İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CONSTRUCTION AND EXPRESSION OF A BI-FUNCTIONAL PEPTIDE BY USING GENETIC ENGINEERING METHODS FOR BIO-NANOTECHNOLOGIES

M.Sc. Thesis by
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Programme: Molecular Biology – Genetics and Biotechnology

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BİYO-NANOTEKNOLOJİ İÇİN ÇİFT İŞLEVLİ BİR PEPTİDİN GENETİK MÜHENDİSLİĞİ YÖNTEMLERİ İLE OLUŞTURULMASI VE ÜRETİLMESİ

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TABLE OF CONTENTS

ABBREVIATIONS

A, Ala : Alanine
APS : Amonium peroxodo disulfate
bp : Base pair
dH$_2$O : Distilled water
DNA : Deoxyribonucleic acid
dsDNA : Double stranded DNA
G, Gly : Glycine
GBP : Gold Binding Peptide
H, His : Histidine
I, Ile : Isoleucine
IPTG : Isopropyl-β-D- thiogalactopyranoside
K, Lys : Lysine
kb : Kilobase
L, Leu : Leucine
LB : Luria Bertani
M, Met : Methionine
Na-Ac : Sodium acetate
Ni-NTA : Nickel-Nitrilotriacetic acid
OD : Optical density
P, Pro : Proline
PAGE : Polyacrylamide Gel Electrophoresis
PCR : Polymerase chain reaction
Q, Gln : Glutamine
QBP : Quartz Binding Peptide
S, Ser : Serine
SDS : Sodium dodecyl sulfate
ssDNA : Single stranded DNA
T, Thr : Threonine
TBE : Tris-borat –EDTA
TEMED : N,N,N',N'-Tetramethylethylenediamine
Tris base : Hydroxymethyl aminomethane
W, Trp : Tryptophane
X-Gal : 5-Bromo-4-chloro-3-indolyl-D-galactoside
Y, Tyr : Tyrosine

LIST OF TABLES

LIST OF FIGURES

Page no

BİYO-NANOTEKNOLOJİ İÇİN ÇİFT İŞLEVLİ BİR PEPTİDİN GENETİK MÜHENDİSLİĞİ YÖNTEMLERİ İLE OLUŞTURULMASI VE ÜRETİLMESİ

ÖZET

Doğada bulunan organizmaların çoğunluğu proteinlerin inorganik maddelerle etkileşimlerinden oluşan inorganik-organik melez yapılara sahiptir. Bu melez sistemlerin özellikleri arasında destek dokuları ve koruyucu katmanlar oluşturmak, iyon ve yük transferinde görev almak, çeşitli optik, manyetik ve mekanik özellikler geliştirmek gibi fevkalâde işlevler vardır. Bu özellikler ve işlevler, biyoteknoloji ve biyo-nanoteknoloji uygulamaları için genetik mühendisliği kullanılarak değiştirilmiş ve geliştirilmiş yapıların tasarlanması için çok büyük esin kaynağı olmaktadır.

İşlevsel inorganik malzemelerin kontrollü olarak oluşmalarını ve düzenlenmelerini sağlamak için, inorganik yapılara bağlanabilen peptitlerin biyo-bağlayıcı ve moleküler yapı taşları olarak kullanılma potansiyelleri vardır. Faj gösterim kütüphanesi ya da hücre gösterim kütüphanesi gibi tümleşik biyolojiye dayalı moleküler kütüphaneler inorganik yüzeylere bağlanabilen proteinlerin elde edilmesinde en önemli yöntemlerden birisi haline gelmektedir. Seçilen peptidler genetik mühendisliği yöntemleri ile daha da geliştirilip modifiye edilebilirler ve kontrol edilebilir işlevselliklerle moleküler araçlar olarak kullanılabilirler. Gösterim kütüphaneleri ile seçilen peptidler genellikle 7-14 amino asit uzunluğundaki kısa peptidlerdir. Bağlanma ilgisini artırmak için peptid dizilerinin ardışık tekrarları genetik mühendisliği yöntemleri ile oluşturulabilir. Kısa peptidlerin tekrarlı yapıları daha iyi bir ilgi göstererek kontrol edilebilir oluşum işlemini sağlayabilirler. Artırılan yüzey tanıma bölgeleri peptidin bağlanma gücünde ve/veya özgüllüğünde bir artışa imkan sağlayabilir.

Bu çalışmada, çift işlevli peptidin oluşturulması için hem quartza bağlanan peptidin (QBP1-PPPWLPYMPPWS) hem de altına bağlanan peptidin (GBP1-MHGKTQATSGTIQS) üçlü ardışık tekrarı kullanılmıştır. İki peptid dizisi birbirine 10 amino asitten oluşan (GGGGSGGGGT) esnek bir bağlayıcı ile bağlanmıştır. Peptidin üretimi için, *E. coli'* ye dayalı bir ekspresyon sistemi olan TAGZyme pQE-2 vektör sistemi kullanılmıştır. Vektör oluşturulmuş ve çift işlevli peptid Luria Bertani besiyerinde eksprese edilmiştir. Üretilen proteinler İmmobilize Metal Afinite Kromatografi yöntemi ile saflaştırılmıştır. İki peptidin birbirine bağlanması sonucunda peptidlerin işlevsellikleri korunmuştur. Genetik mühendisliği ile geliştirilmiş *in vivo* yapının, inorganik malzemelerin kullanıldığı biyonanoteknoloji uygulamaları için yapısal olarak işlevsel ve çok yönlü bir moleküler araç olması beklenmektedir. Üretilen peptid, altın nanotaneciklerini silis üzerine immobilize ederek biyosensör uygulamalarında ve başka nanoteknoloji uygulamalarında kullanılabilir.

CONSTRUCTION AND EXPRESSION OF A BI-FUNCTIONAL PEPTIDE BY USING GENETIC ENGINEERING METHODS FOR BIO-NANOTECHNOLOGIES

SUMMARY

Many organisms found in the nature have inorganic-organic hybrid structures due to the interactions of proteins with inorganic materials. These hybrid systems have excellent functions like providing support tissues and protective coatings, taking place in ion transfer, developing various optical, magnetic and mechanical properties. These properties and functions are inspiring for designing genetically engineered systems for biotechnology and bio-nanotechnology applications.

For the controlled formation and assembly of the functional inorganic materials, inorganic-binding peptides have an enormous potential to be used as bio-linkers and molecular building block. Combinatorial biology-based molecular libraries, like phage display or cell surface display libraries, are increasingly becoming one of the major methods to obtain inorganic specific peptides. Selected peptides can be further engineered, modified through genetic engineering methods and synthesized to utilize as molecular tools with controlled functionalities. Peptides selected by the display libraries are generally short ones, between 7-14 amino acids. To increase binding affinity, tandem repeats of the sequence can be generated by genetic engineering. Repeated structures of short peptides may show a better affinity and provide a controlled assembly process. The increased surface recognition sites could result in an enhancement in binding strength and/or specificity of the peptide.

In this study, three tandem repeats of both quartz-binding peptide (QBP1-PPPWLPYMPPWS) and gold-binding peptide (GBP1-MHGKTQATSGTIQS) was used to develop the bi-functional construct. Two peptide sequences were linked by a flexible linker, composed of 10 amino acids (GGGGSGGGGT). For the expression of the peptide, an E. coli-based expression system TAGZyme pQE-2 is used. The vector was constructed and the bi-functional peptide was expressed in LB medium. Expressed proteins were purified by using Immobilized Metal Affinity Chromatography. Both functionalities of the peptide are conserved after linking two well-characterized peptides. The genetically engineered in vivo construct is expected to be a structurally functional and a versatile molecular tool for bionanotechnology applications with inorganic materials. The peptide can be used to immobilize gold nanoparticles on silica for biosensor applications and for other nanotechnologies applications.

1. INTRODUCTION

1.1. Recombinant Fusion Proteins

Proteins are a diverse class of biomolecules important for all the cells due their feature to participate nearly all of the essential processes. This is why they are plenty in the cells. Enzymes serve as the proper molecular surface supplier and promoters for the chemical reactions to take place in the cells. Pumps and channels pumps formed by the proteins enclosed in the plasma membrane are important in the control of transfer into and out of the cell. Some of the proteins are important in signal transduction mechanisms and some of them take place in structural activities. Some proteins are important for immune response and protect cells from extreme environmental conditions or outer effects. Some are essential in stabilizing DNA molecules and some take place in defense mechanisms. All these variable properties of proteins rely on the fact that they are highly specific and selective towards their ligands [1, 2].

The functional properties of proteins are defined by the three-dimensional conformations they possess. Proteins have a very important feature that is they spontaneously fold into very well-determined and elaborate three-dimensional structures, which are inspired by the amino acid residues they are composed of. This self-folding ability of proteins composes the passage from one-dimensional sequence information to three dimensional biological functions. This is why proteins are so predominant in the world of biochemistry [2].

The fusion protein technology has become a very important tool in several areas of biochemical research studies [3]. Fusion proteins are constructed by end-to-end fusions of whole genes that encode the whole functional proteins and can also be named as hybrid proteins [4]. The construction of recombinant fusion proteins has been used to increase the expression of soluble proteins, to facilitate the purification [3, 4, 5] and immobilization [6] of the proteins. They are practical in the display of proteins on the surfaces of cells and phage, cellular localization (e.g. targeted delivery of toxic activities to disease-causing cells), metabolic engineering, in the study of protein folding and protein–protein interactions in such systems as the yeast two-hybrid system [4]. Also its applications in biotechnology area include the immunoassays using hybrid molecules composed of antibody fragments or antibody binding domains, enzymes or green fluorescent proteins, the selection and production of antibodies and the engineering of bi-functional enzymes [3]. For example, insulin was fused with β-galactosidase for better expression. A streptavidin tag, a polyarginine tag, a His_6tag and other small affinity tags have been used to facilitate purification. Thioredoxin is used as solubility tag to overcome the problems of insolubility [5]

One of the most widely used applications of recombinant fusion proteins is in the field of protein expression and purification. The principle of this application is simple; the gene that encodes the protein of interest is linked to a sequence that codes for a protein or peptide which binds to a specific ligand. These peptides can be simple short sequences like poly(histidine) or whole proteins like glutathione S-transferase or the maltose binding protein. Many of these expression and purification systems are commercially available [6, 7, 8]

1.1.1 Bi-functional Recombinant Proteins

Construction of recombinant proteins, especially multifunctional fusions, has a great potential in biotechnology. The idea of making a hybrid protein can be utilized in the production systems showing bi-functionality, especially in enzyme technology [9]. In molecular biology and biotechnology, enzymes having two or more combined activities with suitable stability, have found extensive application areas [10]. The natural diversity of the enzymes provide some of them to have bi-functional activity but most of the fusion enzymes have been done by the *in vitro* fusion of individual enzymes according to their evolutionary qualities and well-defined structures [11]. Artificial fusion enzymes generated by end-to-end fusion or by joining whole genes of intact functional proteins with a linker have been described to show bi-functionality [10].

The fusion of structural genes encoding enzymes catalyzing sequential reactions is advantageous. This will provide simple expression of a single recombinant unit containing multiple genes and one step purification of recombinant proteins. Physical proximity of the enzymes due to their genetic fusion might guide to increased rates of sequential enzyme reactions. This can be supplied by directly transfer of reaction intermediates to the active sites of the next enzymes and avoiding the diffusion of the intermediates [9].

If the fusion proteins are to be used to assemble organized molecular layers, a detailed knowledge of the kinetics and thermodynamics of binding mechanism is essential [7].

1.2. Linker Regions in Fusion Proteins

The construction of a functional fusion protein generally needs a linker region that provides an extended conformation to permit maximum stability of the proteins or the domains of proteins. The choice of the peptide linker sequence is especially important for retaining the functionalities of the fused proteins [3, 12]. The proper amino acid composition and the general folding of the linker should be considered [12]. Several studies about the choice of the linker regions suggest mainly that the flexibility and hydrophilicity of the linker region are important not to interfere with the functions of the domains by disturbing them. Spatial separation of the functional domains of a fusion protein by the appropriate linker region is effective on the independent working of the bi-functional domains [3]. The linker sequences with a higher tendency to interfere with domain folding [14] or to form α-helical or β-strand structures [12] are not favorable due to their effect by limiting the flexibility of the fusion protein and, therefore, affecting the functionality. Hence, such secondary structural elements should be avoided while designing a linker region [12]. Linker design becomes more important if a multi-domain protein is designed *de novo*. The control of the distance and the direction of the functional domains are significant to maximize the functions of interest [3, 13].

1.2.1. Amino Acids and Their Properties

The amino acid composition of the linker sequences is the most important criterion in the linker design and selection. For the design of flexible linker, glycine and serine amino acids are widely used to minimize the steric hindrance and to provide enough space for the components of the fusion protein to remain their native conformation [6, 15 16]. The rationale of using serine and glycine-rich sequences or similar sequences as linker peptides in immunological applications depend on the understanding that they are very flexible, show no rigid structure, and thus induces insignificant immune response *in vivo*. These linker peptides consisting of only glycine and serine amino acids have been utilized in the construction of several fusion proteins for therapeutic applications. For example, interleukin-2 is linked to the Fc of IgG by GGGSGGG linker sequence for expanding its short half-life and overcoming systemic toxicity, extracellular domain of interleukin-13 receptor is linked to the Fc of IgG by GSG linker sequence for treating antibody-mediated immune disorders. Some other studies have also signified that similar linker peptides consisting of repeating units of GGGGS are often used for preparing VH–VL linked single-chain antibodies for therapeutic applications and *in vivo* imaging [17]. A flexible peptide linker composed of $(Gly_4Ser)_3$ was used to link the variable regions of the heavy chain and the light chain of a recombinant antibody single-chain Fv [14] and to link two copies of epitopes to increase the immunoreactivity of the fusion peptide by increasing the distance between the two epitopes [18]. Studies on these diverse constructs have abundantly verified that the linker peptides with glycine and serine residues preserve functional properties of the fused proteins and show no immunogenecity in vivo [17]. Amino acids with cyclic structures, like Proline, lead to rigid linker sequences and provide a longer distance between the functional domains of the proteins [19].

