclusion chromatography
IEC	Ion exchange chromatography

PEG	Polyethylene glycol
NHS	N-hydroxysuccinimide
LB	Luria Broth
F.A.	Formic acid
EDCI	1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide
ACN	Acetonitrile
t	Time
T	Temperature
FT-IR	Fourier transform infrared
^{1}NMR	Proton nuclear magnetic resonance

SUMMARY

Antimicrobial peptides (AMPs) have a powerful attention as candidate molecules for biotechnological drug industry. Large scale production of them can be a significant challenge if only the chemical synthesis techniques are considered. Bioproduction approaches can be developed by using different fusion partners, co-expressed with peptides, to overcome challenges during expression and purification of the AMPs. In this study, for the production of TN1 and TN3 AMPs, different gene constructs were designed by using different fusion partners and strategies for the removal of them and introduced to the selected vectors by using ligation independent cloning (LIC) approach. Bacterial systems were used to express proteins and protein expression conditions were investigated to obtain soluble / insoluble systems. Protein extraction and purification from inclusion bodies were investigated by using several approaches. Soluble His-tagged proteins were purified and removal of the fusion partners were studied in different conditions by using formic acid and TEV protease. Cleaved products were examined according to their size and their activities. Furthermore, strategies have been developed to improve TN1 and TN3 by conjugating with biocompatible polymer chains to make them more stable to proteases and effective. Cystine modified peptides from their both N and C terminus were synthesized by solid phase peptide synthesizer and characterized with reverse-phase (RP) HPLC. By using orthogonal functionalization, peptides were conjugated to PEG polymer chains and to different linker molecules to obtain single or multivalent peptide-PEG structures. Resulting conjugates were analyzed by RP-HPLC.

Keywords: Antimicrobial Peptides, Bioconjugation, Biotechnological Drug, Ligation Independent Cloning, Peptide Synthesis, Polymer-Peptide Conjugate.

ÖZET

İlaç Olarak Kullanılmak Üzere Tasarlanan Antimikrobiyal Peptitlerin Yüksek Kapasitede Ekspresyonları ve Saflaştırılmaları için Yöntemlerin Geliştirilmesi

Antimikrobiyal peptitler (AMP'ler) biyoteknolojik ilaç endüstrisinde aday moleküller olarak güçlü bir ilgiye sahiptir. Büyük ölçekli üretimleri için sadece kimyasal sentez teknikleri dikkate alındığında önemli zorluklar ile karşılaşılabilir. AMP'lerin biyolojik olarak üretimlerinde, ekspresyonları ve saflaştırılmaları sırasındaki zorlukların üstesinden gelebilmek için füzyon partneri denilen proteinler ile beraber eksprese olacakları metotlar geliştirilmektedir. Bu çalışmada, TN1 ve TN3 AMP'lerinin üretimleri için farklı füzyon partnerleri kullanılarak gen yapıları tasarlanmış ve bu partnerlerin ekspresyon sonrası uzaklaştırılabilmesi için farklı kesim yöntemlerinin uygulanmasını sağlayacak olan özel bölgeler bu tasarımların içinde yer alarak ligasyondan bağımsız klonlama tekniği için seçilen vektörler içerisine yerleştirilmiştir. Bakteriyel sistemler kullanılarak proteinler eksprese edilmiş ve ekspresyon koşulları çözünebilir / çözünemeyen protein sistemlerinin eldesine göre incelenmiştir. İnklüzyon cisimcikleri içerisinden proteinlerin ekstraksiyonu ve saflaştırılması için farklı teknikler denenmiştir. Çözünebilir fraksiyondaki His-tag'li proteinler saflaştırılmış ve füzyon partnerlerinin uzaklaştırılması için formic asit ve TEV proteazı ile kesim için farklı koşullar denenmiştir. Kesilen protein parçaları, boyut ve aktivitelerine göre incelenmiştir. TN1 ve TN3 peptitlerini proteazlara karşı dirençli hale getirmek ve daha etkin olmalarını sağlayabilmek için biyouyumlu polimerler ile konjugatlarının eldesi için farklı stratejiler geliştrilmiştir. N ve C uçlarından sistein amino asiti ile modifiye edilmiş olan peptitler katı faz peptit sentezleyici ile sentezlenmiş ardından ters faz yüksek basınçlı sıvı kromatografi (RP-HPLC) sistemleri ile karakterize edilmiştir. Peptitler, PEG polimer zincirleri ve tek veya çift peptit bağlanmasına imkan tanıyan farklı ara bağlayıcılar ile konjuge edilmiştir. Elde edilen konujagatlar, RP-HPLC ile analiz edilmiştir.

Anahtar Sözcükler: Antimikrobiyal Peptitler, Biyokonjugasyon, Biyoteknolojik İlaç, Ligasyondan Bağımsız Klonlama, Peptit Sentezi, Polimer-Peptit Konjugatı.

1. AIM OF THE STUDY

The ultimate goal of this work is to produce peptides with antimicrobial properties. For this, several approaches were used to express these special peptides with high yield and capacity with a non-disrupted antimicrobial character while they were expressed in bacterial systems. Different fusion partners were supplied with different cloning vectors. Special gene fragments were designed and introduced to these vectors, obtained recombinant plasmids were transformed into BL21 expression lines, and then expression profiles were examined. Purification studies were carried out depending on the expression behavior of the constructs with different fusion partners.

In this study, the fusion of AMPs (TN1 and TN3) with the different fusion partners was attempted as an approach for their large-scale production and could also be used for the production of the analogs of these AMPs.

Within the scope of the thesis, strategies have been also developed to improve the peptides, developed as the original drug and whose antimicrobial character has been proven in previous studies, by conjugating with biocompatible polymer chains to make them more stable and effective. AMPs were synthesized by solid phase peptide synthesizer, N and C terminal groups were activated with cystine and were linked to PEG polymer chains using different orthogonal functional linker molecules. Thus, it is aimed to increase the bioavailability of these peptides with therapeutic effect both by reducing proteolytic degradation and increasing their stability, increasing circulation speed in the body and increasing their half-lives. Another unique aspect of the developed strategies is that it allows to reveal which ends of the peptides have antimicrobial effect and interact with the bacterial membrane.

2. INTRODUCTION

Increasing antibiotic resistance among the infection related microorganisms to the antibiotics, has created a great attention in the wide world. Whenever a new antibiotic is designed, microorganisms become resistant against them, rapidly. Therefore, developing antibiotics which microorganisms cannot become easily resistant against them is crucial, especially for biopharma industry.

In nature, over the millions of years, from the insects to humans, a wide range of organisms create some peptide-form antibiotics which are a form of their innate immune system, bacteria cannot become resistant them. Mechanism of action, production strategies and a functionalization approach by bioconjugation strategy of the antimicrobial peptides (AMPs) were discussed in the following sections.

2.1. Antimicrobial Peptides (AMPs)

AMPs have been identified in a vast variety of animals and plants, at least 3200 AMPs have been identified up to 2020 according to the Antimicrobial Peptide Database tool, with a role in defense against pathogens from the beginning of the life [1-5].

Several AMP families have been found including cathelicidins, defensins and etc. [1,6]. They are generally short amphiphilic peptides with a cationic net charge and form α-helical structure in common. These peptides are thought as helping molecules for the producing microorganisms to protect themselves from competitors or their hosts [1,6,7].

One of the most important common features of AMPs is their amphophilic character, which is necessary for penetration into the membranes of pathogenic microorganisms. The presence of negatively charged groups on bacterial membranes also create electrostatic interaction with positively charged amphipathic groups of AMPs with the membranes. This alters the membrane structure and creates pores in the membrane [6]. At the beginning, AMPs cover the outer membrane and then pass through the peptidoglycan part and inner membrane of the Gram-negative bacteria, finally reach to the cytoplasm of the microorganism [8,9].

There are several offered key mechanisms in literature. (Figure 1). Membrane destruction occurs in several ways; in the barrel-stave" and "toroidal pore" models, pores are formed by AMPs in the membrane, while micelles are formed with membrane components. The mechanisms can alter with the peptide concentration, pH or the temperature [1, 6, 10].

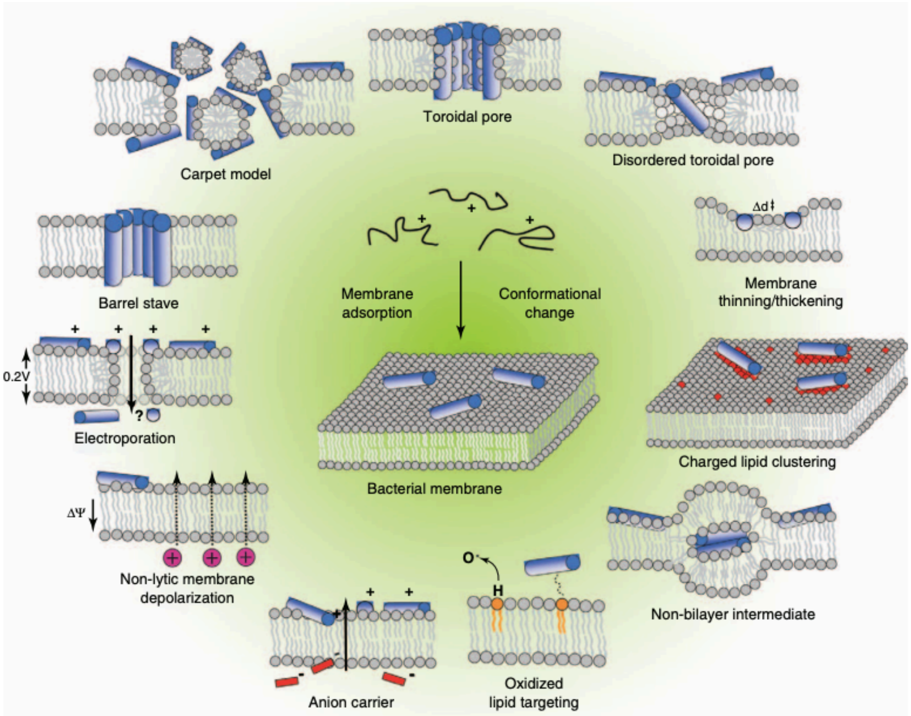


Figure 1. Effects of AMPs to the bacterial cell membrane [11].

Unubol et.al showed some preliminary studies to develop some peptides which mimic the natural antibiotics and they were found with valuable antimicrobial activity in vitro (Figure 2). Molecular modelling and dynamic simulation strategies, which aim to mimic the bacterial membrane and cytoplasm, study the pathway of these AMPs and try to understand the activities of newly designed special candidate AMPs.

Antimicrobial Peptides	*S. aureus* **ATCC 29213**	*E. coli* **ATCC 25922**	*K. pneumoniae* **ATCC 10031**	*P. aeruginosa* **ATCC 27853**
TN1: RLLRLLLLRLLR	16	2	16	16
TN2: KLLKLLKLLL	64	16	512	>512
TN3: RLLRLLRLLL	8	4	16	16
TN4: RLLRLLRLLLLLL	>512	>512	512	256
TN5: RLLLRLLLRLLLR	>512	>512	>512	>512
TN6: RLLRLLLRLLR	4	4	8	32
TN7: RLLRLLRLL	256	64	256	64

Figure 2. Minimal Inhibitory Concentration (MIC) (μg/ml) values of TN AMPs [12].

AMPs have many potential aspects as antimicrobial drugs. They are active in a broad spectrum while inducing little resistance and can coordinate immune response. In contrast to bacterial growth in the presence of commonly prescribed antibiotics, the growth of bacteria in the presence of AMPs does not easily give rise to the selection of pathogenic drug-resistant mutant strains [6]. This is because AMPs rapidly kill microbes by a variety of mechanisms, including (i) fatal depolarization of the normally energized bacterial membrane, (ii) creation of physical holes that cause cellular contents to leak out, (iii) degradation of the cell wall, (iv) disturbance of membrane functions, and/or (v) damaging of critical intracellular targets after internalization of the AMPs [13, 14, 15].

Moreover, AMPs activate the host's innate (nonspecific) immune response without acting as a foreign antigen target of the host's adaptive immune system [6, 13].

2.2. Production Strategies of AMPs

AMPs have a great potential to enhance a significant innovation and form the basis for a new class of antibiotics. Since, AMPs are usually short peptides, chemical synthesis could be one approach for producing them. Nevertheless, for the commercialization of the AMPs, a cost- effective and scalable method of active production is necessary [16]. To produce large amounts of recombinant proteins, bacterial expression of heterologous proteins is applied as an easy and inexpensive tool [2, 17].

Under the scope of this thesis 2 main strategies were used to produce the AMPs.

2.2.1. Solid-Phase Peptide Synthesis (SPPS) of AMPs

While mimicking the AMPs from nature, especially peptides with the short sequences can synthesis in a short time for their complete characterization and for activity screening [1, 18]. SPPS is a major method to synthesize the peptides for many years [16, 19]. However, large scale peptide production using SPSS and especially for long peptide chains (larger than 35 amino acids) have always been an important challenge in terms of the complexity of the chemical reaction steps for each amino acid residue [16, 20]. High volume usage of the toxic reagents for its coupling and activation reactions can cause severe environmental issues [16].

Traditional approach for SPPS with F-moc protection/deprotection chemistry has been applied for this study and described in further section.

2.2.1.1. F-moc Protection/Deprotection Chemistry Approach.

SPPS strategy by using an automated machine which uses the microwave energy to produce a peptide of interest with high efficiency and purity according to the F-moc chemistry methodology was used. In this method, basically, a peptide is produced on a specific solid support by beginning with its C terminus followed through to N terminus of the peptide. At the beginning, the first amino acid of a sequence binds to the solid phase with its COOH group which is in C-terminus and when the reaction starts, deprotection takes place. It means the protective group which is at the N terminus of this first attached amino acid is washed away with proper chemicals. Therefore, the following amino acid can bind to the first one with its COOH group. It means coupling step can succeed. At this time, the amino group at the N terminus of the second amino acid should also be protected with a proper protective group to block the unwanted reactions. Amide bond is formed with this reaction between the two amino acids. And the chemicals which are used for this reaction are washed away with dimethylformamide (DMF). Therefore, only the dipeptide with an amide bond remains on the solid resin. The second round can take place when the protective of the N terminus of this dipeptide washed away. This cycle continues for every step of the process until the sequence of interest is achieved. At the last step of the synthesis, after the deprotection step, the peptide of interest is cleaved from the solid support, and at the same time, from its side-chain protective groups with a proper cleavage conditions (chemicals, temperature etc.) [19, 21].

2.2.2. Production with Recombinant DNA Technology

Because of the high costs of the chemical production of the AMPs in the industrial scale, which also creates environmental pollution by using high toxicity chemicals, production with recombinant DNA technology will overcome these problems with its high production capacity of a peptide of interest.

Although, different hosts can be used to produce recombinant proteins with different strategies, *Escherichia coli* is one of the major hosts for producing them because of it enhances rapid, cheap, and high yield protein production. Molecular tools vary to use the manipulate the well-characterized genetics of *E. coli* [22, 23]. But as a bacterial system, *E. coli* has disadvantageous while producing complex proteins, due to simple nature of bacteria limiting post-translational modifications and proper formation of disulfide bonds. However, the benefits of cost and ease of use of *E. coli* make a convenient system to produce simple proteins like peptides by enhancing to design new strategies [22]. Protein aggregation can occur as a result of lack of the correct folding of proteins while expressing complex or toxic proteins to the host cell and it leads the formation of the inclusion bodies (IBs) [22, 24]. IBs can be advantageous for some aspects explained in further.

DNA cassettes are designed for the high expression and purification of the peptides for the recombinant production in the bacterial systems. A major disadvantage to produce an AMP by using bacterial system is AMPs are toxic to the bacteria. As a result, AMPs are convenient to proteolytic degradation. And AMPs are also small molecules which creates a problem about degradation again and also another disadvantage to purify them effectively. To overcome such problems, we are using different fusion partners to create fusion proteins, by binding our peptide of interests to the special fusion partners, to hide AMPs' toxic effect to the bacteria and to develop effective purification strategies which will give higher yields of the peptide of interest [2, 25]. The high toxicity of the peptides usually leads to expression in IBs or in aggregate form. To overcome this situation, the use of a fusion partner is highly recommended [2, 26].

There are several fusion partners (e.g., solubility-enhancing, aggregation-promoting, and self-cleavable carriers, etc.), cleavage methods and optimization options for AMPs production in *E. coli* [2, 27]. A high amount of solubility enhancer tags, including the maltose-binding protein (MBP), N-utilizing substance A protein (NusA), thioredoxin (Trx), glutathione-S-transferase (GST), small ubiquitin-related modifier (Sumo), Green fluorescent protein (GFP), take place in literature [16, 22, 28]. There are several hypotheses on the way of solubility enhancer partners, although they remain unclear: (i) fusion proteins can form micelle-like structures, (ii) partners attract chaperones, (iii) fusion partners can have an intrinsic chaperone-like activity, net charges of fusion partners [22]. On the other hand, aggregation-promoting partners (e.g., PurF fragment and ketosteroid isomerase) also investigated [29, 30].

Researchers suggest that low cultivation temperature (i.e., 30 °C) and low IPTG (Isopropyl β-D-1-thiogalactopyranoside) concentration (i.e., 0.1 mM) drive the protein expression to the soluble fraction [27]. Although, fusion partners can be selected as their solubility-enhancing properties from the literature, trials should be done for each of the target protein due to fusion partners do not act equally against used target proteins [16, 22]. Addition to use a fusion partner, it has also shown that reinforcing the formation of inclusion bodies to protect the expressed peptides from proteolytic degradation by host proteases [13, 24]. In this strategy, using of an anionic fusion partner has been suggested to neutralize the charge of the cationic peptides. As a result, it has been shown neutralized activity of the peptides while protein expression and drive the accumulation of the expressed proteins to the IBs through electrostatic interactions [13, 30, 31]. Whereas, protein purification from IBs is always a challenge, opposite to its advantages while protein expression steps, needing denaturation–reduction and subsequent refolding steps should be done to obtain active AMPs [16, 24].

Another important point while using the fusion partners, to increase the relative yield (%) of the resulted peptides, to be obtained after the whole purification steps, selection of the smaller fusion partners has been suggested [13]. Furthermore, fusion partner can aid to purify proteins with their special properties. Affinity tags are reliable to purify proteins in high purity. Dual- tagging strategies are also useful approaches to obtain higher purity proteins by specific isolation method to their tags [16, 22, 28].

His6 affinity tag consists of six consecutive histidine residues and coordinates the transition metal ions, such as Ni^{2+} or Co^{2+} immobilized on beads or a resin for immobilized metal affinity chromatography (IMAC), with the help of the histidine imidazole ring [22].

GST fusion protein also serves as an affinity tag and enhances a purification on immobilized glutathione. Although many studies show great success, GST fusions can be quite convenient to proteolytic degradation leading insufficient production level of the peptides [27, 32, 33]. Sumo fusion tag has been demonstrated to improve folding and solubility of the target proteins [16, 27, 34]. Sumo protein has also own protease (Sumoase) enabling removal of non-residual removal of the Sumo fusion. GFP and Sumo fusion partners require additional tags for purification. In common, His-tag is used additionally to aid for purification of the fusion proteins [16, 27, 35].

We study on the different fusion partners to develop different production strategies. They are differing from each other with their size, charge distribution which affect the purification strategies and production levels of our cationic, antimicrobial, small peptides. For instance, histidine repetitives can be used to purify with nickel affinity columns by adding them near to the peptide coding region [2]. Besides, fusion proteins like GST can be directly purified by using glutathione affinity chromatography [36]. Enzymatic or chemical cleavage strategies can be used to separate protein/peptide complex by inserting a cleavage site, specific to the interested strategy, to between the protein and peptide sequences [37]. For the removal of the different fusion partners, chemical cleavage approaches can enhance reduced cost compared to enzymatic cleavage techniques. After the cleavage, depending on the method, some additional amino acid residues can remain at the beginning or end of the peptide of interest [16, 38]. Many cleavage reagents are described. The basic chemicals used for fusion cleavage are Cyanogen bromide (CNBr), formic acid and hydroxylamine. CNBr is more frequently used among them. It recognizes methionine residues and cleaves from their C-terminus [24]. Because of its recognition site only methionine specific, that can cause troubles handling the methionine rich sequences in the partner or linker regions and limit its usage [39]. Formic acid recognizes Asp-Pro sequence and cut between them while hydroxylamine interacts Asn-Gly sequence [24, 40]. Recognizing residue pairs makes them more site-specific reagents compared to CNBr cleavage. Chemical cleavage can be hazardous and convenient to side-chain modifications [16, 27]. There are several enzymatic cleavage options. Tobacco etch virus (TEV) protease recognizes GluAsnLeuTyrPheGln-(Gly/Ser) motif, while Sumo protease interacts with Sumo's tertiary structure instead of small sequence motifs and enhances the non-residual production of the target protein [16, 34]. The other enzymatic cleavage reagents like enterokinase, factor Xa and thrombin are more expensive and sensitive to chaotropes and pH. Formic acid was chosen for hydrolysis instead of thrombin or factor Xa proteases, to reduce production costs. And TEV protease was used due to the nature of the selected special cloning vector. Released peptides can be purified with reverse-phase high performance liquid chromatography (RP-HPLC) or ion exchange chromatography (IEC) techniques depending on the related protein mixture [27].

2.2.2.2. Ligation Independent Cloning (LIC) Approach.

Ligase independent cloning (LIC) is a relatively easy, rapid, robust and cheaper technique, developed in the early 1990s, to obtain constructs to be used in expression [41]. That differs from restriction enzyme/ligase cloning. Briefly, related linearized cloning vector can be designed with specific 10-15 base single stranded overhangs created by 3' to 5' exonuclease activity of T4 DNA polymerase with only one dNTP in the reaction mix and the appropriate inserts (PCR products or synthesized oligonucleotides) with complementary overhangs are used to create new plasmids by annealing of the insert and the vector without using of ligase or alkaline phosphatase and by mixing the DNA fragments. 4 nicks from mixed fragments are repaired by host cell during transformation procedure. LIC is very efficient to obtain only the desired products and advantageous in terms of many different vector choices [42, 43, 44, 45, 46, 47].

Detailed information about the LIC method and the special LIC vectors were introduced in Experimental section.

2.3. Bioconjugation Strategy

In recent years, it is a quite common strategy to use therapeutic peptides and proteins by conjugating with polymers to increase their stability and effectiveness to cure diseases. Depending on the chemical properties of used polymers, solubility range in water, stability properties, half-life in the blood and pharmacodynamic effects of the active molecules to be attached can be adjusted according to the aim and application [48].

Especially the small peptide fragments have a quite reduced stability, due to the enzymatic degradation occurs easily while systemic circulation in the body. As a result, active molecules can show only a small impact while treating diseases. That circumstance causes the reduced bioavailability of the therapeutic peptides. Whereas, many studies in the literature show long polymer chains can protect the peptides and proteins from enzymatic degradation and make a contribution to increase their stability. As a result, functional polymeric structures through their reactive groups making possible to conjugate the peptides and proteins attracts great attention [49].

In many studies, polyethylene glycol (PEG) polymer is used by its solubility degree in water and hydrophilic structure. Although the linear PEG chains are not biodegradable, the chains with small molecular weight and branched polymeric structures can be modified as biodegradable by using different bonds (ester, carbonate, disulfide etc.). Further, because of the elimination of the non-specific protein bindings property of the PEG polymer chains, they can help to reduce the attacks for the active molecule from the immune system. Thus, while it is circulating in the blood by covering proteins and peptides, it can reduce the interaction with enzymes and the other blood proteins and the risk of the degradation by the enzymes [50, 51].

As a protein-based therapeutic with PEG, which was demonstrated in 2002 and officially released in the USA in 2015. This drug formulation, called Neulesta and commercially available under the name Neupogen®, was actually obtained by conjugating the PEG chain to a therapeutic protein molecule. This protein-based cyto-regulator, called filgrastim, is a therapeutic that has been released by the company Amgen. Filgrastim, a protein that increases the white blood cells, is actually a recombinant human granulocyte colony-stimulating factor and is used to increase neutrophils to prevent the immune system from decreasing, especially in patients undergoing chemotherapy. By connecting to a single PEG chain, the half-life of the filgrastim protein in the human body has increased from 3-4 hours to 15-80 hours. In addition, given the increased stability of the protein, polymer binding to proteins and improving their pharmacokinetic properties in this way has been intensively studied today as a highly promising strategy [52, 53].

To obtain a conjugate for two molecules, chemical and physical features of them should be determined, at first. The related molecules can be engineered to create a convenient functional group to be attacked to a crosslinker or a reagent in the reactions. Generally, to obtain molecules, chemically compatible with each other, commercial reagents are used. Site specific bioconjugation can be challenging because of the difficulties while dealing with multiples of the same functional group, within the protein sequence, having an active role in reactions [48, 54]. Knowledge of the primary and secondary structures of the protein is necessary to overcome the difficulties of the site specific bioconjugation. Hence, the target residue can be selected as less abundant amino acid for these reactions. Tryptophan and cysteine amino acids are the common targets because of their natural infrequency. Using the molecules as heterobifunctional reagents carrying another functional group which is reactive against another target molecule is a quite common strategy [55, 56, 57].

Under the scope of this thesis, peptides having antimicrobial character will be used to conjugate with high molecular weight polymeric biomaterials by using heterobifunctional reagents in order to obtain stabile structures in physiological environments.

2.3.1. PEGylation by using Orthogonal Functionalization

Generally, carboxylic acid groups of the proteins can be modified for conjugation. Carbodiimide EDC can be used to introduce N-hydroxysuccinimide (NHS) ester to their carboxylic acid groups to be able react them with amines. [55]. Also, increased stability and solubility of the intermediate molecules can be achieved by the addition reaction of NHS [58].

Cysteine includes thiol groups reactive against many reagents such as maleimides. Maleimides can react with sulfhydryl groups (thiol) with double bonding by 1,4 Michael addition reaction and form stable and irreversible thioether bonds [56].

Two-step reactions are carried out by using heterobifunctional reagents. Hence, two target molecules can be reacted with the reagent consecutively. This type of reagents serving to carry two functional groups are useful in many ways. They can improve some reaction conditions like solubility [57]. The fact that two different reactions take place at the same time is possible by using functional groups that will not affect each other. In this way, functional groups are carefully selected and are known as orthogonal groups that allow at least 2 different reactions to be carried out at the same time [59].

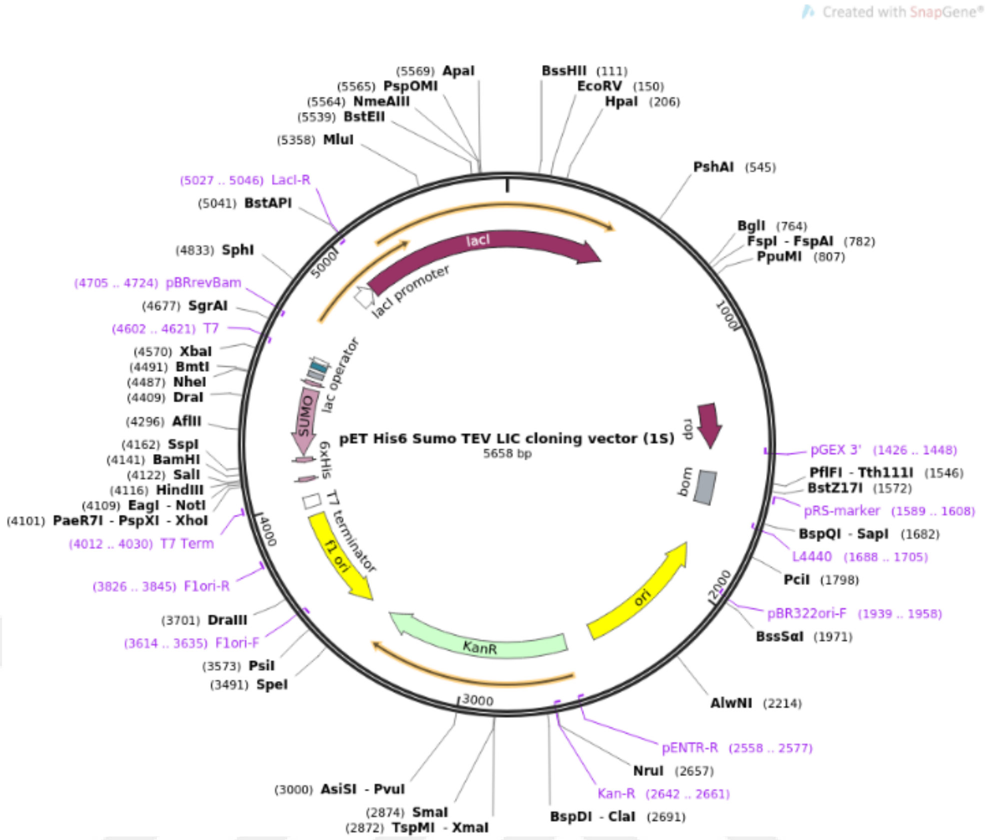
In this study either NHS active or activated linker molecules were used to conjugate PEG and cysteine containing peptides. Two different reactions (1,4 Michael addition and NHS) were carried out by orthogonal functionalization approach.

3. EXPERIMENTAL

This section introduces detailed information about the gene and protein constructs that have been designed, prepared, expressed, purified and characterized with different approaches in this study. The final part of this section includes different types of bioconjugation reactions of AMPs and purification and characterization strategies of them were also explained.

3.1. Synthesis and Cloning of the Genes by Ligation Independent Cloning (LIC) Approach

This section describes the base of the methods to produce and purify AMPs by enhancing the high capacity production. By using LIC strategy, different systems were developed to produce some peptides, known have antimicrobial character [12], with recombinant DNA technology. By the reasons described in the Introduction section, different fusion partners were selected to combine with AMPs and the special LIC vectors (from Addgene) already combined with these selected fusion partners were used. All of these pET vectors (pET His6 GFP TEV LIC cloning vector (1GFP; Addgene #29663), pET His6 TEV LIC cloning vector (1B; Addgene #29653), pET His6 Sumo TEV LIC cloning vector (1S; Addgene #29659), pET His6 GST TEV LIC cloning vector (1G; Addgene #29655)) contain kanamycin resistance, His6 tag to enhance to purify proteins by using nickel affinity column and TEV protease recognition site to cleave the fusion partner from the desired sequence. (Figure 3).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pET His6 Sumo TEV LIC cloning vector (1S)
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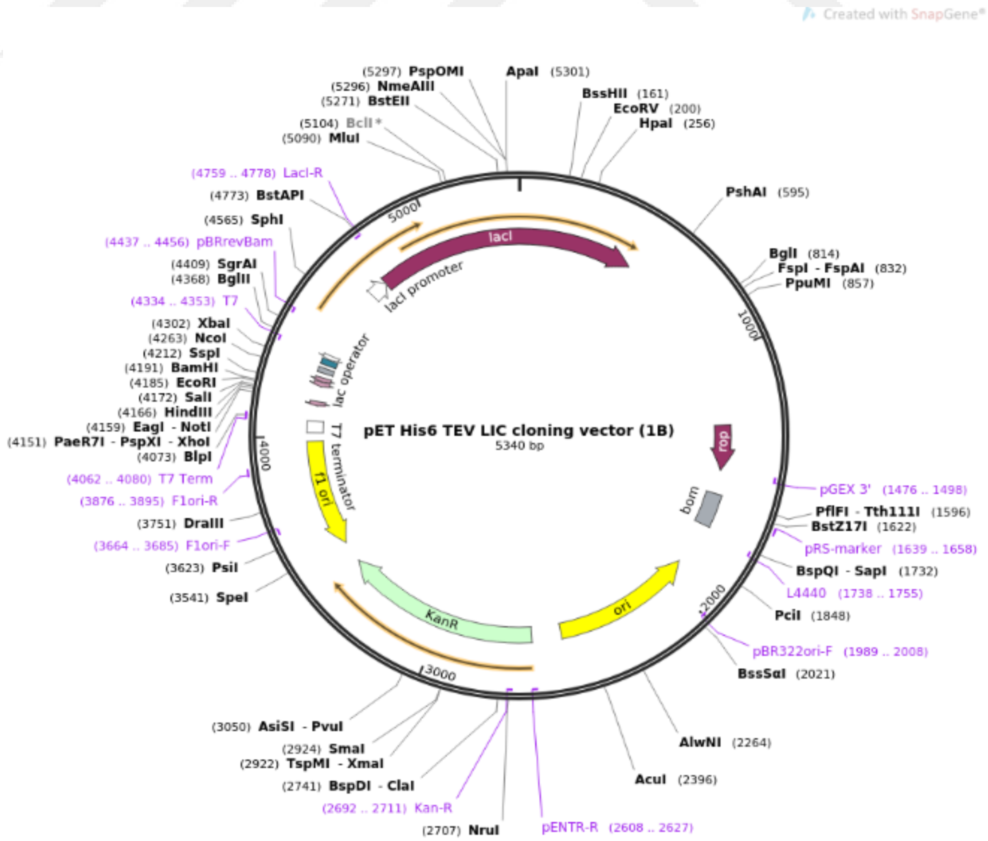
(5569) ApaI
(5565) PspOMI
(5564) NmeAIII
(5539) BstEII
(5358) MluI
(5027 .. 5046) LacI-R
(5041) BstAPI
(4833) SphI
(4705 .. 4724) pBRrevBam
(4677) SgrAI
(4602 .. 4621) T7
(4570) XbaI
(4491) BmtI
(4487) NheI
(4409) DraI
(4296) AflIII
(4162) SspI
(4141) BamHI
(4122) SalI
(4116) HindIII
(4109) EagI - NotI
(4101) PaeR7I - PspXI - XhoI
(4012 .. 4030) T7 Term
(3826 .. 3845) F1ori-R
(3701) DraIII
(3614 .. 3635) F1ori-F
(3573) PsiI
(3491) SpeI
(3000) AsiSI - PvuI
(2874) SmaI
(2872) TspMI - XmaI
BssHII (111)
EcoRV (150)
HpaI (206)
PshAI (545)
BglI (764)
FspI - FspAI (782)
PpuMI (807)
pGEX 3' (1426 .. 1448)
PflFI - Tth111I (1546)
BstZ17I (1572)
pRS-marker (1589 .. 1608)
BspQI - SapI (1682)
L4440 (1688 .. 1705)
PciI (1798)
pBR322ori-F (1939 .. 1958)
BssSαI (1971)
AlwNI (2214)
pENTR-R (2558 .. 2577)
NruI (2657)
Kan-R (2642 .. 2661)
BspDI - ClaI (2691)
lacI
lacI promoter
lac operator
SUMO
6xHis
T7 terminator
f1 ori
KanR
ori
rop
bom
1000
2000
3000
4000
5000

(a)


Created with SnapGene®
pET His6 TEV LIC cloning vector (1B)
5340 bp
(5297) PspOMI
(5296) NmeAIII
(5271) BstEII
(5104) BclI*
(5090) MluI
(4759 .. 4778) LacI-R
(4773) BstAPI
(4565) SphI
(4437 .. 4456) pBRrevBam
(4409) SgrAI
(4368) BglII
(4334 .. 4353) T7
(4302) XbaI
(4263) NcoI
(4212) SspI
(4191) BamHI
(4185) EcoRI
(4172) SalI
(4166) HindIII
(4159) EagI - NotI
(4151) PaeR7I - PspXI - XhoI
(4073) BlpI
(4062 .. 4080) T7 Term
(3876 .. 3895) F1ori-R
(3751) DraIII
(3664 .. 3685) F1ori-F
(3623) PsiI
(3541) SpeI
(3050) AsiSI - PvuI
(2924) SmaI
(2922) TspMI - XmaI
(2741) BspDI - ClaI
(2692 .. 2711) Kan-R
(2707) NruI
ApaI (5301)
BssHII (161)
EcoRV (200)
HpaI (256)
PshAI (595)
BglI (814)
FspI - FspAI (832)
PpuMI (857)
pGEX 3' (1476 .. 1498)
PflFI - Tth111I (1596)
BstZ17I (1622)
pRS-marker (1639 .. 1658)
BspQI - SapI (1732)
L4440 (1738 .. 1755)
PciI (1848)
pBR322ori-F (1989 .. 2008)
BssSαI (2021)
AlwNI (2264)
AcuI (2396)
pENTR-R (2608 .. 2627)
lacI
lacI promoter
lac operator
T7 terminator
f1 ori
KanR
ori
rop
bom
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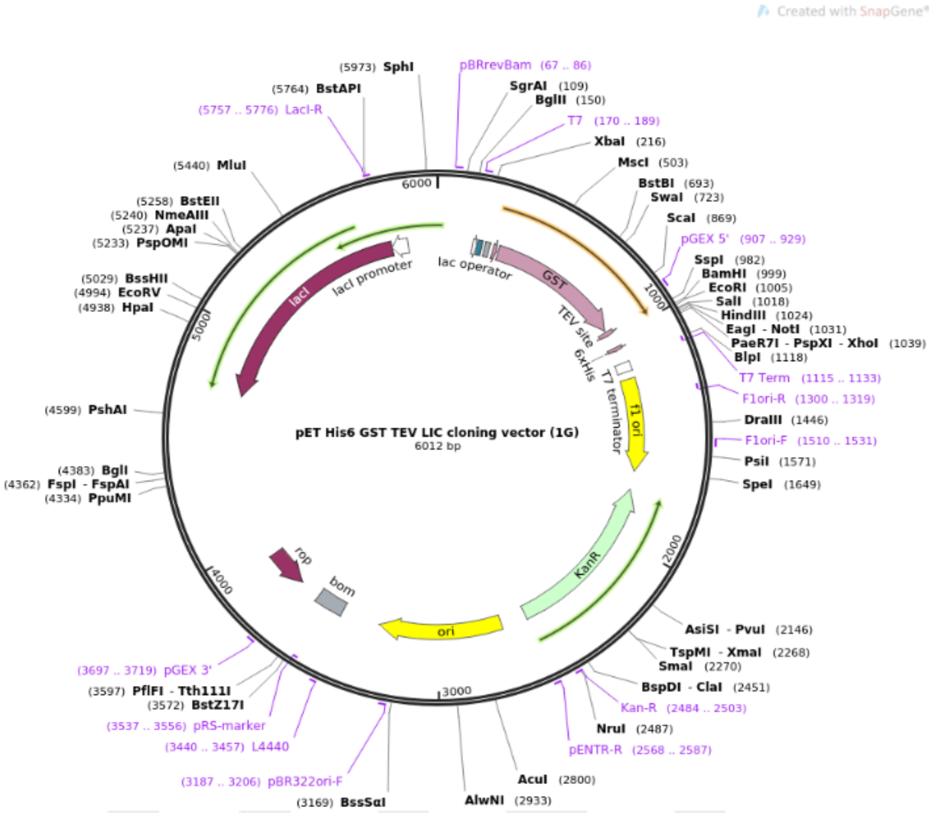
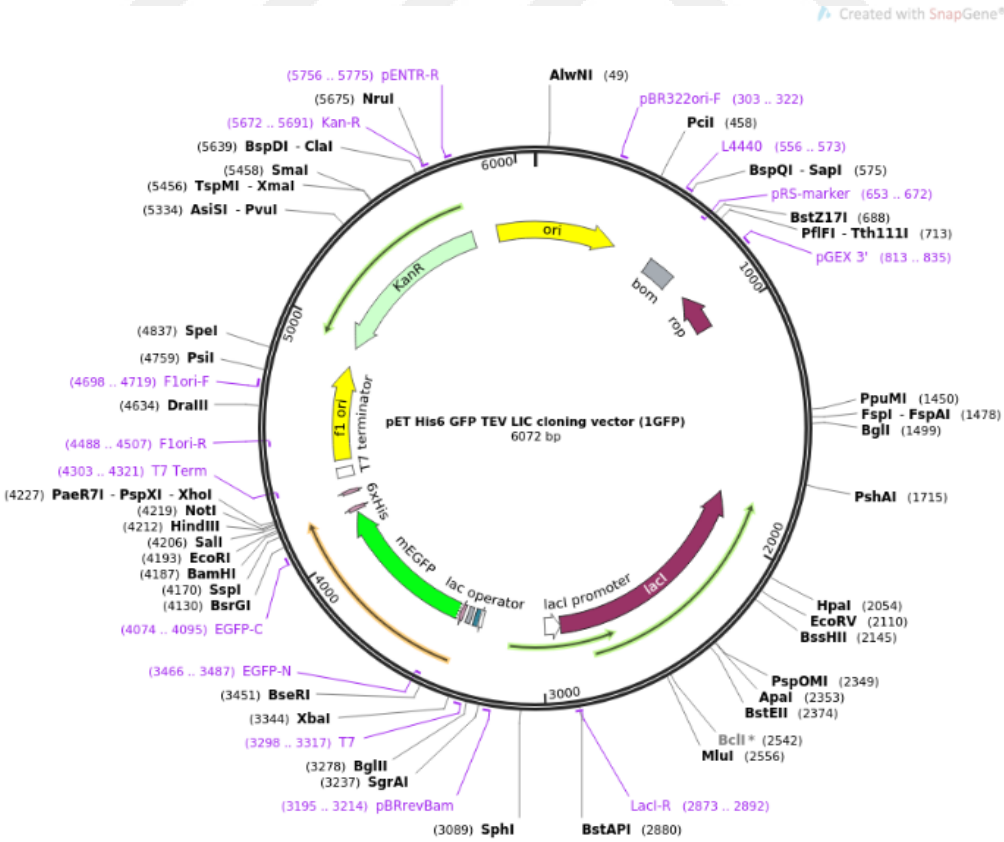
2000
3000
4000
5000

(b)



(c)



(d)

Figure 3. (a) 1S, (b) 1B, (c) 1G, (d) 1GFP LIC vectors (Addgene)

GST partner were used to obtain proteins in the soluble form after the protein expression [32] and it also enhances to purify proteins by using glutathione affinity chromatography. Sumo and GFP partners were used only to investigate the solubilization tendency of the related proteins and as a result of that, expression and purification efficacy were studied and compared. Vector of 1B contains only the His-tag without any extra fusion partner.

Briefly, suggestions from Addgene were applied and the empty plasmid vectors linearized with SspI, recognizes and cuts from the 5'AAT/ATT sequence, thereby the "LIC region", which is also close to the TEV restriction site, opens for cloning (Figure 4).

Figure 4. SspI restriction site

Then, to the vector, T4 DNA Polymerase was applied for the "chew back" reaction with presence of dGTP; meaning that T4 DNA Polymerase will remove bases from the 3' end of the cut site until the first Guanine base is reached as shown in Figure 5.

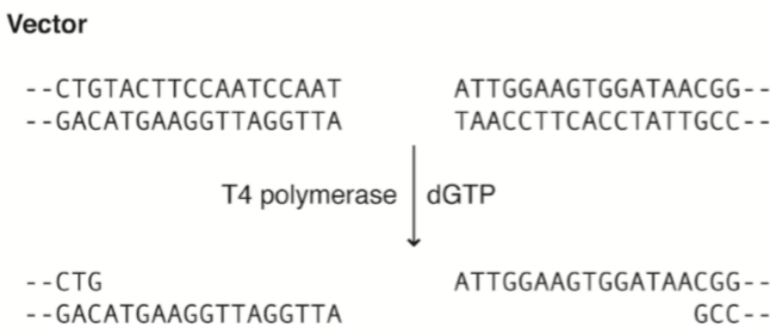


Figure 5. T4 DNA polymerase reaction with the presence of dGTP

Beside of that, similarly, insert of interest should also be treated with T4 DNA Polymerase with presence of dCTP, therefore, complementary strands can be obtained. Due to our peptides cannot be a PCR product, two oligomers were designed as the forward and reverse primers according to the created overhangs of vector under

T4 DNA Polymerase, so they can fit each other; T4 DNA Polymerase were not applied to our insert to create overhangs, they were designed with their appropriate overhangs and annealed each other.

In this cloning strategy, ligation occurs inside the bacteria with its natural enzymes. When the designed forward and reverse primers were used for this strategy, after the purification with TEV cleavage, the resulted peptides will be having two additional amino acid residues at their N-terminus; a Serine (S) (TCC) from TEV cleavage site, an Asparagine (N) (AAT) from the SspI restriction site. To obtain non-residual peptides, some of the inserts were designed with additional sequences which are compatible with some chemical cleavage strategies.

Under the scope of this study, two different AMPs (TN1 and TN3) were selected and studied while designing some strategies to produce them via recombinant DNA technology. For this purpose, different expression and purification approaches were followed. Briefly, different fusion partners (to alter solubilization, purification or visualization of the proteins) were used with different removal strategies (enzymatically or chemically) of these partners. Cleavage methods were also considered about the non-residual and high amount of the resulted peptides. Overall 17 different constructs were designed and obtained. After the purification studies, with some of the selected ones were studied and the remaining ones will be studied in the future studies.

In the following sections, these methods were described in detail.

3.1.1. Designing and Preparation of the Gene Fragments

Gene fragments were designed to use with LIC vectors. For this method, gene fragments should have "LIC fusion tags" for their forward and reverse primers to anneal the prepared LIC vectors, properly.

Forward - 5'TACTTCCAATCCAATGCA3'

Reverse - 5'TTATCCACTTCCAATGTTATTA3'

For the TN1 and TN3 production 3 different gene fragments were designed for each. The related sequence was determined with codon optimization to produce in *E. coli* by using idtDNA codon optimization tool, each time. For the CNBr chemical cleavage, Methionine (M) residue was introduced to the N- terminus end of the peptide [39]. And for the formic acid chemical cleavage trials, the inserts were designed with an aspartic acid (D) and a proline (P) at their N-terminus [2, 40].

The very first design for TN1 production named as SNA-TN1 (5' to 3'):

LIC_TN1_fwd:

TACTTCCAATCCAAT*GCA*CGCCTGCTGCGCCTGCTGCTGCTGCGCCTGCTG CGCTAATAAC

LIC_TN1_rev:

TTATCCACTTCCAATGTTATTAGCGCAGCAGGCGCAGCAGCAGCAGGCGC AGCAGGCGTGC

(The sequence with green represents the stop codon)

```
Delta G: -106.41 kcal/mole  Base Pairs: 46
5'  TACTTCCAATCCAATGCACGCCTGCTGCGCCTGCTGCTGCTGCGCCTGCTGCGCTAATAAC
                        ||||||||||||||||||||||||||||||||||||||||||||||
3'                      CGTGCGGACGACGCGGACGACGACGACGCGGACGACGCGATTATTGTAACCTTCACCTATT
```

Figure 6. idtdna OligoAnalyzer. Annealed primers (LIC_TN1_fwd and LIC_TN1_rev) for SNA-TN1

The sequence shown in red was suggested sequence from the Addgene to be applied to primers while designing to anneal them to the prepared vectors. For the first forward primer, the GCA sequence was also introduced to apply suggestions of "LIC fusion tags". In here, G base to cease the T4 Pol exonuclease activity but T4 Pol was not used to chew back the insert, so the GCA sequence only gives an additional unnecessary residue (Alanine). First trials were made with these 2 oligonucleotides as described in the following sections. And after the successful annealing of vector and the raw insert, other inserts were designed and produced. Briefly, inserts, which have compatible 5' and 3' overhangs for prepared vectors, were obtained by annealing of the two oligonucleotides with each other.

For the CNBr chemical cleavage trials, inserts were designed with an additional M amino acid sequence (ATG; underlined) at the N-terminus of the peptide sequence named as M-TN1 (5' to 3'):

TN1_5Met_fwd:

TACTTCCAATCCAATATGCGCCTGCTGCGCCTGCTGCTGCTGCGCCTGCTG
CGCTAATAA

TN1_5Met_rev:

TTATCCACTTCCAATTTATTAGCGCAGCAGGCGCAGCAGCAGCAGGCGCA
GCAGGCGCAT

(The sequence with green represents the stop codon)

```
Delta G: -105.2 kcal/mole  Base Pairs: 45
5' TACTTCCAATCCAATATGCGCCTGCTGCGCCTGCTGCTGCTGCGCCTGCTGCGCTAATAA
                  |||||||||||||||||||||||||||||||||||||||||||||
3'                TACGCGGACGACGCGGACGACGACGACGCGGACGACGCGATTATTTAACCTTCACCTATT
```

Figure 7. idtdna OligoAnalyzer. Annealed primers (TN1_5Met_fwd and TN1_5Met_rev) for M-TN1

By using the same approach, for the second peptide (TN3), inserts were also designed and produced named as M-TN3 (5' to 3'):

TN3_5Met_fwd:

TACTTCCAATCCAATATGCGGCTGCTCCGGCTGCTCCGGCTGCTCCTGTA
ATAAATGGAGCACTGA

TN3_5Met_rev:

TTATCCACTTCCAATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCA
GCCGGAGCAGCCGCAT

(The sequence with green represents the stop codon and the purple one was added to obtain the increased delta G value.)

```
Delta G: -110.19 kcal/mole  Base Pairs: 51
5' TACTTCCAATCCAATATGCGGCTGCTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGA
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
3'                TACGCCGACGAGGCCGACGAGGCCGACGAGGACATTATTTACCTCGTGACTTAACCTTCACCTATT
```

Figure 8. idtdna OligoAnalyzer. Annealed primers (TN3_5Met_fwd and TN3_5Met_rev) for M-TN3

For the other chemical cleavage strategy, by using formic acid, D (GAC) and P (CCG) residues were introduced to the N-terminus of the peptide sequences. By using the designing approach described previously for the (named as) DP-TN1 peptide (5' to 3'):

TN1_5AspPro_fwd:

TACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCTGCTCCGGCT GCTCCGGTAATAA

TN1_5AspPro_rev:

TTATCCACTTCCAATTTATTACCGGAGCAGCCGGAGCAGGAGCAGCCGGA GCAGCCGCGGGTC

(The sequence with green represents the stop codon)

```
Delta G: -112.31 kcal/mole  Base Pairs: 48
5' TACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCTGCTCCGGCTGCTCCGGTAATAA
                  ||||||||||||||||||||||||||||||||||||||||||||||||
3'                CTGGGCGCCGACGAGGCCGACGAGGACGAGGCCGACGAGGCCATTATTTAACCTTCACCTATT
```

Figure 9. idtdna OligoAnalyzer. Annealed primers (TN1_5AspPro_fwd and TN1_5AspPro_rev) for DP-TN1

By using the same approach, for the second peptide (TN3), inserts were also designed and produced named as DP-TN3:

(5' to 3'):

TN3_5AspPro_fwd:

TACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCGGCTGCTCCT GTAATAAATGGAGCACTGA

TN3_5AspPro_rev:

TTATCCACTTCCAATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCA GCCGGAGCAGCCGCGGGTC

(The sequence with green represents the stop codon and the purple one was added to obtain the increased delta G value.)

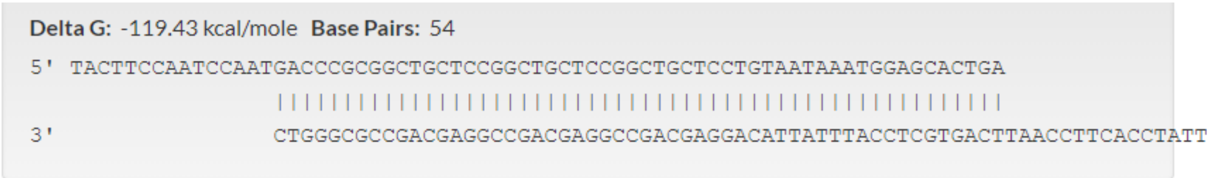


Figure 10. idtdna OligoAnalyzer. Annealed primers (TN3_5AspPro_fwd and TN3_5AspPro_rev) for DP-TN3

All of the designed oligonucleotides were supplied as 50nmol scale and HPLC-purified from Eurofins Scientific.

Melting temperature (Tm) values were 83 °C, in average. Forward and reverse primers (300 pmoles of each) were annealed by using PCR with an incubation with 3 min at 95 °C, 5 min at 74 °C and then used at room temperature. PCR amplifications were carried out by using a thermal cycler (Thermo Electron Corp.) through the all experiments.

Codes were determined to name for these 5 different inserts as given in bold;

- SNA-TN1 (**TN1**),
- M-TN1 (**1**),
- DP-TN1 (**2**),
- M-TN3 (**3**),
- DP-TN3 (**4**).

3.1.2. Preparation of the LIC Vectors

As described in the previous sections, LIC vectors were supplied from Addgene in *E. coli* DH5α bacteria inoculated to the Luria Broth (LB) - agar stab. After the isolation of the vectors, they cut with SspI restriction enzyme and treated with T4 DNA Polymerase.

3.1.2.1. Plasmid Isolation

Bacteria were inoculated to the LB-agar plates containing 100 µg/ml kanamycin to obtain single colonies and incubated at 37 °C for overnight. Obtained single colonies were picked and inoculated to the LB-Broth with 100 µg/ml kanamycin and incubated at 37 °C, 200 rpm, for overnight without reaching the optical density to 0.6 at 600nm. At this step, every culture was stocked as a frozen at 50% glycerol (1:1) at -86 °C. By using the Zymo Research MiniPrep plasmid purification kit, according to its procedure, plasmids vectors (1GFP, 1G, 1B, 1S LIC vectors) were isolated in the elution solution and examined on 0.8% TAE (40 mM Tris acetate, 2 mM EDTA, pH 8.5) agarose gel electrophoresis (according to the protocol at A.2.) run at 80 V by SYBR Gold nucleic acid dye.

Thermo Scientific, NanoDrop 2000c was used to obtain DNA density for each of the vector type.

3.1.2.2. SspI Digestion

Purified circular different types of vector plasmids were get linear forms by cutting SspI restriction enzyme after the several optimization trials with Thermo Scientific SspI enzyme. Procedure was optimized as; 10X Buffer G, 1 µg plasmid, 1 µl SspI enzyme and completed with nuclease-free water to the total volume of 20µl. Vectors were treated with, Thermo Scientific SspI with an incubation at 37 °C for 10 hours and an inactivation period at 65°C for 20min. After the digestion, products were examined on 0.8% TAE agarose gel electrophoresis by SYBR Gold nucleic acid dye. Gel purified with Omega E.Z.N.A gel purification kit according to its procedure. Obtained pure and linear vectors were measured by Thermo Scientific, NanoDrop 2000c.

3.1.2.3. T4 DNA Polymerase Treatment

Purified linear vectors were treated with Novagen T4 DNA Polymerase for the chew-back reaction (Figure 5). The procedure was optimized as; 4 µl T4 Polymerase 10x reaction buffer, 1µl dGTP (100mM), 2 µl DTT (100mM), 200 ng vector DNA and nuclease-free water until the 32 µl with a total reaction volume of 40 µl. The reaction was carried out with an incubation at 25 °C for 30 min and an inactivation at 75 °C for 20 min.

3.1.3. Incorporation of the Gene Fragments with the LIC Vectors

As described, each of the oligonucleotide pairs were annealed and the plasmid vectors were prepared after the T4 Pol. reaction.

3.1.3.1. Annealing of the Vectors and Inserts without using Ligase

Different approaches were tried. At the molar ratios of 1:3, 1:5 and 1:7 (vector: insert) were applied. All of the trials were carried out with following steps. Prepared vectors and the inserts were mixed in a PCR tube and incubated at 25 °C for 5 min. After the incubation, 1 µl 25mM EDTA (pH 8) was added with gently mixing and incubated at 25 °C for 5 min again. Prepared (according to the procedure at A.1.) *E. coli* DH5α competent cells was transformed with prepared mixture of the vectors and inserts. T4 Pol. treatment was also controlled with a mixture included only each related vector prepared under T4 Pol. treatment under the same reaction condition.

3.1.3.2. Transformation of DH5α Competent Cells

By using prepared mixtures described above, *E. coli* DH5α competent cells were transformed.

Each experiment was carried out with control groups. For this, only the competent cells, each related vector prepared under T4 Pol. treatment, competent cells with each related circular plasmid vector were used.

During the transformation, all of the steps were carried on ice. 2,5 µl of prepared vector-insert from the reactions were added to 100 µl of *E. coli* DH5α competent cells and gently mixed and incubated for 30 min on ice. Heat shock was applied at 42 °C for 45 sec. The tubes were incubated on ice for 2 min. 450 µl LB-Broth was added. After the incubation for 1 hour, 200 rpm at 37 °C, 100 µl from each mixture was spreaded to the LB-agar plates containing 100 µg/ml kanamycin for an incubation at 37 °C for 16 hours.

Inside of the bacteria, inserts and different vectors were expected to anneal as:
(Sequences in yellow: overhangs of the T4 DNA Pol. treated plasmid vector. The red ones represent LIC fusion partners introduced to inserts while underlined Stop codon. Sequences in purple were added to optimize the primers.)

SNA-TN1 (5’ to 3’):

```
5’-CTGTACTTCCAATCCAATGCACGCCTGCTGCGCCTGCTGCTGCGCCTGCTGCGCTAATAACATTGGAAGTGGATAACGG-3’
3’-GACATGAAGGTTAGGTTACGTGCGGACGACGCGGACGACGACGCGGACGACGCGATTATTGTAACCTTCACCTATTGCC—5’
```

M-TN1 (5’ to 3’):

```
5’-CTGTACTTCCAATCCAATATGCGCCTGCTGCGCCTGCTGCTGCGCCTGCTGCGCTAATAAATTGGAAGTGGATAACGG-3’
3’-GACATGAAGGTTAGGTTATACGCGGACGACGCGGACGACGACGCGGACGACGCGATTATTTAACCTTCACCTATTGCC-5’
```

DP-TN1 (5’ to 3’):

```
5’-CTGTACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCTGCTCCGGCTGCTCCGGTAATAAATTGGAAGTGGATAACGG-3’
3’-GACATGAAGGTTAGGTTACTGGGCGCCGACGAGGCCGACGAGGACGAGGCCGACGAGGCCATTATTTAACCTTCACCTATTGCC—5’
```

M-TN3 (5’ to 3’):

```
5’-CTGTACTTCCAATCCAATATGCGGCTGCTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGG-3’
3’-GACATGAAGGTTAGGTTATACGCCGACGAGGCCGACGAGGCCGACGAGGACATTATTTACCTCGTGACTTAACCTTCACCTATTGCC—5’
```

DP-TN3 (5’ to 3’):

```
5’-CTGTACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGG-3’
3’-GACATGAAGGTTAGGTTACTGGGCGCCGACGAGGCCGACGAGGCCGACGAGGACATTATTTACCTCGTGACTTAACCTTCACCTATTGCC—5’
```

3.1.3.3. Positive Colony Selection by Colony PCR

After the overnight incubation, colony PCR was carried out to examine the obtained colonies after the transformation.

Each colony was directly inoculated to a PCR mixture (PCR Hot-Start Master Mix II (5X), GeneMark) explained at Table 1 and also LB-Broth containing 100 µg/ml kanamycin to set culture at 37 °C, 200 rpm after the confirmation of the related colony. When positive colony was obtained, the related culture was frozen-stocked to preserve each colony. After each PCR (parameters were given at Table 2), reaction products were examined by agarose gel electrophoresis. Different vectors were examined with proper primers.

Table 1. GeneMark PCR Hot-Start Master Mix II (5X) Protocol

Contents	Amount
Nuclease free water	X µl
DNA template	Y µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
PCR Hot-Start Master Mix II (5X)	5 µl
	Total volume 25 µl
Each 25 µl of mixture contains 0,75 µl Hot Start Taq DNA polymerase, reaction buffer, $MgCl_2$ (final concentration 2 mM) 250 µM dNTP and enzyme stabilizer from the Master Mix.	

Table 2. Optimized PCR condition parameters

Steps	Temperature	Time	Cycle
Initial denaturation / inactivates antibody and starts hot-start PCR	95° C	3 min	1
Denaturation	95°C	30 sec	30
Annealing	37-68°C (according to the Tm value of each primer)	30 sec	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

Some regions closed to the cloning region were selected and the primers were designed to amplify related regions with added inserts to different LIC vectors and supplied from Eurofins Scientific.

PetLIC_fwd_536: TCAATGCTTGAAGGAGCGGT
PetLIC_rev_536 : CTCAGCTTCCTTTCGGGCTT
PetLIC_fwd_119: GCTGGCAAGCCACGTTTGGT
PetLIC_rev_119 : GCTTGTCGACGGCGCTCGAA
T7_fwd : TAATACGACTCACTATAGGG

Primers were designed mainly for 1G LIC vector for the first trails. Later, the convenient pairs were used to amplify the sequences on the other LIC vectors. Based on the expected product base pairs after PCR, different densities of the agarose gels were used. As a control, empty plasmid vectors were also amplified with proper primers.
For this purpose;

- For the 1G vector, PetLIC_fwd_119 and PetLIC_rev_119 primers were used to amplify the region with 119 base pair (while the LIC vectors were empty). PCR products were examined on 1.8% TAE agarose gel.
- For the 1S vector, T7_fwd and PetLIC_rev_119 were used to amplify the region with 505 base pair. PCR products were examined on 1.3% TAE agarose gel.
- For the 1GFP vector, T7_fwd and PetLIC_rev_119 were used to amplify the region with 919 base pair. PCR products were examined on 1.8% TAE agarose gel.
- For the 1B vector, T7_fwd and PetLIC_rev_119 were used to amplify the region with 186 base pair. PCR products were examined on 1.8% TAE agarose gel.

PetLIC_fwd_119 and PetLIC_rev_119 primers were annealed at 61 °C while T7_fwd and PetLIC_rev_119 primers were tried to anneal at both 45 °C and 50 °C by using gradient PCR.

Compared to the controls on the agarose gels, positive colonies were selected and their cultures were stocked as frozen (500 µl overnight culture in 500 µl 50% glycerol solution) at -80 °C freezer and also their PCR products were sent for DNA sequencing to Eurofins Scientific for two directions reading. In the first experiments, cloning with 1G vector, DNA sequencing was applied with T7_fwd and pET-RP (5'-CTAGTTATTGCTCAGCGG-3') primers. And also, the second confirmation was enhanced with PetLIC_fwd_119 and PetLIC_rev_119 primers. According to the results given in further sections, DNA sequencing for remaining experiments was carried out with T7_fwd and PetLIC_rev_119 to enhance to amplify common regions in the different plasmids.

3.1.3.4. Transformation of BL21 Expression Line by obtained Recombinant Plasmids

According to the DNA sequencing, positive colonies were obtained and their plasmids were isolated to transform *E. coli* BL21 competent cells.

Plasmid isolation, preparation of *E. coli* BL21 competent cells and transformation methods were applied as explained before. Obtained colonies were stocked as frozen to be used in the protein expression studies.

3.2. Protein Expression

Protein expression was carried out by using IPTG (Thermo Fisher Scientific Inc.) and optimized by using two approaches (slow and fast induction) at the beginning experiments with transformed BL21 cells with 1G-SNA-TN1 plasmid. Slow and fast induction methods were investigated in terms of different fermentation temperatures.

3.2.1. Protein Expression with Slow Induction Method

The overnight culture of 1G-SNA-TN1 in 10 ml LB medium containing 100 µg/ml kanamycin were used to inoculate 1L cultures having 100 µg/ml kanamycin. Samples were induced with 1M IPTG (Thermo Fisher Scientific Inc.), with a final concentration 0.5mM, after optical cell density has reached a value of 0.6-0.8 that was measured using a UV spectrophotometer (Thermo Electron Corp.) at 600 nm. Induced samples were shaken in an incubator at 23 °C, 240 rpm for 16 hours. Time course of the 1 ml samples were taken before induction and every hour after induction. The samples were centrifuged at 5000 x g for 10min using a microcentrifuge and supernatant was discarded. And collected pellets were frozen at -20°C to extract proteins more efficiently.

3.2.2. Protein Expression with Fast Induction Method

As described in the previous section, fast induction method was tried with an expression temperature at 37 °C, rather than at 23 °C. And the cell pellets were collected same as explained in the previous part.

3.3. Protein Extraction and Purification

3.3.1. Protein Extraction

A detergent, B-PER (Thermo Scientific) was used to extract proteins from the cell pellets directly. This reagent enables a protein isolation from *E. coli* without using any mechanical disruption methods. Proteins both in the soluble and insoluble parts (IBs) can be obtained with B-PER. The procedure was applied as recommended at manufacturer's protocol. The frozen pellets after the protein expression were homogenized as 4mL B-PER/gram cell pellet, then incubated at room temperature and centrifugated for 15 min. 15000 x g. As a result, soluble (in the supernatant part) and insoluble (in the pellet) proteins were obtained and monitored by polyacrylamide gel electrophoresis (Bis-Tris, 10x8, %12, Gene Script, SurePAGE™) with 1X Tris-

MOPS-SDS running buffer. 1X MOPS buffer contains 6.06 g Tris Base, 10.46 g MOPS, 1.0 g SDS, 0.3 g EDTA. Loading buffer were prepared given as Appendix 3 (A.3.). Samples were prepared by mixing with 1X loading buffer and by boiling at 95°C for 5 min. 5 µl GenColor Prestained Protein Marker, 10-170 kd (GeneMark) and 20 µl prepared samples were run on gel at 200 V for 25 min. Then, it was stained by using Instant Blue staining reagent (Sigma) for at least 1 hour on the rocking platform at room temperature and visualized on ChemiDoc (Bio-Rad).