1.3. Inorganic Binding Peptides and Genetically Engineered Polypeptides for Inorganics

Until today, a little number of proteins has been discovered to bind inorganic materials. One of the most well-known examples of these proteins is ice-binding protein, which is found in several fish species, plants and insects [20].

An inorganic binding peptide (IBP) is a sequence of amino acids which bind to an inorganic surface specifically and selectively. This surface might be a well defined one, like a single crystal or nanostructure, or a non-descriptive rough one, like a powder. These materials can be synthesized under physiological conditions and in aqueous environments that make them biocompatible. Also, they show quite stable surface structures and compositions. Many studies are done by using peptides that bind to inorganic surfaces. These surfaces could be noble metals as well as oxide semiconductors [21, 22]. The specificity of a protein for a surface may rise from structural recognition mechanisms, like morphology and size, and chemical mechanisms, such as hydrogen bonding, polarity and charge effects [21].

Gold-binding proteins (GBPs) were the first examples of proteins generated via *E. coli* cell surface display method. In one of the studies, it was determined that gold-binding sequences isolated by cell surface display could be taken from an extracellular loop of maltoporin to the N-terminus of alkaline phosphatase by retaining their gold-binding activity. This could propose that this activity is portable and independent of the environmental protein framework. [21, 23].

1. 3. 1. Obtaining Inorganic Binding Peptides

There are several methods in order to acquire surface-specific proteins. The traditional one of these methods is extraction from hard tissues, which include protein isolation, purification, amino acid analysis and sequencing. Another method is to use proteins whose ability to bind to inorganic surfaces is previously known. Many of the proteins bind to inorganic surfaces non-specifically, usually by chemi-sorption. So, their use is limited and mostly depends on chemical properties of the solution. An effective approach to get surface-specific proteins would be designing molecular design of recombinant proteins by using genetic engineering. If the surface topology of the wanted inorganic crystal structure can be predicted, then a molecule that is complementary to this surface and can be designed and this molecule could fit tightly to that structure with a high binding energy. These kinds of designs can be obtained by site-directed mutagenesis of existing proteins, by DNA shuffling or *de novo* selection polypeptide motifs by using phage or cell surface display libraries. [24, 25]

The utilization of combinatorial biology techniques is a rising era for obtaining of inorganic binding peptides [26]. In this approach, combinatorial biology libraries are used for selection of inorganic-binding proteins towards specific inorganic substrate [21, 26, 27]. These huge libraries are made-up of random peptides having the same number of amino acids but different in sequence compositions. “Phage Display” and “Cell Surface Display” are well-known *in vivo* combinatorial biology techniques [28, 29]. The libraries used for i*n vivo* techniques are generally smaller (10^{7-10}) compared to the libraries used for *in vitro* techniques (10^{15}), like mRNA display and ribosome display [21, 27, 30] These display technologies are primarily utilized for the characterization of receptor binding sites, epitope mapping, the study of protein-ligand interactions, the isolation and evolution of proteins or enzymes having improved or altered binding characteristics for their ligands for designing new drugs, enzymes, antibodies, DNA-binding proteins and diagnostic agents [31], and also adapted for the selection of inorganic binding peptides [21, 27]. Until now, phage display has been used in many researches to for the selection of peptides that are specific to gallium arsenide [32], silica [33], silver [34], zinc sulfide [35], calcite [36], cadmium sulfide [37], and noble metals such as platinum and palladium [38]. Cell surface display also has been applied to identify the iron oxide [27, 39], gold [23, 27], zinc oxide, zeolites and cuprous oxide [40] specific binders.

The effectiveness of the display technologies come from the reality that they do not require a previous knowledge of the desired amino acid sequence because they can simply be selected and enhanced if a large enough population of random sequences is available. [21, 27]

Phage display is an effective method used for selection and engineering of polypeptides with wanted binding specificities. The method depends on the reality that if gene fragments coding for polypeptides are fused to M13 coat protein genes, these fusion genes can be incorporated in bacteriophage particles and so, these heterologous proteins can be displayed on their surfaces. In this way, a physical bridge is formed between phenotype and genotype [41, 42].

The first libraries were constructed by inserting a synthetic fragment of DNA into a cloning site engineered into the 5`-end of gene III of an M13 filamentous bacteriophage. Gene III codes for pIII at 5 copies. So, using 5` pIII cloning site allows displaying the random peptide at the same amount of copies on the phage. Some types of the libraries are constrained pIII display libraries, pVIII display libraries, phagemid libraries, C-terminal libraries, modified libraries and scaffolded libraries. Constrained or cyclic libraries, encode the random polypeptide between two cysteine residues. The logical reason behind the cyclic library is to constrain the peptide and so it assumes fewer conformations. So, if a good binder is included in the library, the peptide will be in the accurate conformation for binding rather frequently than a loose linear one. [43] Other display systems are bacteriophage λ, T7 or T4 phage-based display systems but they are not widely used as compared to M13 phage display systems [21, 27].

Similar to phage display, cell surface display depends on the use of chimeric proteins, which is composed of the target sequence fused within (or to) a protein that is naturally located on the surface of the cell to perform display. The DNA sequence of the target region can be randomized to create a library of cells by using molecular biology techniques and each of these sequences will synthesize a different version of the chimeric protein on its surface. By incubating this library with a substrate or ligand, then washing to remove weak binders or non-binders, and reiterating the procedure to enhance for strong binders, a division can be selected from the original library that will show the capability to strongly interact with the desired ligand or substrate. This process is named as biopanning. Later on, amino acid compositions of the selected sequences can be identified by DNA sequencing due to the fact that the chimeric product is encoded within the plasmid of the cell [21, 27].

1. 3. 2. Further Design and Tailoring of Inorganic Binding Peptides

Once the inorganic binding peptides are selected via phage or cell surface display technologies and characterized in terms of their binding affinities, they can be further engineered, modified or redesigned with improved affinities and specificities [44]. To increase binding affinity, tandem repeats of the sequence can be generated by genetic engineering [23]. Repeated structures of short peptides may show a better affinity and provide a controlled assembly process. The increased surface recognition sites could result in an enhancement in binding strength and/or specificity of the peptide [22, 23].

One of the initial assembly studies was focused on the gold-binding peptide (GBP1), which contains 14 amino acids (MHGKTQATSGTIQS). To increase the binding activity, tandem repeats of the sequence were produced by using genetic engineering techniques and it was observed that at least three repeats of the sequence were needed for a binding with higher affinity [21, 22, 23].

Due to their practical applications in biotechnology and materials sciences, the exploration of inorganic binding peptides is increasing. This acceleration leads to research for gaining information about the relationships between the peptide sequences and their binding affinities or specificities. By succeeding this, it will be possible to design novel peptides with selected properties of interest in engineering and medicine applications. This can be called as "knowledge-based" approach to design these inorganic binding sequences with predictable affinities and specificities.

The hypothesis of "knowledge-based" approach is simple when we consider the evolutionarily-related proteins and conserved sequences: "A set of peptides generated by directed evolution through *in vivo* selection to recognize a given solid material will have similar sequences, much as evolutionarily related proteins do". According to this hypothesis, a bioinformatics approach is generated for the classification of the *in vivo*-selected peptides depending on their inorganic-binding properties. The first step of this approach is all-against-all comparisons of experimentally selected peptides with short amino acid sequences, which are categorized for their binding affinity. These comparisons will lead to scoring the alignments by using sequence similarity scoring matrices. Later on, brand new scoring matrices are developed for the optimization of the similarities within the strong-binder sequences and the differences between the strong- and weak-binder sequences. By utilizing these scoring matrices, classification of a given peptide sequence can be performed based on its sequence similarity to a set of experimentally-selected peptides. So, by combining experimental knowledge with bioinformatics analysis it is enabling to design peptides in a directed manner. [45]

1.4. Bi-functional GEPIs

An inorganic peptide that is selected combinatorially and then tailored by genetic engineering tools are called as genetically engineered polypeptides for inorganics (GEPIs). At this point, the functional linking properties of GEPI could be used either alone or as part of another macromolecule, like an enzyme or DNA by means of genetic fusion. On the other hand, a multifunctional hybrid polymeric structure could be developed by chemical fusion of GEPI to a synthetic polymer. These usages of GEPI are the heart of applications in biological materials science and engineering by offering addressable molecular binder, linker or self-assembler of inorganic structures. Just the once inorganic binding domains are identified, they can be linked genetically to obtain bi-functional units including two GEPI constructs to link two different materials to assemble complex nanocomposites and hybrid materials. Due to their specificity, selectivity and self-assembly properties, genetically engineered protein in these hybrid structures would not only supply the vital molecular linkage among the inorganic substrates but also be a central element of the whole structure by supplying functional stability to the hybrid structure [44].

1.4.1 Metal and Metal Oxide Binding Property

Thiol and silane linkages are two major molecular linkers for noble metal and metal oxide surfaces respectively. These two linkages are mostly non-specific and have been widely used in the formation of self-assembled molecules on solid substrates. Self-assembled GEPI monolayers could suggest new ways for designing and engineering novel surfaces for a wide variety of applications [21, 27]

To accomplish nanoscale effects like quantum conduction and photonic signal enhancement, inorganic nanoparticles are required to be immobilized at specific positions on selected substrates in controlled two dimensional or three dimensional geometries. One of the traditional approaches to manufacture quantum dots for producing quantum electronic properties is to use molecular beam epitaxy. In this approach, quantum dots (QDs) are formed at thin film strain centers on semiconductor substrates. These traditional approaches are accomplished under stringent conditions of high temperature, ultra high vacuum and toxic environment. Alternatively, bi-functional GEPIs can be utilized to assembly one nano-inorganic particle onto another inorganic surface and to decorate the surface by homogenously distributed nanoparticles. This proposes that linking two GEPIs could be useful in the fabrication of tailored nanostructures. The recognition activity of the protein would give the capability to control the particle distribution and solution conditions would permit the control of the particle size [44].

It is crucial to keep two different nanoparticles together in solution with a distance by using one molecule. When considered the distance of these nanoparticles, by controlling this distance it is also possible to control the energy transfer between these two nanoparticles. When these two nanoparticles become close or distant to each other, the difference in the radiation, increase or decrease, can be utilized in sensing application. Another application could be the monolayer formation of a nanoparticle on another nanoparticle by linking two different inorganic materials.

1.4.2. Two important examples of IBPs; GBP1 & QBP1

Gold-binding proteins (GBPs) were the first examples of proteins generated via *E. coli* cell surface display method. GBP1 (MHGKTQATSGTIQS) is a 14-mer peptide selected from a peptide library expressed on the outer surface of *Escherichia coli.* In these studies, it was determined that gold-binding sequences isolated by cell surface display could be taken from an extracellular loop of maltoporin to the N-terminus of alkaline phosphatase by retaining their gold-binding activity (Figure 1.1). This could propose that this activity is portable and independent of the environmental protein framework. Despite the higher affinity of many proteins to gold (Au) at low salt concentrations, it was surprising that these gold binder sequences were selected at high salt concentrations, which is known to inhibit and prevent binding of proteins to gold [21, 23]. For the increased binding activity of the peptide, tandem repeats of the sequence were produced by using genetic engineering techniques and it was observed that at least three repeats of the sequence were needed for a binding with higher affinity [21, 22, 23], In other studies, different repeating units of GBP1 was constructed through the post-selection engineering and by using the seven-repeat binder (7R-GBP1) efficient immobilization of gold nanoparticle was achieved [44].

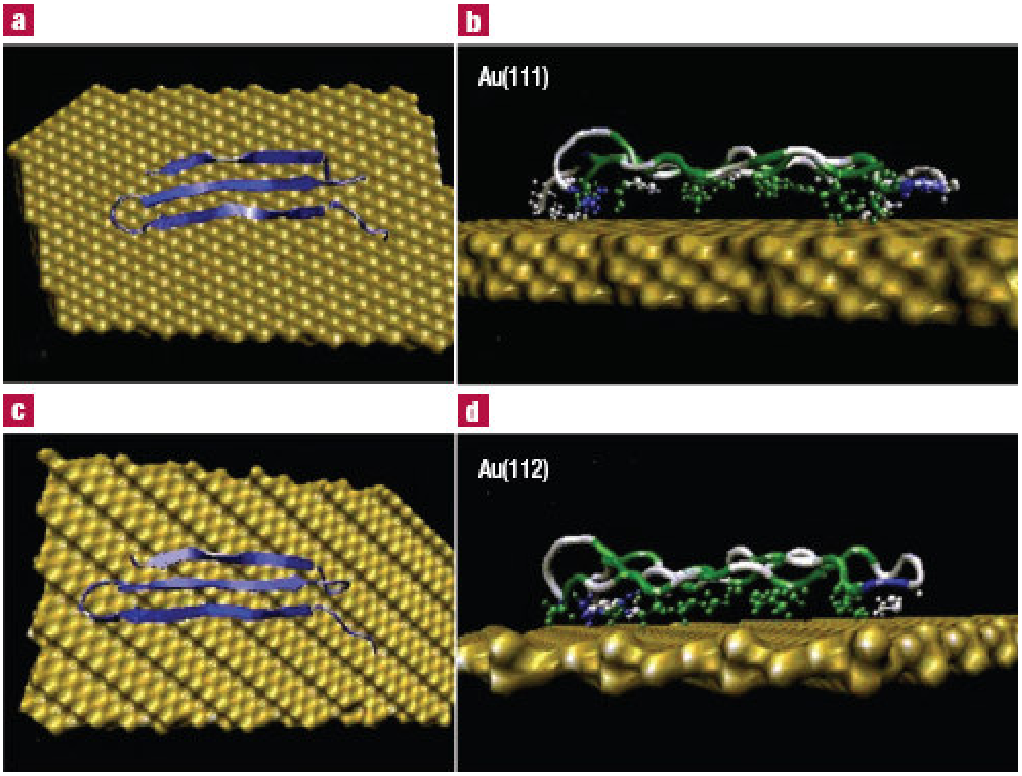


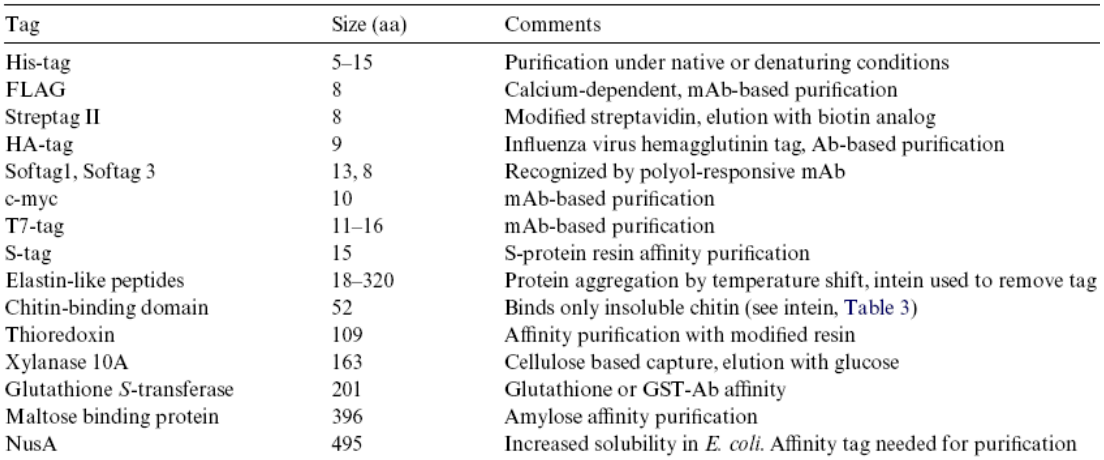
Figure 1.1: A gold-binding protein (3-repeat GBP1) on Au(111) and Au(112) surfaces. Viewed from above (a and c) and edge-on (b and d), respectively. The coloring corresponds to residue type: polar residues are highlighted in green, charged in blue, and hydrophobic in white [21]

The Quartz-binding proteins (QBPs) are 12-mer peptides, which were first selected using phage display combinatorial peptide library, and then characterized to classify them according to their inorganic affinity. After this classification, the sequence similarity characteristics of the strongly-binding peptides were used and a set of *de novo* designed quartz binders were generated. Finally, these *de novo* peptides allowed *in silico* selection of strong-binding QBPs as the candidate of construct formation [44, 45]. Therefore, these QBP sequences are designed peptides in a directed mode by combining experimental knowledge with bioinformatics analysis [45].

1.5. Histidine-Tagged Expression Systems

The expression and purification of recombinant proteins are important in molecular biology studies, especially in the characterization studies of the proteins. Despite the development and usage of several heterologous expression systems for the production of recombinant proteins, their purification can still be difficult. Besides the classical purification methods, these proteins can be purified as fusion proteins by adding specific and small affinity tags to their sequence by recombinant DNA technology (Table 1.1). These affinity tags make the purification of the recombinant fusion proteins easier by applying affinity chromatography methods [46]. The usage of these affinity tags for the purification of recombinant proteins and native protein complexes have turned out to be highly accepted systems for many reasons. First of all, their efficiency and yield is much higher when purification from crude extracts without prior steps for removing nucleic acids or other cellular debris is considered. Following, they are useful for the purification of individual proteins and particularly protein complexes due to their mild elution conditions. Significantly, they permit the purification of miscellaneous proteins with comprehensive protocols when in comparison with conventional chromatographic tools [47].

Table 1.1: Affinity and solubility tags for recombinant proteins [48]



Tag	Size (aa)	Comments
His-tag	5–15	Purification under native or denaturing conditions
FLAG	8	Calcium-dependent, mAb-based purification
Streptag II	8	Modified streptavidin, elution with biotin analog
HA-tag	9	Influenza virus hemagglutinin tag, Ab-based purification
Softag1, Softag 3	13, 8	Recognized by polyol-responsive mAb
c-myc	10	mAb-based purification
T7-tag	11–16	mAb-based purification
S-tag	15	S-protein resin affinity purification
Elastin-like peptides	18–320	Protein aggregation by temperature shift, intein used to remove tag
Chitin-binding domain	52	Binds only insoluble chitin (see intein, Table 3)
Thioredoxin	109	Affinity purification with modified resin
Xylanase 10A	163	Cellulose based capture, elution with glucose
Glutathione *S*-transferase	201	Glutathione or GST-Ab affinity
Maltose binding protein	396	Amylose affinity purification
NusA	495	Increased solubility in *E. coli.* Affinity tag needed for purification

Attachment of an oligo-histidine tag (also called as His-tag or 6xHistag) to a terminus, either N- or C- terminus, of the target protein is an excellent instance of very first and successful protein engineering application in order to develop a unique and comprehensive purification system [49].

These systems depend on the significant selectivity and affinity of the nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices, which are chelated metal ions used as affinity ligands towards proteins which have been fused to 6 consecutive histidine amino acid residues (Figure 1.3) [46, 48]. One of the commercially available expression systems based on the Histidine-tag is The QIAexpress® System of QIAGEN [46].

Histidine tag-based purification system has some advantages over other affinity-tag chromatography systems. The interaction of the 6xHis tag with Ni-NTA matrices is independent from the conformation of the target protein to be purified. The 6xHis tag is much smaller than other frequently used affinity tags and can be used in any expression system because it does not obstruct the function and structure of the target recombinant protein. The 6xHis tag is uncharged at physiological pH and does not obstruct secretion. Furthermore, binding, washing, and elution steps of the purification are highly reproducible, and do not affect the structure of the recombinant protein [46]. Another important point is that, the binding specificity of the system facilitates the purification of proteins and peptides under both native and denaturing conditions [48].

1. 5. 1. Immobilized Metal Affinity Chromatography

Immobilized-metal affinity chromatography (IMAC) is a commonly used method for quick and effective purification of recombinant proteins since 1970s. It was first introduced by Porath and his colleagues in 1975 as a new methodology for protein purification based on the affinity and binding of specific amino acids to immobilized metal ions and named it as Metal Chelate Affinity Chromatography. Later on, the method was engineered by the usage of a unique matrix Nickel(II)-Nitrilotriacetic acid (frequently known as Ni-NTA) which selectively binds to numerous neighboring histidine residues [49].

In IMAC, the adsorption of the target protein is succeeded by the coordination among immobilized metal ions, which serve as affinity ligands, and electron donor groups of the target protein surface. Metal ions are immobilized by the coordinative binding to the chelating molecules, which are covalently bound to the chromatography matrix. Intermediate metal ions, like Ni(II), Cu(II), Zn(II) and Co(II) are the most frequently used ones by coordinating nitrogen, sulfur and oxygen. With these metal ions, adsorption to the matrix is achieved at pH ranges sustaining the nitrogens of the imidazole ring in the histidine amino acids in the – non-ptotonated form, commonly in neutral or faintly basic medium. Also, buffers with high ionic strength (0.1 - 1 M) are commonly used in order to reduce the unspecific electrostatic interactions with the matrix. Elution is achieved either by protonation or by ligand exchange via competitive molecules, like ammonium salts or imidazole (Figure 1.2) [49].

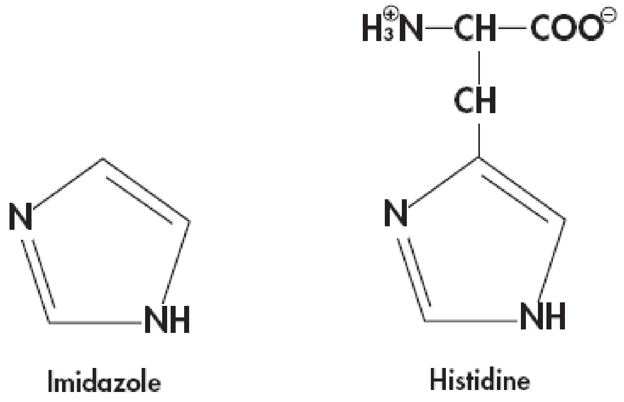


Figure 1.2: Chemical structures of histidine and imidazole [46]

Despite the introduction of several different chelators to be used in IMAC, commercially available matrices commonly use a tridentate chelator iminodiacetic acid (IDA), tetradentate chelators nitrilotriacetic acid (NTA) and carboxymethylated aspartate (CM-Asp). Here, the terms of tridentate or tetradentate implies the number of occupied coordination bonds between the metal ion and the chelating agent [49]. Tetradentate chelating agents bind to the metal ions more firmly and less leakage occurs, however, tridentate chelating agents show higher binding affinities towards the target proteins but cannot bind tightly to metal ions, leading to low yields, impurity and metal-ion contamination of isolated target proteins (Figure 1.3) [46, 49].

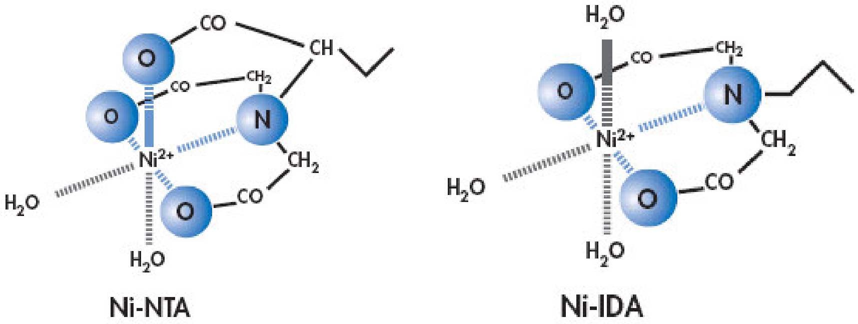


Figure 1.3: Comparison of the interactions of different metal chelate matrices with nickel ions [46]

1. 5. 2. TAGZyme pQE-2 Vector

TAGZyme pQE-2 vector is designed for the expression for N-terminally polyhistidine-tagged (6xHis-tagged) proteins in *E. coli.* The polyhistidine tag is designed to encode the sequence of Methionine-Lysine-Histidine$_6$ (MKH$_6$), that means a short Histidine tag and the N-tetrminus motif composed of Methionine-Lysine brings a high level of protein expression and the lowest level of methionine processing in *E. coli* [50].

pQE-2 vector is a low-copy plasmid and based on the T5 promoter transcription-translation system. It contains an optimized promoter-operator element consist of phage T5 promoter, which is recognized by the *E. coli* RNA polymerase. It also contains two *lac* operator sequences which increases *lac* repressor binding and guarantees efficient repression of the T5 promoter, a synthetic ribosomal binding site, RBSII, for high translation rates, polyhistidine-tag sequence at the N-terminus, multiple cloning site and translational stop codons two strong transcriptional terminators (t_0 from phage lambda and T1 from the *rrnB* operon of *E. coli*, in order to prevent read-through transcription and ensure stability of the expression product, β-lactamase gene (*bla*) for resistance to ampicillin at 100 µg/ml and ColE1 origin of replication (Figure 1.4) [50].

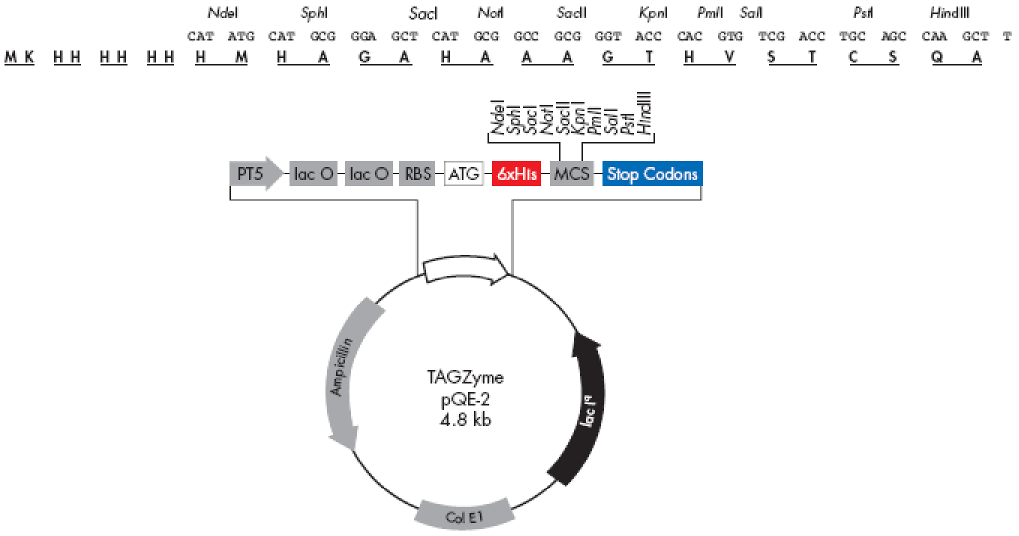


Figure 1.4: pQE-2 vector with restriction maps and sequences for the cloning regions [50]

1.6. Aim of the Study: Construction of a Bi-functional Peptide by Recombinant Fusion of GBP1 & QBP1 with a Flexible Peptide-Linker Region

Once the inorganic binding peptides are selected via phage or cell surface display technologies and characterized in terms of their binding affinities, they can be further engineered with improved affinities and specificities. Next, they can be utilized as presenting a single function or combined to express as multifunctional constructs. In this study, our aim is to design a bi-functional peptide with gold and quartz binding ability. Both of the peptides are engineered ones with respect to their binding affinities. Gold binding peptide (MHGKTQATSGTIQS) is selected by cell surface display and well characterized, and then engineered as three repeat to have a high specificity for gold surface. Quartz binding peptide (PPPWLPYMPPWS) is a second generation *de novo* designed peptide via bioinformatics tools based on the phage display selected and well characterized ones. Both of the peptides are linked in multiple repeat forms to each other through different flexible aminoacid sequences, which consist of Glycine, Serine and Threonine. Following the vector construction, the peptides are expressed using *in vivo* systems. Next after the expression, the peptides are purified by using Immobilized Metal Affinity Chromatography (IMAC) and their bi-functional activities are tested by different tools.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Bacterial Strains

2.1.1.1 *E. coli* Top10 Electrocompetent Cells

F- mcrAΔ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG strain which is supplied within pCR 2.1-TOPO TA Cloning Kit (Catalog # K4500-01, Invitrogen).

2.1.1.2 *E. coli* Top10 Chemically Competent Cells

F- mcrAΔ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG strain which is supplied within pCR 2.1-TOPO TA Cloning Kit (Catalog # K4500-01, Invitrogen).