3.3.2. Western Blot Analysis of Expressed Proteins

After the expression studies were examined on the gels, related proteins were confirmed by tagging with proper antibodies. Within these studies, all of the proteins included His-Tag and some of them (1G plasmids) had GST-Tag. They were visualized with Western Blotting by semi-dry method described in A.4. in detail. As a transfer buffer, 10X tris/glycine buffer (25 mM tris, 192 mM glycine, pH 8.3) from Bio-Rad was used as 1X diluted with 20% methanol in ultra-pure water.

3.3.3. Inclusion Body Solubilization - Denaturation - Refolding and Membrane Filtration

After the examination of the protein expression studies with different fusion partners, as described in the results section, expression of all related proteins by using 1G plasmids were optimized in the insoluble fraction.

After expression, extraction of the proteins from inclusion bodies were done with frozen cell pellets from 1L cultures of different 1G plasmids. 1G-SNA-TN1 and 1G-M-TN1 systems were used to optimize the procedures.

Under denaturing conditions with 6M Guanidine HCl, pellets were tried to solubilized. By using BACHEM-peptide calculator software, isoelectronic point (pI) properties were determined (Figure 11 and Figure 12).

His6-GST-TEV SITE-Cloning Region

MGSSHHHHHHGSSMSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELG
LEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAY
SKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAF
PKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKGIEENLYFQSNARLLRL
LLLRLLR **STOP**

Molecular weight (Mr):	29811.71 g/mol
Isoelectric point:	7.0
Net charge at pH 7.0:	-0.1
Average hydrophilicity:	0.0
Ratio of hydrophilic residues / total number of residues:	37 %

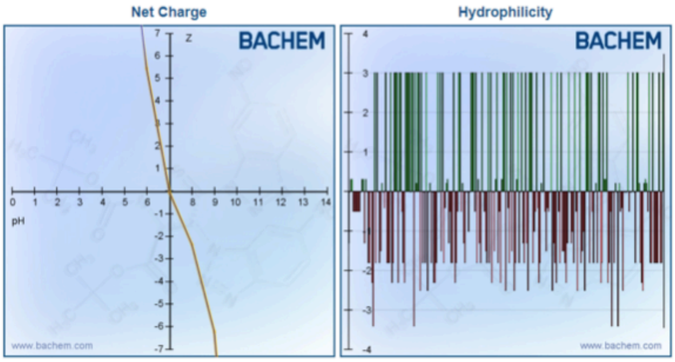


Figure 11. The expected expressed protein structure of the system with SNA-TN1 peptide fused with GST protein. BACHEM-peptide calculator software was used.

His6-GST-TEV SITE- Cloning Region

MGSSHHHHHHGSSMSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELG
LEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAY
SKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAF
PKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKGIEENLYFQSN
MRLLRLLLLRLLR

Molecular weight (Mr):	29871.83 g/mol
Isoelectric point:	7.0
Net charge at pH 7.0:	-0.1
Average hydrophilicity:	0.0
Ratio of hydrophilic residues / total number of residues:	37 %

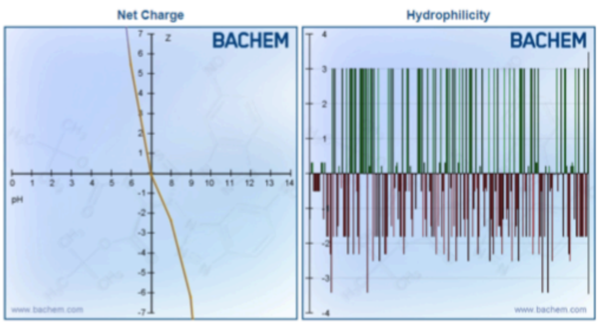


Figure 12. The expected expressed protein structure of the system with SNM-TN1 peptide fused with GST protein. BACHEM-peptide calculator software was used.

According to the pI properties (zero at pH 7) of each protein structure, pH value of the refolding buffer (50 mM K_2PO_4, 100 mM NaCl) were adjusted as 10.3 to keep the expressed protein in the soluble state because of their charges. By examining the pI properties, dialysis conditions were also tried to optimized. Methods were applied as given at A.5. and the samples taken from the steps were examined by SDS-PAGE.

3.3.4. Purification of GST-Tagged Proteins

After the extraction of the proteins from inclusion bodies discussed at results section, lyophilized sample of the GST-SNM-TN1 protein, as a representative, were tried to purify by Pierce Glutathione Spin Columns (with 0.2mL resin bed and 0.8 total volume, Thermo Scientific) for further purification with a given protocol at A.6. At the beginning of the procedure, the protein samples were dissolved in 30mM ammonium bicarbonate (pH 12) appropriate to pI properties of the related protein (Figure 11).

Samples taken from the purification steps were examined by SDS-PAGE.

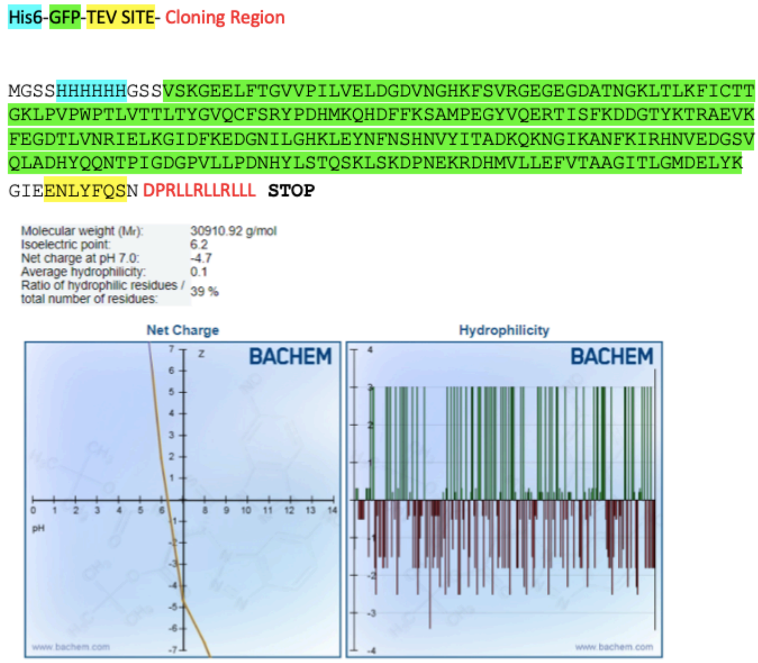
3.3.5. Purification of His-Tagged Proteins

As discussed at Results section, further purification of the expressed proteins both in soluble and insoluble fractions, all included His-Tag, were tried the accomplished by PureProteome Nickel Magnetic Bead System (Merck). This system enhances to bind the tags with metal-affinity to the magnetic beads and they are captured with a magnetic stand, while un-bound proteins are removed by proper washing buffers. By a convenient condition, the proteins, captured to the magnetic beads, are eluted. This purification approach can be used under both native and denaturing conditions. For the denaturing condition, buffers including urea or guanidine should be used in both equilibration and washing of the magnetic beads and elution of the samples to prevent precipitation of the proteins. Washing and elution buffers include imidazole with respective amounts to enhance the gradually elution of the captured protein.

After the expression, frozen cell pellets from 1L cultures of 1G-DP-TN1, 1GFP-DP-TN1, 1GFP-DP-TN3, 1S-DP-TN1 systems were used to optimize the purification method and cleavage studies described in further sections. Two methods given in A.7. and A.8. were applied to isolate the proteins from the frozen pellets. With reduced viscosity and without any aggregation, samples were prepared as convenient to use for magnetic beads. The purification protocol (A.9.) was applied to purify His-Tagged proteins and samples taken from the steps were examined by SDS-PAGE.

3.3.6. Protein Concentration Determination

Pierce BCA Protein test was used to determine protein concentrations through the all experiments. To remove elution buffer, not compatible with BCA test, according to pI properties of each protein (Figure 13) buffer was selected as 100mM ammonium bicarbonate pH 7.8 and the protein standards for BCA was also prepared by selected buffer.

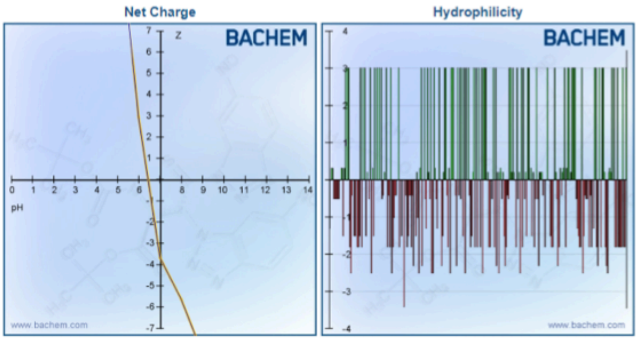


(A)

His6-GFP-TEV SITE- Cloning Region

MGSSHHHHHHGSSVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVK
FEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSV
QLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKD PNEKRDHMVLLEFVTAAGITLGMDELYK
GIEENLYFQSN DPRLLRLLLLRLLR **STOP**

Molecular weight (Mr):	31180.27 g/mol
Isoelectric point:	6.3
Net charge at pH 7.0:	-3.7
Average hydrophilicity:	0.1
Ratio of hydrophilic residues / total number of residues:	39 %

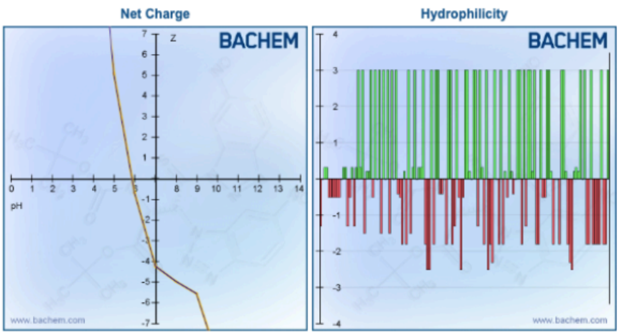


(B)

His6-Sumo-TEV SITE- Cloning Region

MGSSHHHHHHGSSMASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRR
LMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGGIEENLYFQSN D
PRLLRLLLLRLLR **STOP**

Molecular weight (Mr):	15948.07 g/mol
Isoelectric point:	5.9
Net charge at pH 7.0:	-4.2
Average hydrophilicity:	0.4
Ratio of hydrophilic residues / total number of residues:	48 %



(C)

Figure 13. The expected expressed protein structure of the system with (A) DP-TN1 (B) DP-TN3 peptides fused with GFP and (C) DP-TN1 peptide fused with Sumo protein. BACHEM-peptide calculator software was used.

Buffer exchange was done by Protein Desalting Spin Columns (Thermo Scientific) according to the manufacturer's procedure.

BCA test was applied according to the manufacturer's microplate procedure and signals were read at 562 nm wavelength.

3.4. Removal of Fusion Partners

3.4.1. Chemical Cleavage by Formic Acid

Purified proteins from 1GFP-DP-TN1, 1GFP-DP-TN3 and 1S-DP-TN1 were used to optimize the chemical cleavage studies by formic acid (F.A.).

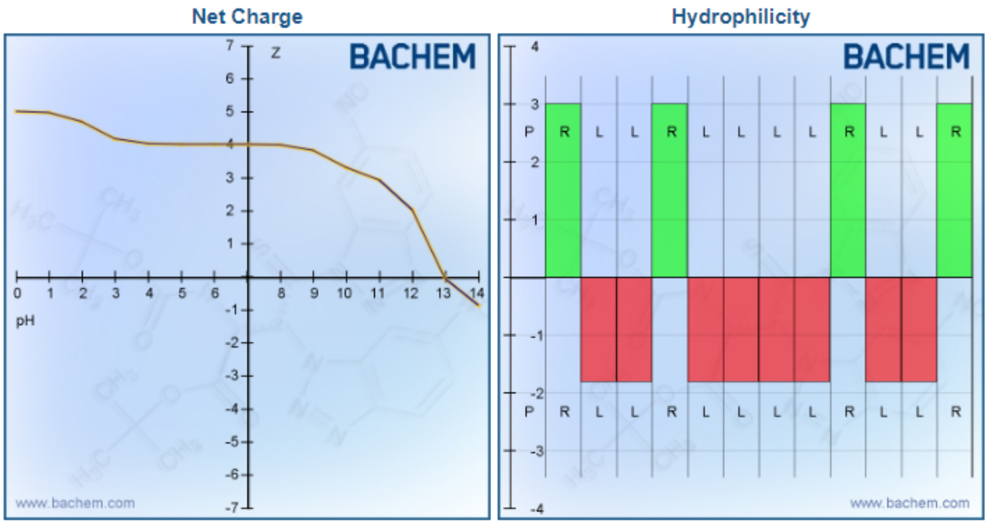
According to the literature [2, 40], depending on their concentrations, determined by BCA, each 2-50 µg of purified proteins were separated to the microcentrifuge tubes and lyophilized. Different concentrations of 50 µl F.A. were applied with different time courses at different temperatures (Table 3) and the chemical reactions were stopped by adding 150 µl ultrapure water. Samples were freeze dried.

Table 3. Formic acid cleavage conditions

Sample	Protein Construct	F.A.%	Temperature (°C)	Time (hours)
1	1GFP-DP-TN1	70%	37 °C	48h
2	1GFP-DP-TN1	50%	50 °C	24h
3	1GFP-DP-TN1	50%	50 °C	12h
4	1GFP-DP-TN1	37%	30 °C	12h
5	1GFP-DP-TN1	37%	37 °C	12h
6	1GFP-DP-TN1	37%	45 °C	12h
7	1GFP-DP-TN1	37%	50 °C	12h
8	1GFP-DP-TN1	37%	45 °C	0.5h
9	1GFP-DP-TN1	37%	45 °C	1h
10	1GFP-DP-TN1	37%	45 °C	1.5h
11	1GFP-DP-TN1	37%	45 °C	2h
12	1GFP-DP-TN1	37%	45 °C	2.5h
13	1GFP-DP-TN1	37%	45 °C	3h
14	1GFP-DP-TN1	37%	45 °C	3.5h
15	1GFP-DP-TN1	50%	50 °C	48h
16	1GFP-DP-TN3	50%	50 °C	12h
17	1GFP-DP-TN3	50%	50 °C	24h
18	1GFP-DP-TN3	50%	50 °C	48h
19	1S-DP-TN1	50%	50 °C	24h
20	1S-DP-TN1	50%	50 °C	12h

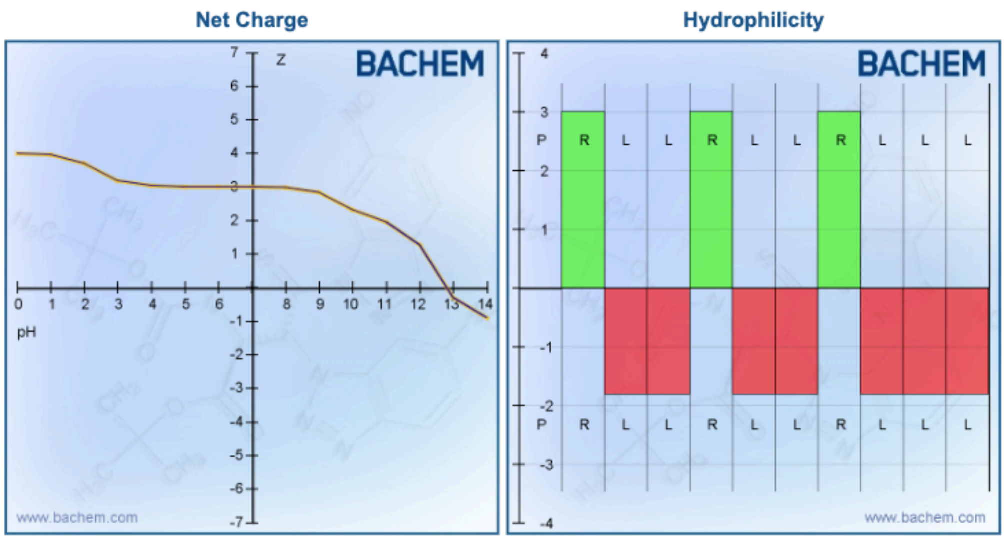
Cleavage products were dissolved in a proper buffer (100mM ammonium bicarbonate pH 7.8) compatible with pI numbers (Figure 14) of the expected cleaved products. Each 30 µl of cleaved products (approx. 10 µg) were examined by SDS-PAGE. Antibiogram experiments and MS analysis were applied to characterized cleavage products (peptides) as described in the further sections.

Molecular weight (Mr):	1645.16 g/mol
Isoelectric point:	13.0
Net charge at pH 7.0:	4.0
Average hydrophilicity:	-0.2
Ratio of hydrophilic residues / total number of residues:	31 %



(A)

Molecular weight (Mr):	1375.81 g/mol
Isoelectric point:	12.8
Net charge at pH 7.0:	3.0
Average hydrophilicity:	-0.3
Ratio of hydrophilic residues / total number of residues:	27 %



(B)

Figure 14. The expected cleaved peptide structure of the system for (A) P-TN1 (B) P-TN3 peptides. BACHEM-peptide calculator software was used.

3.4.2. Enzymatic Cleavage by Tobacco Etch Virus (TEV) Protease

ProTEV Plus (Promega) enzyme was used to separate the fusion partners from the peptide of interest. Suggested protocol (ProTEV Buffer, 20X: 5µl; 100mM DTT: 1µl; fusion protein 20µg; ProTEV Plus (10u): 2µl; total volume up to 100 µl with water and incubate samples at 30°C for 6h) was used. And experiments were carried out by using His-tagged purified and 20µg lyophilized 1S-DP-TN1 complex as a representative system for the cleavage by TEV protease. After the reaction, samples were analyzed on SDS-PAGE.

3.5. Characterization of Peptides

3.5.1. Antibiogram Experiments with Cleaved Products

After the F.A cleavage reactions of the samples described above, antibiogram tests were carried out by using 10µl of each dissolved in 100mM ammonium bicarbonate pH 7.8 compatible with pI numbers (Figure 14) of the expected cleaved products. Samples were applied 0.5McFarland *E. coli* culture as drops.

3.5.2. Size Exclusion Studies of the Cleavage Reaction Products

As a representative, the first trial for the formic acid cleavage (Table 3) was analyzed by SEC UPLC. ACQUITY UPLC Protein BEH SEC 200 Å, 1.7 µm, 4.6mm X 300 mm, 1/pkg column was used under column temperature 30 °C while the sample's 5°C and the column was equilibrated by 20mM sodium phosphate pH 7.4 buffer as a mobile phase and the sample was loaded. Samples were prepared by 27 µg protein mixture trying to dissolve in 90 µl mobile phase. Because of the solubilization problem, the sample was centrifuged at 2000 x g for 2 min and remaining fraction in the supernatant, expected to include the peptide as soluble in this mobile phase (pH 7.4) compared to its pI point (Figure 14-A), was injected to the column as 5 µl. The signals were analyzed at 214 and 280 nm wavelengths.

3.6. Preparation of Polymer-Peptide Conjugates

It is aimed to increase the efficacy and stability of synthetic peptide molecules, which are produced against infections caused by bacteria that have developed resistance against commonly used antibiotics and resemble natural antibiotic structures. In the previous studies, two of the small peptide sequences whose synthetic structure was characterized and proven antimicrobial properties [12] were selected, and it was desired to be modified with polymer chains. The amino acid sequences and short use names in Figure 15 were modified by adding the cystine amino acid separately from both N- and C- terminals and were then be linked to the polymeric structure.

No	Short Name	AMP	Modified AMP	Representative Structure
1	TN1-NC	RLLRLLLLRLLR	**C**-RLLRLLLLRLLR	
2	TN1-CC	RLLRLLLLRLLR	RLLRLLLLRLLR-**C**	
3	TN3-NC	RLLRLLRLLL	**C**-RLLRLLRLLL	
4	TN3-CC	RLLRLLRLLL	RLLRLLRLLL-**C**	

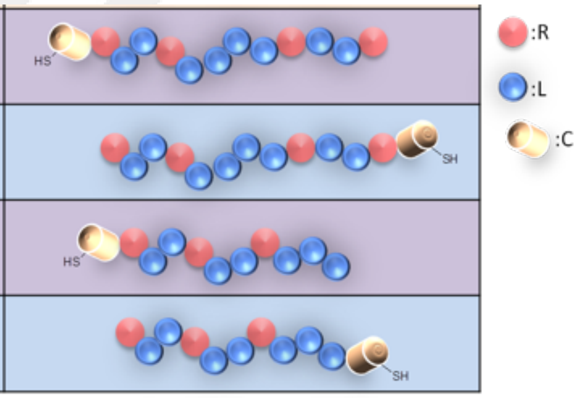


Figure 15. Structures of modified AMPs with cystine amino acid.

At this point, both the protein / peptide to be used as the active ingredient and the functional groups in the structure of the polymer should be chosen carefully. In this study, peptide and polymer chains were joined using intermediate linker molecules with suitable reactive groups. Linear PEG polymer chain with free primary amine group at one end and methoxy on the other end will be attached to the NHS activated carboxylic acid group of the interconnecting molecule. Then, peptides added to the cystine amino acid to the end group will be conjugated to the maleimide reagent group in the structure of the interconnecting molecule.

As shown in Figure 16A, when a linker containing 1 NHS and 1 maleimide group is used, a polymer chain will be conjugated with a peptide molecule. Part B shows the intermediate linker and polymer-peptide conjugate that can allow two of the same peptides to be linked to a polymer chain. The design of these structures makes it possible to bind polymers without affecting the functional groups in the chemical structure of the peptide sequence, which exhibits antimicrobial action, and to create a multivalence effect, especially in conjugates to which two peptides bind. Therefore, it is envisaged that the binding of a peptide to the bacterial membrane will have a much higher chance of binding, and the therapeutic effects of the peptides will be increased (Figure 17).

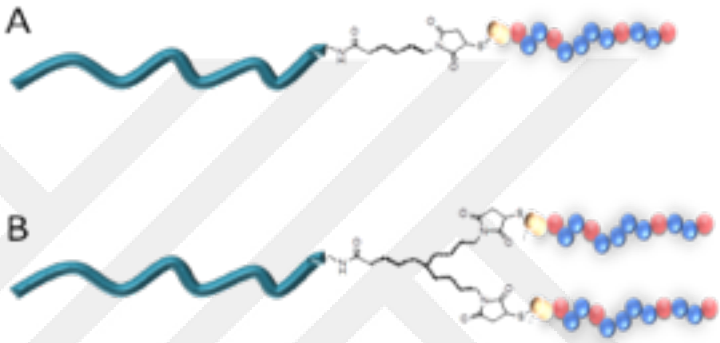


Figure 16. Representative structures of single (A) and double (B) peptide-polymer conjugates planned to be obtained.

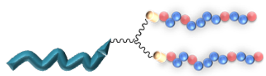
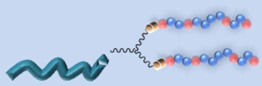
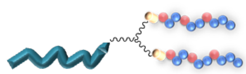
No	Conjugate Code	Bi-functional interconnecting molecule	Tri-functional interconnecting molecule	Modified AMP
1	PP1-NC			**C**-RLLRLLLLRLLR
2	PP1-CC			RLLRLLLLRLLR-**C**
3	PP3-NC			**C**-RLLRLLRLLL
4	PP3-CC			RLLRLLRLLL-**C**

Figure 17. Structures for the peptide-polymer conjugates planned to be obtained.

By using orthogonal functionalization approach described in the introduction section, it is possible to obtain polymer-peptide conjugates in one step.

The first set of the designed polymer-peptide conjugates is the version that will contain a single peptide sequence. A peptide sequence will be conjugated to a PEG polymer chain. For this, a hetero-functional interlinking molecule (Mal-PEG8-NHS ester, BroadPharm) was selected (Figure 18). This small molecule (Mwt: 618.6 gmol-1) with an NHS-activated carboxylic acid group on one end carries the active maleimide group on the other end.

Figure 18. Structure of Mal-PEG8-NHS ester which is a PEG derivative containing a maleimide group and an NHS ester group.

It was planned to obtain the conjugate by adding the free thiol function bearing the free amine group at one end and the peptide bearing the free thiol function from the cystine amino acid at one end to the solution of this molecule to be purchased as prepared by dissolving in anhydride (moisture-free) DMF.

In the reaction medium to be made slightly basic using triethyl amine (Et_3N, Sigma), the amines at the end of the PEG chains are expected to attack NHS-activated carboxylic acid and form an amide bond. At the same time, the thiol in the peptide structure is expected to become a better nucleophile by leaving its hydrogen in the basic medium and to enter 1,4-Michael addition reaction with the maleimide in the structure of the interconnecting molecule (Figure 19).

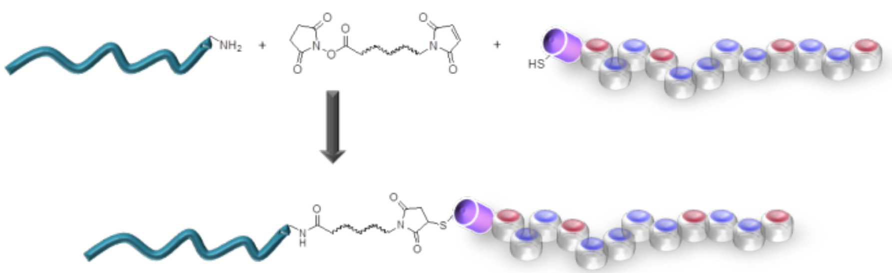


Figure 19. A scheme for conjugating of peptide with thiol to PEG.

In order to increase the anti-microbial activity of the conjugates targeted to be prepared, the strategy that was enable the binding of two more peptides per polymer chain is shown in Figure 20. For this purpose, an intermediate linker molecule (Bis-Mal-Lysine-PEG4-acid, BroadPharm) containing one carboxylic acid and two active maleimide groups (Figure 21) and a small molecular weight (Mwt: 695.7 gmol-1) was used. The carboxylic acid group of this intermediate-binding molecule NHS was activated using an active agent called EDCI (1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide, Sigma). Again, for orthogonal reactions designed for polymer-peptide conjugation to be performed in DMF and at room temperature, twice as many peptides were used than in the previous procedure.

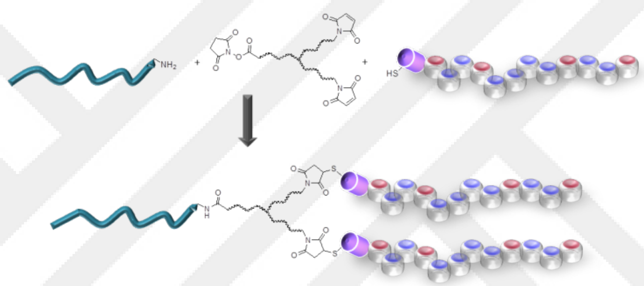


Figure 20. A scheme for conjugating of two peptides with thiol to PEG.

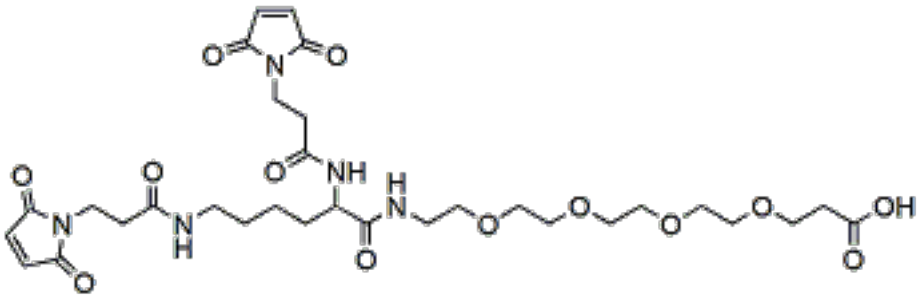


Figure 21. Structure of Bis-Mal-Lysine-PEG4-acid which is a PEG derivative containing two maleimide groups and an NHS ester group.

The amine-functional PEG chain was attached to one end of the interconnecting molecule using the above-mentioned reaction types. Thus, peptide sequences containing one free thiol group at one end were also be treated with the maleimide groups of the intermediate binding molecule. In this way, it was possible to protect two peptide sequences with a single polymer chain and increase its stability.

3.6.1. Synthesis and Characterization of Thiolated Peptides (C-TN1, TN1-C, C-TN3 and TN3-C)

Thiolated amide-end peptides (C-TN1, TN1-C, C-TN3 and TN3-C) were synthesized by using CEM-Liberty Blue Solid-State Peptide Synthesizer by F-moc chemistry as 0.1mmol scale on Rink Amide-ProTide resin (Cem Coorp.) and cleavage procedures to release synthesized peptides and remove protected groups were also applied according to the standard usage manual of the device. Synthesized peptides were analyzed by analytical RP-HPLC by using hydrophobic C18 column (Agilent, AdvanceBio Peptide Plus, 2.1 x 150 mm, 2.7-Micron with Column ID) equilibrated by 5% mobile phase (acetonitrile and ultra-pure water, 0.25% TFA) at 0.2ml/min flow rate and observed the signals at 214 nm.

3.6.2. PEGylation Reactions by Orthogonal Functionalization

3.6.2.1. Synthesis of Single Peptide-PEG Conjugate

As a representative reaction, TN1-NC (1650,18 Da) was used. Stoichiometric calculations were applied for 5mg of the linker molecule (Mal-PEG8-NHS ester, BroadPharm, Mwt: 618.6 gmol-1) used as 1 equivalence and according to this, bounded peptide and polymer also were 1:1. Total reaction was carried out by using 40mg solid in 1.5 ml reaction volume including peptide, polymer (PEG: Methoxypolyethylene glycol amine, Mwt: 2000Da, Sigma-Aldrich) and the linker molecule. Calculated amounts of peptide and the linker was firstly mixed in (anhydrous) DMF (Sigma) in a round bottom volumetric flask and stirred using a small magnet for 2 hours at room temperature for the 1,4-Michael addition reaction. Then, PEG, dissolved in anhydrous DMF and Et_3N (0,726 g/cm^3) were added to the reaction mixture and incubated by stirring for overnight. All experiments were applied under nitrogen gas and at room temperature.

3.6.2.2. Synthesis of Multivalent Peptide-PEG Conjugate

As a representative study, TN1-NC (1650,18 Da) was used and the acid-ended linker was tried to bound to PEG-amine with amide bonding by using addition of HATU and DIPA chemicals. Stoichiometric calculations were applied for 5mg of the linker molecule (Bis-Mal-Lysine-PEG4-acid, Mwt: 695.7 gmol-1) used as 1 equivalence and according to this, bounded peptide and polymer were 2:1 while Et_3N agent was also 2 equilivalence because of the usage of double peptides. Total reaction was carried out by using 53mg solid in 2.5 ml reaction volume including peptide, polymer (PEG: Methoxypolyethylene glycol amine, Mwt: 2000Da, Sigma-Aldrich), ve HATU (Merck, Mwt: 380.23 gmol-1), DIPA (Merck, Mwt: 101.19 gmol-1) and the linker molecule. Calculated amounts of peptide and the linker was firstly mixed in (anhydrous) DMF (Sigma) in a round bottom volumetric flask and stirred using a small magnet for 2 hours at room temperature for the 1,4-Michael addition reaction. Then, PEG, dissolved in anhydrous DMF, Et_3N (0,726 g/cm^3), HATU ve DIPA were added to the reaction mixture and incubated by stirring for overnight. All experiments were applied under nitrogen gas and at room temperature.

Another study was applied for the reaction setup for NHS coupling of acid-ended linker. Acid group of the linker (Bis-Mal-Lysine-PEG4-acid, Mwt: 695.7 gmol-1) was NHS activated by using EDCI (Sigma, Mwt: 191.7 g/mol) with Et_3N (0,726 g/cm^3) and stoichiometric calculations were applied as described before; 52mg solid in 2.2 ml reaction volume. Firstly, peptide was added to the reaction and incubated for 2 hours. And then PEG (Mwt: 2000Da, Sigma-Aldrich) was added and incubated for overnight.

3.6.3. Purification of Conjugates

3.6.3.1. Dialysis Method

After reactions were completed, unreacted impurities were removed by dialysis using 3500 MW cutoff (RC, SpectrumLabs) dialysis membrane. Reaction solution, placed in the dialysis bag, was dialyzed at room temperature and at least 24 hours by using DMF as a receptor solution and then lyophilized. These solid conjugates were packed under nitrogen gas and stored at +4 ° C.