2.1.2. Cloning Vectors

2.1.2.1. pDrive Cloning Vector

pDrive cloning vector (given in Appendix A) was purchased from QIAGEN (Catalog # 231122) for cloning of PCR products containing the single Adenine (A) overhang at each end. Adenine nucleotide was added at 3'- ends of blunt-ended PCR products using QIAGEN A-Addition Kit (Catalog # 231994, Qiagen).

2.1.2.2. pCR 2.1-TOPO Cloning Vector

pCR 2.1-TOPO cloning vector (given in Appendix B) was purchased from Invitrogen (Catalog # K4500-01) for cloning of PCR products containing the single Adenine (A) overhang at each end. Adenine nucleotide was added at 3'- ends of blunt-ended PCR products using QIAGEN A-Addition Kit (Catalog # 231994, Qiagen).

2.1.3. Expression Vectors

2.1.3.1. pQE-2 Expression Vector

TAGZyme pQE-2 vector is designed for the expression for N-terminally polyhistidine-tagged (6xHis-tagged) proteins in *E. coli.* The polyhistidine tag is designed to encode the sequence of Methionine-Lysine-Histidine$_6$ (MKH$_6$) (See Appendix for vector map) and was purchased from QIAGEN (Catalog # 32932).

2.1.4 Enzymes

2.1.4.1. Restriction Enzymes

Pst I (CTGCA↓G, NEB Inc), ***Eag*** **I** (C↓GGCCG, NEB Inc) and ***Acc65*** **I** (G↓GTACC, NEB Inc) restriction endonucleases and their reaction buffers were purchased from New England Biolab (Catalog # R0140S, Catalog # R0505L and Catalog # R0599L respectively).

2.1.4.2. *Pfu* DNA Polymerase

Pfu DNA Polymerase (recombinant) is a DNA polymerase of *Pyrococcus furiosus*, a hyperthermophillic archaebacterium. The enzyme catalyzes the incorporation of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg^{2+} at 70-80°C. Different than *Taq* DNA Polymerase, *Pfu* DNA Polymerase has 3'→5' exonuclease (proofreading) activity that enables the polymerase to correct nucleotide incorporation errors (Catalog # EP0502).

2.1.4.3. i-*Taq* DNA Polymerase

i-*Taq* DNA polymerase (recombinant) is DNA polymerase of *Thermus aquaticus.* The enzyme catalyzes the incorporation of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg^{2+} at optimally 72°C and was purchased from Intron Biotechnology (Catalog # 25021).

2.1.4.4. Quick T4 DNA Ligase

Quick T4 DNA ligase enables the ligation of cohesive end or blunt end DNA fragments in 5 minutes at room temperature (25°C) and was purchased from New England Biolabs (Catalog # M2200S).

2.1.5. DNA Molecular Weight Markers

DNA molecular weight standard markers (given in Appendix C) were obtained from MBI Fermentas.

2.1.6. Oligonuclotides

Oligonucleotides given below were synthesised by Alpha DNA (Genova) company.

3RQBP1 oligo	5`-TAAGGATCA*GGTACC*CCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCTCCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCTCCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCTTAATAA*CTGCAG*ACTAC-3`
3RQBP1 F	5`-TAAGGATCAGGTACCCCTCCTC-3`
3RQBP1 R	5`-GTAGTCTGCAGTTATTAAGACCAAGGA-3`
3RGBP1L F	5’-GCGGCCGCGCAAATGCATGGAAAAACTCAGGCA-3’
3RGBP1L-R	5’-GGTACCTCCACCGCCGCTACCACCTCCACCGCTCTGGATAGTCCCGCTG -3’

Oligonucleotides given below were supplied by the kits purchased from the companies.

M13 -40 (pDrive)	5'-GTTTTCCCAGTCACGAC-3'
M13 -20 (pCR 2.1)	5´-GTAAAACGACGGCCAG-3´
M13 Rev (pCR 2.1)	5´-CAGGAAACAGCTATGAC-3´
Promoter (pQE-2)	5'-CCCGAAAAGTGCCACCTG-3'
Type III/IV (pQE-2)	5'-CGGATAACAATTTCACACAG-3'
Reverse (pQE-2)	5’- GTTCTGAGGTCATTACTGG-3’

2.1.7. Bacterial Culture Media

2.1.7.1. LB (Luria-Bertani) Medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0 with 10 M NaOH and sterilized by autoclaving for 15 min. under 2 atm at 121°C.

2.1.7.2. LB Agar Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g bactoagar (Acumedia) were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized by autoclaving.

2.1.7.3. SOC Medium

SOC medium containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose was supplied within pCR 2.1-TOPO TA Cloning Kit (Catalog # K4500-01, Invitrogen).

2.1.7.4. 2XYT Medium

16 g. tryptone, 10 g. yeast extract and 5 g. NaCl were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0 with 10 M NaOH and sterilized by autoclaving.

2.1.8. Stock Solutions

2.1.8.1. Ampicillin Stock Solution

100 mg/ml ampicillin sodium salt was dissolved in deionized water, filter-sterilized and stored in dark at -20°C.

2.1.8.2. Xgal/IPTG Stock Solution

1.25 g IPTG (Sigma) and 1 g Xgal (Fermentas) were dissolved in 25 ml DMF (Riedel-de-Haen). The solution was stored at –20°C in the dark.

2.1.8.3. 1 M IPTG Stock Solution

2.38 mg. IPTG was dissolved in 10 ml. deionized water, filter sterilized and stored at -20°C.

2.1.8.4. Glycerol Stock Solution

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to have 80 % (w/v) solution. It was sterilized for 15 minutes under 1.5 atm at 121°C.

2.1.9. Buffers

2.1.9.1. Na-Ac Buffer

3 M of Na-Ac (Riedel-de-Haen) was dissolved in 65 ml distilled water. pH was adjusted to 5.2 and distilled water was added up to 100 ml.

2.1.9.2. 10X TBE Buffer (1000 ml)

108 g. of Tris base, 55 g. boric acid, and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water, its pH was titrated to 8.3 and sterilized for 15 min. under 1.5 atm at 121°C.

2.1.9.3 QIAExpressionist Lysis Buffer (1000 ml)

7.8 g. $NaH_2PO_4.2H_2O$, 17.54 g. NaCl and 0.68 g. imidazole were dissolved in 1. liter deionized water, its pH was titrated to 8.0 by NaOH.

2.1.9.4 QIAExpressionist Wash Buffer (1000 ml)

7.8 g. $NaH_2PO_4.2H_2O$, 17.54 g. NaCl and 1.36 g. imidazole were dissolved in 1. liter deionized water, its pH was titrated to 8.0 by NaOH.

2.1.9.5 QIAExpressionist Elution Buffer (1000 ml)

7.8 g. $NaH_2PO_4.2H_2O$, 17.54 g. NaCl and 17 g. imidazole were dissolved in 1. liter deionized water, its pH was titrated to 8.0 by NaOH.

2.1.9.6 10X TAGZyme Buffer (500 ml)

15.6 g. $NaH_2PO_4.2H_2O$ and 43.83 g. NaCl were dissolved in 500 ml. deionized water, its pH was titrated to 6.3 by NaOH, filter sterilized with a 0.2 µm filter and stored at 4°C.

2.1.9.7 2X SDS-PAGE Sample Buffer

0.09 M Tris-Cl pH 6.8, 20% glycerol, 2% SDS, 0.02% bromophenol blue and 0.1 M DTT was mixed to prepare 10 ml. of sample buffer.

2.1.10. Lab Equipments

Lab equipments are given in Appendix D.

2.2. Methods

2.2.1. Bi-functional Peptide Vector Construction

The vector for the production of the bi-functional peptide was constructed by polymerase chain reactions, restriction endonuclease digestions and ligation reactions. For 3RQBP1; an oligonucleticde and a set of primers (Forward and Reverse) were designed and synthesized by Alpha DNA (Genova). For 3RGBP1; a set of primers were designed and synthesized by Alpha DNA (Genova). At following subsections the protocols of the vector construction steps is given and discussed in detail

2.2.1.1. Amplification of Double Stranded DNA (3RQBP1) from an Oligonucleotide

QBP1 was not cloned into any vector as a multiple repeat before. The only DNA sequence is found in an M13 phage genome, cloned by Deniz Şahin. Concerning the difficulties of multimerization procedures (time consuming and expensive), an oligonucleotide was designed. The oligonucleotide was designed to have the three repeat of the QBP1 sequence, two restriction endonuclease recognition sequences and two stop codons at the end of the peptide sequence. A forward and a reverse primer was also designed for the amplification of the 3RQBP1 oligonucleotide. *iTaq* DNA Polymerase was used for the polymerase chain reaction.

Polymerase Chain Reaction

Template DNA (1 µg)	2 µl
Forward primer (10 pmole)	1 µl
Reverse primer (10 pmole)	1 µl
10X PCR Buffer	2 µl
dNTP mixture (2.5 mM each)	2 µl
Sterile distilled water	11.8 µl
i-Taq DNA polymerase(5 U/ µl)	0.2 µl
Total Volume	20 µl

Reaction Conditions

94°C	2 min.	
94°C	20 sec	35 cycles
55, 58, 60 °C	10 sec	
72°C	30 sec	
72°C	5 min	

- **Gel Extraction Procedure**

Using MinElute Gel Extraction Kit, PCR products were extracted from the agarose gel (Catalog # 28604,Qiagen).

1. DNA fragment was excised from agarose gel with a clean scalpel. Gel slices were put into microfuge tubes and their weight was calculated.
2. A 3-gel volume of QG buffer was added to 1 volume of gel slice. (600 µl for 100 mg gel). When gels were excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer was applied.
3. Sample was incubated at 50°C for 10 min. by vortexing every 2 min.
4. After the gel completely dissolved, the color of the mixture was checked. QG buffer contains a pH indicator and gives a yellow color at pH<7.5, the optimum pH for DNA adsorption. If the color of the mixture was not yellow, 10 µl of 3 M sodium acetate, pH5.0 was added.
5. After the gel was dissolved, 1 gel volume of room temperature isoproponal was added and mixed by inverting the tube several times.

6. Sample was applied to min-elute column and centrifuged for 1 min. at 13000 rpm.
7. Flow-trough was discarded from collection tube.
8. 500 µl QG buffer was applied to column and centrifuged for 1 min. at 13000 rpm.
9. Flow-trough was discarded from collection tube and centrifuged for an additional 1 min.
10. Spin column placed in a 1,5 ml. microfuge tube and 10 µl Elution Buffer buffer was applied to elute the DNA. After 1 min. incubation tube was centrifuged for 1 min.
11. Eluted DNA was stored at –20°C for further use.

2.2.1.2. Cloning into a TA Vector

TA cloning vectors contain a U overhang at each ends providing easy and efficient ligaton of PCR products with an A overhang. Unpaired A residues are added to PCR products during PCR reactions generated by *Taq* or any other non-proofreading polymerases. In this study, pDrive Cloning Vector was used combined with an A-Addition procedure.

- **A-Addition Reaction**

Gel extracted PCR product	4 µl (80 ng)
5X QIAGEN A-Addition Master Mix*	2 µl
Sterile Distilled Water	4 µl
Total Volume	10 µl

* QIAGEN® A-Addition Kit from Qiagen. (Catalog # 231994, Qiagen)

Reaction conditions

Incubated at 37°C for 30 min.

- **Ligation Reaction**

	Tube 1	Tube 2
pDrive Cloning Vector (50 ng/ µl)	1 µl	1 µl
PCR product from A Addition Reaction	1 µl	2 µl
Sterile Distilled Water	3 µl	2 µl
Ligation Master Mix, 2X*	5 µl	5 µl
Total Volume	10 µl	10 µl

* PCR Cloning Kit from Qiagen. (Catalog # 231122, Qiagen)

Reaction conditions

The samples were incubated for 90 min. at 16°C and 10 min. at 70°C for ligase inactivation.

- **Preparation of Xgal/IPTG-Amp plates**

100 µl Xgal/IPTG solution and 50 µl ampicillin solution were put into 50 ml liquid warm LB agar and was poured onto plastic sterile petri dishes.

- **Transformation Procedure for Plasmids**

1. 10 µl ligation product was mixed with 40 µl *E. coli* Top10 electrocompetent cells in a prechilled electroporation cuvette.
2. Cuvette was placed into electroporator and 1800 Volts was applied.
3. 250 µl 2XYT medium was added to the cuvette and whole mixture was transferred into a microfuge tube. The tube was incubated at 37°C, 200-250 rpm for 45 min on an orbital shaker.
4. After incubation, transformation mixture was spread onto LB agar plates containing Xgal/IPTG-Ampicillin by using a spreader with two dilutions (100 and 200 µl).
5. Plates were incubated overnight (17 h.) at 37°C.

2.2.1.3. Plasmid DNA Purification

Following overnight incubation, white colonies were selected and grown overnight in 5 ml. LB liquid media containing 5 µl Ampicillin. 850 µl of this bacterial culture was mixed with 150 µl 80% glycerol and stored at –80°C. Plasmid DNA was purified from 3 ml for sequencing and further applications by using Roche High Pure Plasmid Isolation Kit (Catalog # 1 754 785). Purification procedure is described in detail below.

1. 3 ml of the culture is centrifuged 60 sec. at 6000 g.
2. The supernatant is discarded.
3. The pellet is resuspended in 250 µl Suspension Buffer + RNase.
4. 250 µl Lysis Buffer is added, mixed gently by inverting the tube 3 to 6 times and incubated at 15-25 ºC for 5 min.
5. 350 µl chilled Binding Buffer is added, mixed gently and incubated on ice for 5 min.
6. The samples are centrifuged at 13.000 g. for 10 min.
7. High Pure Filter Tubes are inserted into Collection Tubes.
8. Supernatant is transferred to High Pure Filter Tube and centrifuged at 13.000 g. for 1 min.
9. The flow-through is discarded.
10. 500 µl Wash Buffer I is added and centrifuged at 13.000 g for 1 min.
11. The flow-through is discarded.
12. 700 µl Wash Buffer II is added and centrifuged at 13.000 g for 1 min.
13. The flow-through is discarded and an additional 1 min. centrifugation at 13.000 g. is done.
14. The filter tube is placed in 1.5 ml microcentrifuge tube and 100 µl Elution Buffer is added.
15. The samples are centrifuged at 13.000 g. for 1 min. and purified plasmid DNA was stored at -20°C.

2.2.1.4. Plasmid DNA Sequencing

Plasmid DNA	1 µl (200 ng)
M13 -40 Primer	3.2 µl
5X Sequencing Buffer*	1 µl
Big Dye Terminator*	2 µl
Sterile Distilled Water	2.8 µl
Total Volume	10 µl

* Big dye® terminator v 3.1 cycle sequencing Kit from Applied Biosystems.

Reaction conditions

95°C	5 min.	
95°C	1 min.	30 cycles
50°C	30 sec	
60°C	4 min.	

- **NaAc-Ethanol Precipitation (Purification) of PCR Products**

1. 1 µl 3M pH 4.6 sodium acetate (cold) and 25 µl 95 % ethanol (cold) were mixed for each sample.
2. 26 µl mixture was added into each PCR product and samples were incubated on ice for 15 min.
3. After incubation, samples were centrifuged for 15 min. at 13000 rpm.
4. Supernatant was discarded and DNA pellet was washed with 250 µl cold %70 ethanol.
5. Samples were centrifuged for 15 min. at 13000 rpm.
6. Ethanol was discarded, the residual ethanol was evaporated by incubating at 95°C.
7. DNA pellet was dissolved in 20 µl formamide.
8. Samples were denatured by putting at 95°C for 3 minutes and then at -20°C for 5 min.

ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.1.5. Amplification of Double Stranded DNA (3RGBP1+Linker) from pSURE 30Q Vector

3RGBP1 was previously cloned into p53Q vector, which was successfully done by Sibel Çetinel. So; a forward and a reverse primer was designed to amplify this sequence by introducing two restriction sites and a 10 aa-length linker for linking two IBPs. For the amplification of the 3RGBP1+Linker, a proofreading polymerase; *Pfu* DNA Polymerase was used.

Polymerase Chain Reaction

Template DNA (1 µg)	2 µl
Forward primer (10 pmole)	1 µl
Reverse primer (10 pmole)	1 µl
10X Pfu Buffer with $MgSO_4$	5 µl
dNTP mixture (2.5 mM each)	1 µl
Sterile Distilled Water	39.5 µl
Pfu Polymerase (2.5 U/µl)	0.5 µl
Total Volume	50 µl

Reaction Conditions

95°C 3 min.

$$\left.\begin{array}{ll} 95^{\circ}C & 30\ sec. \\ 50^{\circ}C & 30\ sec. \\ 72^{\circ}C & 2\ min. \end{array}\right\}\ 30\ cycles$$

72°C: 5 min.

- **Gel Extraction Procedure**

Using MinElute Gel Extraction Kit, PCR products were extracted from the agarose gel (Catalog # 28604,Qiagen).

1. DNA fragment was excised from agarose gel with a clean scalpel. Gel slices were put into microfuge tubes and their weight was calculated.

2. A 3-gel volume of QG buffer was added to 1 volume of gel slice. (600 µl for 100 mg gel). When gels were excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer was applied.

3. Sample was incubated at 50°C for 10 min. by vortexing every 2 min.

4. After the gel completely dissolved, the color of the mixture was checked. QG buffer contains a pH indicator and gives a yellow color at pH<7.5, the optimum pH for DNA adsorption. If the color of the mixture was not yellow, 10 µl of 3 M sodium acetate, pH5.0 was added.

5. After the gel was dissolved, 1 gel volume of room temperature isoproponal was added and mixed by inverting the tube several times.

6. Sample was applied to min-elute column and centrifuged for 1 min. at 13.000 rpm.

7. Flow-trough was discarded from collection tube.

8. 500 µl QG buffer was applied to column and centrifuged for 1 min. at 13.000 rpm.

9. Flow-trough was discarded from collection tube and centrifuged for an additional 1 min.

10. Spin column was placed in a 1,5 ml microfuge tube and 10 µl Elution Buffer was applied to elute the DNA. After 1 min. incubation tube was centrifuged for 1 min.

11. Eluted DNA was stored at –20°C for.

2.2.1.6. Cloning into a TA Vector

TA cloning vectors contain a U overhang at each ends providing easy and efficient ligaton of PCR products with an A overhang. Unpaired A residues are added to PCR products during PCR reactions generated by *Taq* or any other non-proofreading polymerases. Because Pfu polymerase, which is a proofreading polymerase was used in amplifying 3RGBP1+Linker, an A-Addition procedure is needed. In this study, pCR®2.1-TOPO Cloning Vector was used.

- **A-Addition Reaction**

Gel extracted PCR product	5 µl (150 ng)
5X QIAGEN A-Addition Master Mix*	2 µl
Sterile Distilled Water	3 µl
Total Volume	10 µl

* QIAGEN® A-Addition Kit from Qiagen. (Catalog # 231994, Qiagen)

Reaction conditions

Incubated at 37°C for 30 min.

- **Ligation Reaction**

TOPO Vector (10 ng/ µl)	1 µl
PCR product from A Addition Reaction	3 µl
Sterile Distilled Water	1 µl
Salt Solution*	1µl
Total Volume	6µl

* pCR®2.1-TOPO TA Cloning Kit from Invitrogen (Catalog # K4500-01, Invitrogen)

Reaction conditions

The samples were incubated for 5 min. at room temperature (25°C) and put on ice for transformation.

- **Preparation of Xgal/IPTG-Amp plates**

100 µl Xgal/IPTG solution and 50 µl ampicillin solution were put into 50 ml liquid warm LB agar and was poured onto plastic sterile petri dishes.

- **Transformation Procedure for Plasmids**

1. 1 vial of TOP10 chemically competent cells was thawed on ice.
2. 2 µl of the TOPO® cloning reaction was added into the vial of competent cells, mixed gently and incubated on ice for 30 min.
3. The cells were heat-shocked for 30 seconds at 42°C without shaking.
4. The tubes were immediately transferred to ice.
5. 250 µl of room temperature S.O.C. medium was added to the cells.
6. The tube was tightly capped and shaked horizontally at 200 rpm at 37°C for 1 hour.
7. 4 dilutions (25, 50, 75 and 150 µl) were spread from the transformation reaction onto pre-warmed LB agar plates containing Xgal/IPTG-Ampicillin by using a spreader.

   The plates were incubated overnight at 37°C.

2.2.1.7. Plasmid DNA Purification

Following overnight incubation, white colonies were selected and grown overnight in 5 ml. LB liquid media containing 5 µl Ampicillin. 850 µl of this bacterial culture was mixed with 150 µl 80% glycerol and stored at –80°C. Plasmid DNA was purified from 3 ml for sequencing and further applications by using Roche High Pure Plasmid Isolation Kit (Catalog # 1 754 785). Purification procedure is described in detail below.

1. 3 ml of the culture is centrifuged 60 sec. at 6000 g.
2. The supernatant is discarded.
3. The pellet is resuspended in 250 µl Suspension Buffer + RNase.
4. 250 µl Lysis Buffer is added, mixed gently by inverting the tube 3 to 6 times and incubated at 15-25 ºC for 5 min.
5. 350 µl chilled Binding Buffer is added, mixed gently and incubated on ice for 5 min.
6. The samples are centrifuged at 13.000 g. for 10 min.
7. High Pure Filter Tubes are inserted into Collection Tubes.
8. Supernatant is transferred to High Pure Filter Tube and centrifuged at 13.000 g. for 1 min.
9. The flow-through is discarded.
10. 500 µl Wash Buffer I is added and centrifuged at 13.000 g for 1 min.
11. The flow-through is discarded.
12. 700 µl Wash Buffer II is added and centrifuged at 13.000 g for 1 min.
13. The flow-through is discarded and an additional 1 min. centrifugation at 13.000 g. is done.
14. The filter tube is placed in 1.5 ml microcentrifuge tube and 100 µl Elution Buffer is added.
15. The samples are centrifuged at 13.000 g. for 1 min. and purified plasmid DNA was stored at -20°C.

2.2.1.8. Plasmid DNA Sequencing

Plasmid DNA	1 µl (100 ng)
M13 -20 Primer	3.2 µl
5X Sequencing Buffer*	2 µl
Big Dye Terminator*	2 µl
Sterile Distilled water	1.8 µl
Total Volume	10 µl

* Big dye® terminator v 3.1 cycle sequencing Kit from Applied Biosystems.

Reaction conditions

95°C 5 min.

$$\left.\begin{array}{ll} 95^{\circ}\mathrm{C} & 1\ \text{min.} \\ 50^{\circ}\mathrm{C} & 30\ \text{sec.} \\ 60^{\circ}\mathrm{C} & 4\ \text{min.} \end{array}\right\}\ 30\ \text{cycles}$$

- **NaAc-Ethanol Precipitation (Purification) of PCR Products**

1. 1 µl 3M pH 4.6 sodium acetate (cold) and 25 µl 95 % ethanol (cold) were mixed for each sample.
2. 26 µl mixture was added into each PCR product and samples were incubated on ice for 15 min.
3. After incubation, samples were centrifuged for 15 min. at 13000 rpm.
4. Supernatant was discarded and DNA pellet was washed with 250 µl cold %70 ethanol.
5. Samples were centrifuged for 15 min. at 13000 rpm.
6. Ethanol was discarded, the residual ethanol was evaporated by incubating at 95°C.
7. DNA pellet was dissolved in 20 µl formamide.
8. Samples were denatured by putting at 95°C for 3 min. and then at -20°C for 5 min.

ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.1.9 Cloning of 3RQBP1 into pQE-2 Expression Vector

During the amplification of the 3RQBP1 coding sequence, two restriction sites to each ends of the sequence was also introduced. These restriction sites were recognized by the enzymes PstI and Acc65I. pDrive plasmid vector having the 3RQBP1 sequence and the expression vector pQE-2 were digested with these restriction enzymes and the DNA fragments were extracted from the gel. Cleaned and concentrated insert DNA was ligated into pQE-2 vector. Procedure is described in detail below.

- **Restriction Enzyme Digestion**

Digestion of pQE-2

pQE-2 DNA	10 µl
10X NEB Buffer 3	1.5 µl
BSA	0.15 µl
PstI (20 U/ µl)	0.75 µl
Acc65I (10 U/ µl)	0.75 µl
Sterile Distilled Water	1.85 µl
Total Volume	15 µl

Reaction conditions

Incubated at 37°C for 7 h. and then at 80°C for 20 min. for enzyme inactivation. Digestion reaction was run on a 1% agarose gel and the DNA band corresponding to our vector DNA fragment was extracted from the gel.

Digestion of pDrive

pDrive DNA	20 µl
10X NEB Buffer 3	3 µl
BSA	0.3 µl
PstI (10 U/ µl)	1.5 µl
Acc65I (10 U/ µl)	1.5 µl
Sterile Distilled Water	3.7 µl
Total Volume	30 µl

Reaction conditions

Incubated at 37°C for 7 h. and then at 80°C for 20 min. for enzyme inactivation. Digestion reaction was run on a 2.5% agarose gel and the DNA band corresponding to our desired DNA fragment was extracted from the gel.

➢ Ligation into pQE-2

Component	Volume
Insert (Digestion product) (3:1)	1 µl (5 ng)
Digested pQE-2 Vector	1.5 µl (50 ng)
Sterile Distilled Water	7.5 µl
2X Quick Ligation Buffer*	10 µl
Quick T4 DNA Ligase	1 µl
Total Volume	21 µl

* Quick Ligation™ Kit from New England BioLabs (Catalog # M2200S)

Reaction conditions

Incubated at room temperature (25°C) for 15 min. and then chilled on ice.

➢ Transformation

1. 1 vial of TOP10 chemically competent cells was thawed on ice.
2. 4 µl (10 ng) of the ligation reaction was added into the vial of competent cells, mixed gently by pipetting up and down and incubated on ice for 30 min.

3. The cells were heat-shocked for 1 minute at 42°C without shaking.
4. The tubes were immediately transferred to ice and chilled for 5 min.
5. 300 µl of room temperature S.O.C. medium was added to the cells.
6. The tube was incubated at 200 rpm at 37°C for 1 hour.
7. 4 dilutions (25, 50, 75 and 150 µl) were spread from the transformation reaction onto pre-warmed LB agar plates containing Xgal/IPTG-Ampicillin by using a spreader.
8. The plates were incubated overnight at 37°C.

2.2.1.10. Plasmid Purification

Plasmid DNA from white colonies were purified by using Roche High Pure Plasmid Isolation Kit (Catalog # 1 754 785) with the procedure given in detail above (2.2.1.3)

2.2.1.11. Sequencing of Plasmid DNA

Purified plasmid DNA samples were sequenced by using Big dye® terminator v 3.1 cycle sequencing Kit from Applied Biosystems with the procedure given in detail above (2.2.1.4). ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.1.12. Cloning of 3RGBP1+Linker into pQE-2 Expression Vector

During the amplification of the 3RGBP1 from pSB3053 vector, a linker sequence composed of 10 amino acids (GlyGlyGlyGlySerGlyGlyGlyGlyThr) was added to the C terminus of the peptide sequence. Glycine and Threonine residues at the end of the linker sequence were chosen as they have a nucleotide sequence of GGTACC, which is the same as the recognition site of the restriction enzyme Acc65I. So, the other restriction site recognized by EagI was also introduced during amplification. pCR 2.1-TOPO plasmid vector having the 3RGBP1+Linker sequence and the expression vector pQE-2 were digested with these restriction enzymes and the DNA fragments were extracted from the gel. Cleaned and concentrated insert DNA was ligated into pQE-2 vector. Procedure is described in detail below.

- **Restriction Enzyme Digestion**

Digestion of pQE-2

pQE-2 DNA (3RQBP1 inserted)	10 µl
10X NEB Buffer 3	2 µl
BSA	0.2 µl
EagI (10 U/ µl)	1 µl
Acc65I (10 U/ µl)	1 µl
Sterile Distilled Water	5.8 µl
Total Volume	20 µl

Reaction conditions

Incubated at 37°C for 7 h. and then at 65°C for 20 min. for enzyme inactivation.

Digestion reaction was run on a 1% agarose gel and the DNA band corresponding to our vector DNA fragment was extracted from the gel.