3.6.4. Characterization of Conjugates

The chemical characterization and the degree of purity of the polymer-peptide conjugates to be obtained was measured by using analytical RP-HPLC by using hydrophobic C18 column (Agilent, AdvanceBio Peptide Plus, 2.1 x 150 mm, 2.7-Micron with Column ID). Lyophilized samples were dissolved in 17% acetonitrile and the column was equilibrated by 1% mobile phase (acetonitrile and ultra-pure water, 0.25% TFA) at 0.2ml/min flow rate and observed the signals at 280 nm.

4. RESULTS AND DISCUSSIONS

4.1. Synthesis and Cloning of the Genes by Ligation Independent Cloning (LIC)

This section introduces the detailed results and discussions about cloning of the genes, responsible to produce TN1 and TN3 AMPs, by LIC to obtain protein constructs to be used in different expression and purification systems.

4.1.1. Designing and Preparation of the Gene Fragments

As described in the Experimental section, gene fragments were designed as different oligonucleotides to use with LIC vectors and obtained from Eurofins Scientific. G-C% and melting temperature properties were taken under consideration to enhance primers without binding non-specifically.

Under the suggestions of manufacturer, "LIC fusion tags" were introduced to each oligonucleotide designed as the forward and reverse primers.

Forward - 5'TACTTCCAATCCAATGCA3'
Reverse - 5'TTATCCACTTCCAATGTTATTA3'

The very first design for TN1 production named as SNA-TN1 was applied with original suggested LIC fusion tags. But for the forward primer, the GCA sequence was unnecessary because of our approach using oligonucleotides by annealing each other without using T4 DNA Polymerase treatment to the insert fragments as suggested also in the LIC manual. In here, for the GCA sequence, G base to cease the T4 Pol exonuclease activity but T4 Pol was not used to chew back the insert, so the GCA sequence only gives an additional unnecessary residue (Alanine).

First experiments were carried out without using T4 DNA Polymerase treatment to the insert. After successful annealing of the oligonucleotides, designed to produce SNA-TN1, and incorporating into the 1G LIC vector, other insert fragments were designed without additional GCA sequence. As a result, Alanine residue coming from this sequence was removed by altering the designing approach.

As described in the Experimental section, annealed insert fragments (300 pmoles of each with an incubation with 3 min at 95 °C, 5 min at 74 °C) by obtained with proper overhangs to anneal also with the prepared LIC vectors.

4.1.2. Preparation of the LIC Vectors

As described in the Experimental section, different LIC vectors were prepared to use in cloning. The LIC plasmids were isolated from *E. coli* DH5α bacteria, cut with SspI restriction enzyme and, after the gel purification of the linear plasmids, treated with T4 DNA Polymerase.

4.1.2.1. Plasmid Isolation

Plasmids were successfully isolated described in the Experimental section.

4.1.2.2. SspI Digestion

Plasmids were cut with SspI enzyme (Thermo Scientific) and each LIC vector run on 0.8% TAE agarose gel electrophoresis (Figure 22, 23, 24) and gel purified by Omega E.Z.N.A gel purification kit as described in the Experimental section.

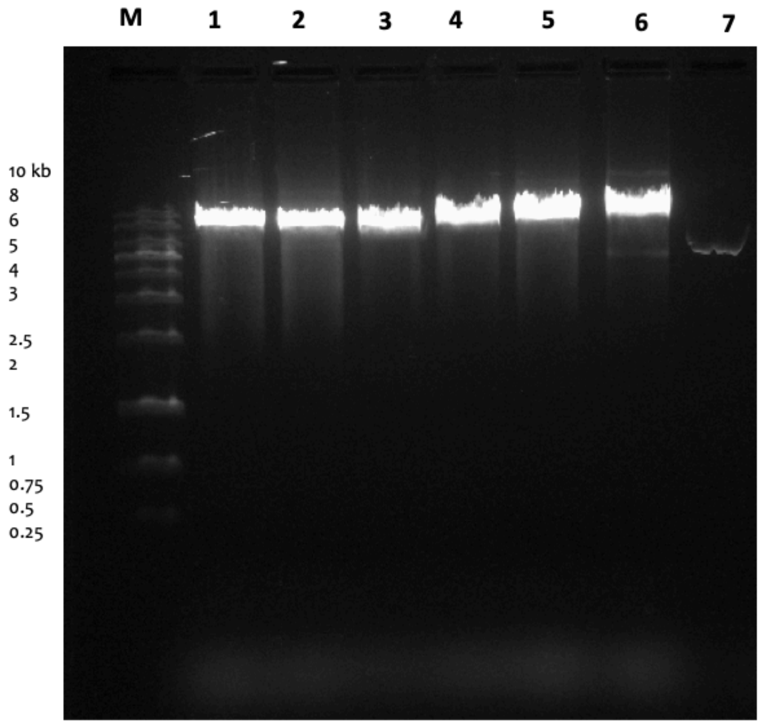


Figure 22. SspI digestion of 1G LIC plasmids isolated by Zymo Research MiniPrep Kit. M: Marker. 1,2,3,4,5,6: digested linear plasmid. 7: un-cut plasmid as a control. 0.8%TAE agarose gel electrophoresis.

As seen at Figure 23, different plasmid isolation kits were tried through the experiments and Zymo Research and GeneMark Plus Miniprep kits were found compatible with SspI digestion studies and Zymo Research MiniPrep was applied as optimized protocol for all other experiments.

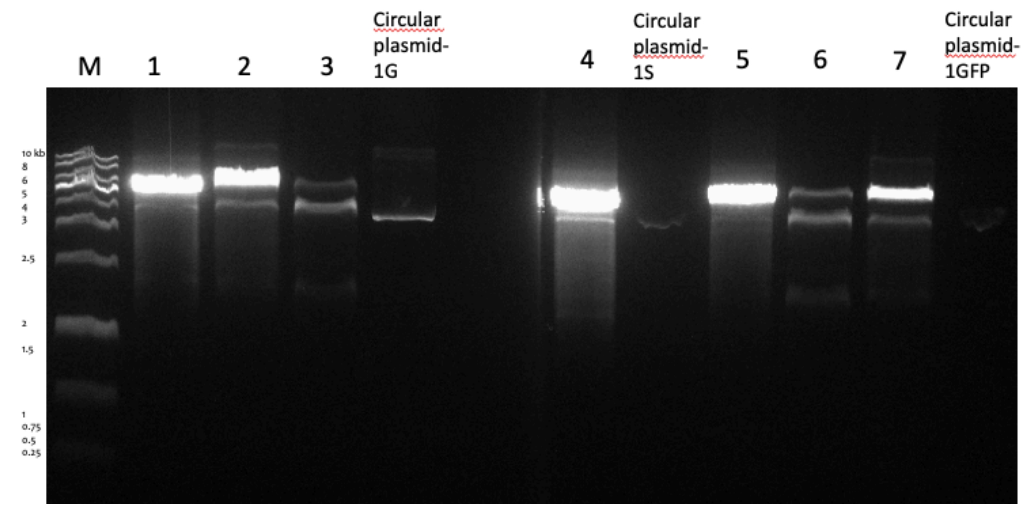


Figure 23. 1,2,3: SspI digestion of 1G LIC plasmids. 4: SspI digestion of 1S LIC plasmids. 5,6,7: SspI digestion of 1GFP LIC plasmids. 1,4,5: plasmid isolation by Zymo, 2: plasmid isolation by GeneMark Plus and 3,7: plasmid isolation by GeneMark. 0.8%TAE agarose gel electrophoresis.

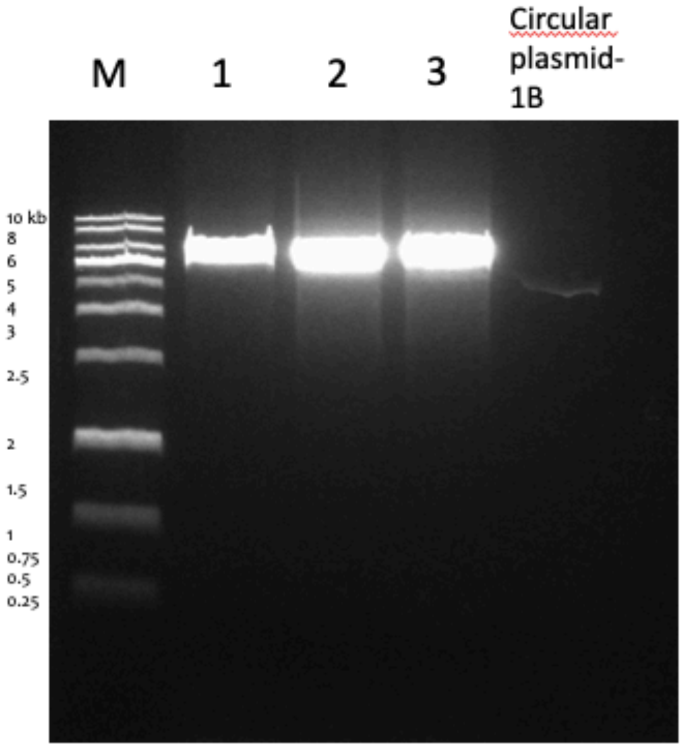


Figure 24. 1,2,3: SspI digestion of 1B LIC plasmids isolated by Zymo Research MiniPrep Kit. 0.8%TAE agarose gel electrophoresis.

4.1.2.3. T4 DNA Polymerase Treatment

Obtained pure and linear vectors were measured by Thermo Scientific, NanoDrop 2000c and 200 ng of each were treated with T4 DNA Polymerase (Novagen) as described in the Experimental section.

4.1.3. Incorporation of the Gene Fragments with the LIC Vectors

In contrast to the traditional cloning methods, LIC strategy enhances the ligation of the vector and insert fragments inside of the bacteria, by its natural enzymes, without using any additional ligase enzyme. By using LIC, cost effective approach was applied. Annealed oligonucleotides with appropriate overhangs were mixed with treated vector plasmids, directly. And vector and insert mixtures were applied to transform competent DH5α cells as described in the Experimental section.

LIC strategy was found cost and time efficient (10 min total time) for incorporation of small gene fragments into LIC vectors when compared to the traditional cloning methods.

4.1.3.1. Positive Colony Selection by Colony PCR and DNA Sequencing

Overnight grown cells were examined by colony PCR on agarose gel as described under Experimental section in detail. Control groups were used to compare with colonies (empty plasmids were amplified with primers) and to analyze the specificity of the primers (primers were tried under the reaction).

First trials were done with a method suggested at LIC manual [42] after without using a molar ratio, only with a mass ratio, 1:3 (insert: vector), T4 DNA polymerase treated vector and insert were mixed and incubated on ice for 30 min. And mixture was used to transform competent DH5α cells. With an overnight incubation, colonies were obtained and examined by colony PCR. For this, following primers were used to analyze cloning of 1G-SNA-TN1 construct:
PetLIC-fwd - 536: TCAATGCTTGAAGGAGCGGT
PetLIC-rev - 536: CTCAGCTTCCTTTCGGGCTT
These primers were designed to amplify a region, has 536 base pairs, on the 1G plasmid vectors. Because of the SNA-TN1 insert construct has 61 bases, amplification of a region with 597 base pairs were expected. PCR protocol were applied and obtained PCR products were examined with agarose gel electrophoresis (Figure 25). Colonies were examined as positive or negative by comparing the control (1). Positive colonies were expected as having more bases (in this case additional 61 bases), as a result, were expected to see above the control on the gel. Colonies in the points of 3,5 and 6 were picked (above 1) and sent for DNA sequencing with PetLIC-fwd and rev– 536 primers. And no positive colonies were obtained.

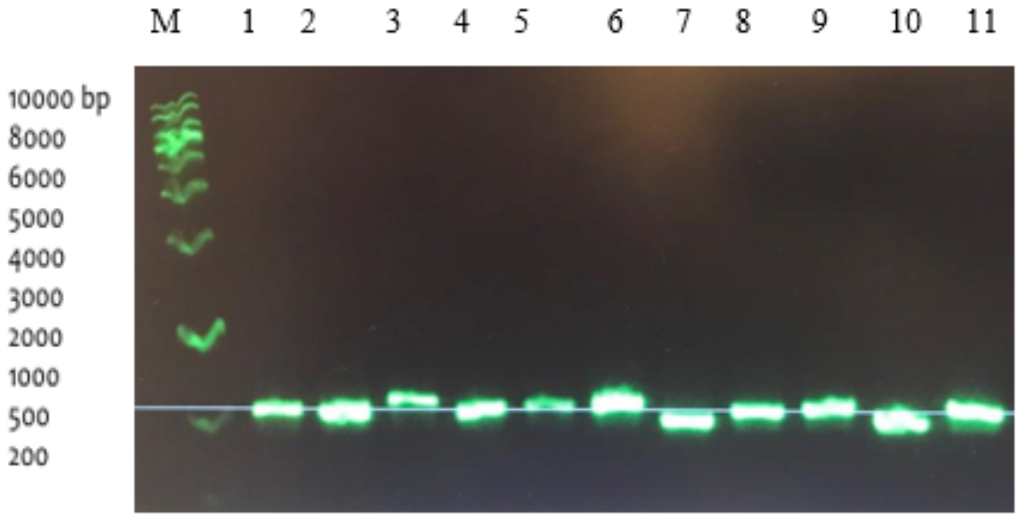


Figure 25. Examination of colony PCR of 1G-SNA-TN1 by using primers amplifying 536 base pairs. M: marker, 1: control by using empty plasmid, 2-11: examined colonies. 1.2 %TAE agarose gel electrophoresis.

Annealing approach of the vector and insert fragments was changed and optimized given as Experimental section. And primers were also designed as shorter sequences to obtain smaller PCR products giving a chance to observe 61 bases long sequences on the gels, having higher densities, with more separated bands.

For the 1G-SNA-TN1 construct, another annealing method, described in the Experimental section, was applied and after the transformation, colonies were obtained and examined (Figure 26) by the following primers:

PetLIC_fwd_119: GCTGGCAAGCCACGTTTGGT

PetLIC_rev_119: GCTTGTCGACGGCGCTCGAA

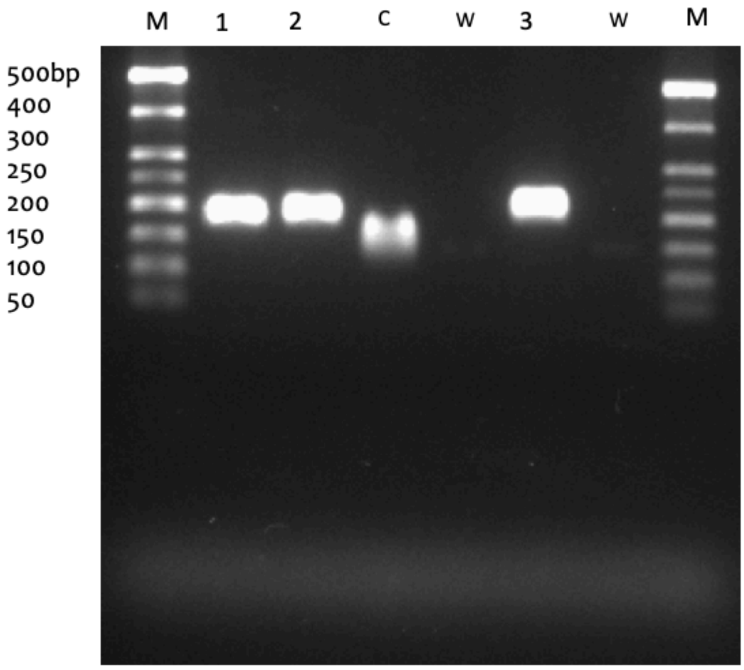


Figure 26. Examination of colony PCR of 1G-SNA-TN1 by using primers amplifying 119 base pairs. C: control by using empty plasmid, w: control by using water, 1-3: examined colonies. 1.8 %TAE agarose gel electrophoresis.

The colonies shown in 1,2,3 were picked and their plasmids and PCR products were sent for DNA sequencing in two directions by T7_fwd and pET_RP, and also by PetLIC_fwd_119 and PetLIC_rev_119 primers. They were all obtained as positive colonies as a result of the sequencing and as a representation, sequencing of plasmids (Figure 27) with T7_fwd and pET_RP, and also PCR products (Figure 28) with PetLIC_fwd_119 and PetLIC_rev_119 for one of the positive colonies were showed.

His6: green
GST: blue
TEV site: yellow
Insert

T7-Fwd Primer

GGTTGAGGAAATTCCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTTCTTCTCACCATCACCATCACCATGG
TTCTTCTATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTGAAGAAAAAT
ATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTT
ATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAA
AAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTG
AAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGT
GATCATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCAAAA
TTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGG
CTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAAGGGATCGAGGAAAACCTGTACTTCCAATCCAATGCA**CGCCTGCTGC**
GCCTGCTGCTGCTGCGCCTGCTGCGCTAATAACATTGGAAGTGGATAACGGATCCGAATTCTATATTCTCGAAAAAGCTTGCGGC

Figure 27. Examination of the selected colonies for 1G-SNA-TN1 by using DNA sequencing by T7_fwd and pET_RP primers. The regions with green: poly-histidine, blue: GST, yellow: TEV recognition site, underlined: TN1 peptide.

GGGCATCTCAAGGATCGAGGAACCTGTACTTCCAATCCAATGCA**CGCCTGCTGCGCC**
TGCTGCTGCTGCGCCTGCTGCGCTAATAACATTGGAAGTGGATAACGGATCCGAATT
CGAGCGCCGTCGACAAGCA

Figure 28. DNA sequencing for PCR products of colonies for 1G-SNA-TN1 by PetLIC_fwd_119 and PetLIC_rev_119. Underlined: TN1 peptide.

After the optimization of the methods (annealing of the vectors and insert, colony PCR, examination on agarose gel and DNA sequencing) by using 1G-SNA-TN1 construct, other vector and inserts were used. As described in the Experimental section, 4 types of LIC vectors were annealed with 4 different inserts and their obtained colonies were examined by colony PCR. Because of the PetLIC_fwd_119 primer was specific to 1G vector, to examine the constructs with 1S, 1B and 1GFP vectors, T7_fwd was used as the forward primer, while PetLIC_rev_119 as reverse primer, again. As a result of amplifying with these primers, for the constructs with 1S, amplified regions were 500 base pairs, for the 1GFP ones, were 919 base pairs, and also for the 1B ones, were 186 base pairs not convenient to examine on 12% SDS-PAGE as explained in the further.

T7_fwd and PetLIC_rev_119 primers were tried to anneal at 45° C and 50° C by gradient PCR. And both temperatures were found convenient to amplify regions when examined the size of the amplified products and primers were observed with no false attraction with each other by using control groups (Figure 29 and 30).

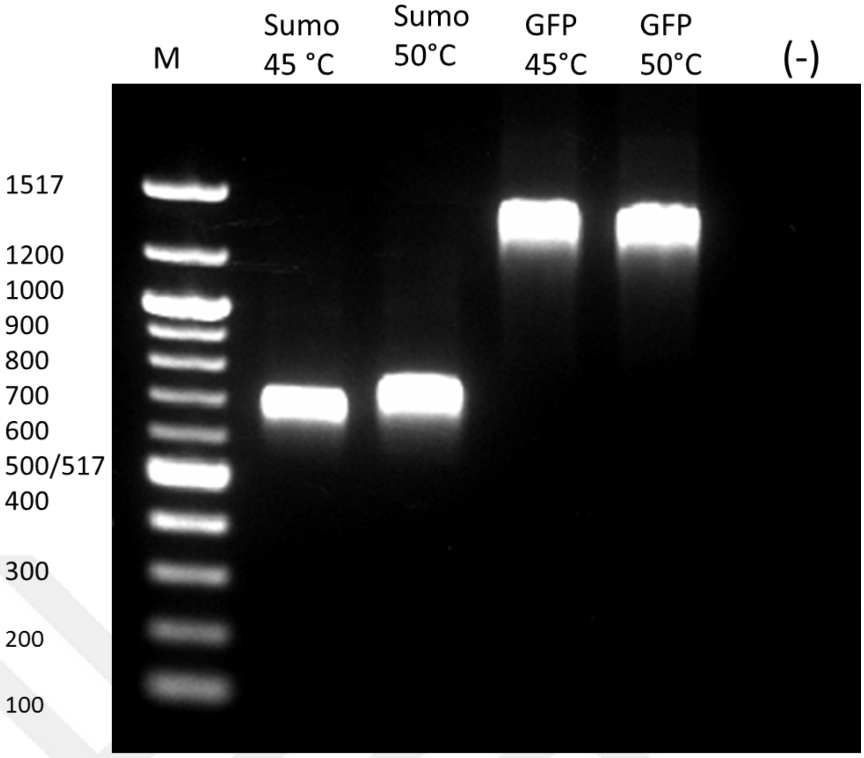


Figure 29. The annealing temperature trials for T7_fwd and PetLIC_rev_119 by amplifying plasmids of 1S and 1GFP vectors. M: marker, (-): negative control with water. 1.3%TAE agarose gel electrophoresis.

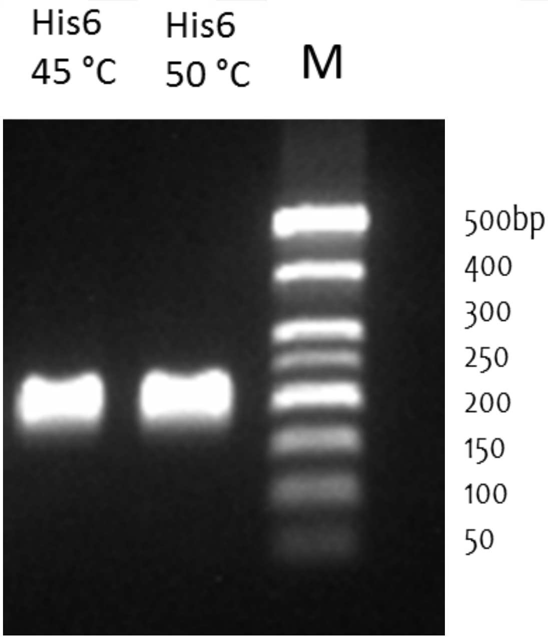


Figure 30. The annealing temperature trials for T7_fwd and PetLIC_rev_119 by amplifying plasmids of 1B vector. M: marker, (-): negative control with water. 1.8%TAE agarose gel electrophoresis.

As optimized PetLIC_fwd_119 and PetLIC_rev_119 primers were annealed at 61° C and used for colony PCR of other constructs with 1G vector. And they also examined with T7_fwd and PetLIC_rev_119 primers as a control. For the all constructs, 2 colonies from each were examined after the transformation and they were showed as "insert number.colony number" (Figure 31 and 32). As shown at Figure 31, compared to the Figure 32, when smaller PCR fragments, as a result of reduced length of the amplified region, were run on the agarose gel, making comparison with control groups were found more precisely. Examined all colonies were thought as positive and 1.2, 2.2, 3.2, and 4.2 were picked and sent for DNA sequencing.

Insert fragments have;

- M-TN1 (**1**), 60 base pairs
- DP-TN1 (**2**), 66 base pairs
- M-TN3 (**3**), 63 base pairs
- DP-TN3 (**4**), 69 base pairs

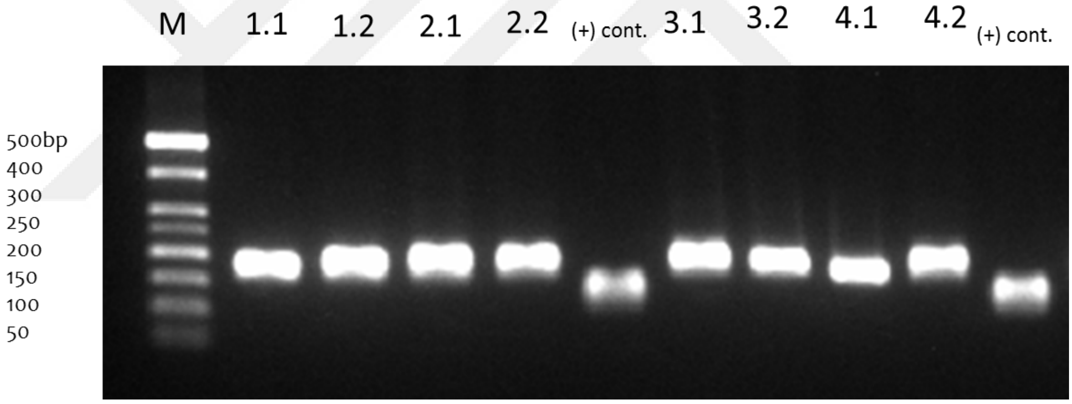


Figure 31. Examination of colony PCR of 1G vector annealed with 4 different inserts by using primers amplifying 119 base pairs. + cont.: control by using empty plasmid, -cont.: control by water, "insert number.colony number": examined colonies. 1.8 %TAE agarose gel electrophoresis.

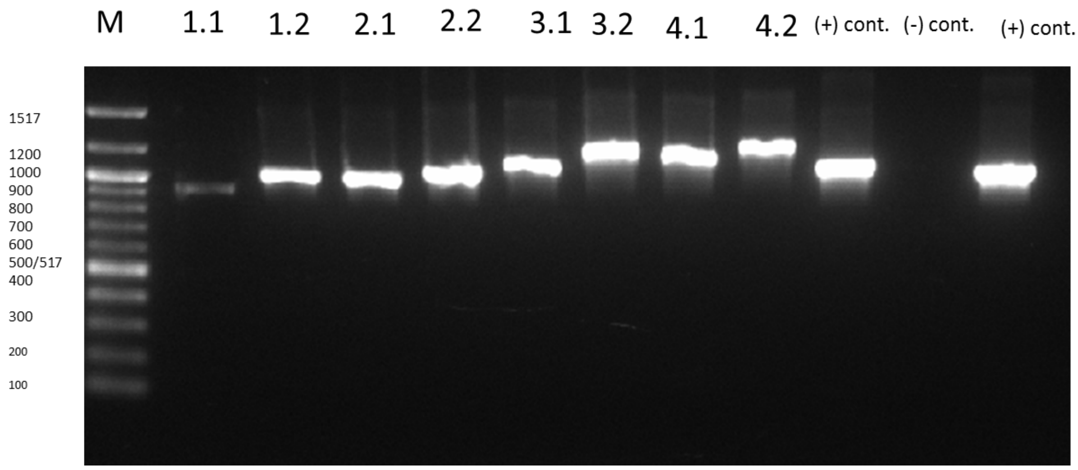


Figure 32. Examination of colony PCR of 1G vector annealed with 4 different inserts by using T7_fwd and PetLIC_rev_119 primers. cont: control by using empty plasmid, "insert number.colony number": examined colonies. 1.3%TAE agarose gel electrophoresis.

Colony PCRs for the constructs with 1B vector were examined by T7_fwd and PetLIC_rev_119 primers (Figure 33). Examined all colonies were thought as positive and 1.2, 2.2, 3.2, and 4.1 were picked and sent for DNA sequencing.

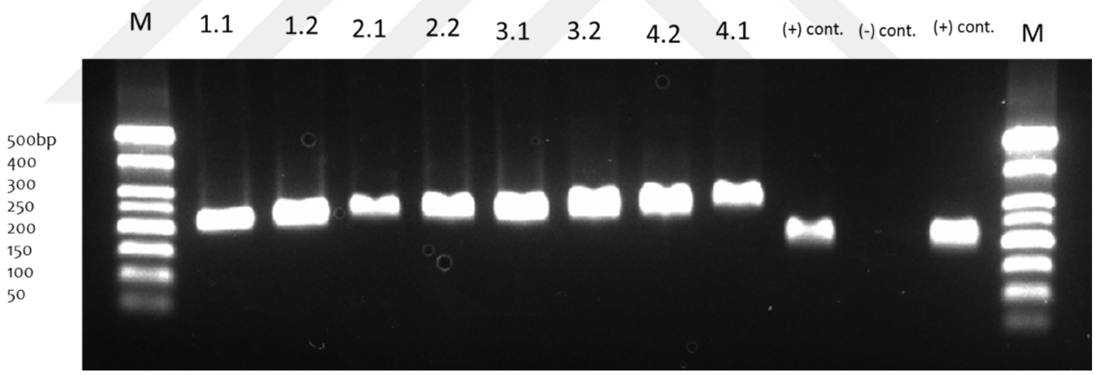
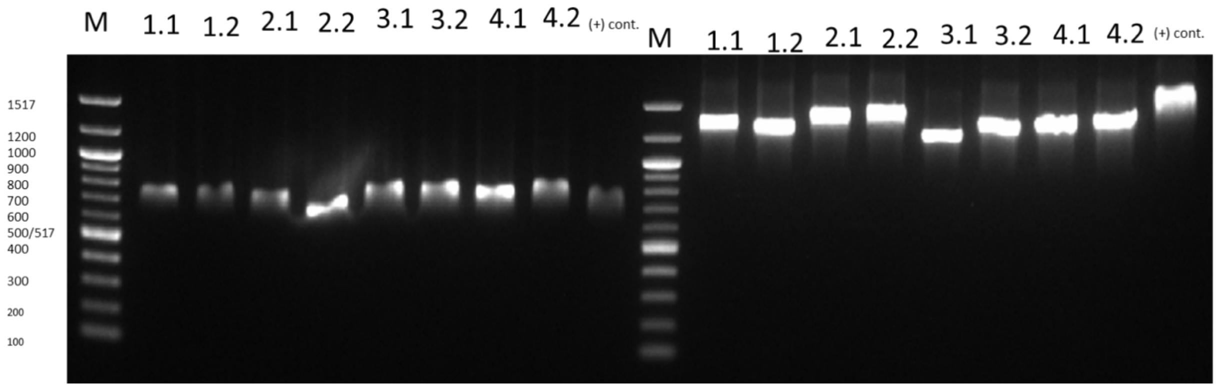


Figure 33. Examination of colony PCR of 1B vector annealed with 4 different inserts by using T7_fwd and PetLIC_rev_119 primers. + cont.: control by using empty plasmid, -cont.: control by water, "insert number.colony number": examined colonies. 1.8%TAE agarose gel electrophoresis.

As shown at Figure 34, Colony PCRs for the constructs with 1S and 1GFP vectors were examined by T7_fwd and PetLIC_rev_119 primers. For the examination with 1S vectors, when compared to the control, all examined colonies were found to be larger. But for the 1GFP constructs, there was no stabile size compared to the control (that was also shown in repeated experiments: Figure 35), so, picking colonies for sequencing was made randomly. As a result, for the 1S and 1GFP constructs, colonies with in numbers of 1.2, 2.1, 3.2, and 4.2 were picked and sent for DNA sequencing.



(A) (B)

Figure 34. Examination of colony PCR of 1S (A) and 1GFP (B) vectors annealed with 4 different inserts by using T7_fwd and PetLIC_rev_119 primers. +cont.: control by using empty plasmid, "insert number.colony number": examined colonies. 1.3%TAE agarose gel electrophoresis.

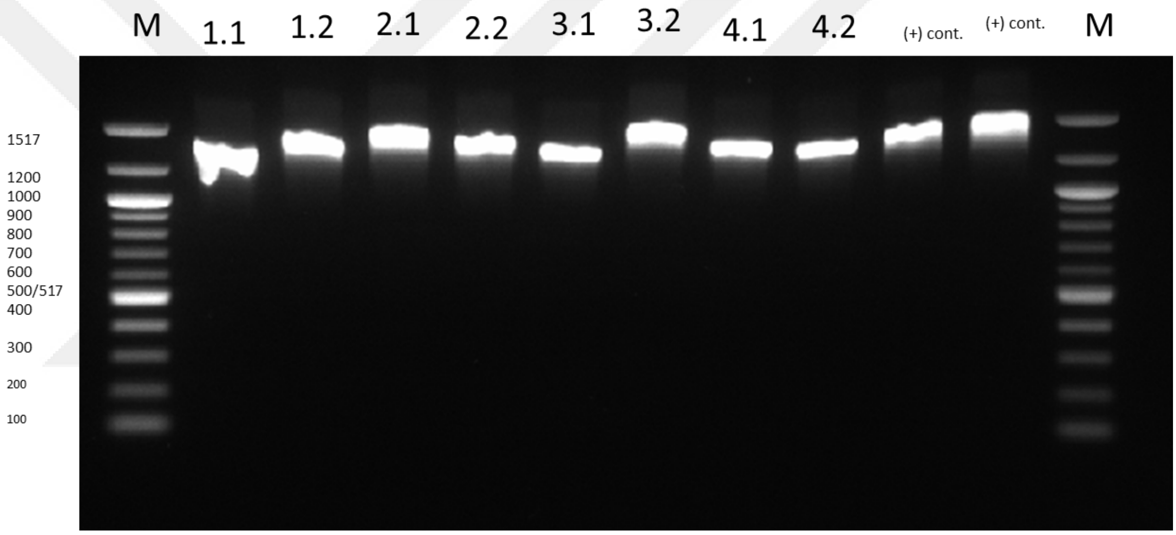


Figure 35. Examination of the colonies with 1GFP vector (annealed with 4 different inserts) after the plasmid isolation for each. Plasmids were amplified with T7_fwd and PetLIC_rev_119 primers. +cont.: control by using empty plasmid, "insert number.colony number": examined colonies. 1.3%TAE agarose gel electrophoresis.

Picked colonies were all frozen stocked. And PCR products of picked colonies were sent for DNA sequencing from two directions by T7_fwd and PetLIC_rev_119 primers. Amplified regions, from two directions, for all constructs were shown in Appendix section (A.11.).

All colonies, sent for DNA sequencing, were found to be positive and their plasmids were isolated and used to transform competent BL21, expression strain, cells as described in Experimental section, in detail.

4.2. Protein Expression, Extraction and Western Blot Analysis of Expressed Proteins

Protein expression from transformed BL21 cells was carried out by using IPTG as described in Experimental section. Optimization of the protein expression (by means of time, temperature and fraction) was tried with 1G-SNA-TN1 transformant cells by using two different induction approaches. For the other constructs, one of them was applied as the optimized induction method.

After the protein expression, collected samples, taken before and every hour after induction, were analyzed on SDS-PAGE to visualize maximum point of the expression of interested protein and whether it was in soluble or insoluble part to determine the purification approaches. Expected size of the related protein complexes were visualized by SDS-PAGE and proven by Western blotting according to their special fusion partners (His6 or GST-tag).

1G vector includes sequences for 0.84 kDa His-Tag, 25.5 kDa GST, 0.9 kDa TEV protease recognition site and 1.73 kDa NA-TN1 (N: asparagine, additional amino acid was due to SspI restriction enzyme recognition site, while A (Alanine) amino acid was coming from designing strategy proper to suggested LIC system), 0.25 kDa GSS residue and also 1.55 kDa TN1 peptide. As a result, by 1G-SNA-TN1 system, interested protein was produced as 29 kDa.

Other constructs formed by different LIC vectors have parts given in kDa; 25.5 kDa GST, 26.7 kDa GFP, 11.2 kDa Sumo and including all common; 0.84 kDa His-Tag, 0.9 kDa TEV protease recognition site, 0.13 kDa AAT (N: asparagine) residue coming from N-terminus of all inserts, 0.25 kDa GSS residue, and according to incorporated inserts:

1. M-TN1 → 60 bp **M**RLLRLLLLRLLR (1,68 kDa)
2. DP-TN1 → 63 bp **DP**RLLRLLLLRLLR (1,76 kDa)
3. M-TN3 → 66 bp **M**RLLRLLRLLL (1,41 kDa)
4. DP-TN3 → 69 bp **DP**RLLRLLRLLL (1,49 kDa)

4.2.1. Protein Expression with Slow Induction Method

The overnight culture of 1G-SNA-TN1 was used for slow induction method carried out at 23 °C and induction with 0.5mM final IPTG concentration. Time course of the 1 ml samples were taken before induction and every hour after induction. The samples were centrifuged and supernatant was discarded. And collected pellets were frozen to extract proteins more efficiently. B-PER (Thermo Scientific) was used to extract proteins as described in the Experimental section. Extracted protein samples both from soluble and insoluble fractions for each step of the induction experiments were analyzed on SDS-PAGE by means of their sizes (Figure 36). 29 kDa protein complex for the 1G-SNA-TN1 system was examined on the gel. By 0.5mM IPTG induction, after 3 hours, related protein band was observed and after 5-6 hours, the band became saturated meaning protein expression was completed within these hours. After the induction, within 6 hours, protein expression was obtained at maximum. Essential time for protein expression was optimized.

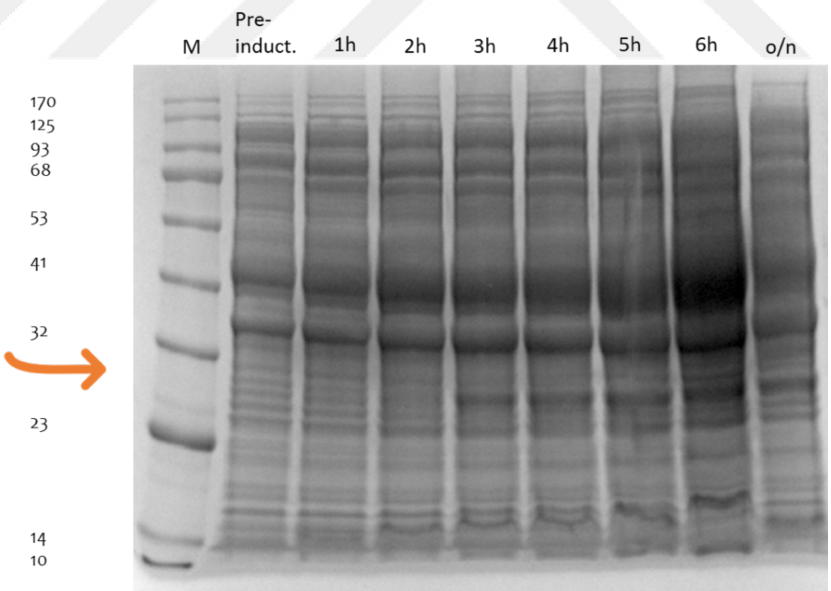
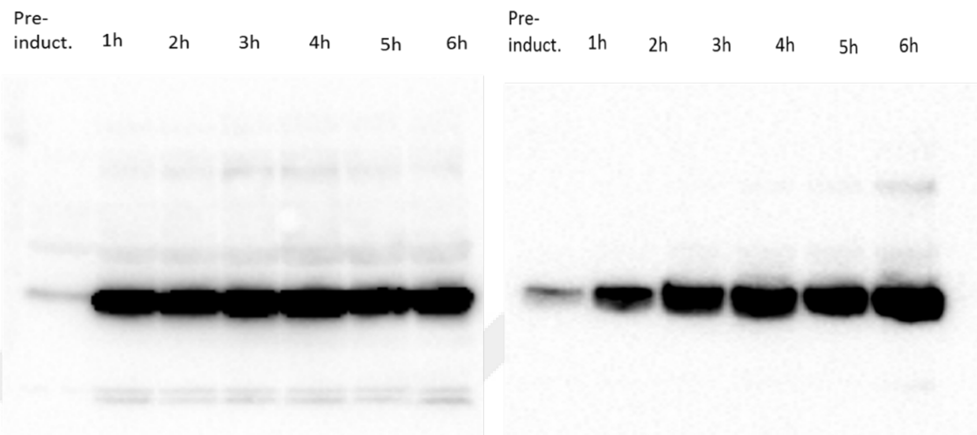


Figure 36. Protein expression with slow induction protocol of 1G-SNA-TN1 system. SDS-PAGE analysis after extraction of the proteins in soluble fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, o/n: taken sample after overnight incubation of inducted culture. The orange arrow represents the interested protein complex (29kDa).

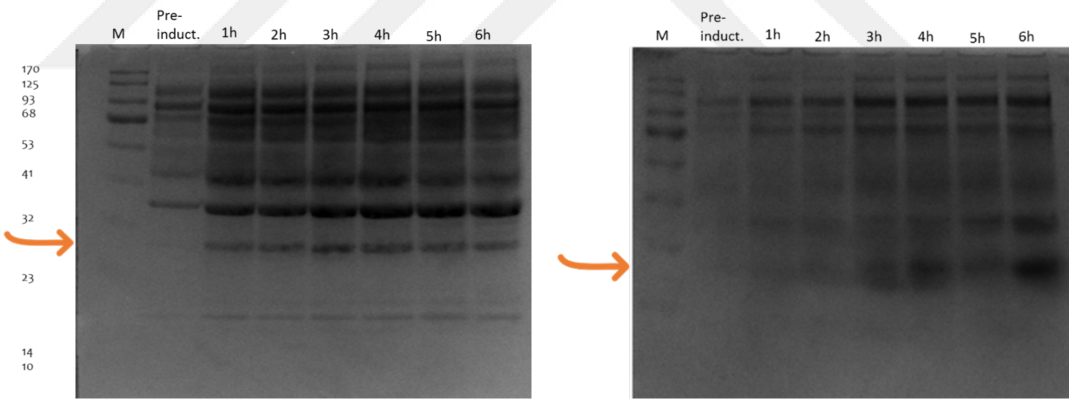
As described in the Experimental section, for the systems with 1G vector, because of having GST fusion partner, anti-GST antibodies were used to tag GST complex. By

SDS-PAGE analysis, desired protein complex was observed at 29 kDa band, as expected and confirmed by western blotting (Figure 37). After transferring to the membrane for blotting, the polyacrylamide gels were also stained and double confirmed the size of the desired protein complex (Figure 38).



(A) (B)

Figure 37. Western blotting with soluble (A) and insoluble (B) fractions of 1G-SNA-TN1 system by Anti-GST antibody. Pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of IPTG induction, o/n: taken sample after overnight incubation of inducted culture.



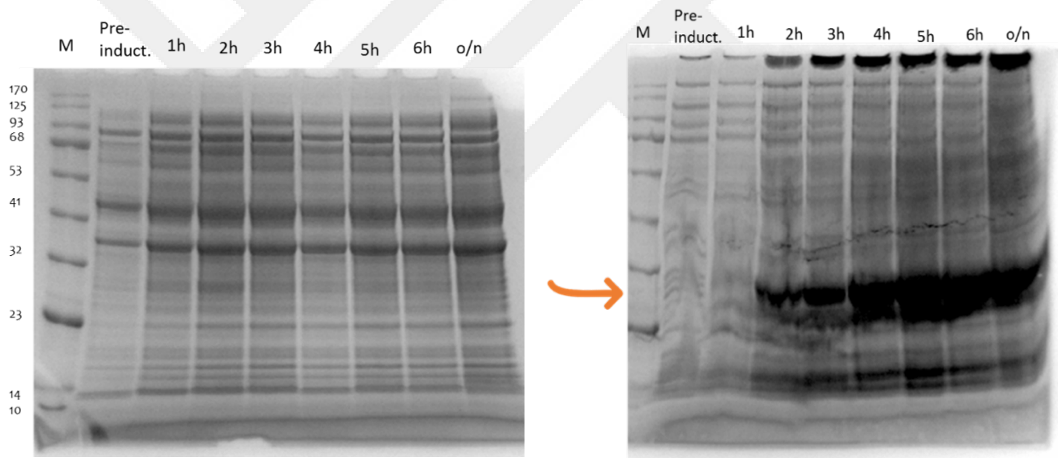
(A) (B)

Figure 38. Protein expression with slow induction protocol of 1G-SNA-TN1 system. SDS-PAGE analysis after transferring for blotting of Soluble (A) and insoluble (B) fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction. The orange arrow represents the interested protein complex (29kDa).

As seen in Figure 37, by GST fusion partner under the slow induction method, related protein complex was observed both in soluble and insoluble fractions. To optimize and obtain the interested protein complex completely in one fraction (soluble or insoluble), to gain higher yield from the protein extraction, fast induction method was also investigated.

4.2.2. Protein Expression with Fast Induction Method

Another induction method was examined by using 1G-SNA-TN1 system. As described in Experimental section, expression was carried out at 37 °C and induction with 0.5mM final IPTG concentration. Collected samples from expression steps were examined by extraction of the proteins from both soluble and insoluble parts by using BPER as described in methods section for each fraction. As seen at Figure 39, under fast induction conditions with GST fusion partner, 1G-SNA-TN1 protein complex was directed to insoluble part completely. Purification of desired proteins from IBs was accepted as challenging, but to produce anti-microbial characters of the peptides in bacteria, expressed in IBs seem to be advantages to protect proteins from degradation by proteases [31].

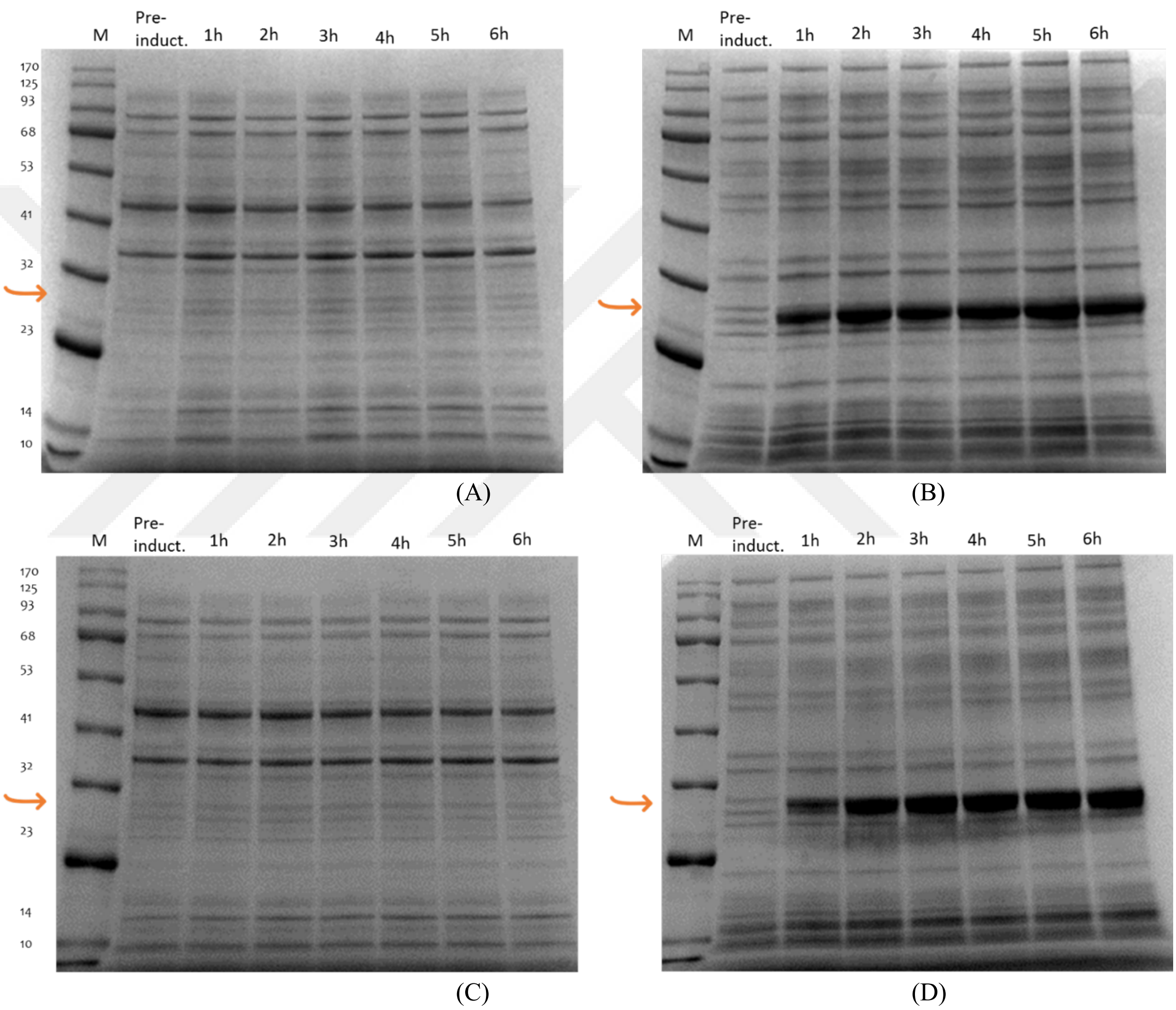


(A) (B)

Figure 39. Protein expression with fast induction protocol of 1G-SNA-TN1 system. SDS-PAGE analysis after extraction of the proteins in soluble (A) and insoluble (B) fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, o/n: taken sample after overnight incubation of inducted culture. The orange arrow represents the interested protein complex (29kDa).

Other constructs formed by other LIC vectors having different fusion partners were also expressed by fast induction method as optimized for systems with GST fusion partner. Proteins from both soluble and insoluble parts were extracted and analyzed by SDS-PAGE.

Figure 40 shows expression studies by constructs with 1G LIC vector. Under fast induction method, related proteins were found in insoluble part completely as expected with expression studies of 1G-SNA-TN1. Generally, lower temperature leads to obtain the proteins in soluble form [22]. After the comparison of slow and fast induction methods, in terms of temperature, for the 1G LIC vector, expression of related proteins was observed in insoluble form as a contrast.



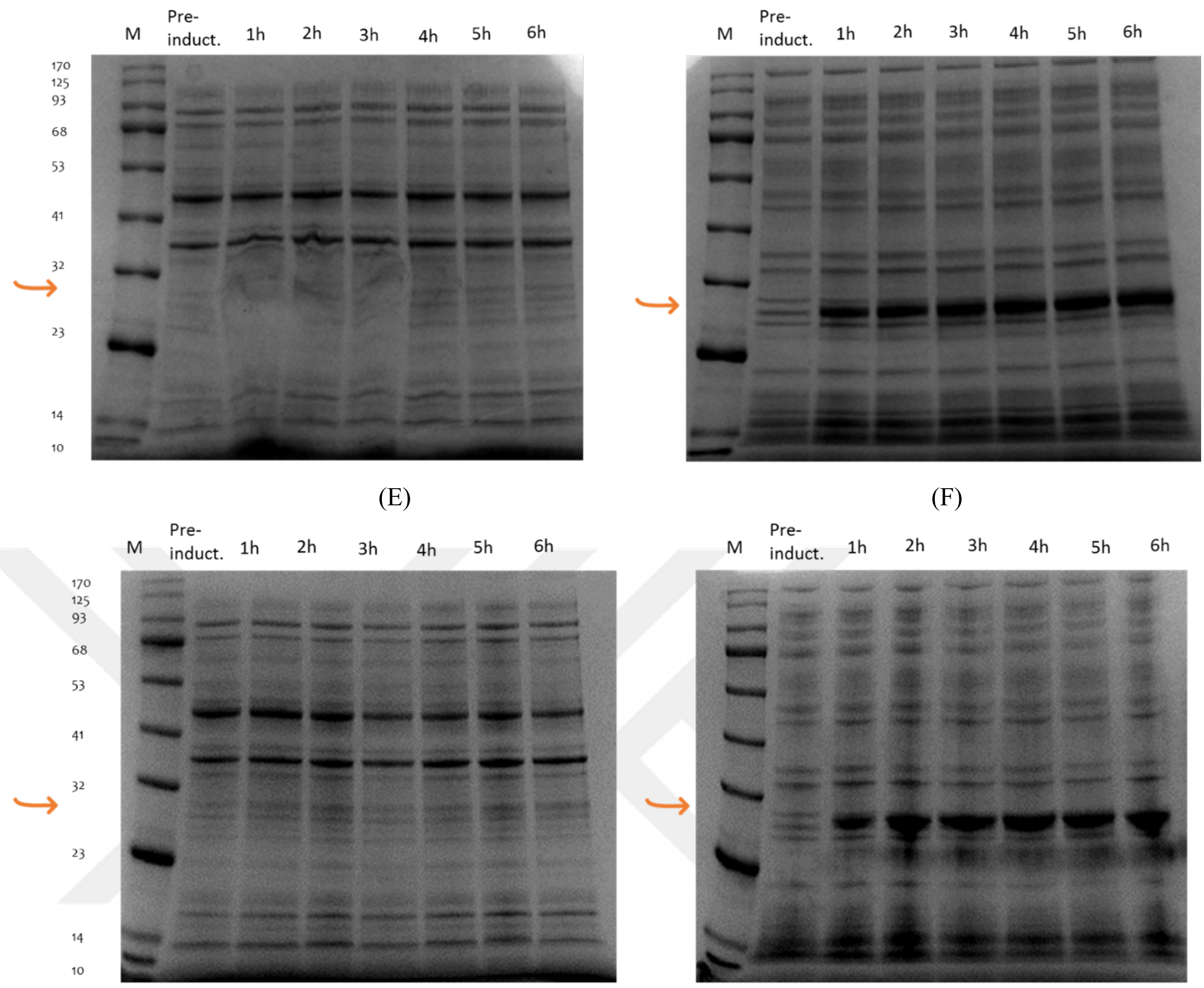
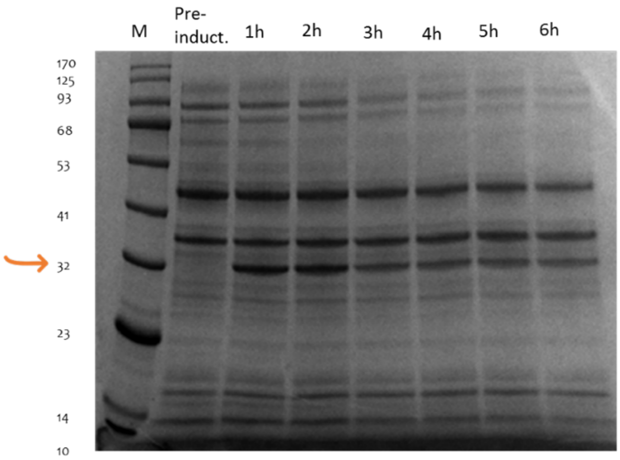
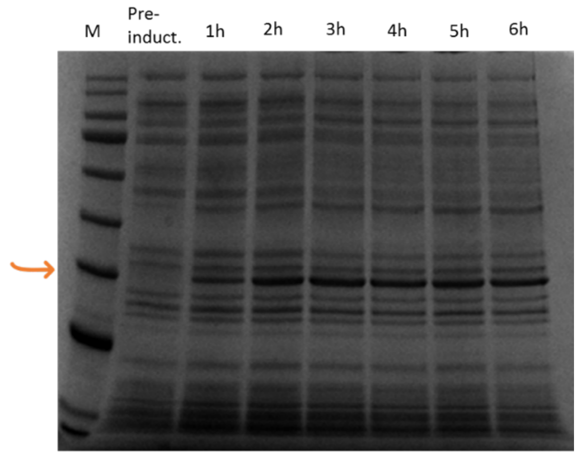


Figure 40. Protein expression with fast induction protocol of 1G-M-TN1 (A, B): 29.3 kDa, 1G-DP-TN1 (C, D): 29.38 kDa, 1G-M-TN3 (E, F): 29 kDa, 1G-DP-TN3 (G, H): 29.11 kDa systems. SDS-PAGE analysis after extraction of the proteins in A, C, E, G: soluble, B, D,F,H: insoluble fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, the orange arrow represents the interested protein complexes.

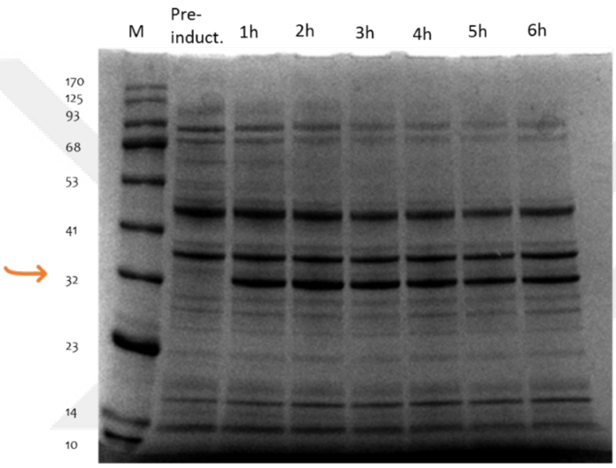
Figure 41 represents expression studies by constructs with 1GFP LIC vector. Under fast induction method, related proteins were found both in soluble (mostly) and also insoluble fractions. In parallel with literature, GFP fusion partner was found to contribute the solubility of the interested proteins [16, 22, 28].


M
Pre-
induct.
1h
2h
3h
4h
5h
6h
170
125
93
68
53
41
32
23
14
10

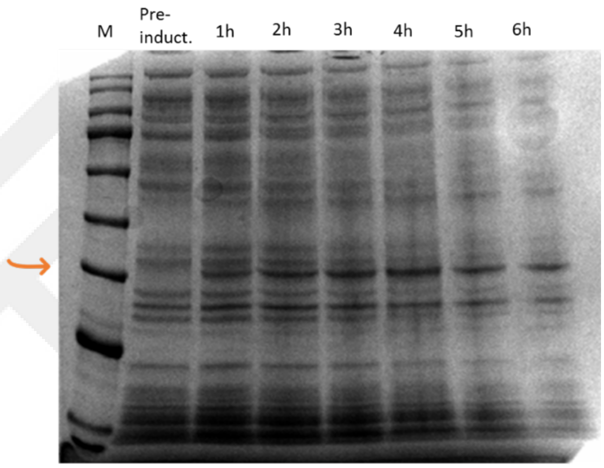
(A)


M
Pre-
induct.
1h
2h
3h
4h
5h
6h

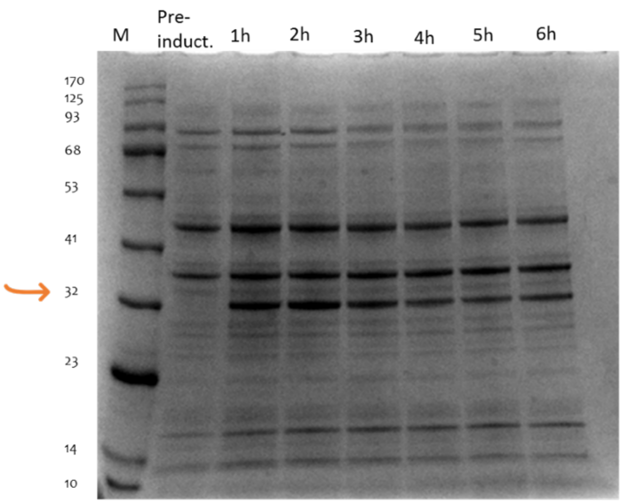
(B)


M
Pre-
induct.
1h
2h
3h
4h
5h
6h
170
125
93
68
53
41
32
23
14
10

(C)


M
Pre-
induct.
1h
2h
3h
4h
5h
6h

(D)


M
Pre-
induct.
1h
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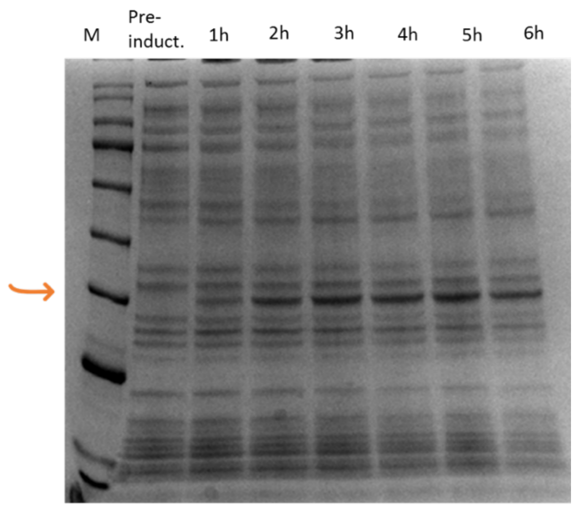
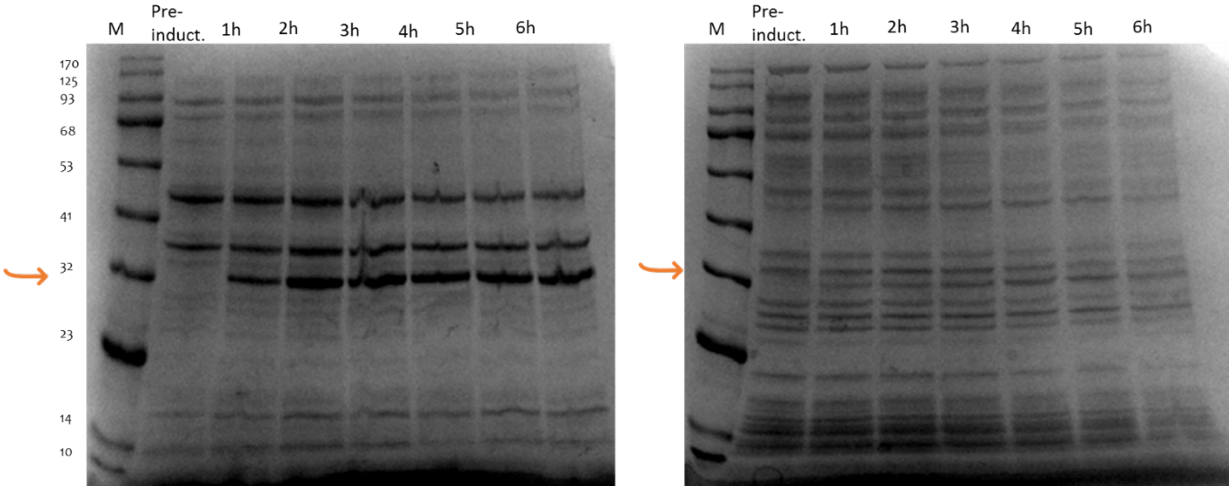
(E)


M
Pre-
induct.
1h
2h
3h
4h
5h
6h

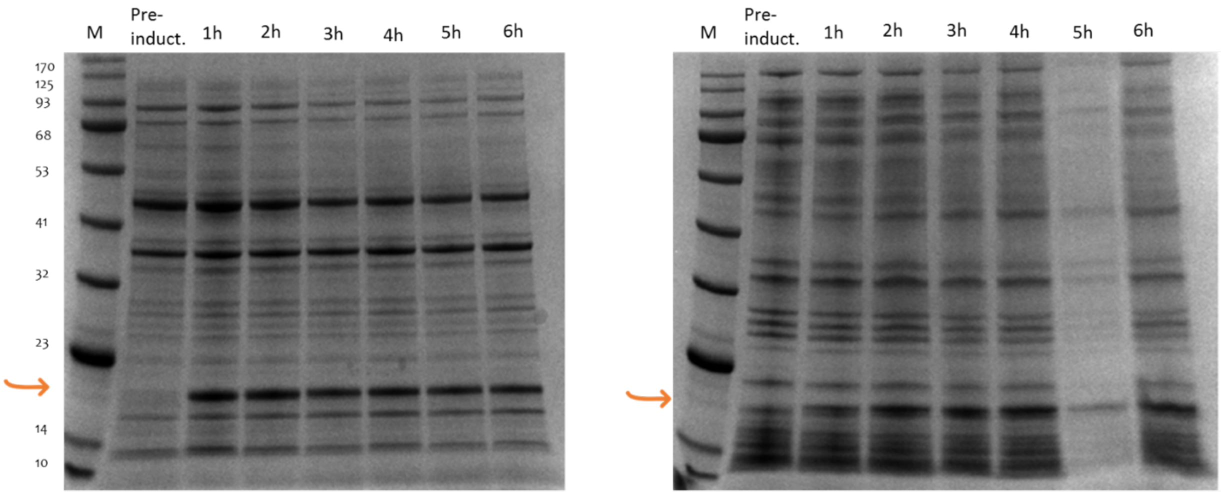
(F)



(G) (H)

Figure 41. Protein expression with fast induction protocol of 1GFP-M-TN1 (A, B): 30.5 kDa, 1GFP-DP-TN1 (C, D): 30.58 kDa, 1GFP-M-TN3 (E,F): 30.23 kDa, 1GFP-DP-TN3 (G,H): 31.05 kDa systems. SDS-PAGE analysis after extraction of the proteins in A, C, E, G: soluble, B, D, F, H: insoluble fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, the orange arrow represents the interested protein complexes.

Figure 42 shows expression studies by constructs with 1S LIC vector. Under fast induction method, related proteins were found both in soluble (mostly) and insoluble fractions. In parallel with literature [16, 22, 28]. Sumo fusion partner was found to contribute the solubility of the interested proteins.