Digestion of pCR 2.1-TOPO

pCR 2.1-TOPO DNA	10 µl
10X NEB Buffer 3	2 µl
BSA	0.2 µl
PstI (10 U/ µl)	1 µl
Acc65I (10 U/ µl)	1 µl
Sterile Distilled Water	5.8 µl
Total Volume	20 µl

Reaction conditions

Incubated at 37°C for 7 h. and then at 65°C for 20 min. for enzyme inactivation.

Digestion reaction was run on a 2.5% agarose gel and the DNA band corresponding to our desired DNA fragment was extracted from the gel.

- **Ligation into pQE-2**

Insert (Digestion product) (3:1)	0.5 µl (12 ng)
Digested pQE-2 Vector	1 µl (50 ng)
Sterile Distilled Water	8.5 µl
2X Quick Ligation Buffer*	10 µl
Quick T4 DNA Ligase	1 µl
Total Volume	21 µl

* Quick Ligation™ Kit from New England BioLabs (Catalog # M2200S)

Reaction conditions

Incubated at room temperature (25°C) for 15 min. and then chilled on ice.

- **Transformation**

1. 1 vial of TOP10 chemically competent cells was thawed on ice.
2. 3 µl (7.5 ng) of the ligation reaction was added into the vial of competent cells, mixed gently by pipetting up and down and incubated on ice for 30 min.
3. The cells were heat-shocked for 1 minute at 42°C without shaking.
4. The tubes were immediately transferred to ice and chilled for 5 min.
5. 300 µl of room temperature S.O.C. medium was added to the cells.
6. The tube was incubated at 200 rpm at 37°C for 1 hour.
7. 4 dilutions (25, 50, 75 and 150 µl) were spread from the transformation reaction onto pre-warmed LB agar plates containing Xgal/IPTG-Ampicillin by using a spreader.
8. The plates were incubated overnight at 37°C.

2.2.1.13. Plasmid Purification

Plasmid DNA from white colonies were purified by using Roche High Pure Plasmid Isolation Kit (Catalog # 1 754 785) with the procedure given in detail above (2.2.1.3)

2.2.1.14. Sequencing of Plasmid DNA

Purified plasmid DNA samples were sequenced by using Big dye® terminator v 3.1 cycle sequencing Kit from Applied Biosystems with the procedure given in detail above (2.2.1.4). ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.2. Expression and Purification of the Peptide

Following construction of the vector for the bi-functional peptide, the expression and the purification of the peptide was done. For the purification process, Ni-NTA IMAC (Nickel-NitriloTriAsetic Acid Immobilized Metal Affinity Chromatography) was used.

- **Culturing in LB medium**

For overnight culture, 20 µl of cells were inoculated into 5 ml LB medium and incubated overnight at 37ºC on an orbital shaker at 200 rpm.. This culture was inoculated into a flask of 50 ml LB medium (%10 inoculation) and was incubated overnight at 37ºC on an orbital shaker at 200 rpm. Then the culture was inoculated into a flask of 1 L LB medium (%5 inoculation) and was incubated at 37ºC on an orbital shaker at 200 rpm until its OD_{600} is 0.5. Then the culture was induced by adding 1 ml of 1M IPTG (1 mM final concentration) and then grown at 37ºC for five hours.

After incubation, the culture was centrifuged at 5000 g. for 15 minutes and the pellets were used for protein extraction.

- **Protein extraction**

The pellets containing the cells were used for the purification of the bi-functional peptide. The details of the procedure are given below;

1. The cells were resuspended in 8 ml lysis buffer.
2. 1 mg/ml lysozyme was added and incubated on ice for 30 minutes.
3. The cells were sonicated on ice (Six 10 s. bursts at 100 W. with a 10 s. cooling period between each burst).
4. The cells were lysed gently by vortexing.
5. The lysate was centrifuged for 20 minutes at 10 000 g.
6. The supernatant was transferred to a fresh tube.
7. 500 µl of Ni-NTA resin was added to the tube and mixed for 30 minutes at 4ºC.
8. The samples were centrifuged for 20 seconds at 5000 g. to pellet the resin, the supernatant was transferred to a fresh tube.

9. The resin was washed 3 times with 4 ml of wash buffer, centrifuged for 20 seconds at 5000 g. between each washing step, the supernatant was removed.
10. The protein was eluted 4 times with 500 µl elution buffer, centrifuged for 20 seconds at 5000 g. between each elution step, the supernatant was transferred to a fresh tube.

- **SDS-PAGE**

Obtained samples were analyzed by using SDS-PAGE. The stacking gel (8%) and the separating gel (20%) were prepared according to the amounts below;

Table 2.1: Ingredients of stacking and separating gel for SDS-PAGE

	Stacking Gel (8%)	**Separating Gel (20%)**
Acrylamide-Bisacrylamide Mixture (30%)	1.3 ml	6.6 ml
Tris-HCl Buffer	1.25 ml (0.5 M, pH 6.8)	2.5ml (1.5 M, pH 8.8)
%10 APS	50 µl	100 µl
%10 SDS	50 µl	100 µl
TEMED	20 µl	20 µl
dH_2O	2.33 ml	680 µl
Total Volume	**5 ml**	**10 ml**

10 µl of each sample was mixed with 10 µl of 2X SDS Sample Application Buffer and loaded into the wells of the gel. The samples were run at 125 V for 3 hours.

3. RESULTS AND DISCUSSION

Peptides obtained from phage or cell surface display libraries are generally short ones, composed of 7-14 amino acids. These kinds of short peptides can have many conformations but, on the other hand, this can result in the reduction of the conformation of its active forms. Multimerization of the short peptide sequences could change their properties by making a more rigid structure, which might be especially important in the controlled assembly. An increase in the number of binding sites in repeating polypeptides might provide an improvement in binding affinity or specificity. The optimal conformation for the surface recognition or desired characteristics of the peptide could be obtained by modifying the repeating forms, as a way of multimerization [16].

In this study, we have studied two inorganic binding peptides; GBP1 & QBP1. Quartz Binding Peptide 1 was previously identified by bioinformatics tools from a large group of strong quartz binders selected by phage display in M13. Gold Binding Peptide was previously identified by cell surface display in *E. coli*. The second-generation peptides, used in these studies were genetically designed as multiple repeats (three) in both of the inorganic binders. Both of the peptide sequences were amplified and cloned into an expression vector by linking them with a peptide linker. The bi-functional peptide was expressed, purified and the functionalities of the both domains have been investigated.

3.1. Construction of the Bi-functional Peptide Vector

3.1.1. Cloning of 3RQBP1 Sequence into pQE-2 Expression Vector

For the construction of the bi-functional peptide vector, first, the cloning of the 3RQBP1 was accomplished. For this, this sequence was amplified by using PCR. The synthesized oligonucleotide, which is 140 base in length, was used as the template. This oligonucleotide has a content of QBP1 sequence as three repeats, two stop codons (TAATAA) at the end of the 3rd repeat of the QBP1 sequence and *Pst*I and *Acc65*I restriction sites at each end. The sequence of the oligonucleotide with primer binding sites and the restriction sites yielding 3RQBP1 DNA are given in Figure 3.1.

```
5`-GGATCAGGTACCCCTCCTCCTTG-3`

5`-GGATCAGGTACCCCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCT
         Acc65I

  CCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCT

                                        STOP  PstI
  CCTCCTCCTTGGTTGCCTTATATGCCTCCTTGGTCTTAATAACTGCAGACTAC-3`
                         3`-AGGAACCAGAATTATTGACGTCTGATG-5`
```

Figure 3.1: The sequence of the 3RQBP1 oligonucleotide. Primer binding sites are shown in blue. Multiple units of 3RQBP1 are shown in red, each repeat in one row. The restriction sites are shown as underlined in different colors; *Acc*65I in green and *Pst*I in purple. Stop codons at the end of the peptide coding sequence are shown in bold.

In the designing of the oligonucleotide, the sequence of the quartz binder region was used as three tandem repeats. Stop codons were placed to the end of the region and the restriction sites introduced to the sequence was selected by regarding some issues. The strategy for the construction of the bifunctional peptide was developed to ligate two domains separately. For this ligation, three different restriction sites given in the multiple cloning site of the pQE-2 vector was chosen (Figure pQE-2). *Pst*I and *Acc*65I was selected for the quartz binder. Here, the reason for choosing *Acc*65I site was because of its protein coding sequence. *Acc*65I nucleotide recognition site is GGTACC which is expressed as Glycine and Threonine (**GT**) respectively. As it was mentioned before, the peptide sequence to be used for linking two GEPIs were chosen as GGGGSGGG**GT.** By selecting *Acc*65I as the restriction site, two residues of the linker region were already cloned into the expression vector. The remaining eight residues of the linker was added to the end of the gold binder region by PCR (will be discussed later).

The forward and the reverse primers were used for the amplification of the sequence and at the end of the reaction a double stranded 3RQBP1 DNA sequence with restriction sites was obtained from a single stranded oligonucleotide. The result of the PCR is analyzed by agarose gel electrophoresis (Figure 3.2)

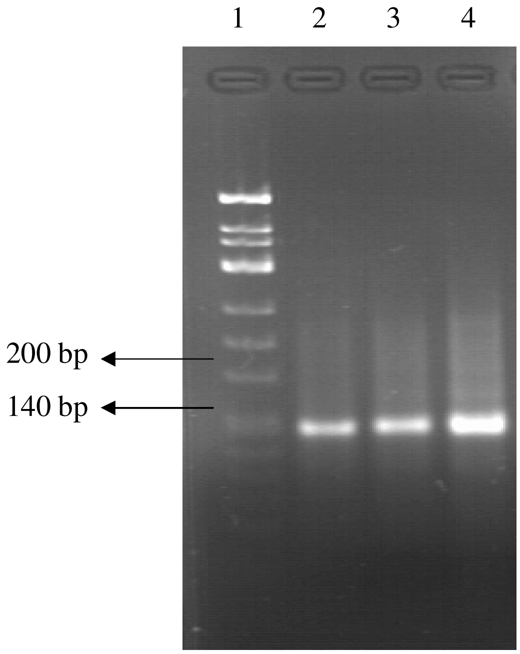


Figure 3. 2: 2.5% agarose gel. Lane 1: Marker 10, PhiX174 DNA/HinfI Marker, Lane 2: PCR product obtained from Tm = 55°C reaction, Lane 3: PCR product obtained from Tm = 58°C reaction, Lane 4: PCR product obtained from Tm = 60°C reaction.

After verification of obtaining the desired DNA fragment by agarose gel electrophoresis, the DNA fragments were extracted from the gel and were cloned into pDrive cloning vector and transformed into *E.coli* competent cells. Top10 *E. coli* electrocompetent cells were used for transformation. pDrive is a TA cloning vector that needs unpaired Adenine residues on 3'-ends of the DNA fragment to be inserted. Although, *Taq* DNA polymerase adds Adenine residues to 3'-ends of amplified DNA sequence with a longer final extension step during PCR, an extra A-Addition step was performed to be sure that unpaired Adenine residues were added to 3'-ends of the amplified 3RQBP1 sequence. By cloning our PCR product into pDrive cloning vector, it was easily checked by DNA sequencing if there was any mutation in our sequence and 20 positive colonies were then grown to have the required amount of insert DNA for ligation into expression vector.

pDrive vector DNA with 3RQBP1 insert was amplified, purified and digested with *Pst*I and *Acc*65I restriction enzymes. Three DNA fragments, which are 126 bp, 54 bp and 3603 bp in length, were obtained after digestion. Digestion mixture was run on 2.5% agarose gel and desired 126 bp fragment which belongs to 3RQBP1 was gel extracted (Figure 3.3.)

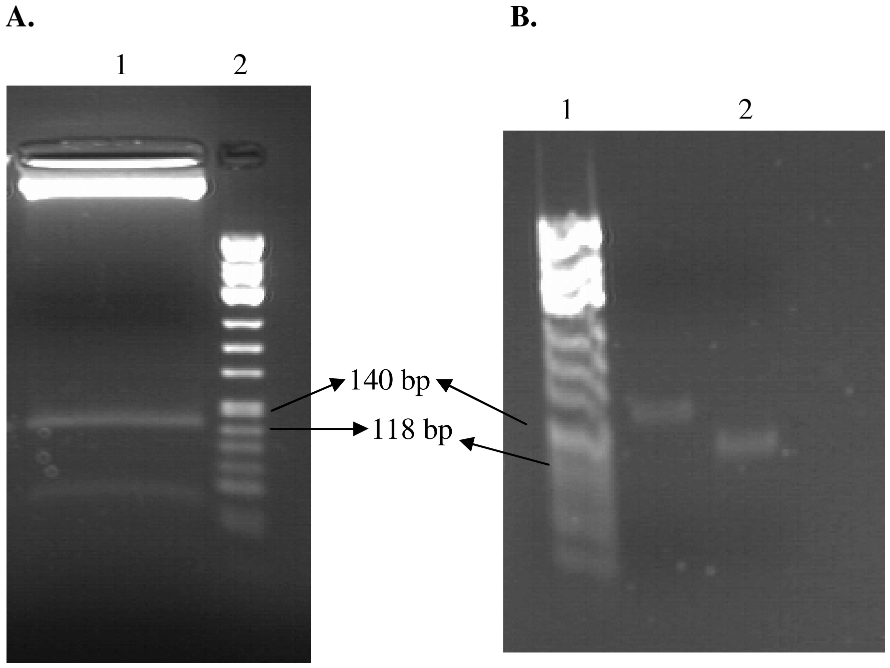


Figure 3.3: **A.** 2.5% agarose gel; Lane 1: DNA fragments obtained after *Pst*I and *Acc*65I digestion of pDrive having 3RQBP1, Lane 2: Marker 10, PhiX174 DNA/HinfI Marker **B.** 2.5% agarose gel; Lane 1: Marker 10, PhiX174 DNA/HinfI Marker, Lane 2: 3RQBP1 after gel extraction.