(A) (B)

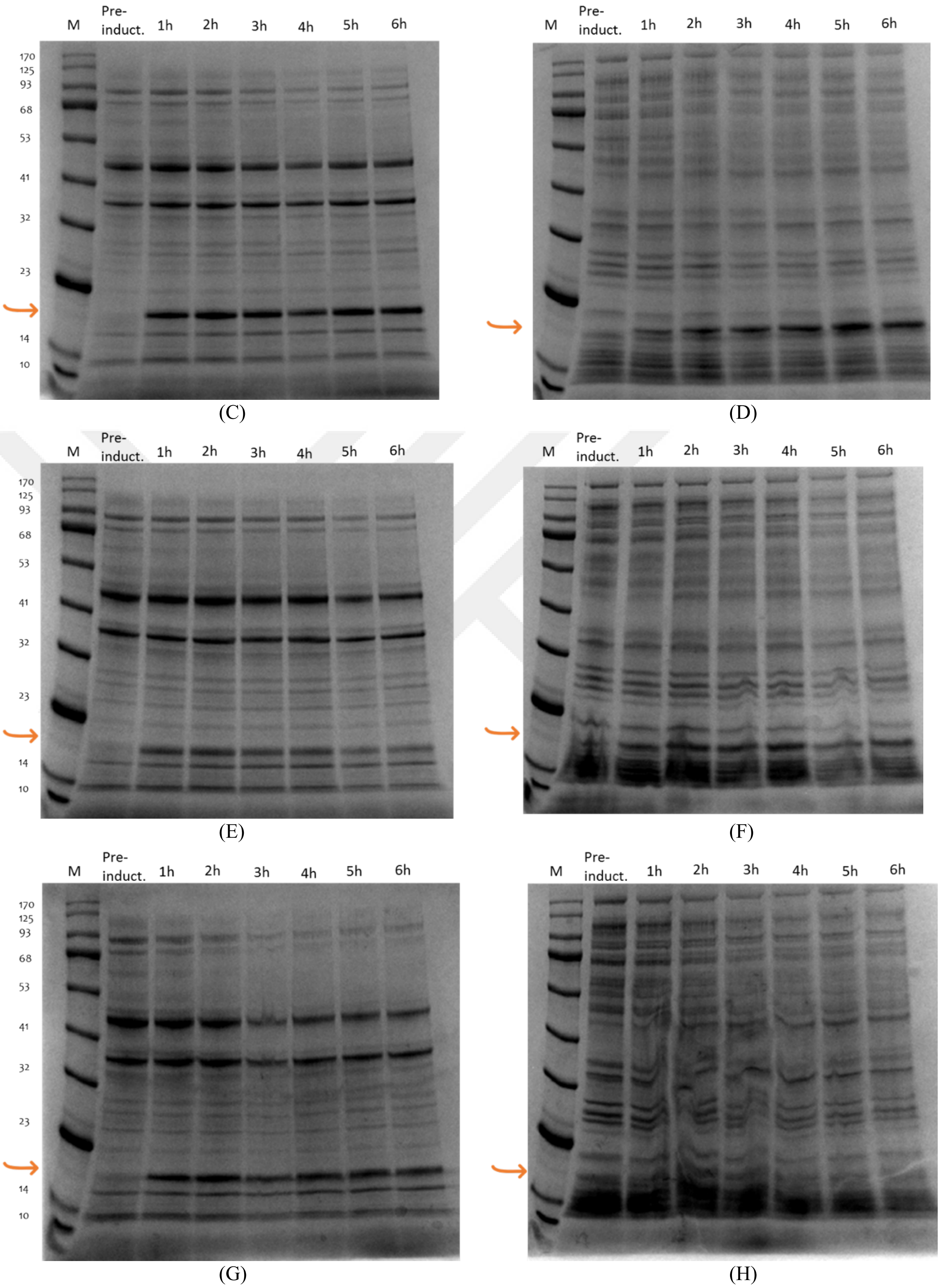
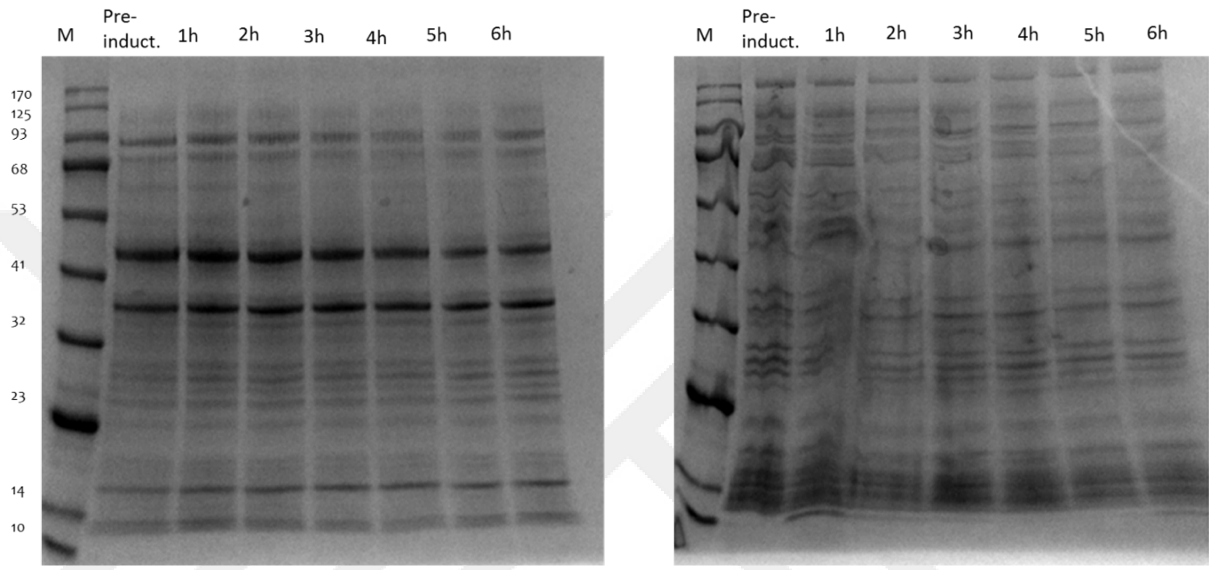


Figure 42. Protein expression with fast induction protocol of 1S-M-TN1 (A, B): 15 kDa, 1S-DP-TN1 (C, D): 15.1 kDa, 1S-M-TN3 (E, F): 14.7 kDa, 1S-DP-TN3 (G,H): 14.85 kDa systems. SDS-PAGE analysis after extraction of the proteins in A, C, E, G: soluble, B, D, F, H: insoluble fractions. M:

GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, the orange arrow represents the interested protein complexes.

Figure 43 shows expression study with 1B-M-TN1 construct. Such a small size as 3.8 kDa protein was not seen on 12% polyacrylamide gel even if it was run a short period of time only to visualize difference between pre-induction sample and on induction samples.



(A) (B)

Figure 43. Protein expression with fast induction protocol of 1B-M-TN1 (A, B): 3.8 kDa, A: soluble, B: insoluble fractions. M: GenColor Prestained Protein Marker:1-170 kDa (GeneMark), pre-induct: taken sample before the IPTG induction, 1h-6h: taken samples after 1-6 hours of 0.5 mM IPTG induction, the orange arrow represents the interested protein complexes.

Soluble and insoluble fractions extracted for all collected samples after the 6 hours of protein expression with 0.5 mM IPTG induction were used to confirm each construct by western blotting using anti-His-Tag antibody due to all constructs have His Tag, in common (Figure 44). 1B-DP-TN1 construct was also tried to tag with this strategy but protein bands were not detected on the 12% polyacrylamide gel, and as a result, not transferred to the membrane.

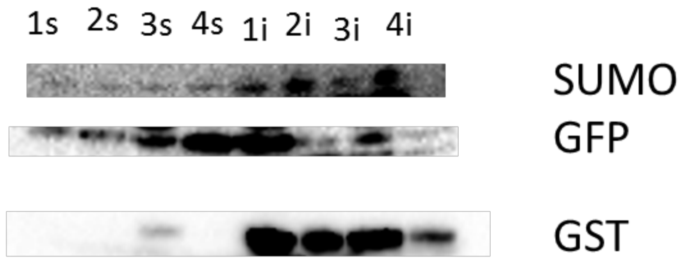


Figure 44. Western blotting of the soluble (s) and insoluble (i) fractions belong to the expression systems (1-4) with Sumo, GFP and GST fusion partners

Protein complexes with GST fusion partner were obtained in insoluble part completely, while ones with other fusion partners, GFP and Sumo, mostly in soluble fractions.

Protein purification strategies were applied for both soluble and insoluble fractions.

4.3. Protein Purification

As described in Experimental section, different strategies were applied to purify related protein structures based on their expression fraction, depending on soluble or insoluble state, and also the specific protein partner fused with desired peptides. Structures with GST protein have a tendency to drive the expression product to the insoluble fraction produced in inclusion bodies, while with other partners (Sumo and GFP) gave an advantage to obtain the proteins in the soluble state.

4.3.1. Inclusion Body Solubilization – Denaturation and Membrane Filtration

1G-SNA-TN1 (29.81 kDa) and 1G-M-TN1 (29.3 kDa) systems were used to optimize the procedures. After 6 hours of protein expression, frozen cell pellets were used and A.5. was applied to extract proteins from inclusion bodies under denaturing conditions. After refolding, the samples taken from the steps were examined by SDS-PAGE. Protein extraction, by using pellets from 1 L culture, was assumed as including 100 mg proteins in total, theoretically. As a result, comparisons and calculations were done easily. After the last step of the procedures, the exact amounts of the proteins were determined and calculations were reverse-engineered.

As seen in Figure 45, 1G-SNA-TN1 system was monitored by SDS-PAGE after the protein extraction from inclusion bodies method by removing soluble fractions with serial dilutions of B-PER procedure. Supernatant and pellet fractions were examined each time and desired protein complex was observed as purer in pellet part and no visible band was obtained in soluble fraction meaning successful removing of the impurities from this fraction. All samples were loaded to the gel as same amounts to compare.

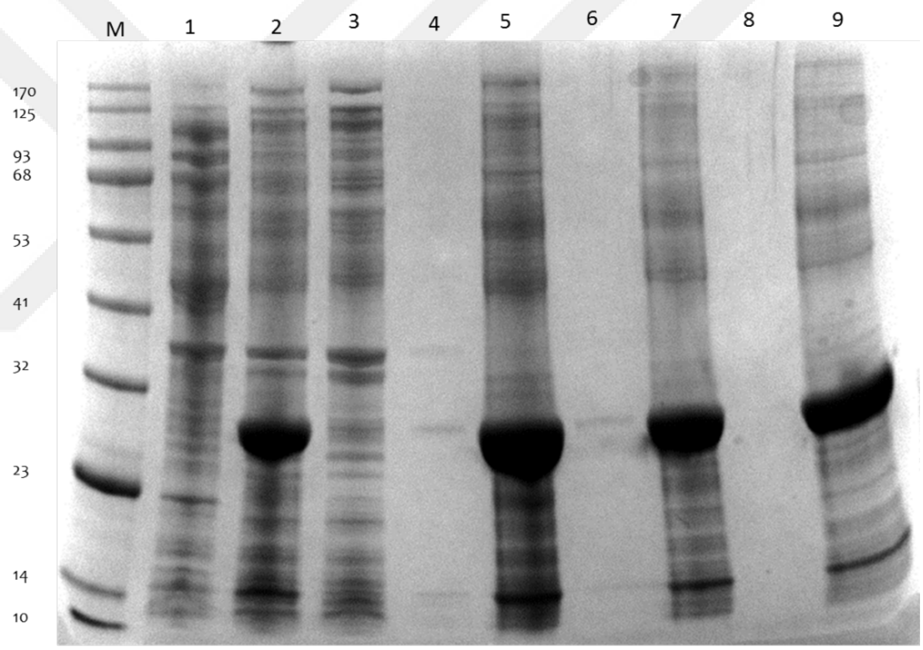


Figure 45. Protein extraction from inclusion bodies for 1L of 1G-SNA-TN1 system. 1,3,4,6,8: supernatant part; 2,5,7: pellet part, 9: the last pellet part solubilized in 6M Guanidine HCl.

After denaturation, proteins were refolded again and dialyzed against an appropriate solution (acetic acid solution, pH 3.3) to keep desired protein in soluble part as explained in Experimental section. After the centrifugation, supernatant part including soluble desired proteins was monitored by SDS-PAGE (Figure 46) and lyophilized.

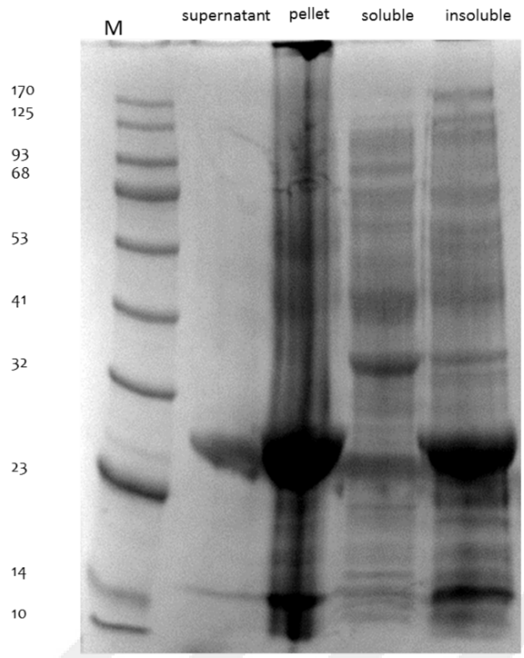


Figure 46. After the dialysis, supernatant and pellet fractions of 1G-SNA-TN1 system after centrifugation. Soluble and insoluble parts, from 6 hours expression, were used as controls of the protein complex.

By comparing almost same amount of supernatant and pellet parts, related protein complex was mostly observed at pellet, but in the supernatant, purer desired protein complex was obtained without any additional purification method such as affinity chromatograph taking extra cost. Such a pure state desired protein could be advantageous in many aspects (cost, time etc.), even if it was obtained in small amounts.

After the lyophilization, obtained purer 13 mg 1G-SNA-TN1 protein mixture were tried to solubilize completely in different solvents (Table 4) to prepare it for further chromatographic purification approaches.

Table 4. Solubilization trails of lyophilized sample mixture containing 1G-SNA-TN1 protein complex with different solvents.

1mg/ml - 100mM ammonium bicarbonate (pH 5)	Not dissolved
2mg/ml ultrapure water	Not dissolved
2mg/ml - 30mM ammonium bicarbonate (pH 8.5)	Not dissolved
0,2mg/ml - 100mM ammonium bicarbonate (pH 5)	Not dissolved
0.13mg/ml – 66,67mM ammonium bicarbonate (pH 3.5)	Not dissolved
1mg/ml – acetic acid (pH 3.3)	Not dissolved
1,11mg/ml – 30mM ammonium bicarbonate (pH 5)	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid	Not dissolved
0,8mg/ml - 30mM ammonium bicarbonate, 28% acetic acid	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid, 0.5%TFA	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid, 0.5%TFA	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid, 1% TFA	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid, 1.5%TFA	Not dissolved
1mg/ml - 30mM ammonium bicarbonate, 10% acetic acid, 2%TFA	Not dissolved
1mg/ml DMSO	Not dissolved
1mg/ml acetonitrile	Not dissolved
2mg/ml 30mM ammonium bicarbonate, 0.16% acetic acid (pH 8.5)	Not dissolved

As seen at Table 4, all cases of basic, neutral, acidic and even catastrophic conditions were applied to dissolve the protein mixture but none of them was successful. That was thought to be due to impurities in the mixture or conformation of the related proteins. For the very final solubilization trial, after the filtration by 0.22 µm filter, microplate procedure of Pierce™ BCA Protein test (BSA standards were also prepared in this solvent: Figure A.1.) was applied to estimate concentration of the proteins remained in the soluble in this solvent. Protein concentration was calculated as 27 µg/ml. Such a high amount of loss in the purified protein from the inclusion bodies was thought related with dialysis conditions applied against acetic acid solution pH 3.3 crossing the pI (pH 7) of the 1G-SNA-TN1 protein seen at Figure 11. Crossing the pI could cause precipitation of the protein and after that, even the conditions were appropriate to dissolve it again, non-reversible changes on conformation of the protein would be possible.

By using pH changing approach, after the refolding step, was only aimed to remove impurities having pI numbers crossing with changed pH would be precipitated and be removed by centrifugation. According to this approach, second trial was designed as refolding conditions (pH 10.3), given at A.5., was changed to pH 8.5 with a buffer (30mM ammonium bicarbonate) not crossing the pI of the 1G-SNM-TN1 protein (similar to 1G-SNA-TN1 protein) shown in Figure 12. The given method in A.5. was applied and samples were taken from some steps to analyze by SDS-PAGE. Supernatant and pellet fractions were examined each time and desired protein complex was observed as purer in pellet part and no visible band was obtained in soluble fraction meaning successful removing of the impurities from this fraction. All samples were loaded to the gel as same amounts to compare (Figure 47).

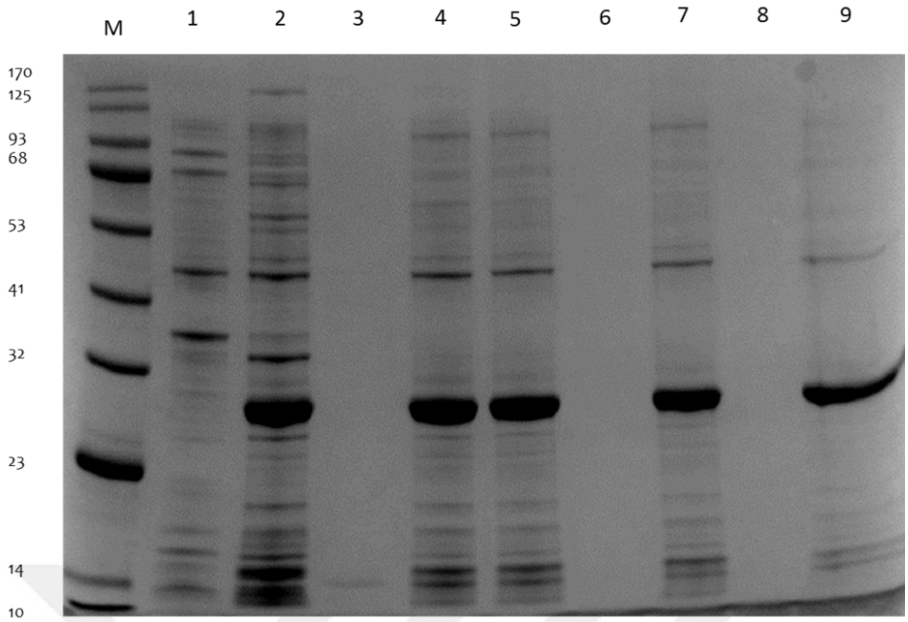


Figure 47. Protein extraction from inclusion bodies for 1L of 1G-SNM-TN1 system. 1,3,6,8: supernatant part; 2,4,5,7: pellet part, 9: the last pellet part solubilized in 6M Guanidine HCl.

By centrifugation after dialysis against 30mM ammonium bicarbonate pH 8.5, supernatant and pellet fractions were analyzed by SDS-PAGE (Figure 48). Related protein complex was observed at pellet part meaning precipitation occurred while dialysis, even if pH changes not crossed the pI of the 1G-SNM-TN1 protein complex.

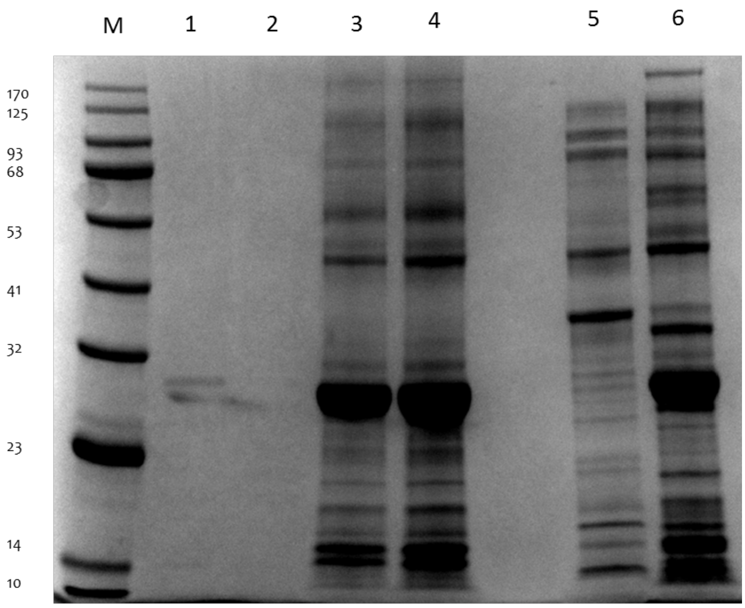


Figure 48. After the dialysis, supernatant (1,2) and pellet (3,4) fractions of 1G-SNM-TN1 system after centrifugation. Soluble (5) and insoluble (6) parts, from 6 hours expression, were used as controls of the protein complex.

When compared to the sample loaded to the gel from supernatant part, sample for pellet fraction was found more concentrated and when calculated theoretically, it was found remaining diluted 1320-fold to load almost same amount of protein (7,6 µg for each) to the gel (Figure 49).

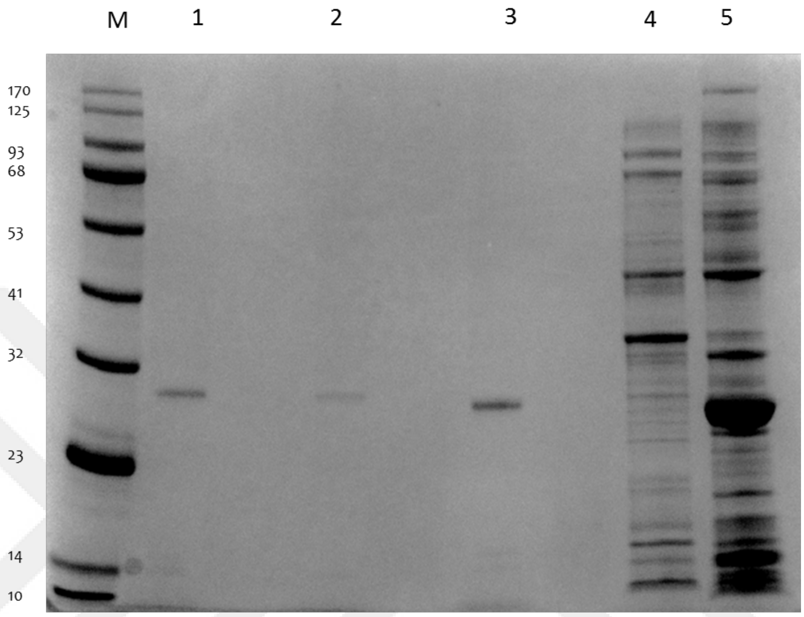


Figure 49. After the dialysis, supernatant (1,2) and pellet (3) fractions of 1G-SNM-TN1 system after centrifugation. Soluble (4) and insoluble (5) parts, from 6 hours expression, were used as controls of the protein complex.

Gained purer sample through supernatant fraction belonging to 1G-SNM-TN1 protein, was lyophilized and tried to solubilize with different solvents (Table 5).

Table 5. Solubilization trails of lyophilized sample mixture containing 1G-SNM-TN1 protein complex with different solvents.

2mg/ml - ultra pure water	Not dissolved
0.77 mg/ml - ultra pure water (pH=13.39)	Dissolved
1mg/ml - 30mM ammonium bicarbonate (pH 12.67)	Dissolved
1mg/ml - 30mM ammonium bicarbonate (pH 8.5)	Not dissolved
1mg/ml - 30mM ammonium bicarbonate (pH 12.0)	Dissolved

At elevated pH points, 1G-SNM-TN1 protein complex was found as soluble. 1mg lyophilized sample of the protein mixture was prepared in 30mM ammonium bicarbonate pH 12.0 and protein concentration was measured by microplate procedure of Pierce™ BCA Protein test (BSA standards were also prepared in this solvent: Figure A.2.) as 530 µg/ml. Different amounts of this protein mixture was loaded to the gel and compared with pellet fraction gained after dialysis in terms of impurity level of the sample (Figure 50). As a result, insoluble protein (1G-SNM-TN1) in IBs was found successfully gained as the soluble protein in the supernatant fraction from dialysis. Impurities were tried to remove by affinity chromatography explained in the further sections.

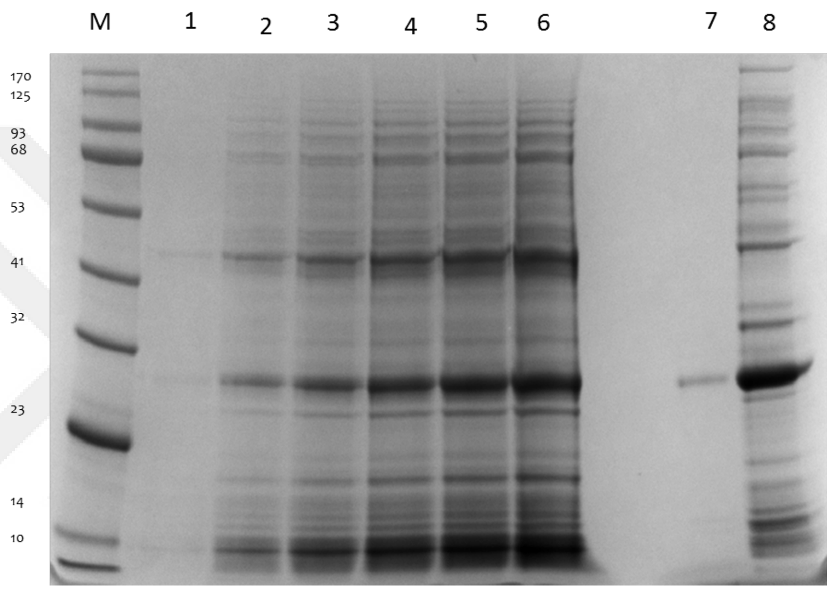


Figure 50. Examination of 530 µg/ml protein mixture, containing 1G-SNM-TN1 protein complex, with different dilutions. 1: 500ng; 2: 5µg; 3: 10µg; 4: 15µg; 5: 20µg; 6: 25µg; 7: pellet (7.6µg); 8: insoluble fraction from 6 hours protein expression.

4.3.2. Purification of GST-Tagged Proteins

Lyophilized sample of 175 mg protein mixture containing 1G-SNM-TN1, extracted from inclusion bodies, was tried to purify by glutathione affinity chromatography as explained in the Experimental section. 175 mg sample was solubilized in 30mM ammonium bicarbonate pH 12.0 buffer and A.6. was applied. While getting ready to load column, when the sample was mixed with equilibration buffer (50mM Tris, 150mM NaCl, pH 8.0), as suggested at the procedure, solubilization problems were observed and homogenous sample was not obtained even by rigorous mixing. At the end, the protein mixture was dissolved in its own buffer (30mM ammonium bicarbonate pH 12.0), completely. Samples were collected from different steps of the purification procedure and analyzed by SDS-PAGE (Figure 51). As seen, related protein (1G-SNM-TN1) was observed as un-bounded sample at very first steps of the purification (and none of it was observed at elution step at the final) meaning GST complex did not bind the glutathione under these circumstances without using any denaturing conditions. That can be also due to unsuccessful refolding of the protein, after IB solubilization step, giving to the GST protein complex a right conformation enhancing to bind the glutathione. Binding conditions can also be a problem because of the prepared protein mixture to be purified could not mixed with equilibration buffer also used to equilibrate the column. That mismatch caused failing of the basic rule of affinity chromatography approaches.

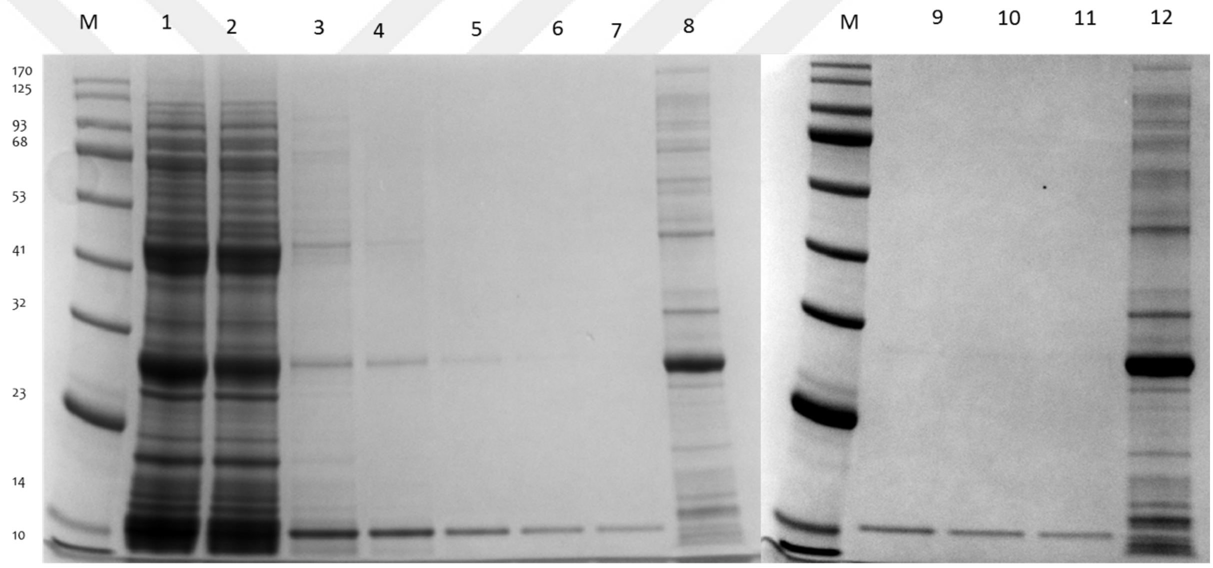


Figure 51. Analysis of glutathione affinity chromatography steps applied to purify a protein mixture containing 1G-SNM-TN1 protein complex. 1: starter protein lysate to be loaded to the column; 2: un-bounded proteins gained after the binding step; 3-7: fractions gained after washing of the column with wash buffer; 8,12: insoluble fraction from 6 hours protein expression as a control; 9-11: eluted fractions after washing of the column with elution buffer.

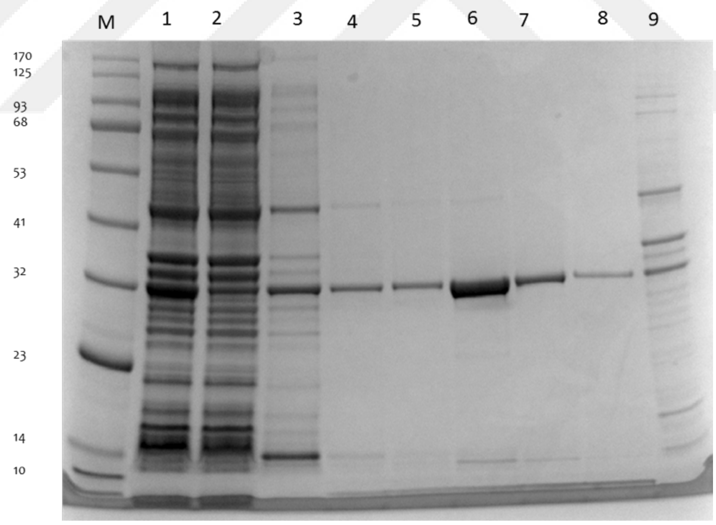
Further investigations are needed to dissolve proteins in the buffers compatible with equilibration conditions of the glutathione affinity columns. Another approach can also be searched for refolding of the proteins. To answer the refolding success of the proteins, after the IB solubilization, another purification approach can be used as described in the next section.

4.3.3. Protein Purification by Nickel Magnetic Bead System

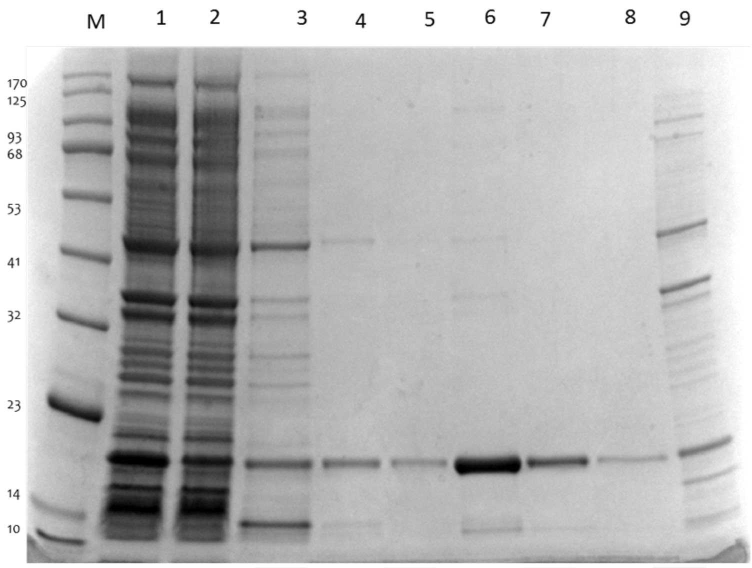
Every protein construct has His6 tag along with their special fusion partners enhancing solubility or aiding to the purification.

As described in Experimental section, further purification of the expressed proteins, both in soluble and insoluble fractions, were applied by PureProteome Nickel Magnetic Bead System (Merck).