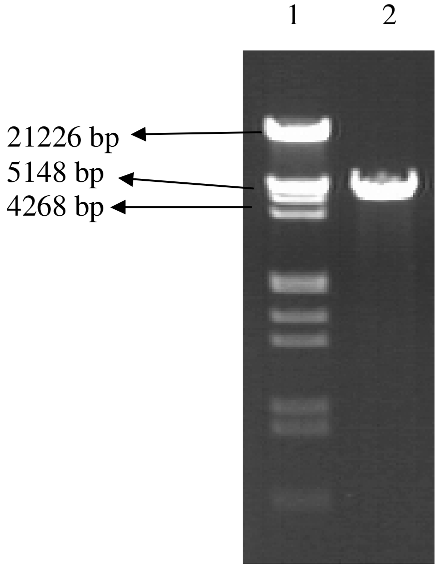


Figure 3.4: 1% agarose gel; Lane 1: Marker 3, Lambda DNA / *Eco*RI+*Hind*III, Lane 2: linear pQE-2 vector obtained after *Pst*I and *Acc*65I digestion.

Following the extraction of the digestion products which had the same sticky ends, the 3RQBP1 DNA fragment (Figure 3.3) was ligated into the pQE-2 vector (Figure 3.4). The ligation product was transformed into Top10 chemically competent cells. White colonies on LB Agar + Ampicillin plates were selected, grown in LB broth medium and plasmid DNA of the bacteria was purified to check their sequence. After confirming with the DNA sequencing results, construction of the pQE-2 vector with 3RQBP1 insert was accomplished.

3.1.2. Cloning of 3RGBP1 + Linker Sequence into pQE-2 Expression Vector

For the construction of the bi-functional peptide vector, cloning of the 3RGBP1+Linker sequence was done later. For this insertion, 3RGBP1 sequence was amplified by using PCR. p53Q vector, which contains a three-repeat GBP1 sequence was used as the template. Forward and reverse primers were used for the introduction of the restriction sites *Acc65*I and *Eag*I and also a linker sequence composed of 30 bases (10 amino acids; GGGGSGGGGT). The sequence of the primer binding sites and the restriction sites yielding 3RGBP1+Linker DNA are given in Figure 3.5.

```
     NotI    Q
5'-GCGGCCGCGCAAATGCATGGAAAAACTCAGGCA-3'
            5'-ATGCATGGAAAAACTCAGGCAACCAGCGGGACTATCCAGAGC

ATGCATGGAAAAACTCAGGCAACCAGCGGGACTATCCAGAGC

ATGCATGGAAAAACTCAGGCAACCAGCGGGACTATCCAGAGC-3'
                  3'-GTCGCCCTGATAGGTCTCGCCACCTCCACCATCGCCGCCACCTCCATGG-5'
                                                                Acc65I
```

Figure 3.5: Amplification of the 3RGBP1 sequence by adding linker region to its end. Primer binding sites are shown in blue. Multiple units of 3RGBP1 are shown in bold, repeat in one row. The restriction sites are shown as underlined in different colors; *Acc*65I in green and *Not*I in gray.

The result of the PCR is analyzed by agarose gel electrophoresis (Figure 3.6).

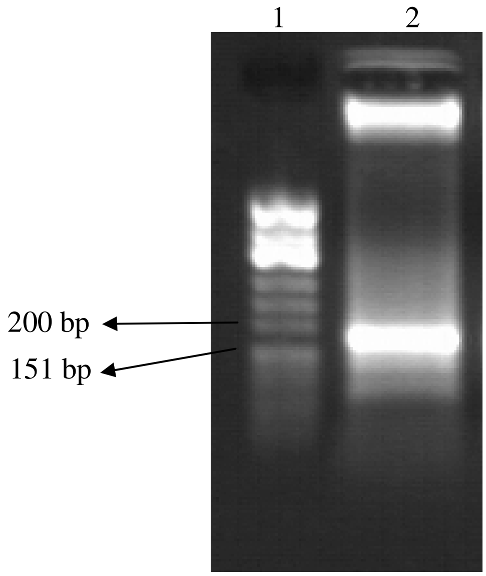


Figure 3. 6: 2.5% agarose gel. Lane 1: Marker 10, PhiX174 DNA/HinfI Marker, Lane 2: PCR product obtained from Tm = 50°C reaction

After confirmation of obtaining the desired DNA fragment by agarose gel electrophoresis, the DNA fragments were extracted from the gel and were cloned into pCR 2.1-TOPO cloning vector and transformed into *E.coli* competent cells. Top10 *E. coli* chemically competent cells were used for transformation. pCR 2.1-TOPO is, like pDrive, a TA cloning vector, that needs unpaired Adenine residues on 3'-ends of the DNA fragment to be inserted. But, the amplification was done by using *Pfu* DNA polymerase, which has a 3'→5' exonuclease (proofreading) activity. So, unlike *Taq* DNA polymerase, no Adenine residues were added to 3'-ends of amplified DNA sequence even with a longer final extension step during PCR. Thus, an A-Addition step was performed to have Adenine residues added to 3'-ends of the amplified 3RGBP1 + Linker sequence. By cloning our PCR product into pCR 2.1-TOPO cloning vector, it was easily checked by DNA sequencing if there was any mutation in our sequence and 24 positive colonies were then grown to have the required amount of insert DNA for ligation into expression vector.

pCR 2.1-TOPO vector DNA with 3RGBP1 + Linker insert was amplified, purified and digested with *Acc*65I and *Eag*I restriction enzymes. Three DNA fragments, which are 168 bp, 3813 bp and 3843 bp in length, were obtained after digestion. Digestion mixture was run on 2.5% agarose gel and desired 168 bp fragment which belongs to 3RGBP1 + Linker sequence was extracted from the gel (Figure 3.7).

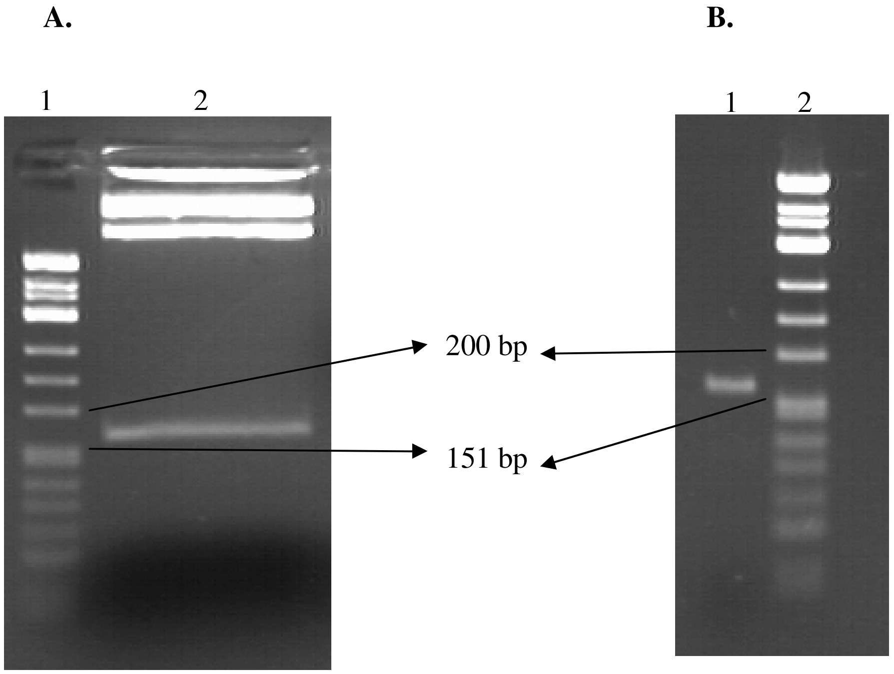


Figure 3.7: **A.** 2.5% agarose gel; Lane 1: Marker 10, PhiX174 DNA/HinfI Marker, Lane 2: DNA fragments obtained after *Acc*65I and *Eag*I digestion of pCR 2.1-TOPO containing 3RGBP1 + Linker sequence **B.** 2.5% agarose gel; Lane 1: 3RGBP1 + Linker after gel extraction, Lane 2: Marker 10, PhiX174 DNA/HinfI Marker

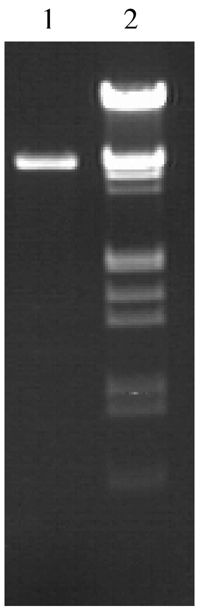


Figure 3. 8: 1% agarose gel; Lane 1: linear pQE-2 + 3RQBP1 vector obtained after *Acc*65I and *Eag*I digestion, Lane 2: Marker 3, Lambda DNA / *Eco*RI+*Hind*III

Following the extraction of the digestion products which had the same sticky ends, the 3RGBP1 + Linker DNA fragment (Figure 3.7) was ligated into the pQE-2 + 3RQBP1 vector (Figure 3.8). The ligation product was transformed into Top10 chemically competent cells. White colonies on LB Agar + Ampicillin plates were selected, grown in LB broth medium and plasmid DNA of the bacteria was purified to check their sequence. After verifying with the DNA sequencing results, construction of the pQE-2 vector with the bi-functional peptide insert was accomplished.

3.2. Expression and Purification of the Bi-Functional Peptide

After verifying the construction of the bi-functional peptide with the sequencing results, expression studies were performed. For this, some pilot experiments were done to get the optimum parameters for expression and purification.

The expression vector used in this study is pQE-2 from Qiagen, which are used for expressing N-terminally Histidine tagged proteins in *E. coli*. Expression of the recombinant proteins encoded by pQE-2 vector is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. Just the once the *lac* repressor is inactivated, the sequences downstream from the promoter region can be transcribed by the RNA polymerase of the host cell. These transcripts are later translated into the recombinant protein [32].

Once this recombinant protein is expressed by the host cell, it will have an affinity tag in its N-terminus composed of 6 Histidine residues. By the help of this tag, expressed recombinant protein can be purified from the culture medium by Affinity Chromatography. The system used in this study is based on the selectivity and the affinity of nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices for the 6xHistidine tagged proteins. Ni-NTA is a tetradentate chelating adsorbent in which Nitrilotriacetic Acid keeps four over six free sites of nickel ion, thus, causing two sites free for the interaction with the Histidine tag (See Introduction, Figure 1.3). The structure of the Histidine amino acid is composed of an imidazole ring as the radical group. This imidazole ring is responsible for the binding of the Histidine tag to the nickel ions that are immobilized by the NTA groups of the matrix. Imidazole can also bind to the nickel ions by itself, so, it is found in the purification buffers at different concentrations. Non-specific binding of the background proteins in the culture is avoided at low imidazole concentrations while 6xHistidine-tagged proteins still bind to the matrix strongly. Hence, low concentrations of imidazole are used in the lysis and wash buffers (10-20 mM) [28].

The Histidine-tagged protein bound to the Ni-NTA matrix can be eluted by increasing the imidazole concentration to 100-250 mM because they can not compete with this high concentration of imidazole and dissociate from the matrix.

In to pilot experiments, the optimum parameters for the expression of the peptide were obtained. The optimum growth temperature for our peptide is always 37 ºC. The IPTG induction is done when the peptides grow to an OD_{600} of 0.5 and the final concentration of IPTG is 1 mM. Following induction, the optimum time for the production of our peptide in culture medium is 5 hours. After 5 hours of growth, the culture was spin down and the pellets containing the cells were used for the purification of the bi-functional peptide (Figure 3.9).

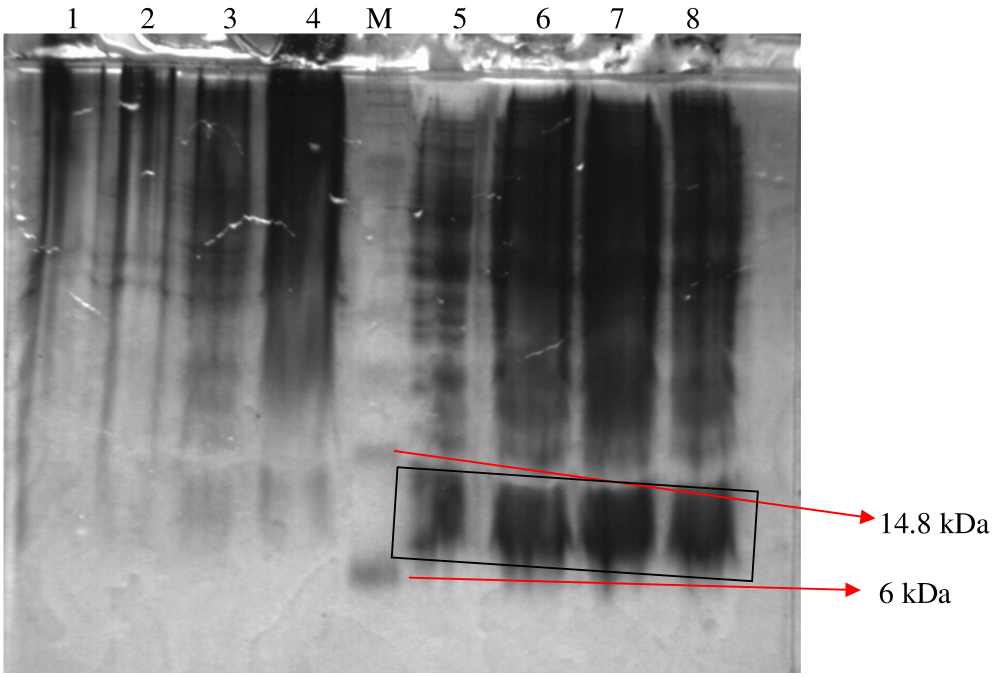


Figure 3.9: %8-20 SDS-PAGE result. Lane 1: Uninduced culture grown for 2 hours, Lane 2: Uninduced culture grown for 3 hours, Lane 3: Uninduced culture grown for 4 hours, Lane 4: Uninduced culture grown for 5 hours, Lane M: BenchMark™ Pre-Stained Protein Ladder, Lane 5: Induced culture grown for 2 hours, Lane 6: Induced culture grown for 3 hours, Lane 7: Induced culture grown for 4 hours, Lane 8: Induced culture grown for 5 hours

In the purification procedure, three different steps were performed for the complete lysis of the cells. First, the cells were resuspended in lysis buffer (which contains 10 mM imidazole). Following resuspension, lysozyme was added to the resuspension and was incubated on ice for 30 minutes. Lyszozyme (EC 3.2.1.17, 129 amino acids, 14,7 kDa) is an enzyme that is extracted from the egg white and acts by damaging the cell walls of the bacteria by hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins [51]. Following enzyme treatment, the cells were sonicated on ice. In this step, the cell membranes are totally disrupted and cellular contents are released.