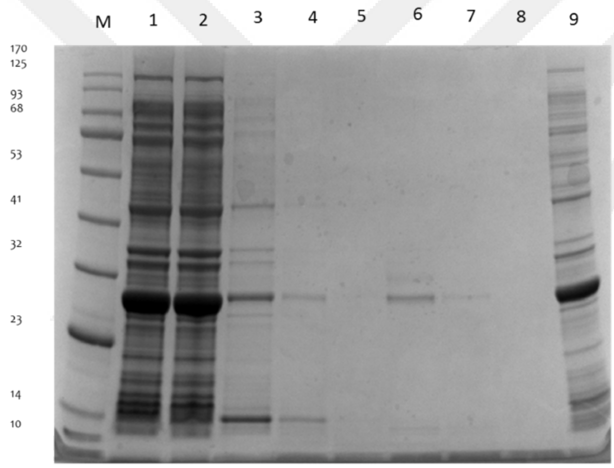
After the expression, frozen cell pellets from 1L cultures of 1G-DP-TN1, 1GFP-DP-TN1, 1GFP-DP-TN3, 1S-DP-TN1 systems were used to optimize the purification method. A.7. (for proteins in 1G, 1S, 1GFP systems) and A.8. (only for proteins with 1G) were applied to isolate the proteins from the frozen pellets. A.9. was applied to purify His-Tagged proteins and samples taken from the steps were examined by SDS-PAGE (Figure 52).



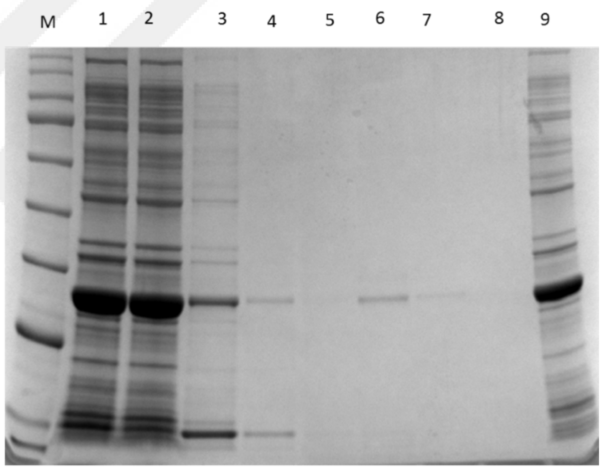
(A)



(B)



(C)



(D)

Figure 52. Purification by PureProteome Nickel Magnetic Bead System (Merck) for 1GFP-DP-TN1 (A), 1S-DP-TN1 (B) and 1G-DP-TN1 (C) protein lysates (1ml each) from total protein extraction method; 1G-DP-TN1 (D) protein lysate (1ml) from insoluble protein extraction method. 1: starter protein lysate to be loaded to the magnetic beads; 2: un-bounded proteins gained after the binding step to the beads; 3-5: fractions gained after washing of the beads with wash buffer; 6-8: eluted fractions after washing of the beads with elution buffer; 9: soluble (A,B) and insoluble (C,D) fractions from 6 hours protein expression as controls.

As seen at Figure 52, nickel affinity chromatography was found successful for protein purification form the systems with GFP and Sumo fusion partners while the proteins in the systems with GST partner was not also purified by this approach both for soluble and insoluble proteins. After the binding to beads step (at the points of 2), related protein complexes were found as un-bound fraction. The protein samples were almost completely lost at this step and a little amount of it were found at the washing steps (3-5). And a really weak protein bands were found at the elution fractions (6-8).

They were valuated as negligible when compared to the lost at the binding step. Fractions at the points of 6,7,8 represent eluted His-tagged proteins.

Figure 53 represents the observation of these 3 consecutive elution steps at day-light by the help of GFP.

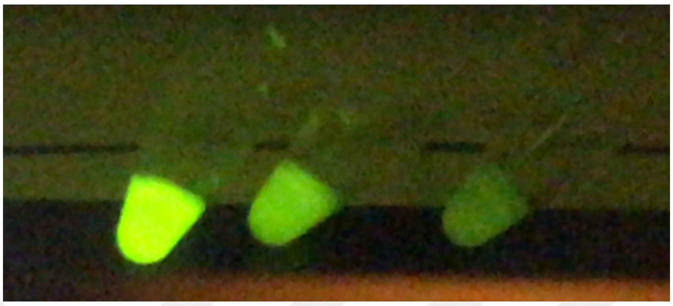


Figure 53. 3 consecutive elution steps at day-light, after the purification by PureProteome Nickel Magnetic Bead System (Merck) for 1GFP-DP-TN1

The original method advised from supplier was optimized to purify more protein by 200 μl of the magnetic beads. 1 ml of the protein lysate was recommended to be loaded to each 200 μl of the beads. Up to 4 ml of the protein lysate from the 1GFP and 1S systems were applied to 200 μl of the beads with a binding of each 1 ml in consecutive incubation periods (A.9.). Figure 54 represents 16 ml protein lysate purification for 1GFP-DP-TN3 system.

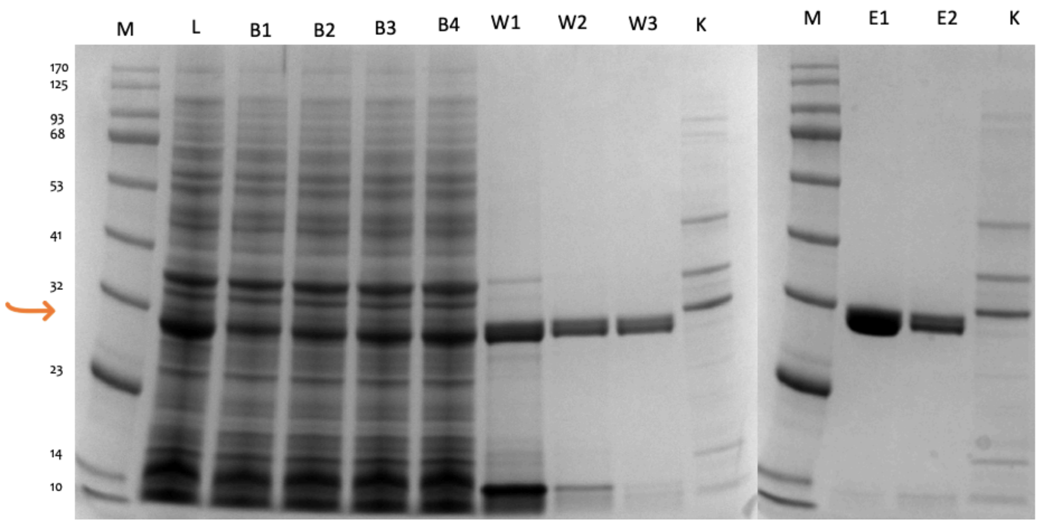


Figure 54. Purification by PureProteome Nickel Magnetic Bead System (Merck) for 1GFP-DP-TN3 protein lysate (16ml) from total protein extraction method. L: starter protein lysate to be loaded to the magnetic beads; B1-B4: un-bounded proteins gained after the binding steps to the beads; W1-W3: fractions gained after washing of the beads with wash buffer; E1,E2: eluted fractions after washing of the beads with elution buffer; K: soluble fraction from 6 hours protein expression as controls. Orange arrow: size of the 1GFP-DP-TN3 protein

GST-tagged proteins were not purified by also these approaches, that can be due to a restriction by the GST conformation to the His-Tag. Detailed research should be done to examine this phenomenon from different aspects.

As a result, purification of 1S and 1GFP systems were optimized with a small amount of impurities can be removed by further purification techniques. Following experiments were applied by using the first elution step (6) of the 1GFP and 1S systems, when needed.

4.3.4. Protein Concentration Determination

The elution buffer of the Nickel Bead System was exchanged to a proper buffer (100mM ammonium bicarbonate, pH 7.8) by using Protein Desalting Spin Columns (Thermo Scientific) as described in Experimental section. Pierce BCA Protein test was used to determine protein concentrations through the all experiments.

As a representative calculation, based on first elution after the 1 ml protein lysate purification of His-tagged proteins of 1GFP system, protein concentration was obtained as 332,14 μg/ml in 130 μl buffer meaning 43,2 μg total purified protein. That amount of protein was purified from 1:24 of 1L of the expression culture meaning from 42 ml of the induced culture. Obtained amount was found as sufficient to apply further experiments trying to obtain related peptides.

Optimization of different steps regarding to the vector selection, protein expression and purification can be studied to increase the yield of the purified proteins.

4.4. Removal of Fusion Partners

Removal of the fusion partners were designed to apply by different strategies as explained in Experimental section in detail. Chemical (formic acid) and enzymatic (TEV) cleavage methods were tried to optimize by using some systems as representatives for optimization studies.

4.4.1. Chemical Cleavage by Formic Acid

First trial was applied by using 28 µg of purified and lyophilized GFP-DP-TN1 protein by applying 50 µl 70% formic acid, mixing and incubating at 37°C for 48 hours. Reaction was diluted by 150 µl ultrapure water and stopped. Then, it was freeze dried [2, 40].

After the formic acid cleavage to cleave between D and P amino acids, expected protein residues for the 1GFP-DP-TN1 system including 3 parts:

1) MGSSHHHHHHGSSVSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGD ATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSA MPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGH KLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGD GPVLLPDNHYLSTQSKLSKD (24968.92 Da)
2) PNEKRDHMVLLEFVTAAGITLGMDELYKGIEENLYFQSND (4602.17 Da)
3) PRLLRLLLLRLLR (1645.16 Da) (related peptide sequence)

After the first cleavage trial, it was evaluated according to size by SDS-PAGE (Figure 55) and size exclusion chromatography (SEC) describing in the further section. The other experiment parameters (Table 3) were designed after no detection of the related peptide while observation by SEC.

Cleavage products were dissolved in 100mM ammonium bicarbonate pH 7.8 compatible with pI numbers (Figure 12) of the expected cleaved products. Each 30 µl of cleaved products (approx. 10 µg) were examined by SDS-PAGE (Figure 55). As seen, such a small fragment (1645.16 Da) was not observed by SDS-PAGE. Only the degradation of the fusion partner could be understood especially at 2 and 3 conditions (Table 3). As a result, additional conditions were applied (the conditions 15-20 at Table 3) to investigate promising conditions for both 1GFP-DP-TN3 and 1S-DP-TN1 systems. Further investigations should apply to characterize the peptide and determine the efficient cleavage conditions.

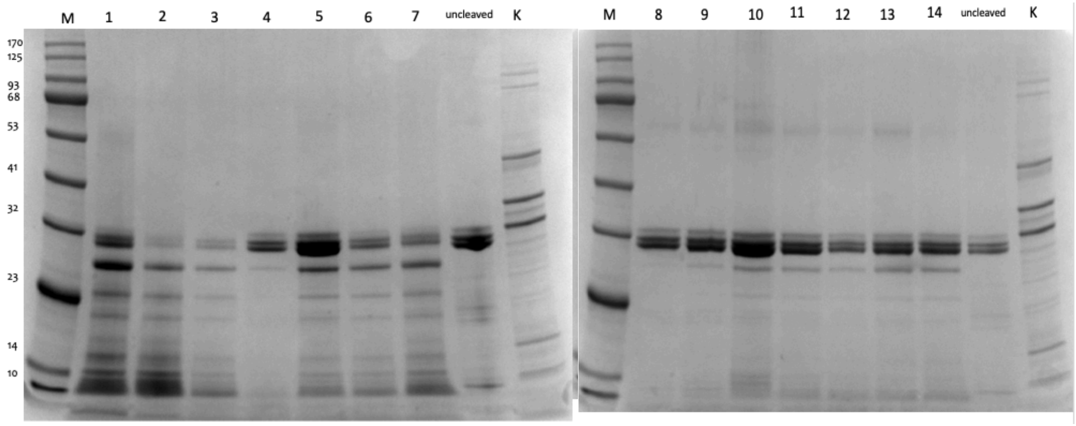


Figure 55. Examination of chemical cleavage trials by formic acid of purified 1GFP-DP-TN1 protein. 1-14: cleavage conditions at Table 3. Uncleaved: purified 1GFP-DP-TN1 protein as a control. K: soluble fraction from 6 hours protein expression as a control.

Antibiogram experiments and MS analysis were applied to characterized cleavage products (peptides) as described in the further sections.

4.4.2. Enzymatic Cleavage by TEV

TEV protease was used to cleavage 1S-DP-TN1 complex, as described in Experimental section. After the reaction, samples were analyzed on SDS-PAGE (Figure 56). The orange arrow represents 1S-DP-TN1 complex, while black is TEV protease and the blue ones can be degradation of TEV protease due to DTT in solution. Further analysis should be applied to determine the cleaved peptides.

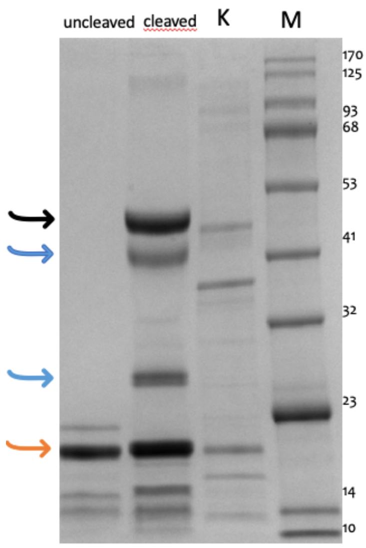


Figure 56. Examination of TEV cleavage of purified 1S-DP-TN1 protein. Uncleaved: purified 1S-DP-TN1 protein as a control. K: soluble fraction from 6 hours protein expression as a control.

4.5. Characterization of Peptides

4.5.1. Antibiogram Experiments with Cleaved Products

The results suggest that the non- charged N-terminal proline, left from formic acid cleavage, did not expect to affect the antibacterial activity of the recombinant protein. Since, AMPs interact by electrostatic forces with the negatively charged bacterial membrane phospholipids, eventually leading to its disruption [2], the same net charge of the produced peptides could not affect their antibacterial activity.

After the F.A cleavage reactions of the samples described above, antibiogram tests were carried out by using 10µl of each dissolved in 100mM ammonium bicarbonate pH 7.8 compatible with pI numbers (Figure 14) of the expected cleaved products. Samples were applied 0.5McFarland *E. coli* culture as drops. Buffer and ampicillin (1mg/ml;10ul) were used as controls (Figure 57). Except ampicillin, no zone was detected. That can be because of the impurities in the protein mixture can mask the antimicrobial effect of the peptides or the obtained peptides can be trace amount or the applied cleavage procedures can be failed. Further analysis (such as LC-MS) should be applied to determine whether cleave the partners, appropriately.

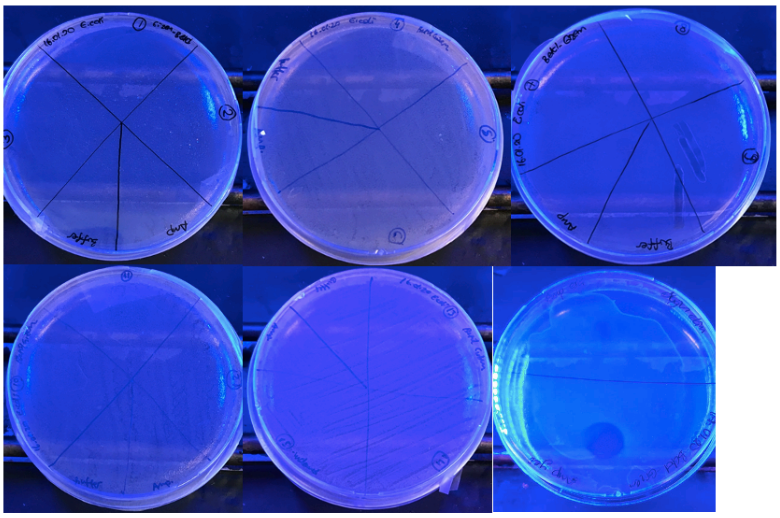


Figure 57. Antibiogram experiments after the formic acid cleavage trials of purified 1GFP-DP-TN1 protein. 1-14: cleavage conditions at Table 3. Uncleaved: purified 1GFP-DP-TN1 protein as a control. Buffer and ampicillin (1mg/ml;10ul) are also control groups.

4.5.2. Size Exclusion Studies of the Reaction Products

As described in Experimental section, the first chemical cleavage reaction (Table 3) was analyzed as a representative and the related peaks were observed with SEC-UPLC system at 214 nm and as a consequence of absence of tryptophan amino acid in used peptide mixture, any signal was not found at 280 nm (Figure 58). As seen, compared to the standard, 2 peaks were obtained between 16.700 and 112 Da standard signals. And to determine the sequence of these 2 peaks, LC-MS-MS analysis was applied and no signal was found to examine the related peptide peaks.

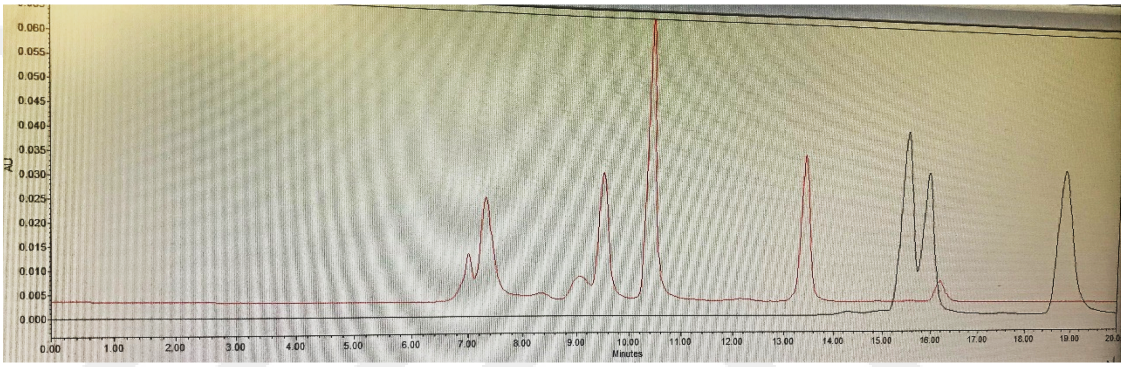
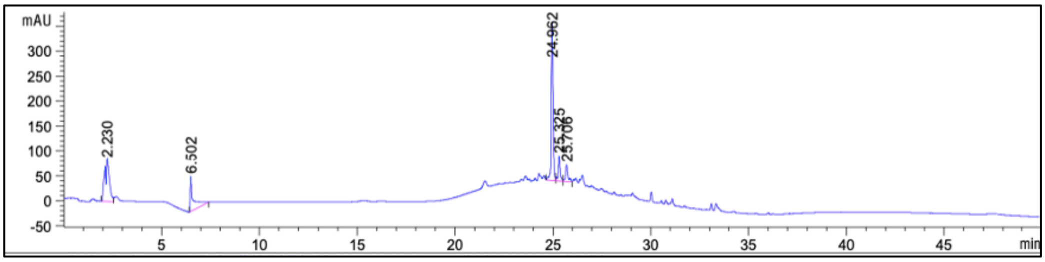


Figure 58. Analysis of the first trial of the formic acid cleavage by SEC UPLC system. The signals were obtained at 214 nm wavelength. The signals in red (666.000, 150.000, 66.400, 16.700 and 112 Dalton, respectively.) represent Waters BEH200 SEC Protein standard mix while in black ones were from sample.

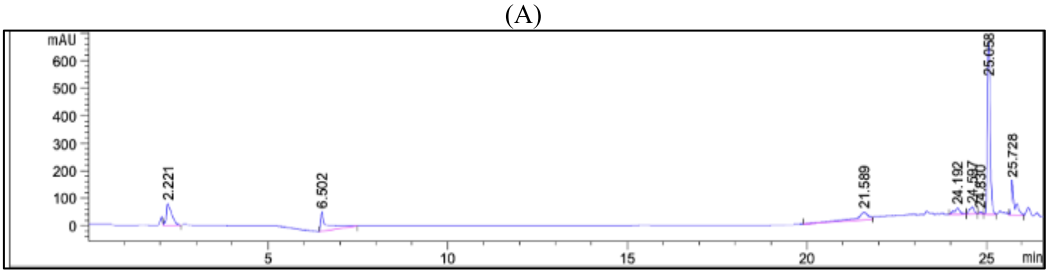
4.6. Preparation of Polymer-Peptide Conjugates

4.6.1. Synthesis and Characterization of Thiolated Peptides (C-TN1, TN1-C, C-TN3 and TN3-C)

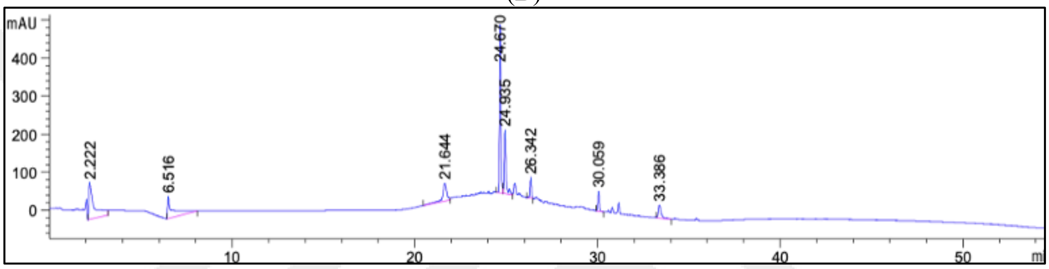
After the synthesis of the peptides, analytical RP-HPLC was used to determine polarity and the impurity level of the peptides. By using gradient method, peptides were observed at 80-81%mobile phase at 214nm with a small number of impurities (Figure 59) can be neglected for conjugation reactions because of their specificity.



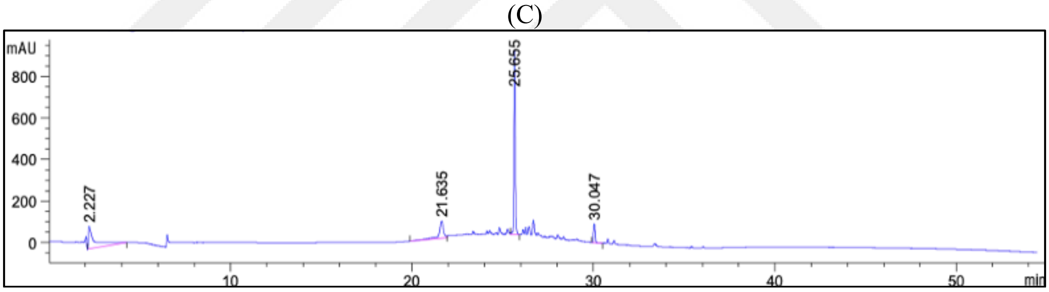
(A)



(B)



(C)



(D)

Figure 59. Analytical RP-HPLC analysis of the synthesized peptides. The signals were obtained at 214 nm wavelength. (A): TN1-NC, (B): TN3-CC, (C): TN1-CC, (D): TN3-NC.

4.6.2. Purification of Conjugates after PEGylation Reactions by Orthogonal Functionalization

4.6.2.1. Dialysis Method

After the conjugation reactions, described in Methods section, for the single peptide system, conjugate size was expected as almost 4300 Da, while for the multivalent system 6000 Da when TN1-NC peptide was used as a representative molecule. Reaction products were expected in high purity when 3.5 kDa dialysis membrane was used. All un-reacted or mis-reacted products can be removed this way, efficiently, simply and cheaply.

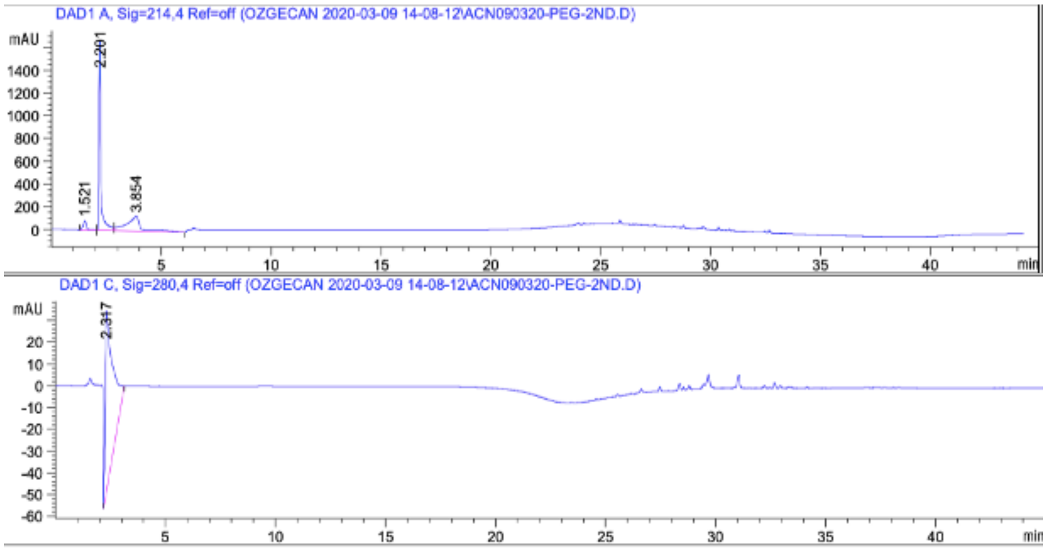
4.6.3. Characterization of Conjugates

The chemical characterization and the degree of purity of the polymer-peptide conjugates to be obtained was measured by using analytical RP-HPLC by using hydrophobic C18 column (Agilent, AdvanceBio Peptide Plus, 2.1 x 150 mm, 2.7-Micron with Column ID). Lyophilized samples were dissolved in 17% acetonitrile and the column was equilibrated by 1% mobile phase (acetonitrile and ultra-pure water, 0.25% TFA) at 0.2ml/min flow rate and observed the signals at 280 nm.

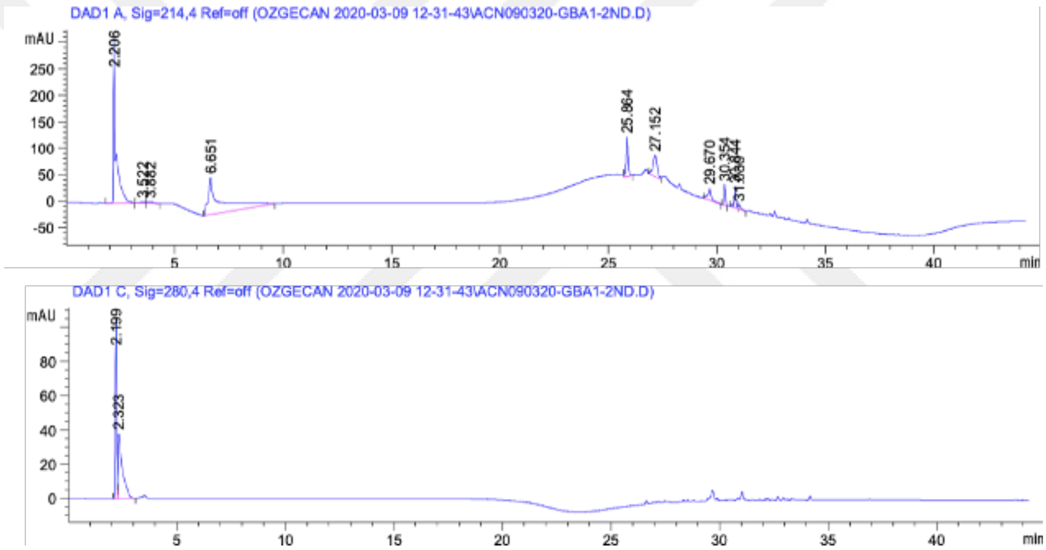
After the purification by dialysis, purified reaction products were examined by analytical RP-HPLC with C18 hydrophobic column. Each reaction was compared with only PEG sample which is extremely hydrophilic and observable at 214nm but not giving any observable signal at 280 nm.

4.6.3.1. Characterization of Single Peptide-PEG Conjugate

Purified single peptide-PEG conjugate was loaded to the column and apart from the observable signals including little impurities at 214nm, a meaningful single peak was observed also at 280nm in contrast to only PEG sample (Figure 60). As a result, the reaction can be interpreted as successful and further characterization techniques should be apply to confirm the structure.



(A)



(B)

Figure 60. Analytical RP-HPLC analysis of the single peptide-PEG conjugate reaction. (A): PEG, (B): TN1-NC single peptide-PEG conjugate signals at 214nm (upside) and 280nm (downside).

4.6.3.2. Characterization of Multivalent Peptide-PEG Conjugate

Obtained conjugate after the first approach, described in Experimental section, including HATU and DIPA for the amidation reaction, purified multivalent peptide-PEG conjugate was loaded to the column and apart from the observable signals including impurities at 214nm, in contrast to the single conjugate system, a meaningful single peak was not observed at 280nm (Figure 61). As a result, the reaction can be interpreted as failed as a result of insufficient reaction conditions for the amidation of acid-ended linker. The other approach, described in Experimental section, including

NHS-coupling of the linker molecule was designed and obtained purified conjugate will be examine in future studies.

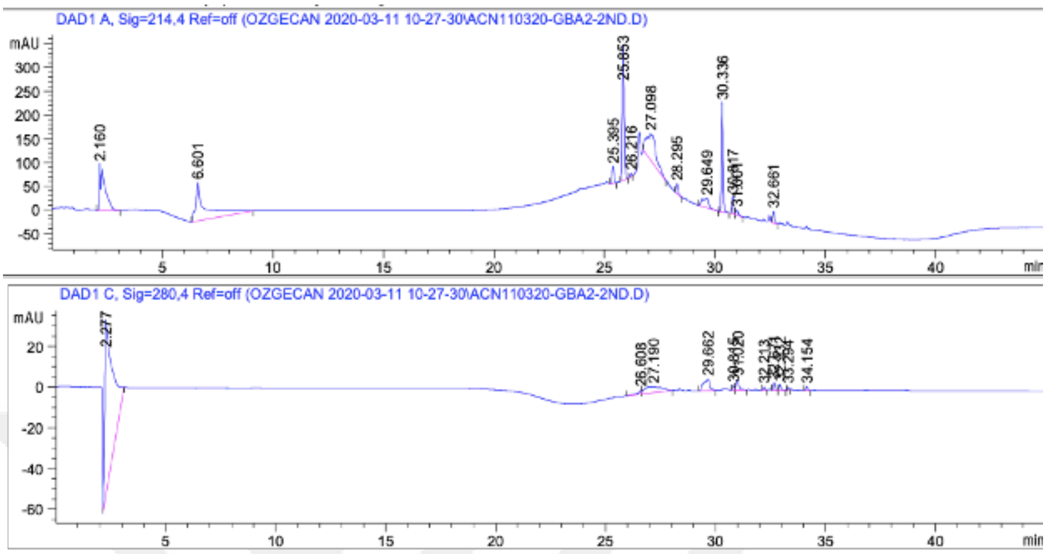


Figure 61. Analytical RP-HPLC analysis of the single peptide-PEG conjugate reaction. TN1-NC multivalent peptide-PEG conjugate signals at 214nm (upside) and 280nm (downside).

CONCLUSION

Production of peptide antibiotics at a large scale is a significant challenge for developing commercial products. Expression of recombinant AMPs is not always successful, either because of their activity to the host and/or proteolysis of the recombinant peptides during synthesis. In this work, the fusion complexes with TN1 and TN3 peptides was successfully expressed, purified by different approaches. To remove the fusion partners, systems with Sumo and GFP fusion partners were used as representative of cleavage studies by formic acid and TEV protease applied in different conditions. Cleaved products were examined according to their size (by using SDS-PAGE, SEC-UPLC) and their activities (by using antibiogram experiments). Due to their small size, SDS-PAGE and SEC-UPLC analyzes could not give valuable information about exact mass of the peptides. As a result of the activity experiments, any antibacterial activity was observed. Further analyzes, such as LC-MS-MS should require right after the cleavage experiments. In future, after the molecular weight determination, peptide concentration will be also calculated and optimization studies will be able to be designed to produce antimicrobial peptides with industrial scale. Bioreactor systems can be designed and optimized to produce AMPs in large quantities along with the optimized downstream reactions and purification methodologies. Developing molecular biology techniques and bioconjugation technology will shed light to designing novel antimicrobial peptides that are suitable to use them as efficient antibiotics.

Proteolytic degradation of such a small peptide is always a challenging situation. New bioconjugation strategies was also designed to obtain more stable peptides in physiological environments. Representative reactions were carried out and obtained conjugate candidates were investigated by RP-HPLC. Further characterization studies should be applied to determine exact structure of the conjugates. It is aimed to use FT-IR spectroscopy to analyze functional groups while using ^{1}H-NMR spectroscopy to determine chemical structure of the polymer-peptide conjugates. In future studies, with the help of these strategies, active terminus of the peptides will also be investigated by using activity experiments.

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APPENDIX

A.1. Preparation of Competent cells

- Inoculate DH5α (or BL21) cells to antibiotic-free 100ml LB-broth.
- Incubate at 37 ^{0}C on a shaker (A_{600} = 0.4-0,6).
- Cool the 50ml centrifuge tubes on ice and pour the bacteria culture and centrifugate at 4000 rpm, 4 ^{0}C for 10min
- Remove the supernatant.
- Gently mix the pellet with 20ml cold 0.1 M $CaCl_2$ (and sterilized by filtrating) and leave on ice for 20 min.
- Centrifugate at 4000 rpm, 4 ^{0}C for 10min.
- Remove the supernatant.
- Gently mix the pellet with 5ml cold 0.1 M $CaCl_2$ (including 15% glycerol).
- Pour into 0.2ml sterile tubes on ice, as 100 ul.
- Apply shock- freeze by using -80 ^{0}C cold pure ethanol and stock the tubes at 80 ^{0}C.

A.2. Agarose gel electrophoresis protocol

Preparing 1.5% agarose gel:

- Add 1.125 g agarose in a beaker containing 75 ml 1X TAE Buffer.
- Stir and heat until clean liquid is formed.
- Allow it to cool down.
- Pour the solution in a cassette and place the appropriate combs for sample injection.
- Allow it to cool down to room temperature.
- Remove the combs and place the gel in a gel box.
- Fill the gel box with 1X TAE solution.

A.3. 6X sample buffer for Polyacrylamide gel electrophoresis

Mix them gently:

- 375 mM Tris-HCl (pH 6.8)
- 9 w% SDS
- 50% Glycerol
- 0.02% Bromophenol blue
- 375 mM DTT

A.4. Semi-Dry Western Blotting

After preparing SDS gels, transfer buffer and running of the samples (as described in the Experimental section):

- Take out the gels out of the glass cost
- Activate the membranes (PVDF) with;

%99,9 Methanol → ddH_2O → 1X transfer buffer

- Put the gel in to the 1X transfer buffer, also.
- Make a sandwich for the transfer (by using semi-dry apparatus).

Using the white thick filter paper, all wet with 1X transfer buffer;

Filter paper → activated membrane → gel → filter paper

- Take out the bubbles between the gel and the membrane.
- Roll out the bubbles above the filter paper with a roller.
- Close and lock the sandwich.
- Put the sandwich to the “BioRad TransBlot” apparatus, set as mixed MW Run, auto setted as 1.3A, 25V, 7 min and run.
- After 7 min, take out the gel and the membrane.
- Stain the gel (if necessary).
- Re- activate the membrane

%99,9 Methanol → ddH_2O → 1X TBS or 1X PBS

- Place the membrane to the box with blocking solution (5% BSA in 1X TBS).
- Block for 2 hours at room temperature on the shaker.
- Prepare the antibody solution with 1^0 antibodies (5% BSA in 1X TBS- Tween20 0.1%).
- Re-collect or discard the blocking solution and put the 1^0 antibody solution.
- Incubate at room temperature for 1 hour on the shaker.
- Re-collect 1^0 antibody solution.
- Wash membranes 3x15min with TBS-T (1X TBS with 0.1%Tween20).
- Prepare the antibody solution with 2^0 antibodies (5% BSA in 1X TBS- Tween20 0.1%).
- Incubate at room temperature for 1 hour with 2^0 antibody solution on the shaker.
- Wash membranes 3x15min with TBS-T (1X TBS with 0.1%Tween20).

- After the last wash, add TBS and scan the membranes with ChemiDoc.
- Prepare substrate solution 1:1 (luminol / enhancer : peroxide solutions) at dark.
- Apply almost 1.5ml solution for full gel membranes at dark and seal them to a plastic bag and place the ChemiDoc. Pose the membranes 1-6 seconds until the band become visual.
- Wash the membranes with TBS and seal them with plastic bag and store at 4 ^{0}C.

A.5. Protein extraction from Inclusion Bodies, Denaturation and Refolding

Protein extraction

- Resuspend the cell pellet in 25 ml BPER.
- Centrifuge at 27000g for 15 minutes.
- Pour off supernatant and resuspend the pellet in 25 ml BPER.
- Add 50 mg/ml lysozyme (125 ul) lysozyme and incubate at room temperature for 5 minutes.
- Add 25 ml 1:10 diluted BPER and centrifuge at 27000g for 15 minutes (Repeat x2).
- Pour off supernatant and resuspend the pellet in 60 ml 1:20 diluted BPER.
- Centrifuge at 27000g for 15 minutes.
- Repeat resuspension and centrifugation steps two more times.
- Resuspend the final pellet in 25 ml 6M Guanidine HCl.
- Homogenize by vortexing, 20 min sonicator and using homogenizer 2 or 3 times (~%80 power; 5 sec repeats).
- Dilute the solution 20-fold using refolding buffer ((50 mM K_2PO_4, 100 mM NaCl, pH 10.3)
- Chill solution at 4C for 4 days on the magnetic stirrer.

Purification and desalting:

- Dialyze the solution against acetic acid (pH 3.3 or 8.5) for 7 days with subsequent repetitions.
- After final dialysis, centrifuge at 27000g for 15 minutes.
- Retain supernatant for lyophilizing.

A.6. Procedure for Spin Purification of GST-Tagged Proteins by Pierce® Glutathione Spin Columns

As a general guideline, 50-200mg of total protein lysate can be loaded onto each milliliter of resin.

Purification Buffers:

- Equilibration/Wash Buffer: 50mM Tris, 150mM NaCl, pH 8.0.
- Elution Buffer: 50mM Tris, 150mM NaCl, pH 8.0 containing 10mM reduced glutathione.

- Equilibrate the column to working temperature. Perform purifications at room temperature.

- Prepare sample by mixing the protein extract (the protein samples were dissolved in 30mM ammonium bicarbonate, pH 12) with Equilibration/Wash Buffer so the total volume equals at least two resin-bed volumes.

- Remove the bottom tab from the Pierce Glutathione Spin Column by gently twisting. Place column into a 1,5 mL centrifuge tube.

- Centrifuge column at 700 × g for 2 minutes to remove storage buffer.

- Equilibrate column with two resin-bed volumes of Equilibration/Wash Buffer. Allow buffer to enter the resin bed.

- Centrifuge column at 700 × g for 2 minutes to remove buffer.

- Add the prepared protein extract to the column and allow it to enter the resin bed. For maximal binding, the sample can be incubated for 60 minutes at room temperature on an end-over-end rocking platform.

- Centrifuge column at 700 × g for 2 minutes and collect the flow-through in a centrifuge tube. If desired, save flow through fraction for downstream analysis.

- Wash resin with two resin-bed volumes of Equilibration/Wash Buffer. Centrifuge at 700 × g for 2 minutes and collect fraction in a centrifuge tube. Repeat this step two more times collecting each fraction in a separate centrifuge tube.

Monitor the absorbance of the washes at 280nm and perform additional washes if necessary, until the absorbance approaches baseline.

- Elute GST-tagged protein from the resin by adding one resin-bed volume of Elution Buffer. Centrifuge at 700 × g for 2 minutes. Repeat this step two more times, collecting each fraction in a separate tube.
- Analyze the collected fractions by SDS-PAGE.

A.7. Total Protein Extraction by the Cell Lysate

- Add 3mL B-PPER to every frozen cell pellet from 125mL cell culture.
- Add lysozyme (0.1mg/ml), DNase I (1U), Tween-20 (0.1%), protease inhibitor (1X) to B-PER solution. And dissolve the pellet completely.
- Incubate on ice for 30 min.
- Homogenize by using homogenizer 5 times (~%40 power; 10 sec repeats).
- With reduced viscosity and without any aggregation, get samples convenient to use for magnetic beads.

A.8. Insoluble Protein Extraction by the Cell Lysate

- Add 3mL B-PPER to every frozen cell pellet from 125mL cell culture.
- Incubate at room temperature for 30 min.
- Centrifugate at 4 C, 20000 × g for 15 minutes.
- Separate soluble and insoluble fractions.
- Add 3mL B-PPER and lysozyme (0.1mg/ml), DNase I (1U), Tween-20 (0.1%), protease inhibitor (1X) to insoluble fraction pellet. And dissolve the pellet completely.
- Incubate on ice for 30 min.
- Homogenize by using homogenizer 5 times (~%40 power; 10 sec repeats).
- With reduced viscosity and without any aggregation, get samples convenient to use for magnetic beads.

A.9. Procedure for Purification of His-Tagged Proteins by PureProteome Nickel Magnetic Bead System (Merck)

- Under denaturing conditions, prepare purification buffers;
    - Equilibration Buffer: 8M urea, 100mM sodium phosphate, 10mM Tris HCl, pH 8.0
    - Wash Buffer: 50mM sodium phosphate, 300mM NaCl, 10mM imidazole, pH 8.0
    - Elution Buffer: 50mM sodium phosphate, 300mM NaCl, 300mM imidazole, pH 8.0
- Use 1 mL of the prepared cell lysate.
- Homogenize magnetic beads by vortexing
- Take 200 µl of the magnetic beads (convenient to use with 200-1100 µg His-Tagged proteins) into 1.5 mL microcentrifuge tube. Place to the magnetic stand (Merck) and collect the beads. Remove storage buffer with a pipette.
- Resuspend the magnetic beads in 500 µl of Equilibration buffer and incubate with gentle mixing for one minute at room temperature.
- Place the tube back into the magnetic stand and remove the buffer.
- Add 1 mL of cell lysate to the magnetic beads and incubate with gentle mixing for 30 min at room temperature.
- Place the tube back into the magnetic stand and remove the lysate and repeat lysate incubation step 3-4 times to enhance additional binding of the proteins to the beads until reaching up to 1100 µg His-Tagged proteins.
- Wash the magnetic beads by adding 500 µl of Equilibration buffer and incubate with gentle mixing for one minute at room temperature.
- Place the tube back into the magnetic stand and remove the buffer and repeat the washing step two more times.
- Elute the bound protein by adding 100 µl of Elution buffer. Incubate with gentle mixing for two min at room temperature.
- Place the tube back into the magnetic stand and transfer the eluted fraction into a clean collection tube.
- Repeat the elution step two more times.

A.10. Calibration Curves for Protein Concentration Determination

Protein concentrations were determined via BCA by comparing the calibration curves related to standards of the proteins which were solubilized in the same solvents as explained in the Experimental section. The calibration curves are presented in the following figures.

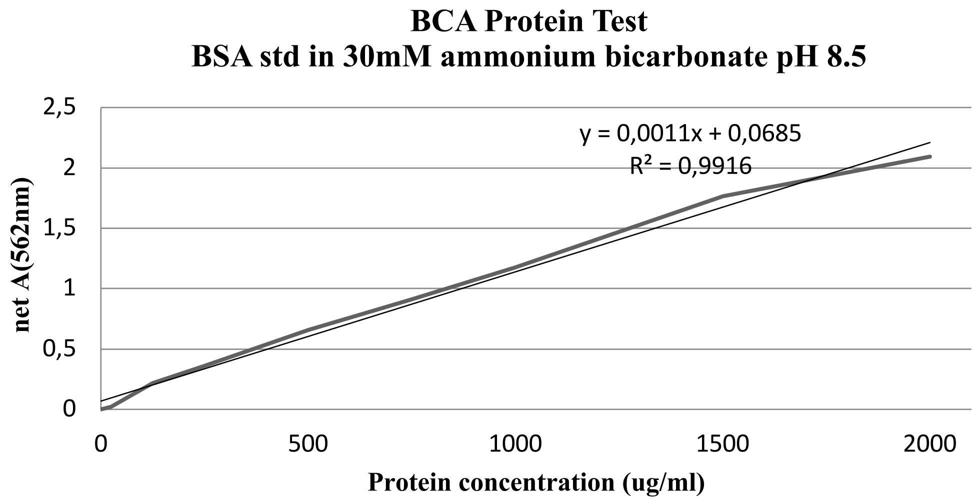

Figure A.1. Calibration curve for Pierce™ BCA Protein test for BSA standard proteins dissolved in 30mM ammonium bicarbonate, 0.16% acetic acid, pH 8.5. Lines represent the linear fit to the UV $_{562}$ absorbance versus concentration data with a given formula.

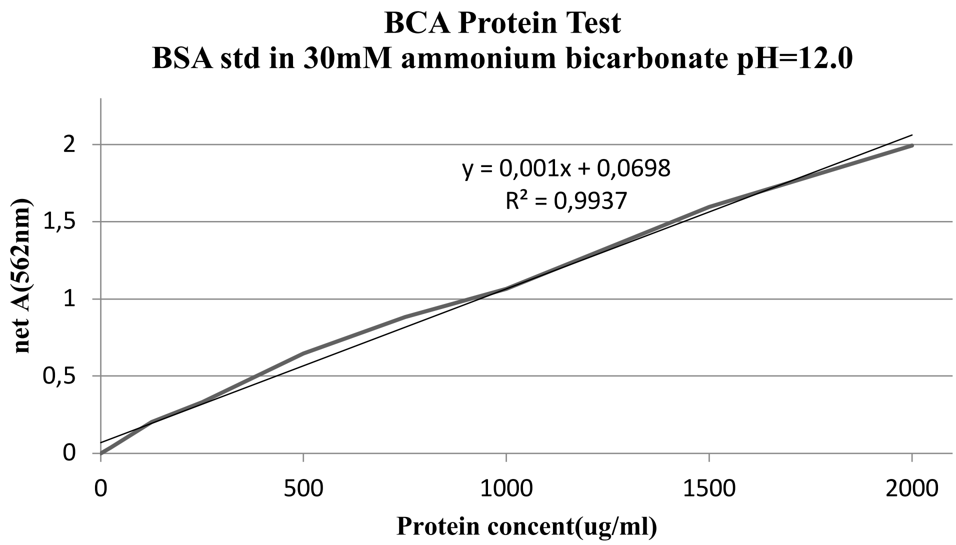

Figure A.2. Calibration curve for Pierce™ BCA Protein test for BSA standard proteins dissolved in 30mM ammonium bicarbonate, pH 12. Lines represent the linear fit to the UV $_{562}$ absorbance versus concentration data with a given formula.

A.11. DNA Sequencing Results

Related peptide sequences with their stop codons were signed with yellow.

His6-GST-TEV SITE-PEPTIDE SEQUENCE

1G_1.2

T7_fwd (TAATACGACTCACTATAGGG)
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CTTCTCACCATCACCATCACCATGGTTCTTCTATGTCCCCTATACTAGGTTATTGGAAAATT
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AAGAAAA

1G_1.2

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1G_2.2

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1G_2.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
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1G_3.2

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1G_4.2

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1G_4.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)

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His6-TEV SITE-PEPTIDE SEQUENCE

1B_1.2

T7_fwd (TAATACGACTCACTATAGGG)
GGGTGAGTTCGTTACCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGG
TTCTTCTCACCATCACCATCACCATGAAAACCTGTACTTCCAATCCAATATGCGCCTGCTGC
GCCTGCTGCTGCTGCGCCTGCTGCGCTAATAAATTGGAAGTGGATAACGGATCCGAATTCGA
GCGCCGTCGACAAGA

1B_1.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
ATGCATAAGTTCATTTATTAGCGCAGCAGGCGCAGCAGCAGCAGGCGCAGCAGGCGCATATT
GGATTGGAAGTACAGGTTTTCATGGTGATGGTGATGGTGAGAAGAACCCATGGTATATCTCC
TTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTAT
AGTGAGTCGTATTAA

1B_2.2

T7_fwd (TAATACGACTCACTATAGGG)
GCGTGCGCTCTTTACCCTCTAGAATAATTTTGTTTACTTTAAGAAGGAGATATACCATGGGT
TCTTCTCACCATCACCATCACCATGAAAACCTGTACTTCCAATCCAATGACCCGCGGCTGCT
CCGGCTGCTCCTGCTCCGGCTGCTCCGGTAATAAATTGGAAGTGGATAACGGATCCGAATTC
GAGCGCCGTCGACAAGA

1B_2.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
GGCGGACAAATCTTCATTTATTACCGGAGCAGCCGGAGCAGGAGCAGCCGGAGCAGCCGCGG
GTCATTGGATTGGAAGTACAGGTTTTCATGGTGATGGTGATGGTGAGAAGAACCCATGGTAT
ATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTC
CCCTATAGTGAGTCGTATTAA

1B_3.2

T7_fwd (TAATACGACTCACTATAGGG)
GGGTGACGGTCTTACCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGG
TTCTTCTCACCATCACCATCACCATGAAAACCTGTACTTCCAATCCAATATGCGGCTGCTCC
GGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGGATCCGA
ATTCGAGCGCCGTCGACAAGA

1B_3.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
GAGGTTAAAGTTCATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCAGCCGGAGCAGCC
GCATATTGGATTGGAAGTACAGGTTTTCATGGTGATGGTGATGGTGAGAAGAACCCATGGTA

TATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATT
CCCCTATAGTGAGTCGTATTAA

1B_4.1

T7_fwd (TAATACGACTCACTATAGGG)
CCGTTCCGCTCAGTTACCCCTCTAGAATATTTTGTTTAACTTTAAGAAGGAGATATACCATG
GGTTCTTCTCACCATCACCATCACCATGAAAACCTGTACTTCCAATCCAATGACCCGCGGCT
GCTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGGA
TCCGAATTCGAGCGCCGTCGACAAGA

IB_4.1

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
GAGGAATCTTCATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCAGCCGGAGCAGCCGC
GGGTCATTGGATTGGAAGTACAGGTTTTCATGGTGATGGTGATGGTGAGAAGAACCCATGGT
ATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAAT
TCCCCTATAGTGAGTCGTATTAA

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1S_1.2

T7_fwd (TAATACGACTCACTATAGGG)
GCGTGACGAACGTTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGG
TTCTTCTCACCATCACCATCACCATGGTTCTTCTATGGCTAGCATGTCGGACTCAGAAGTCA
ATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAG
GTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCT
GATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACG
GTATTAGAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTATT
GAGGCTCACAGAGAACAGATTGGTGGGATCGAGGAAAACCTGTACTTCCAATCCAATATGCG
CCTGCTGCGCCTGCTGCTGCTGCGCCTGCTGCGCTAATAAATTGGAAGTGGATAACGGATCC
GAATTCGAGCGCCGTCCGACAAGCA

1S_1.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
CTCCATTCCTTCCATTTATTAGCGCAGCAGGCGCAGCAGCAGCAGGCGCAGCAGGCGCATAT
TGGATTGGAAGTACAGGTTTTCCTCGATCCCACCAATCTGTTCTCTGTGAGCCTCAATAATA
TCGTTATCCTCCATGTCCAAATCTTCAGGGGTCTGATCAGCTTGAATTCTAATACCGTCGTA
CAAGAATCTTAAGGAGTCCATTTCCTTACCCTGTCTTTTAGCGAACGCTTCCATCAGCCTTC
TTAAAGGAGTGGTCTTTTTGATCTTGAAGAAGATCTCTGAAGATCCATCGGACACCTTTAAA
TTGATGTGAGTCTCAGGCTTGACTTCTGGCTTGACCTCTGGCTTAGCTTCTTGATTGACTTC
TGAGTCCGACATGCTAGCCATAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCATGG
TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAA
TTCCCCTATAGTGGTTCCGTATTAA

1S_2.1

T7_fwd (TAATACGACTCACTATAGGG)
GCGTGACGTCGTTCCGCTAGATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTTC
TTCTCACCATCACCATCACCATGGTTCTTCTATGGCTAGCATGTCGGACTCAGAAGTCAATC

AAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTG
TCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGAT
GGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTA
TTAGAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTATTGAG
GCTCACAGAGAACAGATTGGTGGGATCGAGGAAAACCTGTACTTCCAATCCAATGACCCGCG
GCTGCTCCGGCTGCTCCTGCTCCGGCTGCTCCGGTAAAATTGGAAGTGGATAACGGATCCGA
ATTCGAGCGCCGTCCGACAAGCA

1S_2.1

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
CAGGGGTTTACTTCATTTTACCGGAGCAGCCGGAGCAGGAGCAGCCGGAGCAGCCGCGGGTC
ATTGGATTGGAAGTACAGGTTTTCCTCGATCCCACCAATCTGTTCTCTGTGAGCCTCAATAA
TATCGTTATCCTCCATGTCCAAATCTTCAGGGGTCTGATCAGCTTGAATTCTAATACCGTCG
TACAAGAATCTTAAGGAGTCCATTTCCTTACCCTGTCTTTTAGCGAACGCTTCCATCAGCCT
TCTTAAAGGAGTGGTCTTTTTGATCTTGAAGAAGATCTCTGAAGATCCATCGGACACCTTTA
AATTGATGTGAGTCTCAGGCTTGACTTCTGGCTTGACCTCTGGCTTAGCTTCTTGATTGACT
TCTGAGTCCGACATGCTAGCCATAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCAT
GGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCAC
AATTCCCCTATAGTGAGTCGTATTAA

1S_3.2

T7_fwd (TAATACGACTCACTATAGGG)
GCTGACGTCGTCCGTCTAGATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTTCT
TCTCACCATCACCATCACCATGGTTCTTCTATGGCTAGCATGTCGGACTCAGAAGTCAATCA
AGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTGT
CCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATG
GAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTAT
TAGAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTATTGAGG
CTCACAGAGAACAGATTGGTGGGATCGAGGAAAACCTGTACTTCCAATCCAATATGCGGCTG
CTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGGAT
CCGAATTCGAGCGCCTCCCAACAAGCA

1S_3.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
CAGGATTACGTCATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCAGCCGGAGCAGCCG
CATATTGGATTGGAAGTACAGGTTTTCCTCGATCCCACCAATCTGTTCTCTGTGAGCCTCAA
TAATATCGTTATCCTCCATGTCCAAATCTTCAGGGGTCTGATCAGCTTGAATTCTAATACCG
TCGTACAAGAATCTTAAGGAGTCCATTTCCTTACCCTGTCTTTTAGCGAACGCTTCCATCAG
CCTTCTTAAAGGAGTGGTCTTTTTGATCTTGAAGAAGATCTCTGAAGATCCATCGGACACCT
TTAAATTGATGTGAGTCTCAGGCTTGACTTCTGGCTTGACCTCTGGCTTAGCTTCTTGATTG
ACTTCTGAGTCCGACATGCTAGCCATAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACC
CATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCT
CACAATTCCCCTATAGTGGTTCGTATTAA

1S_ 4.2

T7_fwd (TAATACGACTCACTATAGGG)
GCTTGAGTCGTTCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTTC
TTCTCACCATCACCATCACCATGGTTCTTCTATGGCTAGCATGTCGGACTCAGAAGTCAATC

AAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTG
TCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGAT
GGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTA
TTAGAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATATTATTGAG
GCTCACAGAGAACAGATTGGTGGGATCGAGGAAAACCTGTACTTCCAATCCAATGACCCGCG
GCTGCTCCGGCTGCTCCGGCTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAAC
GGATCCGAATTCGAGCGCCGTCCGACAAGA

1S_4.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
CGCATAGATCATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCAGCCGGAGCAGCCGCG
GGTCATTGGATTGGAAGTACAGGTTTTCCTCGATCCCACCAATCTGTTCTCTGTGAGCCTCA
ATAATATCGTTATCCTCCATGTCCAAATCTTCAGGGGTCTGATCAGCTTGAATTCTAATACC
GTCGTACAAGAATCTTAAGGAGTCCATTTCCTTACCCTGTCTTTTAGCGAACGCTTCCATCA
GCCTTCTTAAAGGAGTGGTCTTTTTGATCTTGAAGAAGATCTCTGAAGATCCATCGGACACC
TTTAAATTGATGTGAGTCTCAGGCTTGACTTCTGGCTTGACCTCTGGCTTAGCTTCTTGATT
GACTTCTGAGTCCGACATGCTAGCCATAGAAGAACCATGGTGATGGTGATGGTGAGAAGAAC
CCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGC
TCACAATTCCCCTATAGTGAGTCGTATTAA

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1GFP_1.2

T7_fwd (TAATACGACTCACTATAGGG)
GGTTAAGAAATTCCCTCTAGAATATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTTC
TTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAGGAGCTGTTCACCGGGG
TGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGCGGC
GAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAA
GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTC
CAGGAGCGCACCATCTCCTTCAAGGACGACGGCACCTACAAGACCCGCGCCGAGGTGAAGTT
CGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAACGTCTATATCACGGCCGAC
AAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTCGAGGACGGCAGCGT
GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG
ACAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCAC
ATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAA
GGGGATCGAGGAAAACCTGTACTTCCAATCCAATATGCGCCTGCTGCGCCTGCTGCTGCTGC
GCCTGCTGCGCTAATAAATTGGAAGTGGATAACGGATCCGAATTCGAGCGCGTTCCGACAAG
AAAAA

1GFP_1.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
AGCCCTTCCTTCAATTTATTAGCGCAGCAGGCGCAGCAGCAGCAGGCGCAGCAGGCGCATAT
TGGATTGGAAGTACAGGTTTTCCTCGATCCCCTTGTACAGCTCGTCCATGCCGAGAGTGATC
CCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCT
CAGCTTGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGG
GGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGACGTTGTGGCGGATC
TTGAAGTTCGCCTTGATGCCGTTCTTCTGCTTGTCGGCCGTGATATAGACGTTGTGGCTGTT

GAAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCA
GCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGTCTTGTAGGTG
CCGTCGTCCTTGAAGGAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAA
GAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGG
TGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTC
AGCTTGCCGTTGGTGGCATCGCCCTCGCCCTCGCCGCGCACGCTGAACTTGTGGCCGTTTAC
GTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCA
CAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCATGGTATATCTCCTTCTTAAAGTT
AAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAAGTCGT
ATTAAA

1GFP_2.1

T7_fwd (TAATACGACTCACTATAGGG)
GGGTGAGAAATTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTT
CTTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGCGG
CGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC
CGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGT
CCAGGAGCGCACCATCTCCTTCAAGGACGACGGCACCTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGC
AACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAACGTCTATATCACGGCCGA
CAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC
GACAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCA
CATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA
AGGGGATCGAGGAAAACCTGTACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCTG
CTCCGGCTGCTCCGGTAATAAATTGGAAGTGGATAACGGATCCGAATTCGAGCGCGCCCCAA
ACAACAAAAA

1GFP_2.1

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
TGGGATATACATCATTTATTACCGGAGCAGCCGGAGCAGGAGCAGCCGGAGCAGCCGCGGGT
CATTGGATTGGAAGTACAGGTTTTCCTCGATCCCCTTGTACAGCTCGTCCATGCCGAGAGTG
ATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTT
GCTCAGCTTGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGA
TGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGACGTTGTGGCGG
ATCTTGAAGTTCGCCTTGATGCCGTTCTTCTGCTTGTCGGCCGTGATATAGACGTTGTGGCT
GTTGAAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCT
TCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGTCTTGTAG
GTGCCGTCGTCCTTGAAGGAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTT
GAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCA
GGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGG
GTCAGCTTGCCGTTGGTGGCATCGCCCTCGCCCTCGCCGCGCACGCTGAACTTGTGGCCGTT
TACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGC
TCACAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCATGGTATATCTCCTTCTTAAA
GTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGGT
CGTATTAAA

1GFP_3.2

T7_fwd (TAATACGACTCACTATAGGG)

GGTGACGTCGTTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTT
CTTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGCGG
CGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC
CGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGT
CCAGGAGCGCACCATCTCCTTCAAGGACGACGGCACCTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGC
AACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAACGTCTATATCACGGCCGA
CAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC
GACAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCA
CATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA
AGGGGATCGAGGAAAACCTGTACTTCCAATCCAATATGCGGCTGCTCCGGCTGCTCCGGCTG
CTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGGATCCGAATTCGAGCGCGCCT
CTCAAACAAGA

1GFP_3.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA) *(In the reverse reading, the peptide sequence was not seen)*

ACAATATTCATTCAGTGCTCCATTTATTACAGGAGCAGCCGGATCAGCCGGAGCAGCCGCAT
ATTGGATTGGAAGTACAGGTTTTCCTCGATCCCCTTGTACAGCTCGTCCATGCCGAGAGTGA
TCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTG
CTCAGCTTGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGAT
GGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGACGTTGTGGCGGA
TCTTGAAGTTCGCCTTGATGCCGTTCTTCTGCTTGTCGGCCGTGATATAGACGTTGTGGCTG
TTGAAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTT
CAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGG
TGCCGTCGTCCTTGAAGGAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTG
AAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAG
GGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGG
TCAGCTTGCCGTTGGTGGCATCGCCCTCGCCCTCGCCGCGCACGCTGAACTTGTGGCCGTTT
ACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCT
CACAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCATGGTATATCTCCTTCTTAAAG
TTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGGTC
GTATTAAA

1GFP_4.2

T7_fwd (TAATACGACTCACTATAGGG)

GGTTAAGAAATTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGTT
CTTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGCGCGG
CGAGGGCGAGGGCGATGCCACCAACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC
CGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGT
CCAGGAGCGCACCATCTCCTTCAAGGACGACGGCACCTACAAGACCCGCGCCGAGGTGAAGT
TCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGC
AACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAACGTCTATATCACGGCCGA
CAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACGTCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC

GACAACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGATCA
CATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA
AGGGGATCGAGGAAAACCTGTACTTCCAATCCAATGACCCGCGGCTGCTCCGGCTGCTCCGG
CTGCTCCTGTAATAAATGGAGCACTGAATTGGAAGTGGATAACGGATCCGAATTCGAGCGCG
TTCCGACAAGCAA

1GFP_4.2

PetLIC_rev_119 (GCTTGTCGACGGCGCTCGAA)
AGCCTTCCTTCAATTCAGTGCTCCATTTATTACAGGAGCAGCCGGAGCAGCCGGAGCAGCCG
CGGGTCATTGGATTGGAAGTACAGGTTTTCCTCGATCCCCTTGTACAGCTCGTCCATGCCGA
GAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGG
TCTTTGCTCAGCTTGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTC
GCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGACGTTGT
GGCGGATCTTGAAGTTCGCCTTGATGCCGTTCTTCTGCTTGTCGGCCGTGATATAGACGTTG
TGGCTGTTGAAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGAT
GCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCT
TGTAGGTGCCGTCGTCCTTGAAGGAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCG
GACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTA
GGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACT
TCAGGGTCAGCTTGCCGTTGGTGGCATCGCCCTCGCCCTCGCCGCGCACGCTGAACTTGTGG
CCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCC
CTTGCTCACAGAAGAACCATGGTGATGGTGATGGTGAGAAGAACCCATGGTATATCTCCTTC
TTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGT
GAGGTCGTATTAAA

CURRICULUM VITAE

Personal Information

Name	Gizem	**Surname**	Boskan
Date of Birth	27.05.1994		
Nationality	T.C.	**Phone Number**	5543781620
E-mail	gzmboskan@gmail.com		

Education

	School / Program	Graduation Date
MSc	Acibadem M.A.A. University / Medical Engineering	Ongoing
BSc	Ege University / Bioengineering	2017
High School	Izmir Ataturk High School	2012

INTERNSHIP

- Otto-von-Guericke-University Magdeburg, Institute of Biology, Department of Genetics & Molecular Neurobiology
  ERASMUS Mobility Grant for Traineeships
  Magdeburg, Germany
  July 2017 - September 2017
- Ege University, Bioengineering Department, Cancer Biology and Genetics Lab.
  Izmir, Turkey
  October 2016 - June 2017
- Dokuz Eylul University, Oncology Institute, Basic Oncology
  Izmir, Turkey

INTERESTS & SKILLS

- Keywords for lab skills: Usage of all basic lab equipments, buffer preparation, gene construct and primer design, Ligation Independent Cloning (LIC), PCR, agarose gel electrophoresis, DNA cloning, bacterial transformation and expression, protein extraction and isolation, SDS-PAGE, Western Blotting, chemical solid state peptide synthesis, HPLC, TLC, different extraction-purification and characterization techniques, chemical reaction set up, PEGylation, dynamic light scattering (DLS), MIC, bacterial culture, mammalian cell culture, drug-dose experiments ect.

- Languages: Turkish (native), English (professional working proficiency, *YDS: 87.50*)

- Analytical Exams: ALES: 85, GRE: 160 (quantitative reasoning)

- Membership: Derinuzay Science & Technology Labs Association, Health and Education Foundation for Children with Leukemia (LOSEV), Ege University Science and Technology Community (EBİLTET), Crossing Paths- Internship Abroad- Mentoring