After lysis of the cells, all the content of the cells are treated with Ni-NTA resin. In this step, our Histidine-tagged peptide is bound to the resin while all the other peptides are not. After washing with wash buffer (which contains 20-50 mM imidazole), other possible non-specific bindings are also removed from the resin. Finally, our peptide is eluted by washing the resin with elution buffer (which contains 250 mM imidazole). Obtained samples were analyzed by using SDS-PAGE (8-20%). Our peptide (11,5 kDa) was purified and can be seen elution fragments. In the flow through samples all the unbound peptides, especially lysozyme, is seen and non-specific bindings removed by washing steps is seen in the wash samples (Figure 3.10).

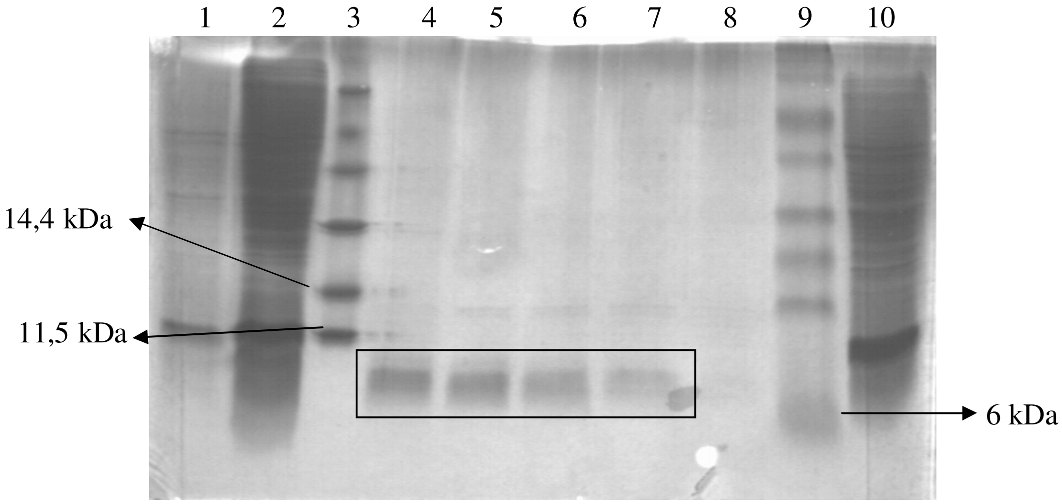


Figure 3.10: %8-20 SDS-PAGE result. Lane 1: Fraction obtained from the first washing step, Lane 2: Cleared lysate, Lane 3: Unstained Protein Molecular Weight Marker, Lane 4: Fraction obtained from the first elution step, Lane 5: Fraction obtained from the second elution step, Lane 6: Fraction obtained from the third elution step, Lane 7: Fraction obtained from the fourth elution step, Lane 8: Empty, Lane 9: BenchMark™ Pre-Stained Protein Ladder, Lane 10: Flow through fraction

4. CONCLUSION

A fusion peptide consisting of a quartz binding peptide and a gold binding peptide, which are previously well-characterized inorganic binding domains, was constructed by using genetic engineering methods and the protein was expressed and purified via Nickel-Nitrilotriacetic Acid Immobilized Metal Affinity Chromatography.

GBP1 is a 14-mer peptide selected from a peptide library expressed on the outer surface of *Escherichia coli* with an amino acid sequence of MHGKTQATSGTIQS. QBP1 is a 12-mer peptide, which were *de novo* designed peptides by *in silico* selection of strong-binding QBPs by using bioinformatics tools. Both the Gold binding peptide and the Quartz binding peptide were used as three tandem repeats with the idea that repeated structures the sequences could show a better affinity and provide an enhancement in binding strength by increasing the surface recognition sites. The peptides were linked to each other by a ten-amino acid flexible peptide linker rich in Glycine content with a sequence of GGGGSGGGGT.

In developing the construction and expression of this peptide, we utilized the molecular biology tools like polymerase chain reaction and immobilized metal affinity chromatography, which are widely-used techniques in vector construction and protein purification.

Our experiments indicated that enabling technologies could be developed for *in vivo* production of peptides at desired amounts with multi-activities and selected bi-functional peptide will be utilized in nanoparticle and surface assemblies in bio/nanotechnologies.

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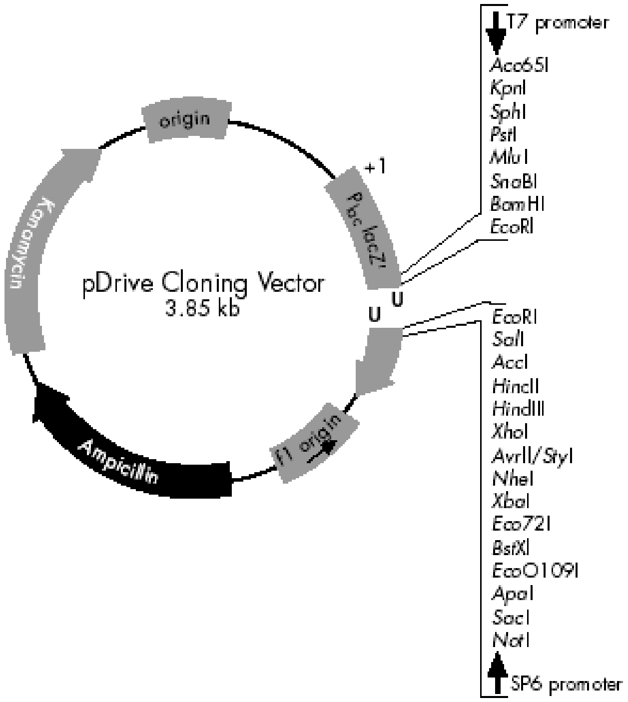
APPENDIX

Appendix A.

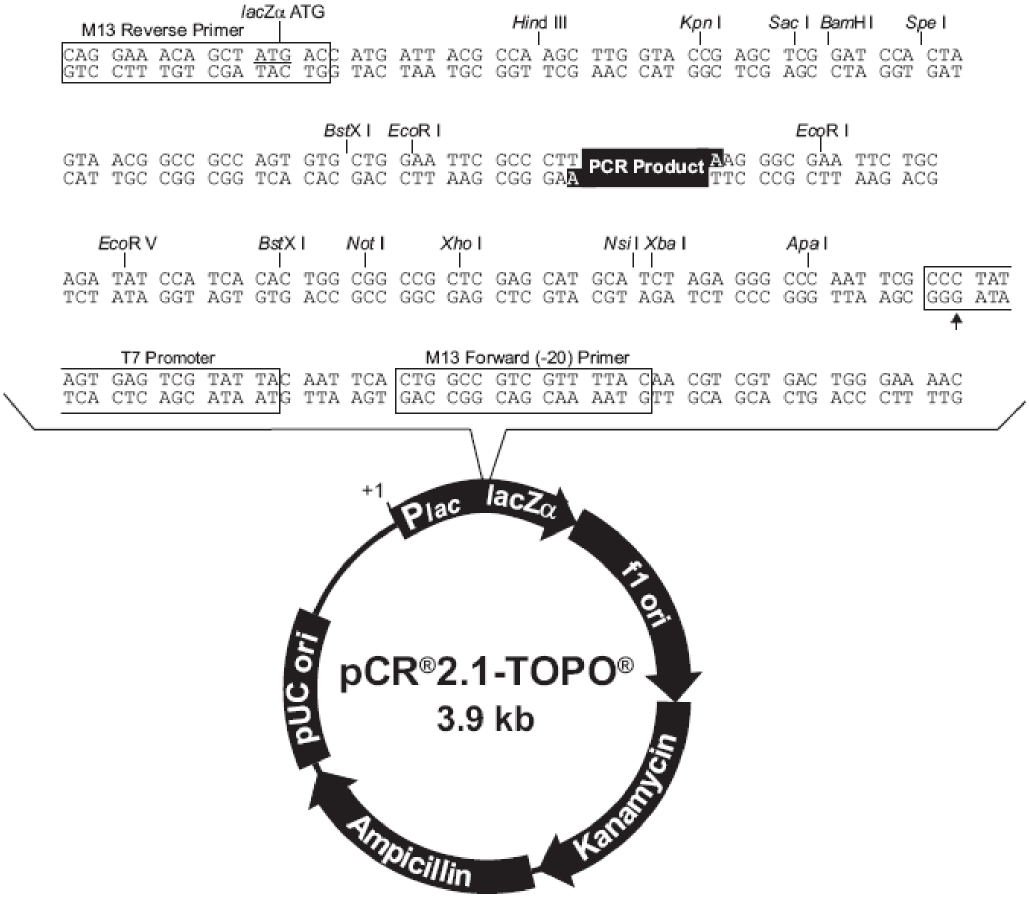
Vector Map of pDrive

Position (bp)	**Element**
266–393	Multiple cloning site
216–593	LacZ α-peptide
239–258	T7 RNA polymerase promoter
start 256	T7 transcription
398–417	SP6 RNA polymerase promoter
start 400	SP6 transcription
1175–2032	Ampicillin resistance gene
2181–2993	Kanamycin resistance gene
3668	pUC origin
588–1043	Phage f1 origin



Appendix B.

Vector Map of pCR 2.1

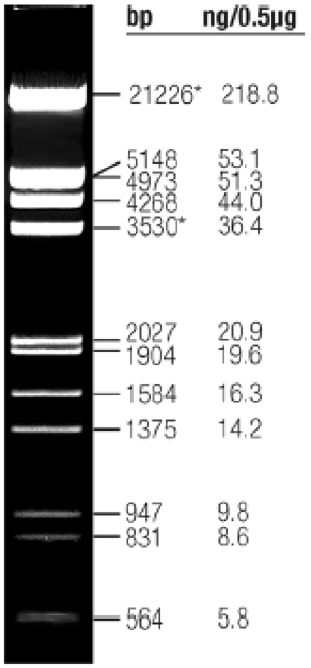


Position (bp)	**Element**
1–547	LacZα fragment
205–221	M13 reverse priming site
234–357	Multiple cloning site
364–383	T7 promoter/priming site
391–406	M13 Forward (-20) priming site
548–985	f1 origin
1319–2113	Kanamycin resistance ORF
2131–2991	Ampicillin resistance ORF
3136-3809	pUC origin

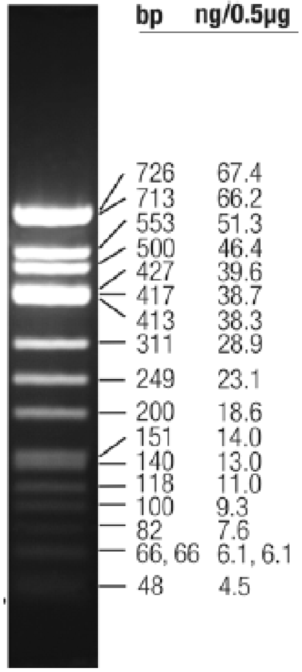
Appendix C.

Molecular Weight Markers

a)



b)



a) λDNA *EcoRI* and *HindIII* digested marker-3, 1.0% agarose, 0.5µg/lane, 8 cm length gel, 1X TAE, 17V/cm. (Catalog # SM0191, NBI Fermentas).

b) ΦX174 DNA *HinfI* digested marker-10, 2.5% agarose, 0.5µg/lane, 8 cm length gel, 1X TBE, 5V/cm. (Catalog # SM0261, NBI Fermentas).

Appendix D.

Lab Equipments

Autoclaves : 2540 ML benchtop autoclave, Systec GmbH Labor-Systemtechnik.
: NuveOT 4060 vertical steam sterilizer, Nuve.

Centrifuges : Avanti J-30I, Beckman Coulter.
: Microfuge 18, Beckman Coulter.

Centrifuge rotors : JA30.50Ti, Beckman Coulter.
: F241.5P, Beckman Coulter.

Deep freezes and refrigerators : Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.
: 2021 D deep freezer, Arcelik.
: 1061 M refrigerator, Arcelik.

Electrophoresis equipments : E-C Mini Cell Primo EC320, E-C Apparatus.
: Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog# 165-3321,Bio-Rad.
: Mini-V 8.10 Vertical Gel Electrophoresis System, Life Technologies GibcoBrl (now Invitrogen), Catalog# 21078.

Gel documentation system : UVIpro GAS7000, UVItec Limited.

Ice Machine : AF 10, Scotsman.

Incubators : EN400, Nuve.

Orbital shaker : Certomat S II, product# 886 252 4, B. Braun Biotech International GmbH.

Magnetic stirrer : AGE 10.0164, VELP Scientifica srl.
: ARE 10.0162, VELP Scientifica srl.

Pipettes : Pipetteman P10, Eppendorf

pH meter : MP 220, Mettler Toledo International Inc.
: Inolab pH level 1, order# 1A10-1113, Wissenschaftlich-Technische Werkstätten GmbH & Co KG.
Power supply : EC 250-90, E-C Apparatus.
Pure water systems : USF Elga UHQ-PS-MK3, Elga Labwater.
Spectrophotometer : DU530 Life Science UV/ Vis, Beckman.
: UV-1601, Shimadzu Corporation.
Sterilizer : FN 500, Nuve.
Transilluminator : UV Transilluminator 2000, Catalog# 170-8110EDU, Bio- Rad.
Vortexing machine : Reax Top, product# 541-10000, Heidolph2.2.

CV

Abdullah Sert was born in Manisa in 1983. He graduated from Fatih Anatolian High School in 2001 and the same year he started to study in Istanbul Technical University Molecular Biology and Genetics Department. He had his Bachelor degree from the same department in 2005 and consequently, he was accepted to Molecular Biology - Genetics and Biotechnology Graduate Program, which is a part of Advanced Technologies Department. Biotechnology, protein engineering, molecular genetics and plant tissue culture are among his professional interest topics.